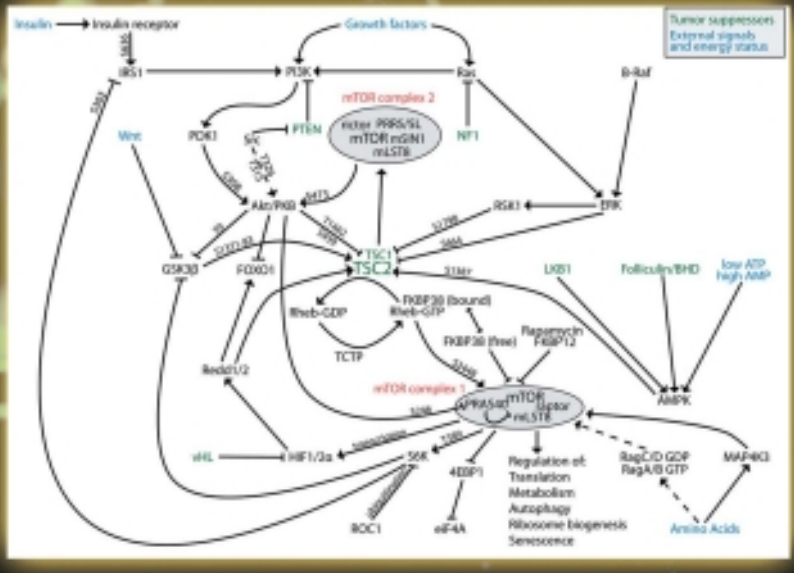
BIOACTIVE PEPTIDES

Applications for Improving Nutrition and Health



Richard Owusu-Apenten

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Contents

Preface

Weight loss is a common reaction to infection, injury, or chronic disease. Many well-known host responses to illness contribute to muscle loss. Ensuring adequate lean body mass is of major concern in health care. Bioactive peptides and medical foods represent new approaches for addressing the metabolic derangements and nutritional deficits that contribute to unwanted weight loss. This book describes the latest research on the application of bioactive peptides for improving nutrition and health. We present the background science in Chapters 1 through 5 on the relations between illness and muscle weight loss. Chapters 6 through 9 deal with the use of bioative peptides to modify aspects of the host response to illness, including inflammation, antimicrobial activity, anabolic dysfunction, and anorexia. The chapter titles are

Evidence from in vivo studies and randomized clinical trials indicate that bioactive peptides can be effective in the prevention of weight loss associated with conditions such as aging, HIV/AIDS, burn injuries, chronic obstructive pulmonary diseases, diabetes, inflammatory bowel disease, kidney failure, and tuberculosis. Some of the approaches currently under development may be suitable for optimizing muscle growth and performance in otherwise healthy subjects. Parts of this book will be suitable reading for health-care professionals, including nutritionists, dietitians, food scientists, and technologists. The discussion will also be of interest to those interested in medical foods, nutraceuticals, and functional foods.

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Richard Owusu-Apenten, PhD, spent his early years in the newly independent ex-British colony of Gold Coast, Ghana, where he attended schools at Akim Oda, Bolgatanga, Kumasi, and Berekum. His interest in biology and chemistry was nurtured by further education in Quintin Kynaston High School, St. John's Wood, followed with BSc (Hons) and PhD degrees from the University of London. Dr. Apenten (Kwasi Owusu-Apenteng) spent four years as a postdoctoral research fellow in China, Wales, and University College London before taking up a lectureship in food science at Leeds University in 1989. From 2001 to 2005, Dr. Apenten had a fruitful stint as an associate professor at Penn State University before returning to the United Kingdom to pursue further interests in nutrition. Dr. Apenten is employed with the School of Biomedical Sciences, University of Ulster, at the Coleraine campus. He is an active member of both the Institute of Food Technology (IFT) and the American Chemical Society (ACS), and a fellow of the Institute of Food Science and Technology (IFST) (United Kingdom).

1 Nutrition and the Host Response to Infection and Injury

> Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.
>
> **World Health Organization**

1.1 NUTRITION AND ILLNESS

1.1.1 Introduction

Illness may be considered a programmed host response to infection and injury that is intended to aid recovery in the long term. However, illness can also lead to a set of "sickness behaviors" that include lethargy, sleepiness, loss of appetite (anorexia), reduced grooming, low regard for personal hygiene, and a state of mild depression. It is thought that sickness behavior is linked with increased levels of cytokines produced in response to invading microbes, injury, or chronic diseases stress.* The short-term host response to illness is powered ultimately by muscle loss. Chapter 1 provides an overview of the relations between nutrition, illness, and weight loss (Section 1.1). The important features of the host response to illness and injury are described in Section 1.2. In Section 1.3, we compare weight loss due to cachexia or anorexia with the effects of calorie restriction. In principle, there are many ways to improve well-being because illness is usually multidimensional. Possible modalities for improving health using bioactive peptide are outlined in Section 1.4. Overall, bioactive peptides and proteins present diverse opportunities for moderating the host response to illness thereby improving nutrition and health. The avoidance of weight loss during illness is an interesting surrogate measure of well-being that might be applied to evaluate bioactive peptides.

* For an introduction to sickness behavior, cf. http://en.wikipedia.org/wiki/Sickness_behavior. Illness-related anorexia is discussed in Chapter 9.

1.1.2 Infection and Undernutrition

Undernutrition and unwanted weight loss are implicated in 53% of the 10 million deaths recorded for children annually.[1,2,*] Undernourished children are more prone to infectious diseases and recover less rapidly from illness. Persistent infection, injury, and stress contribute to growth retardation with long-term consequences on cognitive development. The developmental shortcomings associated with chronic infection arise partly because vital body resources are channeled toward fighting infection rather than growth.[3–6] Low nutritional status also leads to a higher incidence of diseases in aging adults, especially those living in long-term care homes (Section 1.3).

1.1.3 Nutritional Status and Immune Function

The link between undernutrition and infection can be explained in terms of the effect of low nutrient intake on the human immune system.[7–11] The inadequate intake of protein or energy reduces the number of immune cells in the circulation. The concentration of antibodies produced by each immune cell also declines. Defects in immune function increase the likelihood of infectious disease.[†]

1.1.4 The Undernutrition-Infection Paradigm

Infection leads to increases in energy and material demands on the host, which weakens and renders them vulnerable to further disease.[12,13] The so-called undernutrition-infection paradigm provides a justification for public health programs to combat childhood malnutrition (Figure 1.1). Improvements in a child's nutritional status will boost their long-term resistance to infection. This type of prophylactic nutrition could be perhaps more cost effective in the long term compared to drug treatment. Disease prevention is also justifiable on ethical grounds since not all the consequences of illness can be fully reversed by later treatment.

The connection between undernutrition and poor health provides one reason for adopting nutrition support. Patients suffering from infectious disease, accidental injury, or so-called dynamic diseases have all been found to benefit from nutritional therapy, dietary supplementation, or feeding programs that give due consideration to their particular form of ailment. Nutritional support has been shown to reduce the length of hospital stay, reduce the likelihood of medical complications, and improve treatment outcomes for severely ill patients.[14,15]

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* Undernutrition refers to values for anthropometric measurements (body weight, height, skin fold thickness, BMI, etc.) that are below the average expected for subjects of similar age and gender. Malnutrition refers to a diet that is either too low or too high in key nutrients compared to recommended daily allowance (RDA) values. According to the current usage, undernutrition may arise from inadequate food intake as well as from illness.

† Impoverished nutrition can also lead to nutrient deficiency diseases. However, undernutrition cannot be overcome simply by providing excess supplies of nutrients. To assure weight gain requires the underlying causes of muscle wasting are addressed (see Section 3.1.2).

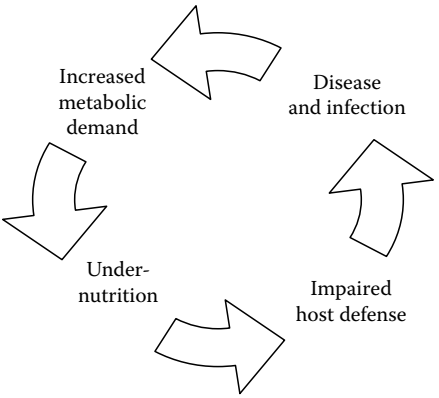


FIGURE 1.1 A schematic presentation of the undernutrition-infection paradigm. (Adapted from Keusch, G.T., *J. Nutr.*, 133, 33S, 2003.)

1.2 HOST RESPONSE TO INJURY

1.2.1 The Ebb and Flow Phases

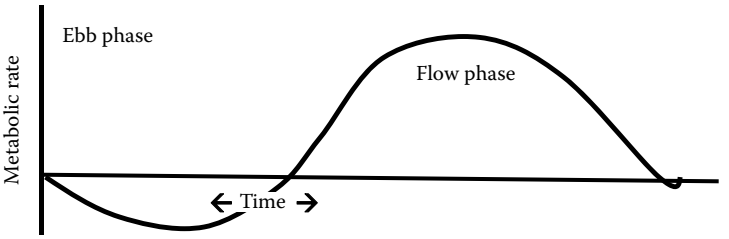
Complex animals including birds, reptiles, and mammals show a programmed response to injury and infection, which form the symptoms of disease. Though intended to aid recovery in the longer term, the response to injury places extra demands on the body that may hinder short-term survival.[16–20] The so-called acute phase response to injury and infection occurs in two stages: the ebb and flow phases. The ebb phase lasting 24–48 h after injury involves a dramatic lowering of body temperature, combined with falls in blood pressure, cardiac output, and oxygen consumption. It is thought that these changes are directed at conserving body reserves in the period immediately following severe injury.

Where the Phase 1 response to injury is not fatal, it is followed by the flow phase characterized by increasing body temperature and rising basal metabolic rate. The flow phase may also be accompanied by a hypermetabolic response sometimes leading to fever. The increase in resting energy expenditure (REE) is driven by existing resources from the body rather than from increases in food intake. On the contrary, the flow phase coincides with a *loss of appetite and a decrease in food-seeking behavior.* There are characteristic changes also in hormone secretion and nerve activity (Figure 1.2). Table 1.1 lists some of the characteristic changes associated with the acute phase response to infection and injury. In principle, the efficiency of recovery may be improved if the time-course or intensity of the acute phase response could be moderated.

1.2.2 Molecular Aspects of the Host Response to Injury

Observations from simple organisms provide important clues about the illness-response in humans and other higher animals.[21,*] Fruit flies show a three-stage response to infection by pathogenic microbes: first there is an innate immune response leading to the

* The fruit fly and zebra fish are thought to provide appropriate models for the human response to illness.



Ebb Phase	Flow-Phase Catabolic	Flow-Phase Anabolic
Duration <24 h	3–10 days	10–60 days
Metabolic response BMR↓, temperature↓, VO↓	BMR↑, temperature↑, nitrogen balance (–)	Nitrogen balance (+)
Hormones Catecholamine, cortisol, aldosterone	Glucagon, cortisol, catecholamine	Growth hormone, IGF

FIGURE 1.2 The ebb and flow phases of the metabolic response to illness. *Abbreviations*: BMR, basal metabolic rate; VO, volume of oxygen used.

TABLE 1.1
Common Features of the Acute Phase Response to Illness

Clinical features
Anorexia
Resting metabolic rate ↑
Fever (body temperature >37°C)
Hypothermia ↑
Muscle loss ↑
Unwanted weight loss ↑

Metabolic or cellular changes
Intestinal absorption ↓
Catabolism of glucose, fat, and protein ↑
Production of immune cells and antibodies
Acute phase response proteins ↑
Protein breakdown ↑
Protein synthesis ↓
Pro-inflammatory cytokines ↑
Inflammation ↑
Catecholamines ↑, glucocorticoids ↑, glucagon ↑

Protein changes
Leucine oxidation ↑
Flux of glutamine and glycine from muscles ↑
Glutamine utilization by splanchnic tissue and immune cells ↑
Glucose formation from glycine ↑
Plasma amino acid levels ↓

production of antimicrobial peptides, phagocytosis by white blood cells, and melanization; second, there follows a series of processes leading to bacterial spread or virulence; and third, scientists observe a group of host–pathogen responses arising from adverse physiological consequences of the preceding two stages. The flies also show a metabolic response to infection characterized by increases in glucose levels in the hemolymph akin to that seen in type 2 diabetes. Finally, sick fruit flies show increased losses of glycogen, body fat stores, and a loss of body protein. The fruit fly response to infection with *Mycobacterium marinum* (a nonpathogenic microbe closely related to *M. tuberculosis*) mirrors the human response to tuberculosis (e.g., weight loss, illness anorexia, and altered anabolic signaling by insulin).[22] In higher animals such as the rat, sickness leads to changes in fecal and urine output, altered sleep patterns, and the advent of sickness behavior (see footnote on p. 1). Relevant observations have also been obtained with mice injected with bacterial liposaccharide (LPS) cell wall extract.

1.2.3 The Infection-Inflammatory Response

In higher organisms, stimulation of the host's innate immune response by invading pathogens occurs in three phases (Chapter 4): first, pathogens are detected by host cell pattern recognition receptors (PRR) mounted on the membranes of macrophages and other frontline cells; second, phagocytes (macrophages, granulocytes, and monocytes) found in the general circulation are attracted to the site of infection; and third, there is the release and/or activation of a variety of mediators designed to neutralize the invading pathogenic microbes. Where infections persist after the innate immune response, pathogens are engulfed and digested by macrophages, dendritic cells, and/or professional antigen-presenting cells (APC). These present fragments of microbial antigens to naïve T-cells as a key part of the adaptive immune response. Components of innate immunity are localized on mucosal surfaces and epithelium where the body comes into contact with infectious agents including, skin, eyes, mouth, GI tract, urinary tract, endometrium, and lungs (Chapter 6).

1.2.4 Neuroendocrine Responses to Illness and Stress

The response to injury, stress, and illness includes changes in the secretion of a variety of hormones that affect weight balance. During illness, there is an increase in the concentrations of catabolic hormones, such as adrenocorticotropic hormone (ACTH) and cortisol, which increase the loss of body mass. The concentrations of anabolic hormones (growth hormone, thyroid hormone, insulin, and insulin-like growth factor-1 [IGF-1]) that help to maintain muscle and body mass decline. Chronic illness leads to marked drops in the plasma levels of growth hormone. The topic is discussed by Van den Berghe and others in relation to new therapies for treating unwanted muscle loss and wasting in critically ill patients.[23–27]

According to the classic fight-or-flight response, stress leads to increased secretion of adrenaline, which has diverse physiological effects, including increased blood pressure, increased heart rate, increased heart stroke volume, and increased respiratory rate. ACTH also stimulates the adrenal gland to produce a less well-known family of stress-related hormones, the glucocorticoids (GC), exemplified by cortisol. The GCs are anti-inflammatory agents and immunosuppressants. The GCs also stimulate gluconeogenesis and fat metabolism, and increase muscle protein

breakdown to form amino acids. Past studies indicate that GCs are also linked with decreasing responsiveness to insulin (insulin resistance), decreased production of IGF-1, increased protein breakdown, and decreased protein synthesis (Chapter 6).

ACTH is released from the anterior pituitary gland under the influence of corticotrophin-releasing hormone (CRH), which is controlled in turn by signals arriving within the hypothalamus. As described above, ACTH is noteworthy because of its effect on the adrenal gland. Within the central nervous system (CNS), the ACTH peptide is converted to melanocyte-stimulating hormone (α-MSH), which controls skin pigmentation (melanization) as well as food intake.

1.3 UNINTENDED WEIGHT LOSS

1.3.1 Sickness-Related Weight Loss

Body protein and fat loss are common responses to illness. The former is due to an increase in the rate of muscle protein breakdown and/or decreased rate of protein synthesis. As noted above, insulin resistance is another common feature of illness-related weight loss. There is reduced glucose uptake into cells despite a high plasma insulin concentration. Tissue resistance to other anabolic hormones and amino acids also occur leading to anabolic dysfunction (Chapter 9). There is accelerated breakdown of body proteins to provide free essential amino acids for the construction of new defense proteins. Some major changes associated with illness wasting are listed in Table 1.2.

TABLE 1.2
Possible Causes for Illness-Related Weight Loss

Examples	Variables
Anorexia	Physical obstruction of GI tract
	Loss of sensory acuity
	Decreased appetite
	Impaired absorption
	Increased nutrient losses from urine and skin
Deficiency in key nutrients	Essential fatty acids
	Essential amino acids
	Antioxidant nutrients
	Vitamins
	Toxic nutrients
Cachexia	
Decreased anabolic agents	Insulin and IGF-1
	Insulin resistance
	Growth hormone resistance
Increased catabolic agents	Gonadal steroidal hormones
	Pro-inflammatory cytokines
	PIF
	LMF
Disease, injury, and illness	

1.3.2 Illness Anorexia

Anorexia or reduced food intake is an integral part of the acute phase response to injury (Figure 1.3). According to evolutionary medicine, low food intake improves host survival during illness by decreasing the availability of essential nutrients for invading pathogens; presumably, host cells are more able to withstand the low nutrient environment compared to the invading bacteria. Loss of appetite and lethargy reduces food-seeking behavior, which lowers the host's voluntary energy expenditure. Paradoxically, injury or infection is often followed by increases in the REE sometimes accompanied by fever. Though the entire acute phase response may have survival value, certain elements (e.g., fever) require moderation. Prolonged anorexia could also be counterproductive as this leads to nutrient deprivation not only for the invading pathogen but also for the host.[28–31,*]

Two forms of pathological anorexia can be differentiated. Anorexia nervosa arises from psychiatric causes whereas illness anorexia refers to the disturbances of food intake associated with physical illness or injury. Illness anorexia produces a deficit of essential nutrients and macronutrients, accounting for the poor nutritional status (undernutrition and decreased body mass index [BMI]) associated with acute and

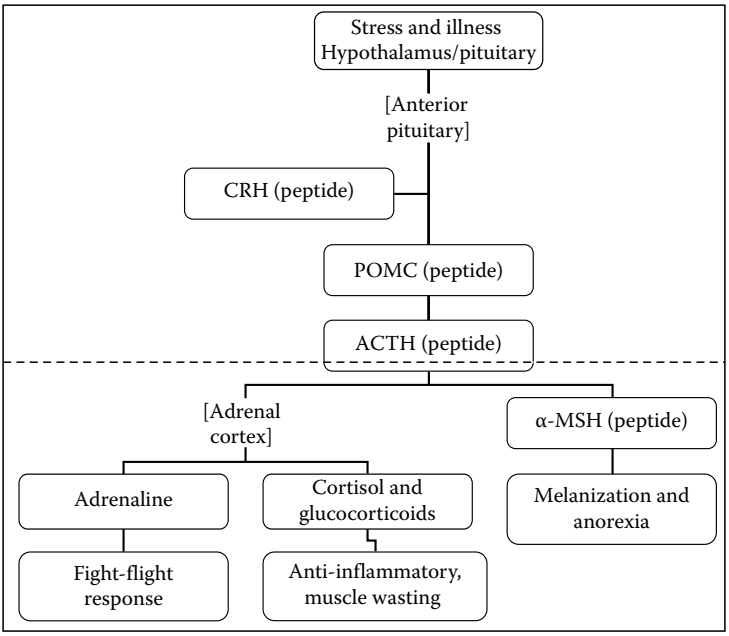


FIGURE 1.3 The hypothalamic/pituitary/adrenal cortical response to stress and illness. Dotted line shows approximate boundary between central and peripheral responses; POMC is pro-opiomelanocortin (Chapter 9).

* The survival value of starvation as a response to acute/chronic illness has been questioned by some experts. Van den Berghe et al. suggest that the chances of surviving extreme injury and acute insults were very low prior to the development of intensive care medicine and that the acute phase response was "discovered" in the modern era partly as a consequence of developments in intensive care medicine.[19–24]

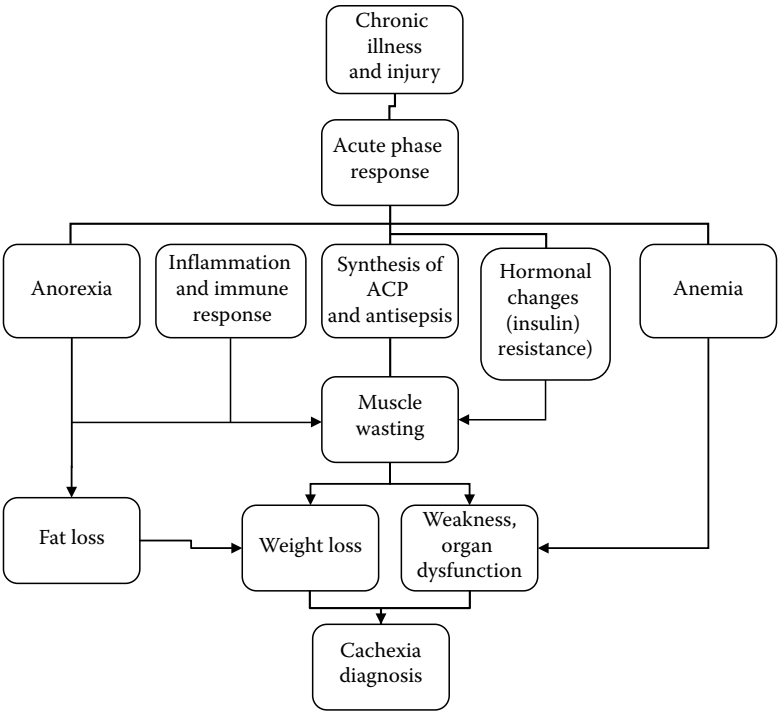


FIGURE 1.4 The acute phase response to chronic illness in relation to anorexia and cachexia. (Adapted from Stephens, N.A. and Fearon, K.C.H., *Medicine*, 36, 78, 2007.)

chronic ill-health (Figure 1.4). In addition to the direct effects related to nutrient intake, anorexia produces psychological changes in the patient's relation to food. Low food intake may also exacerbate the symptoms of fatigue, apathy, and depression.[32–34]

Body weight is thought to be controlled within narrow limits over time.[35,*] Dietary energy intake can be matched with prevailing levels of physical activity and energy utilization. Experiments with animals show that "enforced" weight gain or weight loss can be readily reversed when the animals are given free access to food. It has been suggested that each person has an idealized body weight, and physiological apparatus for ensuring close adherence to this weight. During illness, the ability to maintain body weight is impaired.[36] The results of initial clinical trials suggest that bioactive peptides may be developed for addressing some of the symptoms of illness anorexia (Chapter 9).

1.3.3 Cachexia

Cachexia refers to the extreme loss of muscle protein and fat stores arising from an accelerated rate of catabolism compared to anabolism. Cachexia is

* According to a popular urban legend described by Harris, the average women will gain 11 kg (~22 lbs) between the ages of 25–65 years. The weight increase corresponds to a daily overconsumption of approximately 0.3 g of food over 40 years. Apparently, body weight is normally controlled within narrow limits by matching of caloric intake and expenditure over extended periods of time.

often concurrent with anorexia (Section 1.3.2). Michael Tisdale suggests that weight loss arising from cancer cachexia is not due to decreasing food intake alone, based on several sources of evidence[37–39]: (1) body composition changes in anorexia and cachexia tend to be different, (2) the losses of body fat and muscle observed in cachexia can precede a decline in food intake, (3) anorexia and cachexia may be caused by different conditions, and (4) the percentage of anorexic and cachexic subjects differ within different patient groups. Cancer patients that develop anorexia also exhibit cachexia; the proportion of patients with chronic heart failure (CHF) who show cachexia and those that develop anorexia may differ substantially.[40]

Cancer cachexia features anorexia arising from the disruption of the neuroendocrine system, which controls food intake (Figure 1.4). Anorexic peptides including leptin and melanocortin hormones increase, possibly in response to pro-inflammatory factors produced by tumors cells (Section 9.6.1). There are also changes in the carbohydrate, lipid, and protein metabolism as well as increases in resting metabolic rate that all contribute to a state of overall weight loss. There is a generalized increase in REE during cancer cachexia thought to be linked with increased appearance of brown adipose tissue in sufferers. Lipid mobilization factors (LMF) and proteolysis-inducing factors (PIF) produced by tumor cells stimulate the breakdown of fats and proteins in the host.[41,42] Cachexia observed in sufferers of rheumatoid arthritis shows interesting similarities to cancer cachexia. Rheumatoid arthritis is principally an inflammatory disease leading to the erosion of joint cartilage, joint stiffness, and pain. The increased production of pro-inflammatory cytokine by macrophages stimulates matrix metallo-protease expression from bone cells, which leads to joint damage (Figure 1.5). Interestingly, rheumatoid arthritis is associated with cachexia, increased REE, increased loss of muscle mass, and a state of insulin resistance.[43] Key aspects of cancer cachexia have been reviewed.[37–39,42–44]

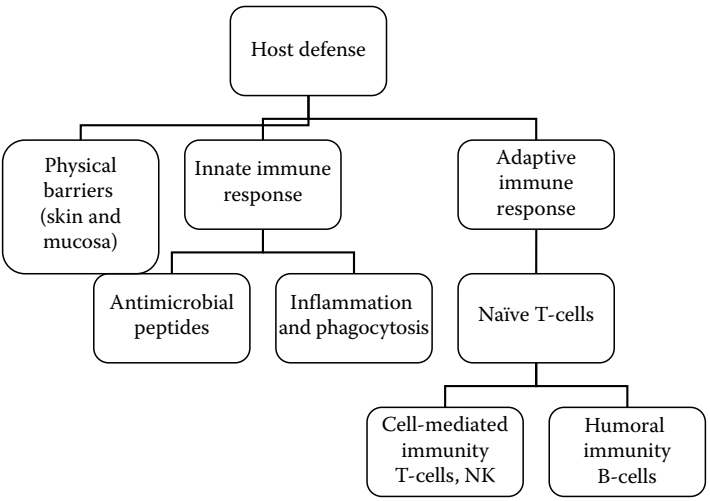


FIGURE 1.5 Summary of host defense systems.

The differences between cachexia and weight loss arising from caloric restriction are discussed in Section 1.3.4

1.3.4 Starvation Weight Loss versus Cachexia

There are differences between weight loss arising from starvation (calorie restriction) and illness-related weight loss from (cancer) cachexia.[45,46] Weight loss from starvation begins 12–18 h after caloric restriction. First, the levels of blood glucose and glycogen from the liver and muscles become depleted. Fat is then utilized as the major fuel for metabolism. Fatty acids are degraded to 2C and 3C compounds and fed through the Krebs cycle, which generates adenosine triphosphate (ATP), the main energy storage compound within the body. There is a loss of adipose tissue while muscle tissue is preserved during mild starvation. During severe starvation, the reserves of body protein are broken down and utilized for gluconeogenesis. It is ironic that starvation (with decreases food protein intake) enhances protein-nitrogen loss from the body (Chapter 4). It is thought that the average 70 kg person may survive for about 2 months without food. The overall response to starvation seems designed to maintain brain function at the expense of less vital organs. The REE is decreased compared to the non-starving state. Table 1.3 lists some of the key differences between starvation weight loss and wasting due to illness-induced anorexia–cachexia. Though starvation and cachexia represent distinct forms of weight loss, there are many practical situations where undernutrition arises due to the combination of decreased food intake and metabolic derangements affecting protein metabolism (Chapter 3). Interestingly also, the different forms of cachexia are preceded by a rise in inflammatory cytokine levels that tend to reduce food intake (Appendix 1.A.1).

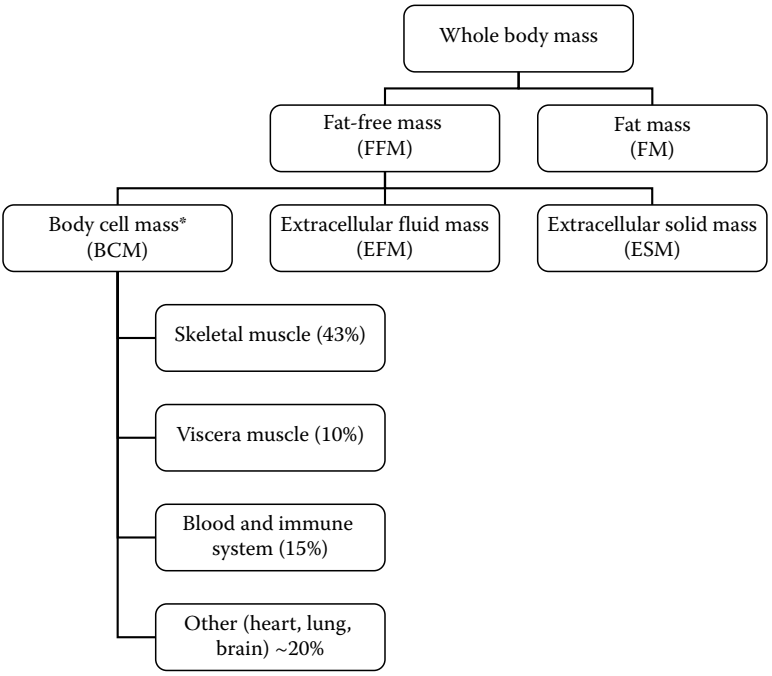
TABLE 1.3
Differences in the Metabolic Responses to Starvation and Cachexia

	Starvation Weight Loss	Illness Wasting
Appetite	Increased	Decreased
REE	Decreased	Increased
Total energy expenditure	*Decreased*	*Decreased*
Acute phase response	No	Yes
Skeletal muscle	Maintained	Decreased
Protein synthesis	Decreased	Increased/decreased
Protein degradation	Decreased	Increased
Adipose tissue	*Decreased*	*Decreased*
Liver size	Decreased, atrophied	Enlarged
Insulin	Decreased	Increased
Serum cortisol levels	Normal	Increased
Responds to simple feeding	Yes	No change

Source: Adapted from Gordon, J.N. et al., *QJM-Int. J. Med.*, 98, 779, 2005.
Note: Compiled from diverse sources, for example, Ref. [42].

1.3.5 Fat-Free Mass and Body Composition during Illness

Weight loss during illness affects body composition, which is the proportion of protein, fat, and water contained in the average body.[47] Body mass is generally divided into fat-free mass (FFM) and fat mass (FM) (Figure 1.6). The FFM is further subdivided into body cell mass (BCM), extracellular fluid mass (EFM), and extracellular solids mass (ESM), which consists of collagen and the inorganic components of bone. The following relation applies: FFM = BCM + ESM + EFM. The BCM corresponds to *metabolically active tissue* and consequently REE is related to the quantity of FFM.[47] Pi-Sunyer noted that plotting a graph of the REE



	Normal Weight		Obese	
Index	kg	%	kg	%
Fat	14.0	20.0	40.3	38.5
Water	41.0	58.6	47.4	45.2
Protein	11.5	16.4	13.2	12.6
Glycogen	0.5	0.7	0.5	0.5
Mineral	3.0		3.4	3.2
Total W	70.0	100.0	105.0	100.0
FFM	56.0	80.0	64.5	61.5

* % Body composition data from Ref. [48].

FIGURE 1.6 Division of body mass into FFM and FM. Body composition for a normal person and for an obese person. (Adapted from Pi-Sunyer, F.X., *Am. J. Clin. Nutr.*, 72, 533S, 2000.)

versus FFM should give a straight-line relation. Therefore, a drop of FFM during aging would produce a decrease in REE. On the contrary, more complex interactions can be envisaged if the slope or intercept of the FFM–REE graph were reset by the onset of disease.[49] Many infectious diseases are characterized by a hypermetabolic response and an increase in the REE.

Losses of FFM are commonly observed due to the three causes[47,49]: (1) dehydration affecting EFM; (2) loss of ESM affecting bone, cartilage, and other extracellular solids matrix; and (3) loss of BCM affecting skeletal muscle proteins, visceral proteins, or immune proteins. The loss of >40% of basal BCM is expected to be fatal whereas more moderate declines are associated with functional impairment that reduce the quality of life. Changes in BCM tend to correlate with the chances of surviving serious illnesses such as cancer and AIDS. The components of BCM, for example, skeletal cell mass, determine muscle strength, balance, mobility, and related physical characteristics. The immune cell mass affects immune response and, finally, the combination of visceral and skeletal cell masses affect energy metabolism.

Modern techniques for assessing body composition include the use of bioelectric impedance[50] or total body potassium[51,52] measurements. Such measurements are considered more accurate compared to total body weight determinations, which do not differentiate between losses of FM and FFM. BCM declines during muscle wasting associated with aging and rheumatoid arthritis. The major amounts of proteins occur as skeletal muscle with the rest evenly distributed between the skin, blood, visceral tissue, etc. Wasting has a disproportionate effect on muscle and visceral protein reserves with the lungs, brain, etc., being least affected.

1.3.6 Weight Loss and Mortality Risk

Unwanted weight loss affecting the BCM is linked with increasing risk of mortality; this relationship first noted for HIV/AIDS patients[53] has been observed in a diverse number of wasting diseases, for example, cancer,[54] CHF,[55] cystic fibrosis,[56] and end state renal disease (kidney failure),[57,58] and in hospitalized elderly patients.[59,60] Declines in FFM appear to compromise essential organs leading to an increased likelihood of death. Not surprisingly, the prevention of weight loss is a fundamental goal during nutritional support and diet therapy.[61,62] As might be expected, the link between high BMI and improved survival might not hold true at extremely high values for BMI, which present other dangers to health. There are reports that a moderate BMI may be beneficial for survival against heart failure with very low- or high-BMI values leading to increased risk of mortality.[63,64]

1.3.7 Premature or Preterm Infants

Preterm infants born at <37 weeks of gestation are usually categorized as low body weight (<2500 g), very low body weight (<1500 g), or extremely low body weight (<1000 g).* Premature babies tend to be small for their gestational age (SGA) and tend

* The MeSH term premature infant is used to describe children <37 weeks gestation. Premature infants are also referred to as underweight infants. Some of the discussion for premature infants may be relevant to normal term, newly born babies (neonates).

to loose weight after birth during a period needed to adapt to the extrauterine nutrition. Preterm babies have limited nutritional (protein and lipid) stores and decreased nutritional status. Such children may be ill and/or otherwise subject to stress. Though the exact causes appear to be uncertain, premature babies show extraordinarily high rates of body protein loss. Current expert advice suggests that preterm children should be given nutritional support containing protein or amino acids using fortified human milk, specialized infant formulas, or transition formulas (Chapter 3). Feeding has to be carefully planned to provide adequate energy intake as well as protein intake.[65–68] The amino acid requirements for preterm babies are further discussed in Chapter 4.

1.4 MULTIMODAL NUTRITIONAL SUPPORT USING BIOACTIVE PEPTIDES

1.4.1 Nutritional Support

Unwanted weight loss is a feature of the host response to illness. The resultant loss of muscle mass can lead to poor treatment outcome, prolonged hospitalization, and increased risk of mortality. Maintaining lean body weight is linked with reduced length of hospital stay, lower incidence of posttreatment complications, and lower overall treatment costs. Oral nutritional supplements and tube feeds are now used as adjuvants for conventional therapy.[69–71] The normalization of body weight is a major goal of nutritional support. An adequate provision of dietary protein (as well as micronutrients) is a major element for encouraging weight gain. The classification of bioactive proteins and protein supplements, and current legislation surrounding medical foods are discussed in Chapter 2. Human dietary protein needs during illness are reviewed in Chapter 3. The aspects of protein synthesis and breakdown, that is, protein turnover within the human body, are discussed in Chapter 4. The major causes of muscle loss and accretion are addressed in Chapter 5.

1.4.2 Anti-Inflammatory Therapy and Wasting

The extreme ends of the BMI scale appear to be linked with increasing levels of inflammation[72] (Figure 1.7), probably caused by different mechanisms.[64,73] Recent research suggests that chronic inflammation contributes to unwanted weight loss associated with chronic renal failure (CRF),[74] HIV/AIDS, and cancer.[75–77] Nonsteroidal anti-inflammatory drugs have been proposed for the treatment of cachexia[78] (and see reviews cited in Section 1.3). It may be proposed that anti-inflammatory peptides and protein supplements may have a role in the development of nutritional support for promoting weight gain.[79] Anti-inflammatory bioactive peptides and protein supplements to address muscle wasting are discussed in Chapter 6.

1.4.3 Infection and Antisepsis

Infection by microbial pathogens leads to host responses that contribute to wasting. Animal scientists have shown that dietary antibiotics promote the growth of livestock.[80,81] Chapter 7 considers the relation between infections, sepsis, and muscle

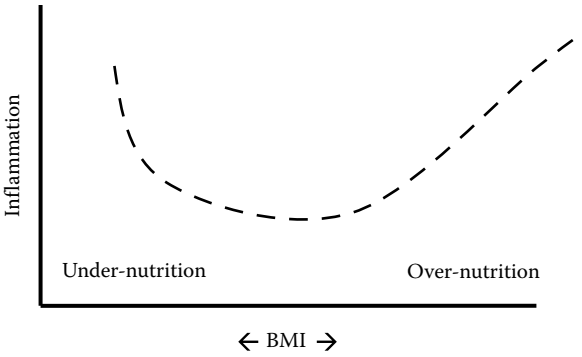


FIGURE 1.7 Schematic diagram showing the possible biphasic relations between body mass index and inflammation.

wasting. We also consider the current applications of antimicrobial bioactive peptides to improve health and nutrition.

1.4.4 Anabolic Dysfunction

A blunted response to anabolic hormones and nutrients (branched chain amino acids) is a feature of many wasting conditions.[82–84] Chapter 8 considers the role of anabolic dysfunction in weight loss and considers the potential for using bioactive peptides and proteins as growth promoters to facilitate weight gain.

1.4.5 Anorexia and Food Intake

Low food intake is a contributory factor for muscle loss during anorexia–cachexia syndrome.[85–88] Chapter 9 provides an introduction to illness anorexia and the potential for applying bioactive peptides and proteins to promote appetite and food intake.

1.4.6 Antioxidant Capacity

Oxidative stress is thought to be a feature of chronic illness, some of which are linked with muscle wasting. The possibility of moderating cell redox status using antioxidant peptides will be considered in various parts of the book in relation to anti-inflammatory action, immune modulation, etc.

1.5 SUMMARY AND CONCLUSIONS

Most organisms demonstrate a programmed response to illness, infection, and injury termed the acute phase response. The host response is responsible for derangements in metabolism and the unwanted loss of lean body mass frequently observed in sick people. The undernutrition-infection paradigm provides a justification for

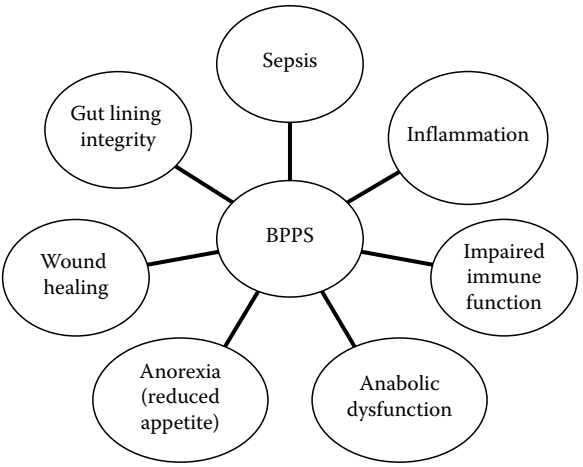


FIGURE 1.8 Multimodal nutritional support using bioactive peptides and protein supplements (BPPS).

the nutritional support of hospitalized patients and other vulnerable groups. In particular, weight gain is considered a surrogate measure for improving well-being (Figure 1.8).

Conventional therapies for unwanted weight loss help to maintain muscle mass, using anabolic steroids,[89,90] recombinant growth hormone,[91] cytokines antibodies,[92,93] and appetite promoters, for example, MEGACE® (megestrol acetate). Advocates for multimodal support suggest strategies should be developed that attempt to correct multiple metabolic derangements arising from illness at the same time. In principle, nutritional strategies could be developed, which facilitate growth, improve inflammatory status, increase appetite, and improve immune response simultaneously.[94–96] Medical foods (Chapter 2) can be envisaged, which address nutritional deficits as well as metabolic derangements associated with illness. There is already some evidence that pharmafoods (formulas containing arginine, omega-3 fatty acids, dietary mRNA, and sulfhydryl amino acids), may be useful for enhancing the immune function of intensive care patients.[97–99]

Bioactive amino acids, peptides, and protein (BPP) supplements offer enormous opportunities for developing novel medical foods for nutritional support[90,100,101] (Figure 1.6). For instance, bioactive peptides might be deployed for nutritional support based on their anti-inflammatory action, immune moderation, antisepsis and antibiotic activity, anabolic action, and for their ability to increase appetite. The range of common BPP currently include protein hydrolysates, amino acids, peptides, protein isolates, as well as composite high-protein foods such as milk, eggs, fish, and meat. Another generation of highly potent BPP especially suited for clinical nutrition includes hormones such as insulin, IGF-1, and growth hormone, which are established biopharmaceutical agents. Other emerging BPP include the newly discovered ghrelin, the growth hormone release peptides, and related secretagogues.

APPENDIX 1.A.1
Wasting Diseases

Cytokine-mediated conditions
Aging
Burns injury
Cancer
Chronic renal failure (CRF)
Chronic pulmonary obstructive disease (COPD)
Heart failure
HIV/AIDS
Inflammatory bowel disease (IBD)
Liver cirrhosis
Obesity
Rheumatoid arthritis
Sepsis
Surgery
Tuberculosis

Other
Denervation
Diabetes
Disuse
Inactivity
Muscular dystrophies
Unloading/microgravity

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2 Bioactive Peptides for Nutrition and Health

2.1 LEGISLATION

2.1.1 Introduction

Bioactive peptides are currently available as foods or medicines depending on their source, method of preparation, as well as presentation. A wide range of intermediate classifications may also be relevant, including terms such as functional food (FF), nutraceutical food, or medical food. The diversity of bioactive peptides potentially available for nutrition and health applications is reviewed in this chapter. International legislation and its applicability on the different categories of medical foods and bioactive peptides are explored in the rest of Section 2.1. Major classes of bioactive peptides occurring within the body or produced for industrial applications are described in Section 2.2. Some current applications supported by clinical trial evidence are reviewed in Section 2.3. In Section 2.4, we consider current limitations of clinical data for bioactive peptides and recommendations for future studies. On the basis of current evidence, legislation is actively being developed to guide future developments of bioactive peptides.

What is the distinction between a food and drug? According to the relevant laws, a food is defined as any "article or drink or components of any such article" which "provide taste, aroma, or nutritive value." By comparison, a drug is "…an article intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease…."* Any agent (*other than* foods or dietary supplements for which alternative legislation exists—see below) that claims to affect the structure and function of the human body is treated as a drug. The so-called structure–function claims can be made for food agents if such claims are related to the nutritional properties of the food agent. In the event that structure–function effects arise due to *nonnutritive* characteristics, the food concerned must be reclassified as a dietary supplement or drug; this rather key distinction between foods and supplements is noteworthy. As will be seen throughout this book, bioactive peptides can produce nutritional as well as nonnutritive health responses, though differentiating between these is not always easy.

* In the United States, legislation surrounding food or drugs is overseen by Food and Drugs Administration (FDA), with the Centre of Food Safety and Applied Nutrition (CFAN) being specifically concerned with foods and dietary supplements. The sections quoted here are based on literature available from these organizations' Web sites. A list of major food and drugs legislation between 1905 and 2005 is available from http://www.fda.gov/opacom/backgrounders/miles.html (accessed July 2008).

2.1.2 Dietary Supplements

Dietary supplements are defined by the United States Dietary Supplement Health and Education Act (DSHEA) of 1994 (Tables 2.1 and 2.2). This legislation amended the existing Federal Food Drugs and Cosmetics Act of 1938 and established standards for dietary supplements. DSHEA also offered industry guidelines for the supplement market sector with the aim of increasing consumer protection.[1–3] The DSHEA legislation of 1994 differentiates dietary supplements from drugs and conventional foods. Accordingly, manufacturers and producers of *dietary supplements* must refrain from claims that their product can be used for the diagnosis, cure, mitigation, treatment, or prevention of disease. Such claims are normally reserved for drugs. On the other hand, new dietary supplements (in contrast to drugs) can be sold without pre-market

TABLE 2.1
Chronology of Terms Applicable to Different Categories of Bioactive Protein Foods

Category	Date Defined
Food for special medical uses	1972 (United States)
Dietary supplements	1984 (United States)
Foods for special dietary uses	1985 (CODEX)
Medical food	1988 (United States)
Nutraceutical[a]	1988
Foods for special medical purposes (FSMP)	1989 (EU)
PARNUT	1989 (EU)
Dietetic foods, dietary foods	1989 (EU)
FPNU	1989 (EU)
FSMP	1991 (CODEX)
FOSHU	1991 (Japan)
FF[a]	
Food supplements	2002 EU

[a] Literature term with no current statutory definition.

TABLE 2.2
A Definition for Dietary Supplement

Dietary supplement: *A product that is intended to supplement the diet; contains one or more dietary ingredients* (*including vitamins; minerals; herbs or other botanicals; amino acids; and other substances*) *or their constituents; is intended to be taken by mouth as a pill, capsule, tablet, or liquid; and is labeled on the front panel as being a dietary supplement*.

Source: Dietary Supplement Health and Education Act of 1994. Public Law 103-417 103rd Congress, available from http://www.fda.gov/opacom/laws/dshea.html#sec3 (accessed July 2008).

TABLE 2.3
The EU Definition for Food Supplement

Food supplement: *Foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities.*

Source: Directive 2002/46/EC of the European parliament and of the Council of June 10, 2002 on the approximation of the laws of the Member States relating to food supplements, available from http://www.fsai.ie/uploadedFiles/Science_and_Health/Directive-2002-46-EC-on-food-supplements.pdf (accessed July 2008).

safety testing, evaluations, tests for efficacy, or prior approval by the FDA. Instead, current U.S. legislation holds manufacturers liable if adverse effects result from the use of their supplements.

Food supplements are defined in the EU by directive 2002/46/EC, enacted June 10, 2002 (Table 2.3). Initially, the EU legislation indicated that food supplements should contain only specific vitamins and minerals listed in Annex I and II of the directive. However, there was recognition also that food supplements could contain other agents (e.g., amino acids, essential fatty acids, fiber, and various plants and herbal extracts). Within the EU, the term "dietary" has a defined legal meaning being reserved only for a special category of dietetic foods (see below).

2.1.3 Foods for Special Medical Purposes

Codex Alimentarius describes foods for special medical purposes (Table 2.4). This product subgrouping is normally classed under the heading of foods for special dietary uses (FSDU) defined in Table 2.5. Food labeling rules require that foods for special medical purposes carry the warning "use under medical supervision" in bold

TABLE 2.4
A Statutory Definition for Foods for Special Medical Purposes

Foods for special medical purposes: *Foods which are specially processed or formulated for the dietary management of patients, to use only under medical supervision… for exclusive or partial feeding of patients with limited or impaired capacity to take, digest, absorb, or metabolize ordinary foodstuffs or nutrients contained therein, or who have other medically determined nutrient requirements, whose dietary management cannot be achieved only by modification of the normal diet, by other foods for special dietary uses, or by a combination of the two.*

Source: CODEX STAN 180-1991, Codex standard for the labelling of and claims for foods for special medical purposes, available from http://std.gdciq.gov.cn/gssw/JiShuFaGui/CAC/CXS_180e.pdf (accessed July 2008).

TABLE 2.5
A Statutory Definition for Foods for Special Dietary Uses

Foods for special dietary uses: *Foods which are specially processed/formulated to satisfy particular dietary requirements which exist because of a physical or physiological condition and/or specific diseases and disorders and which are presented as such. The composition of these foodstuffs must differ significantly from the composition of ordinary foods of comparable nature, if such ordinary foods exist.*

Source: CODEX STAN 146-1985. General standard for labelling of and claims for prepackaged foods for special dietary uses, available from http://www.codexalimentarius.net/download/standards/292/CXS_146e.pdf (accessed July 2008).

letters, in an area separated from other written, printed, or graphic information. FSDU are intended for "special" use, which is to address dietary needs that exist *by reason of a physical*, *physiologic*, *pathologic*, *or other condition*, including, but not limited to, diseases, convalescence, pregnancy, lactation, allergen hypersensitivity, and being underweight or overweight. Clearly, FSDU covers a broad range of applications. The equivalent classification within the EU is defined by directive 1999/21/EC referring to foods for particular nutritional uses (FPNU), which includes six different subcategories (see Section 2.1.6, Table 2.13).

2.1.4 Medical Foods

A medical food is defined in the U.S. Orphan Drug Act Amendments of 1983, the Nutrition Labeling and Education Act (1990), and within the FDA's rule on mandatory nutrition labeling in 1993 (Table 2.6).[4–6,*] Medical foods can be administered by oral or tube feeding. Labeling requirements dictate that packaging for these products contain the following phrases: (a) for the dietary management of a medical disorder (disease, or condition) and (b) to be used under medical supervision. Within the United States, medical foods are obtained through hospitals, clinics, or long-term

TABLE 2.6
A Statutory Definition for Medical Food

Medical food: *Food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation.*

Source: Orphan Drug Act, http://www.fda.gov/forindustry/developingproductsforrarediseasesconditions/overview/ucm119477.htm (accessed July 2008).

* An overview of medical foods legislation and guidelines is available from http://www.cfsan.fda.gov/~dms/medfood.html (accessed July 2008).

TABLE 2.7
Elements for the Statutory Definition of a Medical Food

1. Specially formulated and processed, not naturally occurring foodstuff
2. Feeding by means of oral intake, or enteral feeding by tube
3. Nutritional support intended to meet specific requirements of a disease or condition determined by medical evaluation
4. For patient showing subnormal capacity to ingest, digest, absorb, or metabolize ordinary nutrients, or who have medically determined nutrient requirements, which cannot be achieved by the normal diet alone
5. Used under medical supervision

care facilities supervised by a qualified medical practitioner or dietician. In the United Kingdom, medical foods may be obtained by "prescription" and can then be consumed at home. Some attributes of medical foods overlap with foods for special medical purposes (Table 2.4). However, both categories can be differentiated from other FSDU that may make health claims. Provided that medical foods meet some key elements of the statutory definition (summarized in Table 2.7), these products are exempt from regulations for nutrition labeling, nutrient content claims, as well as health claims. The most common types of protein medical foods are enteral nutrition (EN) products, i.e., products provided through the gastrointestinal (GI) tract, taken by mouth, or provided through a tube or catheter that delivers nutrients beyond the oral cavity or directly to the stomach. In general, medical foods are synonymous with tube feeds. The first medical foods can be traced back to the 1950s and were designed to deal with inborn errors of metabolism, e.g., phenylketonuria (PKU). Children with PKU have a tendency to develop neurological symptoms linked with phenylalanine in the diet. Medicinal foods for PKU sufferers therefore have low phenylalanine content. Other medical foods have been developed for addressing conditions including pancreatitis, osteoarthritis, end-stage renal disease, cachexia, and age-related weight loss.[7–11]

Features of medical foods that distinguish them from health foods, dietary supplements, and drugs are highlighted by Morgan and Baggott[11] and summarized in Table 2.8. First, dietary supplements are legislated under the DSHEA (1994) whereas the medical food category is defined by the earlier legislation (Organ Drug Act, 1988). Second, dietary supplements may be taken by all consumers including those not suffering from overt illness. Medical foods are more drug-like and their use is confined to patients diagnosed as suffering from a certified medical condition. Third, medical foods have to be administered under the supervision of a qualified medical practitioner, which is contrary to the way drugs are managed. Very few drugs, whether prescribed or not, cannot be administered by the consumer. Finally, medical foods, unlike dietary supplements, need to be evidence based and supported by randomized clinical trials in humans. There are an estimated 200 or so medical food products manufactured by 20 main manufacturers.[12] A representative sample of medical foods is listed in the appendices to this chapter.

TABLE 2.8
Differences between Medical Foods, Dietary Supplements, and Drugs

Product/ Attribute	Health Food	Dietary Supplement	Medical Food	Drug
Legislation	HACCP, GMP	DSHEA, 1994	Orphan Drug Act, 1988	Federal Food, Drug and Cosmetics Act, 1934
Consumers	All	Healthy	Ill consumers	Ill consumers
Ingredients	Nutritional	Nutritional	Nutritional, not in ordinary diet	Synthetic or nutritional
Product rationale	Expectation of desired effect	Expectation of desired effect	Corrects metabolic imbalance	Safe, effective for defined patients
Safety requirements	HACCP, GMP	Safe, marketed before 1994	GRAS as food ingredient	Approved daily allowances, toxicity, etc.
Evidence base	None	None	Recognized science, human trials	Preclinical, Phase I, II, and III trials
Physician required	No	No	Yes, or by prescription	Yes, for prescription drugs
Route of administration	Oral	Oral	Oral/enteral IV, parenteral	All routes including topical, oral, IV, etc.
Labeling requirements	Yes	Yes	No	No

Source: Adapted from Morgan, S.L. and Baggott, J.E., *Nutr. Rev.*, 64, 495, 2006.

2.1.5 EU Legislation for Dietetic Foods

EU directive 89/398/EEC relates to FPNU, also called PARNUTS, dietary foods, or dietetic foods (Table 2.9).* PARNUTS are designed to fulfill particular nutritional requirements for three categories of people: (a) those whose digestive processes or metabolism are disturbed, (b) those who are in a special physiological condition and who are therefore able to obtain special benefit from controlled consumption of certain substances in foodstuffs, and (c) infants or young children in good health. PARNUTS correspond to the foods for special dietary purposes defined by CODEX STAN 146-1985 (Table 2.10).

EU legislation is somewhat prescriptive in terms of allowed ingredients for PARNUTS. Directive 2001/15/EC filed as amendment to 89/398/EEC broadened the list of allowed ingredients for PARNUTS to include six categories of ingredients: (a) vitamins, (b) minerals, (c) amino acids (as Na-, K-, Ca-, Mg-salts, or HCl derivatives), (d) carnitine and taurine, (e) nucleotides, and (f) choline and inositol. Directive 2004/5/EC further expanded the list of components for PARNUTS to include the following dipeptides (L-arginine-L-aspartate, L-lysine-L-aspartate, and L-lysine-L-glutamate) and the natural glutathione precursors (*N*-acetyl-L-cysteine

* Material from this section is mainly derived from the EU Web site (Europa) for feed and food safety http://ec.europa.eu/food/food/index_en.htm

TABLE 2.9
EU Definition for Foods for Particular Nutritional Use (PARNUT)

Foods for particular nutritional use: *Foodstuffs which, owing to their special composition or manufacturing process, are clearly distinguishable from foodstuffs for normal consumption, which are suitable for their claimed nutritional purposes and which are marketed in such a way as to indicate such suitability*.

Annex I of directive 89/398/EEC identifies 6–8 subcategories of PARNUTS:

1. Foods for infants and young children
2. Infant formulae and follow-on formulae
3. Processed cereal-based foods and baby foods (weaning foods)
4. Foods intended for use in energy-restricted diets for weight reduction
5. Foods for special medical purposes
6. Foods for sports people

Source: 89/398/EEC, Council Directive of May 3, 1989 on the approximation of the laws of the Member States relating to foodstuffs intended for particular nutritional uses, available from, http://ec.europa.eu/food/food/labellingnutrition/nutritional/d89-398-ec.pdf; Commission Directive 2001/15/EC of February 15, 2001 on substances that may be added for specific nutritional purposes in foods for particular nutritional uses, available from http://www.legaltext.ee/text/en/U61159.htm (accessed July 2008).

TABLE 2.10
Summary of EU Directives Related to Special Foods

Directive or Legislation	Date for Adoption	Food Group
89/398/EEC, 96/84/EC, 1999/41/EC, 2001/15/EC, and CODEX STAN 146-1985	May 3, 1989	PARNUT, foods for particular nutritional uses (FPNU), dietary foods, or dietetic foods
1999/21/EC, CODEX STAN 180-1991	March 25, 1999	Foods for special medical purposes
2002/46/EC, 2006/37/EC	June 10, 2002	Food supplements
2001/15/EC, 2004/5/EC	February 15, 2001 January 20, 2004	Describing substance allowed to be added to FPNU

and *N*-acetyl-L-methionine) though these are only allowed as components of foods for special medical purposes. To facilitate competitiveness and innovation, directive 96/84/EC allowed FPNU, which do not conform to items in Annex 1 of directive 89/398/EEC, to be placed on the market for a trial period of 2 years.

2.1.6 Functional Foods

The FF program dating from 1984 (Japan) led to the creation of the "Food for Specified Health Uses" (FOSHU) category in 1991. The FOSHU initiative examined the health functions of diverse foods focusing on body regulating food factors, body defending food factors, anticancer factors, and so on. The first commercial food

TABLE 2.11
Accepted Definition of Functional Food

Functional foods: *Food that affects beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either improved stage of health and well-being and/or reduction of risk of disease. A functional food must remain food and it must demonstrate its effects in amounts that can normally be expected to be consumed in the diet: it is not a pill or a capsule, but part of the normal food pattern.*

Note: Literature definition with no statutory recognition.

products awarded FOSHU status appeared in 1993. An accepted definition for FF is listed in Table 2.11. Detailed aspects of Japanese legislation related to FOSHU are reviewed by Arai and others (see references in Table 2.12).

TABLE 2.12
International Legislation Related to Functional Foods

Austria[13]
China[14,15]
EU[16–20]
Global or international[21–25]
Japan[26–30]
Latin America[31]
Malaysia[32]
Nigeria[33]
Singapore[34]
Thailand[35]
United States[36–42]

The term "functional food" appeared in a 1993 issue of the journal *Nature* in an article describing the then recent developments in Japan.* A key requirement for FOSHU classification is that this category of product should be "real" foods (not capsules, pills, or powders) and based on naturally occurring compounds. It is further assumed that such foods are consumed as part of the normal diet and that they have a defined function in the human generally related to, improvement of the immune function, prevention of specific diseases, support for recovery, or control of physical and psychological complaints. In addition, it was recognized that some FFs could slow down processes of aging. Table 2.13 lists some general application areas for FFs.

Recent EU perspective on FF was reviewed by Roberfroid and others and summarized in the consensus documents describing among other things, possible definitions, strategy for scientific research and development of FF, and guidelines for FF claims and technological aspects. It is generally assumed that FF should be essentially food in nature (not pills or capsules as might be expected for dietary supplement). The FF should have effect substantiated by scientific community. The effects of FF on body functions should go beyond those expected from nutritional effects, related to improved state of health and well-being and/or reduction of risk (not prevention) of disease. Finally, the FF should be consumed as part of a normal food pattern. The EU consensus document on FF also suggests that these foods may be produced from natural food, to which a component has been added, from which a component has been removed, or where the nature of one or more components has been modified. In addition, an FF could be produced by a process via

* The PubMed database shows an early reference to "functional food" in the article Dymsza HA, 1975. Nutritional application and implications of 1,3 butanediol. *Federal Proceedings* 34:2167–2170.

TABLE 2.13
General Application Areas of Functional Foods

Specific Applications	General Areas
Aging[43]	
Appetite[44]	Gastrointestinal functions
Behavior, cognition, psychological function[45,46]	Redox and antioxidant systems
Cancer[47–49]	Metabolism of macronutrients
Coronary heart disease[50–53]	Development in fetal and early life
Diarrhea[54]	Xenobiotic metabolism
Exercise, and sport athletics[55,56]	Mood, behavior, or cognition
Gastrointestinal health[57–60]	Physical performance
Immune system[61,62]	
Clinical application[63–71]	
Obesity and weight control[72–76]	
Osteoarthritis[77]	
Osteoporosis[78]	
Pediatric medicine[79,80]	
Public health nutrition[81–84]	

which the bioavailability of one or more components has been modified. Clearly this legislation is much informed by the legislation from Japan. However, the EU viewpoint stopped short of prescribing that there should be defined function in relation to specific physiological functions (e.g., immune function). The United States has no legislation definition for "functional food."

2.1.7 Protein and Peptide Medical Foods

Protein medical foods are currently synonymous with enteral feeds produced by several commercial manufacturers (see appendices). Enteral feeding refers to the provision of specially formulated foods via the GI tract under the supervision of a health professional. Nutrients are given orally or via narrow-bore tubing where patients have difficulty swallowing.*,† Some major milestones in the development of tube feeding are reviewed.[85,86] The historical antecedents of tube feeding can be traced to enemas (containing wine, wheat, barley, milk, etc.) for rectal feeding in ancient Egypt to fairly recent developments in Europe during the sixteenth and seventeenth centuries. The first commercial enteral protein feeds appeared in the 1950s. Over 100 enteral feed products are currently available with the majority of products containing blended or homogenized whole foods, or chemically defined mixtures of intact or hydrolyzed food protein and other food constituents. A guide to modern EN

* Enteral nutrition (EN) is defined as nutritional support given via the alimentary canal or any route connected to the gastrointestinal system and includes oral feeding, sip feeding, and tube feeding using nasogastric, gastrostomy, and jejunostomy tubes—PubMed MeSH index.

† Parenteral nutrition (PN)—delivery of nutrients for assimilation and utilization by a patient whose sole source of nutrients is via solutions administered intravenously, subcutaneously, or by some other non-alimentary route—PubMed MeSH index.

products and a selection guide for applications are described by Malone[87] and also by Bolonge and Griffiths.[88] EN and parenteral nutrition (PN) are widely used as adjunct therapies for managing various types of ailments and also for post-surgery support. The possibility of developing EN formulas for moderating host response to injury and infection is discussed in later chapters.

Tube feeding is applied for nutritional support within intensive care units, for the care of preterm infants, for the elderly, and also for cases of oral, head and/or neck cancer, which affects the ability to swallow. EN may be used for partial or complete nutritional support where the patient has a functioning GI tract. For short-term EN feeding, tubes are placed within the stomach or jejunum via the nasal passage. EN may be employed in the home. For long-term feeding, gastrostomy tubing (G-tubes) can be passed directly though the abdominal wall and into the GI tract. A full discussion of EN and PN is beyond the scope of this book and the interested reader is directed to many excellent texts dealing with this topic,[89–91] and a large number of reviews dealing with cost–benefit analysis of tube feeding,[92,93] potential risks,[94] and evidence for EN based on randomized clinical trials.[95,96]

A comprehensive listing of enteral feeds available commercially is provided in Appendices 2.A.1 through 2.A.5. Currently, EN foods can be classified into the following categories: (a) nutritionally complete formulas, (b) nutritionally incomplete formulas, including individual "modular"-type products that may be mixed with other products before use (e.g., protein, carbohydrate, or fat), (c) formulas for metabolic (genetic) disorders in patients over 12 months of age, or (d) oral rehydration products. In parenteral nutrition, tube feeding occurs directly into the major vein. The basic components of total parenteral nutrition (TPN) solutions are protein hydrolysates or free amino acid mixtures, monosaccharides, and electrolytes. Components are selected for their ability to reverse catabolism, promote anabolism, and build structural proteins.

2.2 BIOACTIVE PEPTIDES AND PROTEINS

2.2.1 Bioactive Compounds

The current American Dietetic Association (ADA) definition for bioactive compounds (Table 2.14) is comprehensive, inclusive, and practical. The emphasis on food-derived agents is noteworthy; however, derivation may be by isolation, concentration, or synthesis of equivalent compounds possessing bioactivity similar to natural compounds. By agreement, harmful or toxic agents are excluded from the ADA description. The range of *food sources* encompassed by the ADA definition is also potentially very large, not restricted to plant and animal foods, but also microbial, fungal, amphibian, insect, etc. In principle, the biosphere contains an almost limitless variety of bioactive agents, with potential effects in nutrition and health. The rest of this chapter will focus on bioactive peptides and proteins.* Technological developments covering bioactive peptides are well documented.[97–102] By contrast, applications of bioactive peptides for nutrition and health have not been widely publicized.

* Bioactive compounds occurring in foods include macronutrients (lipids, carbohydrates, and proteins) and micronutrients (vitamins, minerals, and phytochemicals).

TABLE 2.14
Definition of Bioactive Compound

Bioactive compound: *Physiologically active constituents in foods or dietary supplements derived from both animal and plant sources, including those needed to meet basic human nutrition needs, that have been demonstrated to have a role in health and to be safe for human consumption in intended food and dietary supplement uses, to include: Synthetically derived compounds possessing physiochemical or biological equivalency of a naturally occurring bioactive food component, any processed naturally occurring bioactive food component, extracted, concentrated, or modified for the purposes of fortifying foods or other dietary supplements*.

Source: Adapted from American Dietetic Association, Chicago, IL, available from, http://dietary-supplements.info.nih.gov/pubs/bioactivefoodcomponents/American%20Dietetic%20Association%20-%20ADA%20-%20comments%20on%20defining%20bioactive%20food%20components.pdf.htm (accessed July 2008).

Bioactive peptides from food and other sources can produce physiological effects unrelated to their function as nutrients. Currently, the nonnutritive functions of bioactive peptides are not well understood (Section 3.5). From the statutory viewpoint, bioactive peptides for nutritional applications may be classified as dietary supplements or foods for special dietary uses. The nutrition literature refers to functional, biofunctional, nutraceutical, pharmaconutrient, or immunonutrient peptides though such terms lack strict legal meaning.[101] For convenience, the group of bioactive peptides may be extended to include physiologically active amino acids, peptides, and proteins (without regard to size) that produce effects beyond their role as substrates for oxidation and tissue building. Toxic bioactive peptides will be discussed in some instances (e.g., venom peptides) where these have potentially useful therapeutic applications.[103]

2.2.2 Bioactive Peptide–Related Nutritional Phenomena

A PubMed search using the terms peptides and nutritional phenomena produced 14,122 articles and 1633 reviews between the years 1953 and 2008.* The main topics of intersection between peptides and nutritional sciences covers the regulation of food intake, satiety, weight reduction, effects of exercise, cachexia, and control of muscle and fat mass during age-related wasting (sarcopenia). Another area of interest was therapeutic applications of peptides for chronic or lifestyle conditions, many of which are affected by diet. The main classes of peptides related to nutritional phenomenon are listed in Table 2.15 and Appendix 2.A.7.

TABLE 2.15
Major Classes of Peptides for Moderating Nutritional Phenomena

1. Antimicrobial cationic peptides
2. Intercellular signaling peptides and proteins
3. Intracellular signaling peptides and proteins
4. Neuropeptides
5. Oligopeptides
6. Opioid peptides
7. Peptide hormones
8. Cyclic peptides

* Search conducted September 2008, restricted to title and abstract terms, and English as text language.

Endogenous peptides perform a variety of functions within the body ranging from host defense, communication, to integration of metabolism and growth. Antimicrobial peptides are positively charged peptides produced by animals, plants, as well as microorganisms. The strong positive charge allows interactions with and disruption to bacterial membranes. A variety of intercellular signaling peptides are produced within various tissue compartments of the human body allowing localized (paracrine) communication between cells, e.g., adipokines (fat tissue), agouti-related protein (CNS), and cytokines (lymphocytes). Also of interest are the neuropeptides, a range of peptides produced by brain cells or neurons. Interestingly, some gut peptides (e.g., leptin, ghrelin, and CCK) also appear to function as neuropeptides. The oligopeptides are a miscellaneous group of peptides containing between 2 and 12 amino acids.

2.2.2.1 Bioactive Peptides in Body Compartments

Groups of peptides are confined or produced within particular compartments within the body including the gut and GI tract, fat depots, pancreas, and kidneys. The gut peptides are produced by the stomach and intestines where they coordinate physiological actions ranging from gut motility and acid secretion to food intake. Recent research has shown that these hormones may have far-reaching physiological effects, since they also affect the release of other hormones (e.g., insulin and growth hormone) and the general regulation of energy homeostasis. Many low molecular weight bioactive peptides occur in the brain and general CNS where they function as neurotransmitters. The application of gut hormones and CNS peptides for the treatment of conditions such as anorexia are discussed in Chapter 9. The fat depots around the body produce adipokines (adiponectin, leptin, and resistin), which have been identified to effect an important range of physiological phenomenon ranging from inflammation to cardiovascular health.

2.2.2.2 Exogenous Bioactive Peptides Associated with Foods

Some major classes of food bioactive peptides are listed in Table 2.16. Physiologically active peptides were first detected in food materials during the 1980s, when opiate-like exorphins, thyrotrophin-releasing hormone-like peptides and cyclic-hystidinyl-proline (cyclo-His-Pro) were found in samples of some commercial high-protein supplements and protein digestions. The study of bioactive peptides has grown enormously in the past 20–30 years.*

2.2.3 Gene-Encoded Bioactive Peptides

The gene-encoded bioactive proteins and peptides are discussed in this section. Natural selection and agricultural breeding may have favored "medicinal" foods that produce health benefits. Thus, milk contains a vast array of physiologically active dietary proteins (hormones, growth factors, antimicrobial peptides, etc.) that are believed to support the healthy development of the newly born.[104,105] Milk factors also stimulate the growth and proliferation of the developing mucosal cells within the infant. Maternal antibodies from milk provide passive immunity from the mother, against pathogens

* See Table 3.8 and associated text.

TABLE 2.16
Encrypted Bioactive Peptides from Milk Proteins and Other Sources

Bioactivity	Examples	Parent Protein
Antihypertensive	Casokinins	α-Casein, β-casein
ACE inhibitors	Lactokinins	α-Lactalbuminand β-lactoglobulin
Antimicrobial peptides	Lactoferricin B	Lactoferrin
	Isracidin	α_{S1}-Casein
		α_{S2}-Casein
Antithrombotic	Casoplatelins	κ-Casein
Immunomodulatory	Immunopeptides	α-Casein, β-casein, β-lactoglobulin
Mineral binding	Caseinophosphopeptides	α-Casein, β-casein
Opiod agonist	Casomorphins	α-Casein, β-casein
	α-Lactorphins	α-Lactalbumin
	β-Lactorphins	β-Lactoglobulin
Opiod antagonist	Lactoferricin	Lactoferrin
	Casoxins	κ-Casein
	Beta-lactotensin	β-Lactoglobulin

Note: From various reviews.

encountered in the early environment. Insulin-like growth factor-1 (IGF-1), lactoferrin, lysozyme, kappa-casein glycosylated-macropeptide, peroxidase, prolactin, and osteopontin are examples of other milk proteins whose bioactivity may be important for sustaining the growing infant.[106,107] It is feasible that significant quantities of protein nutrients could be absorbed intact and thereafter produce bioactivity at distant sites.

2.2.4 Bioactive Peptides and the Cryptome

Cryptides are short peptide sequences hidden or encrypted within larger protein molecules. Encrypted peptides are released by proteolytic processing of larger molecules. Cryptic peptides can be distinguished from gene-encoded bioactive peptides that are produced in a fully active state within the organism. Enzymic processing of pre-proteins and zymogens illustrates the importance of cryptides in physiological regulation. Activation of pancreatic pre-proteases (trypsinogen, chymotrypsinogen, pro-elastase, etc.) represents another classic example of peptide de-encryption. The activation of nascent bioactive peptides appears to take place within virtually all organs. Encryption is thought to increase the amount of information held within the genome, along with alternative reading frames, and multiple splicing, etc.[108] A recent analysis by Tautz suggests that gene-encoded peptides (containing 11–24 residues) may be more common than thought.[109] The possibility was raised that mRNA leader sequences that code for pre-proteins may contain short (but hitherto unrecognized) functional peptide sequences that could be released inside cells following proteolysis. Though gene-encoded peptides have been reported in insects and crustaceans, they appear to be rare in higher animals.

The earliest groups of bioactive peptides derived from nutritional sources were short peptide fragments generated by digesting milk casein. Encrypted, bioactive peptides were also formed as by-products of food fermentation. Literally hundreds of bioactive encrypted peptides have been discovered over the past 15 years with a range of physiological activities including, antimicrobial, antihypertensive, and immunomodulatory activities. Food-derived bioactive peptides may be produced by in vitro protease digestion of dietary protein ingredients or by bacterial fermentation in vitro or within the gut. Therefore, many of the activities of so-called friendly bacteria may lead to the generation of bioactive peptides and other functional metabolites. For representative reviews over the past 10 years.[99,110–117] There is growing interest in applications of bioactive peptides in pharmacology[118] and nutrition.[119–123]

2.2.5 Commercial Bioactive Peptides

Bioactive peptides are now available from commercial sources for use as supplements, administered by tube feeding via the oral (enteral) or injected (parenteral) routes—under the supervision of a health worker. The subject matter overlaps with the therapeutic use of protein drugs for disease treatment. Some bioactive peptides such as lactoferrin may cross over from nutritional to therapeutic applications. Secondly, many bioactive peptides appear to function via the same pathways employed by conventional therapeutic protein drug molecules.[124,125] The health benefits derive from the ability to enhance antioxidant status, increase antisepsis, boost immune function, and augment the body's anti-inflammatory status. The ranges of products include amino acids and proteins, protein hydrolysates, and food grade immunoglobulins from sera or whey.[126] Biofunctional food proteins (e.g., soybean and whey proteins [WP]) have been used since the 1970s for nutritional support for vulnerable groups including the elderly and malnourished, but essentially the ambulatory. Commercial protein feeds are based on a range of protein foods including, milk casein, WP, milk protein concentrates, egg protein, and soya proteins. The express use of health functional protein deserves greater recognition.

The therapeutic application of proteins dates from the 1920s with the development of crude pancreatic extracts of insulin[127,128] (see[129] for a recent review) (Table 2.17). The fields of so-called biopharmaceuticals expanded with advances in protein chemistry and biochemistry culminating in the determination of protein primary structure in the 1960s. Improved analysis and separation methods for proteins have contributed greatly to this field. The modern era for biopharmaceuticals started with the advent of recombinant technology. Therapeutic proteins are now produced using genetically modified microorganisms.[130]

2.2.6 Nutrigenomics Considerations

Nutrigenomics is defined as the study of gene interactions with *bioactive food components* including nutrients. According to the underlying principles of nutrigenomics (1) bioactive dietary components moderate risk of illnesses via changes to the processes affecting the onset, incidence, progression, and severity of disease; (2) bioactive components act on the genome (in)directly to alter the expression of genes; (3) diet can moderate genetic predispositions to disease arising from gene polymorphisms; and

TABLE 2.17
Therapy Using Polypeptide Pharmaceuticals or Biologicals

Areas of Application	Commentary
Growth promotion	Growth hormone, IGF, insulin
Life-time or long-term replacement therapy	Insulin, growth hormone, factor VIII
Short-term stimulation of endocrine axis	Calcitonin, oxytocin, glucagon
Antitumor agents	Interferon and antibodies
Thrombolytic therapy	Tissue plasminogen activator, urokinase
Blood volume replacement	Serum albumins and plasma proteins
Passive immunization	Immunoglobulin
Active immunization	Vaccines
Miscellaneous	Protease inhibitors, aprotinin, pepsin
Immune modulating agents	Cytokines
Growth and proliferation agents	Growth factors and cytokines

Source: Adapted from Biopharmaceutical, available from http://en.wikipedia.org/wiki/Biopharmaceutical (accessed August 2008).

(4) bioactive components produce effects that are influenced by the balance of health, disease processes, and individuals' genetic background.[131] A major goal in nutrigenomics is the identification of genes that are either upregulated or downregulated by food components.[132–136] The relevance of nutrigenomics concepts to the study of bioactive peptides is further addressed in Section 2.5, where we suggest that the ability to moderate gene expression should be considered one of the major hallmarks for bioactive peptides.

2.3 APPLICATIONS OF PROTEIN SUPPLEMENTS FOR HEALTH

2.3.1 Heterogeneous versus Enriched Supplements

Potential health benefits from protein supplementation and nutritional support are discussed in this section using evidence from randomized human controlled trials (RCT). It is useful to differentiate between heterogeneous and enriched or purified protein supplements. The former are more food-like (e.g., egg protein and milk protein) whereas enriched or purified protein supplements contain one protein (e.g., lactoferrin) or a highly restricted number of proteins. Current RCT show that heterogeneous protein supplements produce modest health benefits probably because these products contain low amounts of bioactive agents diluted by inert protein filler. The efficacy of protein supplements increases if the inert protein is removed, leading to higher concentrations of bioactive component. Unfortunately, the majority of RCT fail to distinguish between different grades of protein supplements thereby blunting results.* Examples of RCT dealing with heterogeneous or enriched protein supplements are summarized in Table 2.18.

* A notable exception is the meta-analysis of soy protein effects on blood cholesterol published by Anderson et al. (Ref. 144).

TABLE 2.18
Randomized Clinical Trials of Heterogeneous Protein Supplements

Condition/Reference	Effects of Protein Supplementation[a]
Blood pressure[137–140]	Protein intake is linked with reduced BP
Cardiovascular risk[141–144]	Soybean protein reduces cholesterol
Bone and hip fractures[145–150]	Reduces medical complications, promotes weight gain, reduces mortality
Chronic diseases and long-term care[151–153]	Little evidence of direct disease effect
Elderly malnourished[154–158]	Promotes weight gain, avoids wasting
HIV/AIDS infection[159,160]	Maintains body weight
Pregnancy[161–165]	No benefits, decreases birth weight
Renal patients[166]	Protein restriction may be beneficial
Resistance exercise, strength sports[167,168]	Weak evidence for beneficial effects
Sports nutrition[169,170]	Protein intake improves performance
Weight loss and food intake[171–181]	High-protein foods increase weight loss and decrease appetite

[a] Randomized controlled trials or meta-analysis.

2.3.2 Bone and Hip Fractures

Nutritional support using dietary protein supplementation may be useful for elderly hip-fracture patients who show an inflammatory response, hypermetabolism, and weight loss. Illness anorexia is also found in hip-fracture victims adding to undernutrition.[182] Increased protein intake may help with bone formation.[183,184] Avenell and Handoll performed a meta-analysis of 21 RCT (1727 participants) dealing with protein supplementation for hip-fracture patients. Eight of twelve RCT showed that protein supplementation reduced the proportion of unfavorable outcomes during treatment and reduced the number of long-term complications for hip-fracture patients though there was no effect on the mortality rate.[148] Hedstrom et al. identified 12 RCT for nutritional support for hip-fracture victims. Fifty percent of the RCT with bone fracture patients found a statistically significant increase in the rates of recovery and a reduction in the average length of stay in hospitals following protein supplementation.[146–148,182–184] The beneficial effects of protein supplementation were attributed to increased plasma levels of growth hormone and insulin-like growth factor-1 (IGF-1) that is known to improve bone accretion.

2.3.3 Elderly Malnourished Patients

Milne et al. discussed 49 RCT (total of 4790 participants) that examined the effect of protein supplementation on malnutrition in the elderly.[186] Of the total of 49 RCT examined, 32 (65%) studies showed that protein supplementation produced a small but statistically significant (2.3%) weight gain in the elderly malnourished patients. Feeding extra protein also produced 26% reduction in the rate of mortality in elderly persons suffering protein undernutrition. There was no evidence of functional benefit or reduction in length of hospital stay with supplements. The ability of protein to reverse aspects of weight loss has been confirmed for hospitalized elderly patients.

2.3.4 Pregnancy

Since developing babies depend on the mother for their nutritional needs, it was suspected that low protein intake could adversely affect fetal development. However, data from RCT did not show a link between low maternal protein intake and gestational weight or poor pregnancy outcome. On the contrary, increased protein intake during pregnancy seemed to be linked with weight loss for both the mother and fetus. Accordingly, there is insufficient evidence for increasing protein intake during pregnancy.[157–161]

2.3.5 Muscle Strength from Resistance Exercise

A number of RCT suggest that WP supplementation may assist in the buildup of muscle tissue and increase muscle strength.* The muscle-enhancing effects of WP have been explained in terms of the high content of leucine and other branched chain amino acids (BCAA) present in WP. WP supplementation before strength exercise is thought to decrease circulating levels of testosterone and growth hormone, but increase levels of insulin,[187] and somehow increase muscle protein synthesis. The muscle-building effect of WP may be related to the content of bioactive peptides and/or leucine supply from these supplements.[123,188]

2.3.6 Insulin-Stimulating Activity

Dietary proteins appear to stimulate insulin secretion to varying degrees, which may be related to their effect of body protein accretion. Human studies reported by Nilson et al. showed that WP increased insulin responsiveness and reduced blood glucose to a greater extent compared to white bread, or supplementation with gluten, cod protein, casein, or cheese (Figure 2.1). WP produced large changes in postprandial levels of several amino acids (leucine, alanine, lysine, valine, isoleucine, and threonine), which are known to possess insulin-stimulating activity. BCAA released by digestion of WP could explain the insulinotropic effect of this particular protein; similar results could also be obtained with skimmed milk. WP may also stimulate the body's incretins—two gut hormones, GLP-1, and GIP, which are known to stimulate insulin secretion or release by beta cells. The analysis of blood samples following WP supplementation showed that levels of GLP-1 were increased but that levels of GIP were not affected. Tessari compared casein and WP and reported different results than discussed above. For instance, WP was found to stimulate the production of insulin compared to casein, whereas the latter produces greater stimulation of GLP-1. The administration of free amino acids (equivalent to casein) lowers plasma glucose compared to either WP or intact casein. Since casein is digested more slowly than WP in the stomach owing to the tendency to coagulate at acidic pH, the above data suggested that the "slowness" of protein digestion could have an important influence on the insulinotropic effect. Fast proteins (e.g., WP) would be expected to stimulate insulin secretion to a greater extent compared to slow proteins such as casein.[196]

* Cross reference, muscle strength in sarcopenia, anabolic effects of WP, leucine, etc.

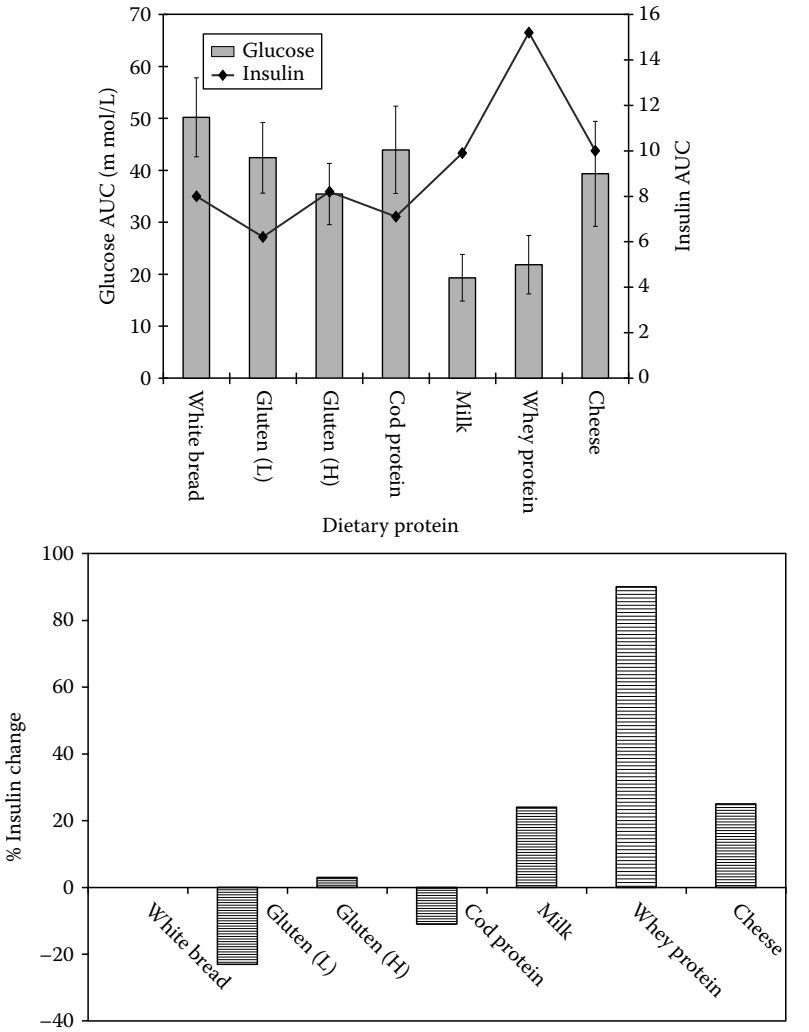

FIGURE 2.1 Effect of different dietary protein sources on glucose and insulin responses in human subjects. Graph axis show area under curve (AUC) 0–90 min after drinks containing lactose supplemented with different protein supplements. White bread is used as standard. (Drawn from Nilsson et al., *Am. J. Clin. Nutri.* 80,1246, 2004.)

2.3.7 Protein Supplementation and Cardiovascular Health

The beneficial effect of soybean protein consumption on cardiovascular health was confirmed by the classical meta-analysis by Anderson, Johnston, and Cook-Newel.[144] Data compiled from 38 clinical trials suggested that consumption of 25 g/day of soybean protein in place of animal protein reduced blood cholesterol and LDL levels by 7.4% or 9.8% for people with a baseline blood cholesterol range of 259.3 or 332.8 mg/dL, respectively. Reductions of 19.6% were observed for those with high blood cholesterol

values (>335 mg/dL). A maximum fall in LDL levels by 24% was possible. In summary, the benefits of soybean consumption were higher for at-risk groups. Bakhit and coworkers reported a linear correlation between the starting serum cholesterol levels and the size of reductions obtained after consuming soybean foods. The consumption of 25 g soybean protein per day reduced serum cholesterol levels for human volunteers only where their initial serum cholesterol was >5.7 mmol/L.[144] Such results led to increased acceptance of the health benefits of soybean consumption in the United States and FDA approved health claims that the consumption of 25 g/day soybean protein protects against cardiovascular disease.[189] The American Heart Association statement for healthcare professions noted that it would be prudent to include 25 g/day of soybean in the diet.[190] Finally, the National Institute of Health is in general support of the benefits of including soybean foods in the diet.[191] Though the mechanism of cholesterol reduction by soy protein are diverse, research from Adams et al. suggests the involvement of soybean-derived bioactive peptide (W008) in blood cholesterol reduction in mice.[192,193] More recent trials using monkeys, however, failed to demonstrate positive effects due to soybean bioactive peptides.[194]

2.4 PERSPECTIVES ON HUMAN TRIAL DATA

2.4.1 Statistical Effects

Proof of efficacy of medical foods should be based on evidence gathered from human trials (Section 2.1.4). However, the therapeutic benefits of bioactive peptides seem rather weak, inconsistent, or easily masked by background noise. There is general dissatisfaction with the quality of RCT for protein supplements documented by the consolidated standards of reporting trials (CONSORT) statement.[195,196,*] Many RCT are thought to be lacking adequate statistical power, suggesting that clinically important effects would not be detected. In a classical paper describing the effect of low statistical power on RCT reporting, the authors examined 71 RCT and found that 67 (95%) had >10% chance of missing a true therapeutic effect, amounting to about 25% change of the outcome measure. A further 70% of all the RCT in the sample studied would miss therapeutic benefits amounting to 50% due to low statistical power.[197] Similar conclusions have been reported by other authors. For example, Mohar et al. examined a total of 383 RCT published in several prominent medical journals (*JAMA*, *Lancet*, and *The New England Journal of Medicine*) during 1975, 1980, 1985, and 1990, and found that only 16% and 32% of the studies had sufficient statistical power to detect 25% and 50% relative increase or decrease in the measured outcome, respectively. Some 70% of RCT that reported negative results did not report the number of participants.[198] The overwhelming agreement is that further RCT of adequate power are needed in order to evaluate protein dietary supplements.

The statistical power or the ability to detect differences between treatments is a function of (1) the size-effect being analyzed, (2) number of study participants, (3) the probability level set for Type II error (i.e., the chance of detecting a false-positive

* A summary of the CONSORT guidelines for designing and reporting randomized clinical trials is available at http://www.consort-statement.org (accessed July 2008).

result), and (4) inherent variability associated with the effect being analyzed. The number of study subjects required for any study should be large in order that small differences can be determined. A low number of participants can be used where the effect under examination is large. The common strategy advocated by medical statistics is to increase the number of study participants and hence increase statistical power.[199,200]

The equivocal clinical results demonstrated for dietary protein supplements may also be explained by the low concentration of bioactive components present in these products. Efficacy is determined by the dosage administered, bioavailability, and potency—or ability to elicit a physiological change. To increase the potency of protein supplements, the bioactive components should be identified and isolated. Most of the applications addressed in this book use enriched protein supplements or purified bioactive peptides.

2.4.2 Health Claims for Foods and Supplements

Current international guidelines for nutrition health claims are available from *Codex Alimentarius* and are briefly summarized in Table 2.19. All foods can carry certain health claims related to well-being. Most commonly, food packages may carry "nutrition claims" related to composition and energy content. In addition, foods may make "heath claims" relating particular foods and health. Finally, reduction of disease claims suggests that consumption of a food may reduce the risk factor for the development of particular diseases. The labels of dietary supplements are allowed to carry health or disease reduction claims, nutrient content claims, or structure–function claims. Perhaps the most important from the standpoint of bioactive peptides are those health claims relating food disease reduction and structure–function claims. Claims for medical foods are required to be supported by scientific evidence of the type summarized in this book.

TABLE 2.19
Nutrition Health Claims under *Codex Alimentarius*

1. *Nutrient contents claim*: A statement that describes the relative amount of a nutrient or dietary substance in a product (e.g., "high in" or "free from").
2. *Health claim*: A statement describing a relationship between a food, food component, or dietary supplement ingredient and health.
3. *Reduction of disease claim*: A statement suggesting that consumption of a food significantly reduces the risk factor in the development of human diseases.
4. *Structure–function claim*: A statement describing how a product may affect the structure and/or function of (particular) organs or systems within the human body; ordinarily, structure–function claims may not mention a specific disease.

Source: Guidelines for nutrition health claims. Codex alimentarius ref. CAC/GL 23 1997. Available from http://www.codexalimentarius.net/web/more_info.jsp?id_st a=351 (accessed June 2008).

TABLE 2.20
Safety Assessment for Bioactive Compounds

All potentially bioactive food components should be evaluated using a scientifically valid risk–benefit model that clearly assesses all physiologic effects, both positive and negative. Bioactive food components shown to be potentially toxic for the intended food or supplement use according to this analysis would not qualify. ADA recommends that the definition reflect the safety of bioactive food components.

Source: American Dietetic Association; available from, http://dietary-supplements.info.nih.gov/pubs/bioactivefoodcomponents/American%20Dietetic%20Association%20-%20ADA%20-% 20comments%20on%20defining%20bioactive%20food%20components.pdf.htm (accessed July 2008).

2.4.3 Safety and Side Effects of Bioactive Peptides and Proteins

Bioactive proteins are well known in the field of toxicology. Many organisms produce defensive or offensive proteins for deterring predators. For instance, many plant proteins are known that are toxic to parasites and most major herbivores. Proteins inhibitors of digestive enzymes appear to have evolved to prevent the consumption of various plants by grazing animals. Other defense proteins include allergens, ribosome inhibitory proteins (RIP), and notorious materials such as ricin, arguably the most toxic natural agent known (Appendix 2.A.6). Many invertebrates (frogs, insects, and crustaceans) and reptiles produce venoms that contain a host of bioactive peptides some of which may find application in nutritional science. The potential toxicity of some useful bioactive peptides requires that each agent is tested for safety under conditions of intended use (Table 2.20). Aside from overtly toxic proteins and peptides, other bioactive peptides may produce adverse side effects when used at unusually high concentrations. The effects are linked with the issues of specificity. Some notable examples of side effects include those associated with growth hormone use (Chapter 9).

2.5 SUMMARY AND CONCLUSIONS

Protein supplementation is routinely applied for the nutritional support of a wide range of clinical situations. This chapter describes legislation and classifications of protein and peptide foods and their effects on health. Current applications for heterogeneous and enriched protein supplements are appraised based on evidence from RCT. The majority of RCT reported for protein supplement show modest health benefits and conflicting results. Equivocal health effects are a feature of heterogeneous protein supplements that contain low levels of biologically active components. Improvements in study design may also increase the sensitivity toward bioactive effects. The advantages of enteral feeds containing bioactive peptides, hormones, and other trophic factors are being gradually recognized.[201–206] In this book, we consider the following hypothesis: (1) the efficiency of nutritional

support—judged by the ability to maintain body weight—can be increased using bioactive peptides to address derangements associated with illness, (2) bioactive peptides may be applied via a wide variety of routes in addition to the oral/enteral route, which is associated with low bioavailability. Nasal administration is interesting since it appears to bypass the blood–brain barrier for peptide, and (3) the prospects of bioactive peptides for nutritional support is potentially very large. Current technological advances render the distinction between food- and nonfood-derived peptide sequences arbitrary because bioactive sequences can be synthesized readily. The bioactive peptides available for nutritional support also include endogenous peptide sequences and material produced by recombinant technology, in addition to exogenous peptides isolated from natural sources.

APPENDICES

APPENDIX 2.A.1
Summary of the Main Categories of Medical Foods

Code B4102	Enteral formula for adults, used to replace fluids and electrolytes
Code 4103	Enteral formula for pediatrics, used to replace fluids and electrolytes
Code B4149	Enteral formula, blended natural food with intact nutrients (commercially prepared)
Code B4150	Enteral formula, nutritionally complete with intact nutrients
Code B4152	Enteral formula, nutritionally complete, calorically dense (>1.5 kcal/mL) with intact nutrients
Code B4153	Enteral formula, nutritionally complete, hydrolyzed proteins (amino acids and peptide chain)
Code B4154	Enteral formula, nutritionally complete for special metabolic needs, excluding inherited disease of metabolism
Code B4155	Enteral formula, nutritionally incomplete/modular nutrients
Code B4157	Enteral formula, nutritionally complete for special metabolic needs, for inherited disease of metabolism
Code B4158	Enteral formula for pediatrics, nutritionally complete with intact nutrients
Code B4159	Enteral formula for pediatrics, nutritionally complete with soy based with intact nutrients
Code B4160	Enteral formula, for pediatrics, nutritionally complete calorically dense (equal to or greater than 0.7 kcal/mL) with intact nutrients
Code B4161	Enteral formula, for pediatrics, with hydrolyzed amino acids and peptide chain
Code B4162	Enteral formula, for pediatrics, special metabolic needs for inherited disease of metabolism
Code B4180	Parenteral nutrition solution, with carbohydrates (dextrose), greater than 50%
Code B4185	Parenteral nutrition solution per 10 g lipids
Code B4189, B4193, and B4199	Parenteral nutrition solution; compounded amino acids with carbohydrates
Code B4172	Parenteral nutrition solution, amino acid, 55% (500 mL/unit)

APPENDIX 2.A.2
Selected Commercial Medical Formulas for Adults

Code B4150: Enteral Formula, Nutritionally Complete with Intact Nutrients	Code-B4152: Enteral Formula, Nutritionally Complete, Calorically Dense (>1.5kcal/mL) with Intact Nutrients	Code B4153: Enteral Formula, Nutritionally Complete, Hydrolyzed Proteins (Amino Acids and Peptide Chain)	Code B4154: Enteral Formula, Nutritionally Complete for Special Metabolic Needs, Excluding Inherited Disease or Metabolism	Code B4157: Enteral Formula, Nutritionally Complete for Special Metabolic Needs, for Inherited Disease of Metabolism	Code B4155: Enteral Formula, Nutritionally Incomplete/ Modular Nutrients
1. Boost	1. Boost Plus	1. Alitraq	1. Advera	1. Cylinex-2	1. Casec Powder
2. Boost high protein liquid	2. Carnation Instant Breakfast Lactose Free Plus	2. Glutasorb RTU	2. Amin Aid Powder	2. Hominex-2	2. Duocal
3. Boost high protein powder	3. Carnation Instant Breakfast Lactose Free VHC	3. L-Emental	3. Boost Diabetic	3. Phenex 2	3. Immunocal
4. Boost w/fiber	4. Deliver 2.0	4. Optimental	4. Choice DM	4. PhenylAde Drink Mix	4. Juven
5. Carnation instant breakfast lactose free	5. Ensure Plus	5. Peptamen	5. Diabetisource AC	5. Phenyl-Free 2	5. MCT Oil
6. Ensure	6. Ensure Plus HN	6. Peptamen 1.5 Diet	6. Glucerna	6. Propimex 2 (powder)	6. Microlipid
7. Ensure fiber w/FOS	7. Nutren 1.5	7. Peptical	7. Immun-Aid		7. Moducal Powder
8. Ensure high protein	8. NutriAssist 1.5	9. Peptamen/Prebio 1	8. Hepatic-Aid II		8. OS2 Powder
9. Ensure HN	10. NovaSource 2.0	8. Peptamen VHP	9. Glytrol		9. PFD 1
10. Fibersource	11. Resource Plus	9. Peptinex	10. Impact		10. PFD 2
11. Fibersource HN	12. ScandiShake	10. Peptinex DT	11. Isosource VHN		11. Phlexy-10 Capsules
12. Isocal	13. TwoCal HN	11. Perative	12. Ketocal		12. Phlexy-10 Drink Mix
13. Isocal HN	13. Subdue	12. Pro-Peptide	13. L-Emental Hepatic		14. Polycose Liquid Phlexy-10 Drink Mix
14. Isolan	15. Tolerex	13. Pro-Peptide VHN	14. Lipisorb Liquid		16. Polycose Powder
15. Isosource	17. Vital HN		15. Magnacal Renal		18. ProCel Powder

(*continued*)

APPENDIX 2.A.2 (continued)
Selected Commercial Medical Formulas for Adults

Code B4150: Enteral Formula, Nutritionally Complete with Intact Nutrients	Code-B4152: Enteral Formula, Nutritionally Complete, Calorically Dense (>1.5 kcal/mL) with Intact Nutrients	Code B4153: Enteral Formula, Nutritionally Complete, Hydrolyzed Proteins (Amino Acids and Peptide Chain)	Code B4154: Enteral Formula, Nutritionally Complete for Special Metabolic Needs, Excluding Inherited Disease or Metabolism	Code B4157: Enteral Formula, Nutritionally Complete for Special Metabolic Needs, for Inherited Disease of Metabolism	Code B4155: Enteral Formula, Nutritionally Incomplete/ Modular Nutrients
16. Isosource HN	19. Vivonex Plus		16. Modulen IBD		20. ProMod Powder
17. Jevity (1.0 cal)	21. Vivonex T.E.N.		17. Nepro		22. Propass Powder
18. Jevity plus (1.2 cal)	23. Vivonex RTF		18. Novasource Pulmonary		24. ProStat 101
19. Nutren 1.0			19. Nutrihep		25. ProStat 64
20. NutriHeal complete			20. Novasource Renal		26. ProViMin
21. Osmolite			21. NutriRenal		27. ReSource Inst Protein Powder
22. Osmolite 1.2 cal			22. NutriVent		28. Ross Carbohydrate Free (RCF)
23. Osmolite 1.2 cal			23. Product 3232A		29. Sumacal
24. ProBalance			24. Pulmocar		
25. Promote			25. Renalcal		
26. Replete			26. Respalor		
27. Replete w/fiber			27. Similac PM 60/40		
28. Resource			28. Suplena (Replena)		
29. Ultracal			29. Traumacal		
30. Ultracal HN plus					

Note: All names listed are trademarked unless otherwise stated.

APPENDIX 2.A.3
Selected Pediatric Enteral Formulas

Code B4158: Enteral Formula for Pediatrics, Nutritionally Complete with Intact Nutrients	Code B4160: Enteral Formula, for Pediatrics, Nutritionally Complete Calorically Dense (Equal to or Greater Than 0.7 kcal/mL) with Intact Nutrients	Code B4161: Enteral Formula, for Pediatrics, Hydrolyzed Amino Acids and Peptide Chain	Code B4162: Enteral Formula, for Pediatrics, Special Metabolic Needs for Inherited Disease of Metabolism
1. Portagen	1. Beginnings Pediatric Drink	1. EleCare Powder	1. Calcilo XD
2. Similac Special Care	2. Carnation Instant Breakfast Junior	2. Enfamil Nutramigen Lipil	2. Cyclinex-1
3. Advance w/Iron 24	3. Kindercal	3. Enfamil Pregestimil	3. Cyclinex 2
	4. Nutren Junior	4. Neocate Infant Formula	4. Hominex-2
	5. Pediasure	5. Neocate Junior	5. Phenex-1
	6. Pediasure w/Fiber	6. Neocate One + Powder	6. Phenex-2
	7. Resource Just For Kids with Fiber	7. Pediatric E028	7. PhenylAde Drink Mix
	8. Resource Just For Kids 1.5 cal	8. Peptamen Jr. w/Prebio 1	8. Phenyl-Free 1
	9. As above + cal with Fiber	9. Peptamen Junior	9. Phenyl-Free 2
		10. Peptamen Junior Powder	10. Periflex
		11. Peptinex DT Pediatric	11. Propimex 1 (powder)
		12. Product 3232A	12. Propimex 2 (powder)
		13. Similac Alimentum Advance with Iron	
		14. Vivonex Pediatric	

Source: Adapted from the list of officially approved enteral formulas from the Michigan Department of Community Health (available from http://www.priorityhealth.com/provider/manual/policies/91278.pdf and http://www.michigan.gov/documents/MSA_01–26_9065_7.pdf (accessed August 2008).

APPENDIX 2.A.4
Selected Enteral and Oral Supplement Substitutions and Classes

Enteral formulas	*Representative Type*
1. Ensure, Boost, Isocal, Nutren, Osmolite, Resource	Osmolite 1 cal
2. Standard, Isosource Standard, NuBasics 1.0	
3. Nutren with Fiber, ProBalance, Ultracal, FiberSource, Ensure with Fiber, Boost with fiber, Ultracal	Jevity 1 cal
4. Nutren 1.5, Boost Plus, Ensure Plus, Ensure Plus HN, Resource Plus, Comply, Isosource 1.5	Jevity 1.5 cal
5. Nutren 2.0, NovaSource 2.0, NuBasics 2.0, Deliver 2.0, Resource 2.0, MedPass 2.0	2 cal HN
6. Replete, Protain XL, Isosource VHN	Promote
7. Replete with Fiber, Protain XL, Isosource VHN	Promote with fiber
8. Pulmocare, NutriVent, Novasource Pulmonary, Respalor	Oxepa
9. Perative, Peptamen, Optimental, Peptinex, Vital HN, Vivonex, Tolerax, faa	Peptamen AF
10. Impact, Pivot 1.5	Crucial
11. No substitute	Portagen
12. Traumacal, Magnacal Renal, RenalCal, NutriRenal, Novasource Renal, Suplena	Nepro Carb Steady
13. Nutren Junior	Pediasure Enteral Formula
14. Kindercal, Nutren Junior with fiber	Pediasure Enteral, Formula with fiber
Peptamen Junior, Peptamen Junior with Prebio, Vital Junior	Peptamen Junior with fiber and Prebio
Oral supplements and classes	
1. Boost, Boost Plus, Equate Plus	Ensure Plus
2. CIB Sugar-Free, Choice DM	Glucerna Shake
3. Enlive, Boost Breeze, NuBasics Juice Drink	Resource Breeze
4. Kindercal, Nutren Junior with fiber	Pediasure
5. Peptamen Junior with fiber and Prebio	Vital Junior

APPENDIX 2.A.5
Major Producers of Medical Devices and Enteral Feeds

Commercial Producers	**Medical Conditions Likely to Require Nutritional Support**
Abbott Laboratories	Acquired immune deficiency syndrome
Alaris Medical Systems, Inc.	Burn injury
American Gastroenterological Association	Cancer
American Home Patient, Inc.	Cancer and diet
American Society For Parenteral & Enteral Nutrition (ASPEN)	Cancer weight loss
Arrow International	Nutritional support of cancer treatment
Astrazeneca PLC	Diabetes mellitus
B. Braun Medical Inc.	

APPENDIX 2.A.5 (continued)
Major Producers of Medical Devices and Enteral Feeds

Commercial Producers	Medical Conditions Likely to Require Nutritional Support
Baxter International Inc.	Nutritional management in chylous leaks
Bristol-Myers Squibb Company	Pulmonary disease
Fresenius Kabi	Nutrition support in the dying patient
Fujisawa Pharmaceutical Company, Ltd.	Vegetative states
Hospira Inc. (Subsidiary of Abbott Laboratories)	Chronic renal failure
Kendall Healthcare Products	Chronic heart failure
Mead Johnson Nutritional Group (a subsidiary of Bristol-Myers Squibb)	Chronic heart disease
Nestlé Nutrition	Generic wasting diseases
Novartis Medical Nutrition	
Oley Foundation	
Ross Products Division of Abbott Laboratories	
Sandoz Incorporated	
SHS North America	
Wyeth Pharmaceuticals	
ZEVEX International, Inc.	

Source: Adapted from Anon (2005). The Market for Clinical Nutritional Products. Kalorama Information (Pub). Available from http://www.marketresearch.com/map/prod/1089382.html (accessed August 2008).

APPENDIX 2.A.6
Toxic Bioactive Peptides and Defense Proteins from Food Sources

Allergens
Amylase inhibitors
Celiac peptides
Casein peptides
Lectins
Lipase inhibitors
Lipid-binding proteins
Protease inhibitors
Ribosomal inhibitor proteins
Enterotoxins
Toxins
Venoms

APPENDIX 2.A.7
Natural Bioactive Peptides from Amniotic Fluid and Breast Milk

Adrenal steroids
Calcitonin
β-Casomorphin
Epidermal growth factor
Erythropoietin
Fibroblast growth factor
Gastrin-releasing peptide (GRP)
GRP-like factor
Growth hormone–binding protein
Granulocyte–macrophage colony–stimulating factor
Hepatocyte growth factor
Insulin
Insulin-like growth factors (IGF)
IGF-binding proteins
Leptin
Nerve growth factor (NGF)
Nucleotides
Oxytocin
Plasminogen activator inhibitor
Prolactin
Prostaglandins
Somatostatin
Thyroid-stimulating hormone
Thyrotropin-releasing hormone
Transforming growth factors
Vasoactive intestinal peptide
Vascular endothelial growth factor

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3 Dietary Protein Requirements for Health

3.1 INTRODUCTION

3.1.1 Protein-Energy Undernutrition

The health benefits associated with protein consumption are well known, but our understanding of the role of bioactive peptides in the human body is limited.[1,2,*] There is no general agreement on which factors contribute to the non-nutritive versus nutritive benefits of dietary proteins. However, it is nonnutritive effects, which define peptide bioactivity.[†] In this chapter, we consider dietary protein requirements in the light of emerging knowledge concerning peptide bioactivity, and take account of recommended daily allowances (RDA) for intake. We also consider possible changes to the RDA during illness. Protein intake and its relation to protein-energy undernutrition (PEU) is explored in the remainder of Section 3.1. The relations between protein quality and their health effects are discussed in Section 3.2. Current advice regarding the RDA for dietary protein and the effect of ill health on the RDA are discussed in Sections 3.3 and 3.4, respectively. In Section 3.5, we consider the hallmarks for peptide bioactivity. Finally, some major types of health benefits associated with bioactive peptides are discussed in Section 3.6.

Persistently low intakes of dietary protein will result in unwanted weight loss. Health professionals refer to PEU as a disorder of nutritional status linked with inadequate protein-energy intake. PEU can also arise from derangements in protein metabolism associated with ill health.[3,‡] PEU occurs frequently for aging adults living in care homes and hospitals.[4–6,§] Muscle loss occurs during aging due to the tendency to reduce food intake, sarcopenia, and cachexia.[7,8] PEU in children is associated with poor diet or infection. The gap between actual protein intake and RDA is likely to increase during illness as a result of anorexia. Illness-related derangements

* Dietary protein serves as source of essential amino acids (EAA) for the body and hence it is normal to use the terms protein and amino acid interchangeably in nutrition discussions.

† See Section 2.2.1 and Section 3.5.

‡ Protein-energy undernutrition (PEU) encapsulates two enormously important ideas: First, protein balance (nutritional status, gain of lean body mass, etc.) depends on dietary protein intake. The human body can only produce so-called nonessential amino acids. Aside from these, new tissue can only be built with the aide of external sources of EAA. Second, poor health leads to mishandling (metabolic derangements) of dietary and tissue protein resources. Therefore, protein balance is also affected by the health of the consumer.

§ Rudman et al. noted that the incidence of anorexia in care homes is related to staffing levels. However, different starvation levels remain, even after staffing levels are corrected for. Aging and illness leads to decreasing appetite and food intake.

in metabolism may also increase protein requirements beyond those needed for healthy subjects (see below).

3.1.2 Detection of Protein-Energy Undernutrition

PEU may categorized as mild, moderate, or severe according to body mass index (BMI) values of 18–18.9, 16–17.9, or <16, respectively (Table 3.1). The intensity of PEU appears to be related to the severity of illness, but is independent of specific types of diseases. Feeding can restore body weight but only partially. Recovery from PEU is more likely if metabolic derangements associated with illness are also addressed. Undernourished subjects require feeding to stabilize body mass while any underlying malady is corrected by natural healing or by drug therapy.* Table 3.1 lists characteristic values for several indices related to undernutrition. The pathological features of PEU include, a loss of skeletal muscle, heart muscle, and respiratory muscle. There are also reductions in the rate of respiration and hypothermia. A decrease in serum albumin levels leads to edema. Lymphocyte numbers may be severely reduced leading to symptoms of immunodeficiency (Table 3.1). Subjects with PEU show increased levels of urea excretion and a negative nitrogen balance (NBL). The causes and consequences of PEU have been reviewed.[9,10]

Trained nutritionists can readily assess protein nutritional status using anthropometric and biochemical measurements (Table 3.2).[11] Physical examination and questionnaires also help in the detection of PEU. A widely used rapid screening method is the malnutrition universal screening tool (MUST). MUST is applied by

TABLE 3.1
Common Indices for Protein-Energy Undernutrition and Their Values

Measurement	Normal	Mild PEU	Moderate PEU	Severe PEU
Normal weight (%)	90–110	85–90	75–85	<75
BMI	19–24[a]	18–18.9	16–17.9	<16
Serum albumin (g/dL)	3.5–5.0	3.1–3.4	2.4–3.0	<2.4
Serum transferrin (mg/dL)	220–400	201–219	150–200	<150
Lymphocyte count (per mm^3)	2000–3500	1501–1999	800–1500	<800
Delayed hypersensitivity index[b]	2	2	1	0

Source: Adapted from Potein-Energy Undernutrition, The Merck Manual, Online Medical Library. Available from http://www.merck.com./mmpe/sec01/ch002/ch002b.html?qt=protein undernutrition&alt=sh (accessed July 2008).

[a] In the elderly, BMI < 21 may increase mortality risk.

[b] Delayed hypersensitivity index uses a common antigen (e.g., one derived from *Candida* sp. or *Trichophyton* sp.) to quantify indurations elicited by skin testing grades: 0 = <0.5 cm, 1 = 0.5–0.9 cm, 2 = ≥1.0 cm.

* Improved staffing levels and the degree of assistance given to the elderly during eating can help to resolve undernutrition. See Refs. [1–6] on the psychological dimensions of the care of the elderly and other at-risk groups.

TABLE 3.2
Assessment of Protein Nutritional Status

Dietary Protein	
Nitrogen Intake	
Biochemical Indices	**Anthropometric Measurements**
Serum albumin	Weight
Transferrin	Skin fold thickness
Retinol binding protein (RBP)	Creatinine production
Prealbumin	Total body nitrogen
IGF-1	Total body potassium
Urinary nitrogen	Impedance densitometry
Urinary urea	Dual energy X-ray absorptiometry (DEXA) scanning
	CT scanning
Nitrogen	
Urea nitrogen	
Total nitrogen output	

giving patients scores between 0 and 2 according to the (a) BMI value, (b) presence of unplanned weight loss, and (c) acute disease effects. Nutritional support is given to patients scoring >2. Other screening tools have been developed with the aim of improving accuracy and ease of detection of undernutrition including the mini nutrition assessment (MNA) questionnaire and the subjective global assessment (SGA) approach. Patients exhibiting undernutrition can undergo nutritional support by oral, enteral, or parenteral nutrition. Standard tests for PEU have been described.[12,13]

3.1.3 Incidence and Consequences of Undernutrition

The incidence of PEU is estimated at between 20% and 50% for long-term hospitalized patients (Figure 3.1). By comparison, ~70% of liver transplant patients are thought to be underweight at admission. Advanced cancer patients are another group with high incidence of PEU.[2–5,14] The incidence of PEU for chronic renal disease patients is reportedly 38%–60%. PEU was found to be higher in female dialysis patients compared to men and tended to increase if patients were also diabetic. Weight loss associated with hemodialysis treatment is due to decreased protein and energy intake. Recent investigations suggest that wasting is the result of cachexia attributable to uremia, acidosis, and an inflammatory response probably linked with interleukin-6 (IL-6) and other proinflammatory cytokines.[15–17] In renal dialysis patients, there was a negative correlation between the indices of muscle mass (thigh muscle area, serum albumin levels, and creatinine excretion) and levels of IL-6 and CRP biomarker for inflammation.[18] Currently, <50% of U.K. hospitals possess nutrition teams comprising doctors, dieticians, nurses, and nutritionists working together to identify and treat undernutrition.[19,20]

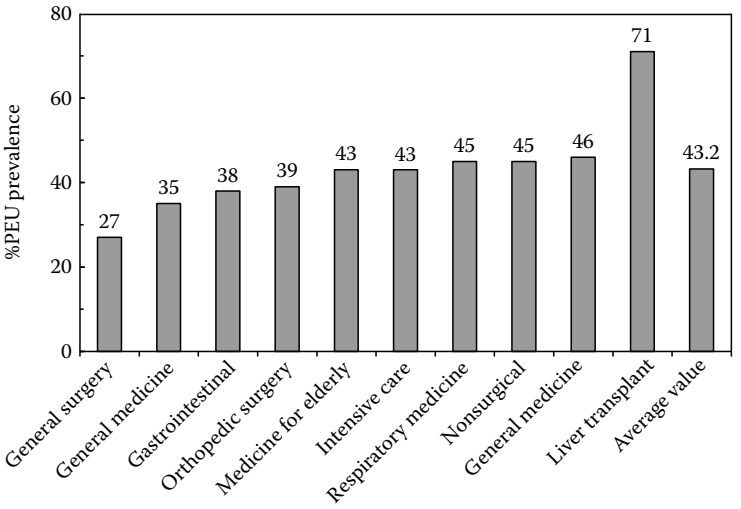


FIGURE 3.1 Incidence of undernutrition in different patient groups in U.K. hospitals. (From Corish, C.A. and Kennedy, N.P., *Br. J. Nutr.*, 83, 575, 2000.)

As discussed earlier, PEU leads to increased susceptibility to infection and reduced rates of recovery from illness (Section 1.3.6). Mortality rates increase with increasing weight loss for AIDS patients,[21] aging adults,[22,23] hemodialysis patients,[24,25] chronic heart failure,[26] and cystic fibrosis sufferers.[27] The adverse health consequences of PEU are due to weakening effects on the skeletal muscle, circulatory system, respiratory system, digestive system, and immune function. Smooth- and cardiac-muscle loss promotes conditions such as heart failure and kidney failure. Skeletal muscle loss leads to frailty, declining strength and coordination, and increases the likelihood of accidents in the elderly. Undernutrition is also accompanied by psychological symptoms such as apathy, depression, and self-neglect—the forms of "sickness behavior." Prospective studies by Sullivan et al.[23,28] showed that poor treatment outcomes were directly linked with indicators of low nutritional status and lean body mass (e.g., >5% weight loss in the last 6 months, low BMI, decreasing mid-arm circumference, and skin-fold thickness). Adverse clinical outcomes persisted long after discharge from hospital.[29]

3.2 DIETARY PROTEIN QUALITY RELATION TO HEALTH

3.2.1 Protein Quality and Nutritive Properties

The RDA for dietary protein reflects the essential amino acid (EAA) profile as well as the digestibility of different proteins. The consumption of high-quality dietary protein is necessary for health.[30] The amount of different EAA present in any dietary protein source can be quantified with a fair degree of accuracy by standard methods (Table 3.3). Combining the EAA profile and digestibility score yields the protein digestibility corrected amino acid score (PDCAAS). To determine the PDCAAS value, the EAA profile for a test protein is normally compared

TABLE 3.3
Essential Amino Acid Requirements

	FAO/WHO/UNU 1985		Milward and Colleagues (1999)		MIT EAA Pattern 2000	
Amino Acid	mg/kg/day	mg/g Protein	mg/kg BW/day	mg/g Protein	mg/kg BW/day	mg/g Protein
Aromatic[a]	14	19	20	33	39	65
Sulfur AA	13	17	16	27	13	25
Lysine	12	16	19	31	30	50
Leucine	14	19	26	44	40	65
Isoleucine	10	13	18	30	23	35
Valine	10	13	14	23	20	35
Threonine	7	9	16	26	15	25
Tryptophan	3.2	5	4	6	6	10

Source: Adapted from Young, V.R. and Borgonha, S., *J. Nutr.*, 130, 1841S, 2000; Fukagawa, N.K. and Galbraith, R.A., *J. Nutr.*, 134, 1569S, 2004.

[a] Tryptophan, tyrosine, and phenylalanine.

to the EAA requirement for preschool human children, published by the FAO/WHO/UNU in 1985. The concentration of each EAA found in the test protein is divided by the EAA value from the FAO/WHO pattern. The lowest numerical fraction is then taken as the limiting EAA score.* Currently, the PDCAAS (=limiting EAA × % digestibility) is considered the gold standard for assessing protein nutritive quality. According to such considerations, we expect the nutritional quality of protein hydrolysates (predigested food proteins) to be higher than the value for intact (unhydrolyzed) proteins. A mixture of amino acids should also exhibit improved nutritional characteristics compared to intact (unhydrolyzed) protein. These expectations are not borne out by experimental evidence, which shows that intact and slowly digested (slow) proteins are better utilized in the body, when compared under similar circumstances.†

3.2.2 Growth Assays for Dietary Protein Quality

A growth assay is easily conducted by feeding dietary proteins to young and actively growing animals (typically, rats and chicks). Changes in body weight are then measured. Results for dietary proteins are then ranked according to values for the protein efficiency ratio (PER), which is the weight gain observed for the test protein compared to the weight gain obtained with a standard casein diet.

* Therefore dietary proteins contain a succession of first, second, etc., limiting EAA. The first limiting EAA in many cereal proteins tends to be lysine, followed by methionine. Mixtures of foods will also have one or more limiting EAA.

† See Section 5.4.3.

Until recently, the PER value was the industry standard index for judging protein quality. However, small and actively growing animals have different EAA requirements from humans. Indeed, the protein requirements and growth characteristics of human infants are impressively different from those of adult humans (Section 3.3). Currently, the growth assay has fallen out of favor as a method for determining dietary protein quality and has been superseded by the PDCAAS. Characteristics of the rat-growth assay for evaluating protein quality have been reviewed[33,34]:

$$\text{PER} \equiv \frac{\text{Weight gain (P1)}}{\text{Weight gain (casein)}} \tag{3.1}$$

3.2.3 Nitrogen Balance and Protein Quality

NBL is the difference between dietary protein intake and the amount of nitrogen excreted (Equation 3.2). NBL reflects the balance between tissue protein synthesis and degradation within the body. Interestingly, NBL reflects the health of the consumer as well as the nutritional quality of dietary protein consumed. Tissue protein synthesis occurs only when all the nine EAA are provided within the diet. Where one or more EAA becomes limiting, then other available AA (both essential and nonessential) are oxidized to produce fuel, CO_2, and urea. Worse still, tissue EAA stores can be mobilized, in an attempt to adjust for inadequate dietary supplies, leading to increases in protein turnover (Chapter 4). A persistent undersupply of a limiting EAA within the diet leads to a negative NBL where the quantity of protein nitrogen excreted exceeds the amount of protein nitrogen consumed. NBL tends toward negative values during EAA deprivation as a result of starvation and also as a result of illness (Section 2.4.2). Equation 3.2 shows a working relation used by nutritionists to estimate nitrogen balance, where 24 h UUN refers to the 24 h urinary urea nitrogen and 4 g refers to an estimated amount of nitrogen lost via fecal, dermal, and non-urea losses[11]:

$$\text{NBL} = \left(\frac{\text{24 h Protein intake (g)}}{6.25} \right) - \text{24 h UUN (g)} + 4\ \text{g} \tag{3.2}$$

Brown and Cline[35] demonstrated that the NBL for pigs could be modified depending on the amount of EAA added as dietary supplement (Figure 3.2). The amount of urea-nitrogen excretion decreased in direct proportion to increasing EAA (lysine) added, reaching a plateau at high levels of addition. This study confirmed that NBL is determined partly by dietary protein quality (EAA profile). For healthy subjects, the NBL takes on a positive value (protein intake > protein excretion), zero value (protein intake = protein excretion), or negative value (protein excretion > protein intake), depending on the dietary protein intake/quality. Current models explaining how dietary EAA availability regulates protein synthesis will be found in Section 3.5.3.

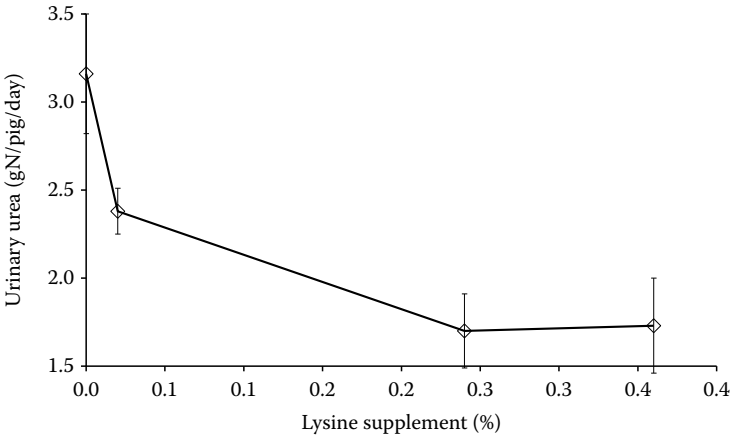


FIGURE 3.2 Effect of the EAA (lysine) intake on the urinary urea-nitrogen excretion from pigs fed a corn diet. (From Brown, J.A. and Cline, R., *J. Nutr.*, 104, 542, 1974.)

3.2.4 Dietary Protein Digestibility Relation to Nitrogen Balance

The RDA for dietary protein is dependent on their digestibility. Fecal digestibility is the difference between the amount of protein consumed and the amount excreted in the stool. Taking note of urinary nitrogen excretion yields the *apparent digestibility*.* Another measure of digestibility termed the "true digestibility" can be determined by correcting nitrogen losses for the amount of human (endogenous) protein that enters the GI tract along with a meal. Dietary protein nitrogen flowing beyond the small intestines is assimilated by colonic bacteria to form microbial protein, which may or may not contribute to human physiological function. Apparently, some of the protein metabolized in the colon may be absorbed, recycled to the upper gastrointestinal tract leading to underestimation of the amounts utilized by the body. Colonic digestion and utilization of dietary protein remains poorly understood at present.[36–39]

$$\text{Apparent(N)digestibility} = \left(\frac{\text{Ingested} - (\text{Fecal} + \text{Urinary})}{\text{Ingested}} \right) \tag{3.3}$$

The process of protein digestion is well known.† The acidic (low pH) environment of the stomach induces protein unfolding to expose peptide bonds to gastric enzymes. Protein digestion begins with substrate attack by pepsin. The polypeptide products

* Apparent digestibility is analogous to nitrogen balance (NBL).

† Digestion is a process of protein degradation that takes place within the GI tract in order to render amino acids available for absorption. Digestibility is an intrinsic property of different proteins that reflects their structure (chemical composition, 1, 2, 3, and 4 structure).

of pepsin digestion undergo further cleavage in the small intestines under the action of proteolytic enzymes (trypsin, chymotrypsin, elastase, and carboxypeptidases A and B) in pancreatic juice. Aminopeptidases from the intestinal brush border then act on the small peptides. Di- and tri-peptides produced by gastrointestinal digestion are absorbed by peptide transporters located in the apical membrane of epithelial cells. An estimated 70–100 g/day of external protein enters the GI tract combined with 50–60 g/day of endogenous protein derived from digestive enzymes and exfoliated cells.

Studies with pure proteins show that digestion ends with the production of amino acids and dietary peptides containing 3–6 AA. The digestive capacity for proteins is not normally exceeded even where intake is increased to 300–350 g/day. Nitrogen excreted in the feces contains bacteria (approximately 20% of fecal mass is bacteria) as well as protein from the intestinal walls, some digestive proteases, and mucins. There are limitations on the interpretations attached to NBL data because of the diverse sources of nitrogen found in the feces. As an alternative to NBL studies, more reliable measurements of protein synthesis, degradation, and turnover can be achieved using isotopically labeled amino acids as tracers (Chapter 5).

3.2.5 Ileal Digestibility and Net Postprandial Protein Utilization

Dietary protein digestion from the stomach to the start of the large intestine is termed ileal digestibility. Estimates of ileal digestibility can be obtained by feeding stable isotope (^{15}N and ^{13}C) labeled dietary proteins to healthy volunteers and sampling digestion products directly from the ileum using nasogastric tubing. These measurements are difficult and costly because of the need for surgical expertise. However, highly precise data can be obtained using nontoxic labeled dietary proteins to differentiate unlabeled (human) proteins entering the GI tract. The tracer methods also provide unprecedented data on the kinetics of protein digestion and absorption.[40,41,*]

The ileal digestibility for raw egg was estimated as 51.3 ± 9.8%, increasing to 90.9 ± 0.8% after cooking compared to a faecal digestibility of ~98%.[42,†] There was a negative correlation between the quantity of $^{13}CO_2$ exhaled and the amount of ^{15}N excreted following the consumption of labeled egg protein, indication that both indices reflect protein digestibility and absorption. During feeding, approximately 40%–50% of human proteins entered the gastrointestinal tract.[42] The levels of endogenous proteins varied according to the protein meal, type of food, and content of dry matter. Reabsorption of endogenous protein is necessary to avoid a negative NBL. The oral ileal digestibility of milk protein was ~95.5% compared to the fecal digestibility of ~98%. Interestingly, 20% of ingested milk

* See Chapter 4, also Section 4.1.3.

† The study compared the ileal digestibility of raw and cooked egg for five human subjects, each fitted with an ileostomy bag. Using protein double-labeled with N(15) and C(13), investigators could monitor both protein utilization as well as oxidation of amino acids (to labeled CO_2).

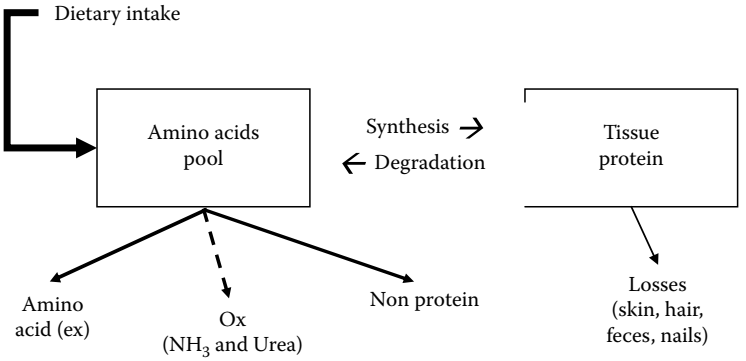
protein underwent oxidative deamination as monitored from the appearance of ^{15}N in the body urea pool versus the amount excreted in urine. The net postprandial protein utilization (NPPU = (Absorbed − Deaminated)/Ingested) was 81.0% measured at 8 h after milk ingestion.[43] The consumption of pea protein was associated with an ileal digestibility of 89.4%. The amount of ^{15}N amino acids within the ileum reached a plateau between 5 and 8 h, though plasma levels of ^{15}N amino acids reached a maximum after 2 h and declined gradually while remaining above prefeeding levels for 24 h. The first ^{15}N-labeled compounds appeared in the urine 60 min after a meal. Approximately, 20%–30% of pea or soybean protein undergoes oxidation and deamination while 72%–78% was directed toward maintenance.[44] Heat treatment of raw pea improved its nutritional characteristics including digestibility without adversely affecting its bioactivity as a cholesterol-lowering agent.[45] The ileal digestibility of ^{15}N-labeled soybean protein isolate was 91%.[46] The low digestibility of buckwheat protein is thought to be related to its function as a cholesterol-lowering agent.[47] In summary, the NPPU for high-quality proteins is 70%–80%, which is the percentage incorporated into body tissue daily. Approximately 20% of dietary protein intake is oxidized.

3.2.6 Dietary and Body Protein Balances and Transformations

The average 70 kg human body contains ~11 kg of total protein distributed between the liver, kidneys, heart, intestines, skeletal muscles, skin, etc.[1,48] The total protein reserves within the body remain relatively constant under normal circumstances. In addition, various body compartments (plasma, intracellular, extracellular compartments) contain only small quantities of free EAA. Major losses of EAA occur predominantly from tissue (muscle)-bound EAA. The ratio of bound amino acid versus free amino acids dissolved in body fluids ranges from 15 (glutamate and glutamine) to 506 (leucine). The free amino acid pool exchanges continually with tissue protein EAA. On average, about 70–100 g dietary protein is metabolized daily, for four basic processes: (a) synthesis of body tissue, (b) excretion as free amino acids, (c) oxidation, or (d) transformation to nonprotein products (Figure 3.3). Coincidentally, a quantity of protein equivalent to 90%–95% of RDA is oxidized daily.* By comparison, approximately 300–350 g of body protein is broken down and resynthesized into new proteins daily.

The contribution of the kidney and liver (viscera) to whole body protein turnover is out of proportion to their size (Chapter 5). A small quantity of protein nitrogen is lost in the form of actual amino acids. Protein-oxidation losses during illness are discussed in Section 3.4.1.

* Only 20% of the daily urea excretion is derived from dietary protein. NPPU estimates for healthy subjects indicate that ~70%–80% of dietary protein is incorporated into body tissue (see Section 3.5). Therefore, ~70%–80% of daily urea excretion is derived from tissue protein degradation. These balance considerations underscore the extent to which the rate of urea excretion is normally disconnected from levels of intake.



Compound	N_2 (g/day)	Protein (N*6.25)	%
Urea	12.8	80.0	84
Ammonia	0.7	4.4	5
Amino acids	0.7	4.4	5
Creatine/creatinine	0.7	4.4	5
Uric acid	0.3	1.9	2
Hippuric acid	0.1	0.6	1
Total	15.3	95.6	100

FIGURE 3.3 Body protein and amino acid metabolism. Losses of free amino acids occur due to excretion (ex), oxidation (Ox), or via nonprotein (nonprotein uses). Physical protein losses occur via the feces, nails, hair, and exfoliated cells via skin and intestines. Numerical data show approximate distribution of nitrogen and protein in urinary constituents in humans on a 100 g protein/day (~16 gN) intake. (Adapted from Lupton, J.R. et al., Protein and amino acids, in *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids, Macronutrients*, The National Academies Press, Washington, DC, 2005, pp. 589–768. Available from http://www.nap.edu/catalog/10490.html, accessed June 07.)

3.3 PROTEIN REQUIREMENTS AND HEALTH

3.3.1 Adults

The RDA for dietary protein is normally estimated by experts from the levels of EAA needed to replace the so-called obligatory loss due to protein metabolism.[48] The typical adult requires about 0.34 g/kg BW/day (20 g of protein) to replace tissue protein losses via the feces, urine, skin, and sweat. The actual daily requirement of protein is set at ~0.6 g/kg BW/day and a further safety margin of about 0.15 g/kg BW/day is then added to allow for uncertainty in present methods. The RDA for dietary protein is therefore set at 0.8 g/kg BW/day (~56 g/day) for an average healthy adult. The RDA is then adjusted for various "special needs" or groups, for example, the young, aging adults, and lactating women (Table 3.4). According to a recent meta-analysis by Rand et al.,[49] the results from 19 clinical trials dealing with NBL measurements suggest that the RDA for dietary protein should be of the order of 0.65–0.83 g/kg BW/day for healthy adults. Values for the RDA were apparently not dependent on gender, adult age, climate, or source of dietary protein.

TABLE 3.4
Recommended Daily Allowance of Dietary Protein for Special Needs

Special Needs	RDA (g/kg BW/day)	Total Intake
Preterm infants	3.0–4[a]	—
Infants 0–6 months	1.52–2.2	—
Infants 6–12 months	1.2–1.6	—
Children 1–13 years	0.87	—
Adolescents	0.85	52 (boys), 46 (girls)
Adults	*0.8*	—
Pregnancy	1.1	25
Lactating	1.3	25

Source: Adapted from Lupton, J.R. et al., Protein and amino acids, in *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids, Macronutrients*, The National Academies Press, Washington, DC, 2005, pp. 589–768. Available from http://www.nap.edu/catalog/10490.html, accessed June 07.

[a] Values of 2.2.–3.0 are given by other literature.

3.3.2 Protein Requirements for Aging Adults (Elderly)

The protein needs for aging adults (55–90 years) is a controversial topic that has been debated for many years.[50–54,*] According to one view, the RDA should be no different for young and aging adults (0.6–0.8 g/kg BW/day). Milward et al. showed that the RDA for dietary protein decreases from 0.99 g/kg BW for children to a final value about 0.69 g/kg BW for all adults. They found no strong case for revising current RDA for the aging adults.[52] However, this view is disputed by others. Campbell et al. showed that a protein intake of ~0.6 g/kg BW/day led to a negative balance and suggested that a protein intake of ~1 g/kg BW/day was needed for positive NBL in aging adults.[50] Fereday et al. found that the protein needs for aging adults (5 men and 5 women; age range 68–91 years) was 0.66 g/kg BW/day equivalent to 0.79 g/kg FFM[†]/day, which is 30% lower compared to values for younger subjects.[55] Later studies by Campbell et al. found that the RDA was not significantly different for 19 aging adult subjects (8 men and 11 women; age range 63–81 years) compared to younger adults (11 men and 12 women; age range 21–46 years). The RDA was 0.61 ± 0.14 for younger adults and 0.58 ± 0.12 g/kg BW/day for ageing adults.[56] Accordingly, they could see little support for increasing RDA values for healthy aging adult subjects from the current value of ~0.8 g/kg BW/day.

* Muscle loss increases above the age of 50 years, leading to a condition described as sarcopenia.

[†] FFM = fat free mass.

Defining protein requirements for sick or convalescent aging adults is also problematic. Wolfe et al. suggest that the RDA for dietary protein should be increased for aging adults from a current value of 0.8 to 1.2–2 g/kg BW/day in order to address symptoms of sarcopenia, undernutrition, insulin resistance, and anabolic dysfunction, *which are less frequent in younger adults.* Another case for increasing the current RDA considers the balance of macronutrient intake required for health. The RDA for dietary protein was estimated at ~2 g/kg BW/day for a 70 kg adult with a total caloric intake of 1500 kcal/day assuming that 15% caloric intake is protein.[57] Refer to the following reviews for further discussions on protein intake for the aging adults[58,59].

3.3.3 Protein Requirements for Exercise

Resistance exercise has been proposed as therapy for muscle wasting. However, the effect of exercise on dietary protein requirements remains uncertain.[60–63] There is no general agreement whether athletes need more dietary protein compared to age-matched, non-trained subjects. The discussions are polarized into three points of view: (a) resistance exercise should be accompanied by increased protein intake to address increased wear and tear for elite athletes, (b) the adult RDA for protein (0.8 g/kg BW/day) is adequate to supply the dietary needs for athletes, and (c) dietary protein intake may be adequate but there may be benefits from increased use of bioactive or "ergogenic" agents (e.g., leucine and hydroxymethyl butyrate). It has also been suggested that optimum protein intake for athletes should take account of different types of (endurance vs. resistance) exercise, the timing of the intake (better before than after), co-ingestion of protein with other nutrients (glucose helps in muscle building), and adequacy of dietary energy supply.

Protein supplementation at the level of 1.2–1.7 g/kg BW/day may be useful or at least not harmful for athletes. Clinical trials published between 1967 and 2001, dealing with dietary supplement use by athletes were examined by Nissen and Sharp.[64] Of the 250 different types of supplements subjected to meta-analysis only two had a significant effect on lean muscle mass, that is, creatine > hydroxymethyl butyrate ≫ protein. Study-related methodological issues and problems were highlighted, particularly centering on the lack of sensitivity and the limitations of nitrogen balance as an index for monitoring dietary protein needs for athletes.[65] It is generally agreed that protein needs—for endurance and resistance exercise—may be different with no specific gains expected in the former case.[66] Overall, there is no general consensus on whether strength exercise should be accompanied by increased protein intake.[58,67] Athletes that tend to have a high caloric intake also consume more than the current RDA for dietary protein by virtue of their need to maintain a balanced macronutrient intake, for example, 15% caloric intake as protein.

3.3.4 Preterm Infants and Children

The RDA for dietary protein for preterm babies is estimated at 3–4 g/kg BW/day compared to 4 g/kg BW/day for infants within the uterus.[68–70] The actual protein

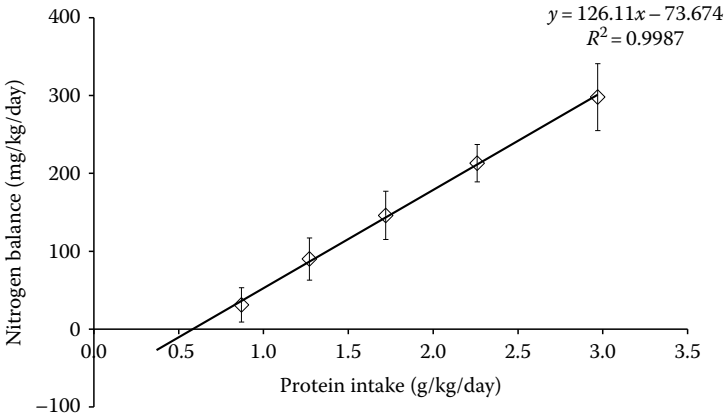


FIGURE 3.4 Effect of dietary protein intake on nitrogen balance for preterm infants fed a specialized formula by mouth. Minimum protein requirements can be read from the $y = 0$ intercept as ~0.58 g/kg BW/day. (From Zello, G.A. et al., *Pediatr. Res.*, 53, 338, 2003.)

requirement necessary to achieve protein balance for preterm babies is estimated at 1–1.5 g/kg BW/day from measurements of nitrogen balance.[71] Lower estimates for the minimal protein needs (0.51–0.75 g/kg BW/day) were obtained using nonradioactive isotopic labels[72] (Section 5.4.1). Preterm babies fed with protein at 0–3 g/kg BW/day increase muscle weight in direct proportion to dietary amino acid intake. This extraordinarily high anabolic drive (growth responsiveness to amino acid feeding) declines with increasing age. The utility of aggressive nutritional support for preterm infants is currently under discussion (Figure 3.4). According to other estimates, protein requirements decline from ~1.5 g/kg BW/day at 0 years of age to 0.8 g/kg BW/day for teenagers and adults. Burrin and Davis suggested that bioactive amino acids may one day be exploited for nutritional support of preterm babies.[73]

3.4 DIETARY PROTEIN AND HOST RESPONSES TO ILLNESS

3.4.1 Urea-Nitrogen Losses during Illness

The quantity of urea excreted in the urine increases during illness in accordance with rising muscle protein breakdown. During illness, muscle EAA are mobilized for the production of defense proteins (acute phase proteins, APP) in the liver. There is also increased conversion of amino acids to glucose to provide cells with energy. These changes lead to a declining NBL value during illness that correlates with weight loss, severity of illness, and the number of days with fever (Figure 3.5). Observations using pair-fed subjects indicate that only ~35% of the changes in NBL during illness were due to decreased food intake arising from anorexia.[74–79,*] The reasons for the accelerated losses of nitrogen from the body during illness are not entirely clear at present. However, most of the urinary nitrogen is derived from amino acid oxidation (see Section 4.1.5).

* In any event, dietary protein intake tends to be reduced due to illness anorexia.

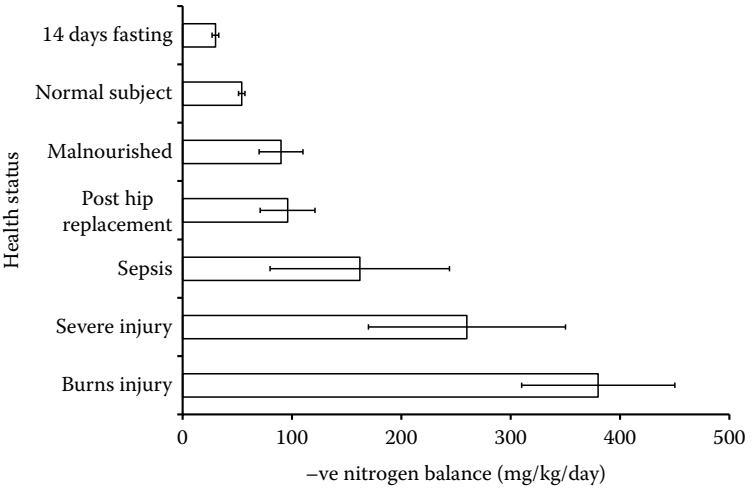


FIGURE 3.5 Effect of illness on nitrogen balance. (From Furst, P., *Nutrition Support for the Critically Ill Patient. A Guide to Practice*, CRC Press, Boca Raton, FL, 2005.)

Derangements in protein metabolism can be considered part of the host response to illness (Chapter 2). For instance, infections by pathogens lead to a need for enhanced barrier function at epidermal surfaces/mucosal layers in the digestive tract and airways. The host immune response is also activated following injury and infection, leading to production of antimicrobial peptides, synthesis of antibodies, and the activation of immune cells.[80] It may be supposed that the host response requires a distinctive mix of EAA, which is derived from muscle stores.[81] Illness has been shown to induce a flow of amino acids (chiefly glutamine and glycine) from muscle to other organs. Glycine is used for gluconeogenesis in the liver whereas glutamine has multiple functions as substrate for GI tract cells and immune cells. Illness decreases plasma levels of most amino acids excepting phenylalanine and tryptophan. Since 70% of the body protein is found in the muscles, mobilization of amino acids during illness has a disproportionate effect on the skeletal and smooth muscles. Loss of skeletal muscle leads to loss of strength and general mobility. Loss of smooth muscle tissue leads to eventual organ failure, affecting most of the systems of the body. Possible consequences of EAA imbalances arising from illness are discussed further below.

3.4.2 Acute Phase Protein Synthesis

Synthesis of APP is a well-known feature of protein metabolism during illness. The positive APPs are plasma proteins whose concentration rises by about 25% within 7 days of an infection.[83–85] By contrast, the concentrations of negative APP decrease in response to illness. Clearly, the formation and degradation of APP in the liver may lead to increased protein turnover during illness (Table 3.5). α-1 anti-trypsin is an enzyme inhibitor whereas ceruloplasmin is a metal chelator for zinc that is required for bacterial growth. In addition to the common APP listed, the concentrations of

TABLE 3.5
A List of Some Acute Phase Proteins Produced in Response to Disease[a]

Protein Class	Positive APP	Negative APP
Protease inhibitors	α_1-Antitrypsin, α_1-anti-chymotrypsin	Inter a-antitrypsin
Coagulation proteins	Fibrinogen, prothrombin	
	Factor VIII, plasminogen	
Complement proteins	C1, C2, B, C3, C4, C5, and C9	Properdin
Transport proteins	Haptoglobin, hemopexin	
Lipoproteins		HDL, LDL
Acute phase proteins	C-reactive protein (CRP)	Serum albumin
	Serum amyloid A, α-acid glycoprotein, Gc-globulin	Prealbumin (transerythrin)
	Ceruloplasmin, fibronectic	Transferrin

[a] Levels of positive and negative APP increase and decrease during illness, respectively.

cytokines, complement proteins, immunoglobulins, and the hormone leptin also rise as part of the body's response to infection. The effect of leptin on food intake is considered in Chapter 9. Blood serum albumin (BSA) and transferrin levels decline following infection or critical injury. The response to injury, infection, and disease is a component of the body's reaction to stress involving the stress hormones and may have survival value.

Research shows that malnourished and infected children are able to mount an APP response, though the magnitude of this response declines with the severity of PEU.[86] Measurement of the rates of synthesis of several APP showed that their levels reflected a balance of synthesis and degradation. Some infections produced increased rates of synthesis of APP whereas others tended to inhibit the rate of APP degradation, especially in the malnourished state with decreased availability of amino acids.

3.4.3 Protein EAA Imbalances during Illness

Dietary protein intake should be increased during periods of illness in accordance with elevations of general metabolism, that is, according to the degree of hypermetabolism observed. The RDA for dietary protein has been set at 1.06 g/kg BW/day, 1.25 g/kg BW/day, 1.56 g/kg BW/day, or 1.87 g/kg BW/day for illness accompanied by zero hypermetabolism, 2%–25% hypermetabolism, 25%–20% hypermetabolism, or >50% hypermetabolism, respectively. Further specific recommendations by the WHO consultative committee on protein and amino acid requirements[1] are summarized in Table 3.6. Beyond these recommendations, it has been suggested that renal failure may require reduced protein intake in order to reduce the strain on the kidneys. Interestingly, protein requirements during renal failure remain controversial because this condition is also associated with the loss of lean body mass that may be treatable by increasing protein intake.[87]

TABLE 3.6
Recommended Daily Allowance for Protein during Illness

Conditions	Additional Need (% RDA)
Untreated tuberculosis	25
Partly treated melioidosis	15
HIV (symptom free)	50
Intestinal parasites	10
Acute bacterial infection	20
Acute diarrhea	30
Sepsis	30
Burns injury	[a]

Source: Lupton, J.R. et al., Protein and amino acids, in *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids, Macronutrients*. The National Academies Press, Washington, DC, 2005, pp. 589–768. Available from http://www.nap.edu/catalog/10490.html, accessed June 07.

[a] Values differ according to % of body surface affected, WHO.

It is generally believed that muscle wasting occurs against a background of increased protein turnover (i.e., increased degradation and protein synthesis) during illness (Chapter 4). The contributions of different organs to net protein synthesis may be altered during illness. The splanchnic organs (liver, spleen, and intestines) show rising levels of protein synthesis whereas muscle protein synthesis decreases during illness.[88–92] The APPs are produced in the liver using glutamine, alanine, and EAA derived from the muscle. Since the average EAA composition of APP is different from the EAA composition of muscle protein, transforming muscle proteins to APP is likely to be highly inefficient. The disparity between EAA profile for APP synthesis and EAA derived from muscle would provide an imbalance of EAA supply versus needs, which could create the tendency for wasting.[93–96]

Considerations of the EAA imbalance arising from illness led to a proposed three-stage strategy for preventing muscle wasting[97]: (a) Determine the pathways activated by particular illnesses—We know that the host response to illness is moderated by increasing proinflammatory cytokines and corticosteroids that together stimulate APP synthesis, activate the innate immune (inflammation) system and tissue oxidative stress. The argument goes that these responses involve the production of a limited set of defense proteins. (b) Assess the EAA requirement for host-response proteins (the average EAA composition of APP can be estimated). Initial estimates suggest that on average, the APP are higher in aromatic amino acids (tyrosine, phenylalanine, and tryptophan) compared to normal

EAA requirements. Oxidative stress can be expected to lead to increased requirements for sulfur amino acids. Glutamine serves as a substrate for immune cells. (c) Provide specific EAA supplements to correct the EAA imbalance associated with host response to illness; for instance, methionine requirements have been shown to be increased during sepsis and aging.[98,99] A brief moment of reflection suggests that this approach is probably oversimplistic. EAA supplementation is discussed in Section 8.5.1.

3.5 PEPTIDES AND PROTEIN BIOACTIVITY

3.5.1 Essential Amino Acid and Dietary Protein Meta-Nutrients

The health benefits of EAA, dietary proteins, and peptides cannot be explained only in terms of their nutritional roles. Therefore, quality indices for dietary protein might be expanded to include their drug-like effects.[75,100–103] As discussed earlier, inadequate intake of EAA leads to muscle wasting, owing to a lack of adequate material for tissue building. Though the overall RDA for protein (g/kg BW) decreases between the ages of 0 and 5 years, reaching a plateau at >5 years of age, the proportion of dietary EAA utilized for maintenance (as opposed to growth) increases between the ages of 0 and 18 years (Figure 3.6). Proper functioning of muscles and organs requires adequate intakes of EAA. During periods of illness, EAA are needed to enable the synthesis of APP, immune components,

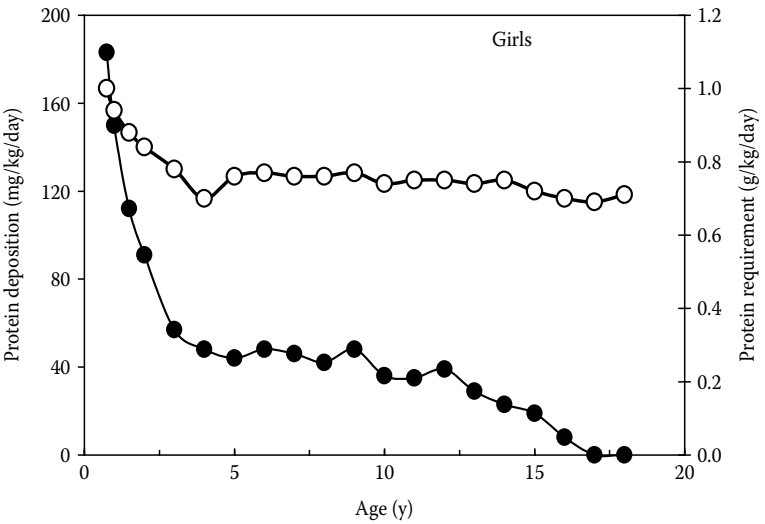


FIGURE 3.6 Effect of aging on protein requirements (open circles) and protein deposition (closed circles). (From Lupton, J.R. et al., Protein and amino acids, in *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty acids, Cholesterol, Protein and Amino Acids, Macronutrients*, The National Academies Press, Washington, DC, 2005, pp. 589–768. Available from http://www.nap.edu/catalog/10490.html, accessed June 07.)

and the initiation of host response to illness (Figure 1.4). Another well-known benefit of an adequate EAA supply is the maintenance of gut barrier function to reduce the risk of host invasion by gut bacteria and septicemia.[104]

Glutamine provides a useful example of the pertinent issues. The level of glutamine in the healthy human body is among the highest of all the amino acids, so this is not an EAA. Nevertheless it is thought that levels of glutamine may be depleted during periods of illness. Investigations spanning several decades suggest that glutamine might be useful as a substrate for gut mucosal cells. It was also suggested that glutamine may function as an exceptional substrate for lymphocytes. Glutamine is also required for transamination and oxidative deamination, which are important reactions for protein-nitrogen transfer, urea synthesis, and gluconeogenesis. Finally, glutamine is important for acid–base homeostasis and for the production of the intracellular antioxidant glutathione[105] (Table 3.7). The therapeutic benefits of amino acids such as glutamine can also be described in terms of well-documented nutritional concepts.[106,*]

TABLE 3.7
Suggested Health Benefits of Glutamine Supplementation

Immune cell substrate
Gut barrier function ↑
Gut inflammation ↓
Gut mucosa growth ↑
Immune cell number ↑
Muscle and plasma glutamine↑
Nitrogen retention ↑
Neutrophil and monocyte bacterial killing ↑
Reactive oxygen species synthesis in monocytes ↑
Substrates for lymphocytes and macrophages ↑
Tissue glutathione ↑
T lymphocyte recovery after chemotherapy ↑

Anti-inflammatory activity (see Chapter 6)
Phospholipase A2 Activity ↓
Cellular adhesion molecules ↓
Nuclear factor kappa beta activation ↓
Activation of tight junction proteins
Endothelial cell nitric oxide synthesis ↓
Proinflammatory cytokine release ↓

Source: Ziegler, T.R., *J. Nutr*., 131, 2578S, 2001; Biolo, G. et al., *Curr. Opin. Clin. Nutr. Metab. Care*, 6, 55, 2001

* Glutamine is a nonessential amino acid though supplies are thought to become restrictive during stress and illness. Perhaps for such reasons glutamine is used widely as a component for medical foods and is generally considered as an immunonutrient (Chapter 3). Potential anti-inflammatory activity of glutamine is discussed in Chapter 6.

There are, however, other attributes of glutamine such as the ability to moderate inflammatory response that require other models, such as the ability to moderate cell signaling. Current research suggests that leucine and glutamine have antagonistic effects on cell growth though acting via similar signaling pathways; whereas leucine increases muscle cell size, glutamine favors an increase in cell numbers.[107,108] To summarize, the nutritional roles of EAA are well established. However, the EAA, conditionally EAA, and food-derived peptides can also produce drug-like, meta-nutritional effects.[31–34,106,108–111] Given that dietary proteins have beneficial effects on health, it may be important to differentiate nutritional from non-nutritional effects. The hallmarks of protein and EAA bioactivity are addressed in the remainder of this chapter.

3.5.2 Leucine and the Branched Chain Amino Acids

The effect of leucine and other EAA intake on muscle protein metabolism has been studied intensively by researchers from the Hershey Medical Center for nearly 45 years. Initial investigations of Milton et al. published in the 1970s showed that perfusion of rat heart with amino acids led to a rise in the formation of polyribosomes.[112,*] The ribosome–mRNA complex is needed to allow information encoded within mRNA to be translated into a string of amino acids within a protein. Later studies using isolated rat diaphragm muscle exposed to amino acids by Fulks et al., and also Maria Buse and Sandra Reid, identified leucine as a key amino acid that stimulates protein synthesis.[113,114] By contrast, a mixture of 18 amino acids did not stimulate muscle protein synthesis when leucine was excluded. These studies suggested also that the anabolic effect of dietary amino acid could be attributed to leucine but not isoleucine and valine. The three branched chain amino acids (BCAA) produced no greater effect than leucine alone.[115] Valine and isoleucine were able to stimulate protein synthesis under some circumstances, for example, with rat diaphragm from diabetic rats. BCAA were also found to decrease muscle protein breakdown, though the effect was less marked compared to stimulation of protein synthesis. The anabolic effects of BCAA are independent of the effects of insulin and are observed when the synthesis of new mRNA is blocked using actinomyin D.[115]

Later studies showed that, amino acids aside from leucine (e.g., methionine, cysteine, threonine, and tyrosine) may also stimulate muscle protein synthesis. However, leucine is by far the most well-understood anabolic amino acid (see below). Isoleucine, valine, arginine, lysine, phenylalanine, tryptophan, and glutamine are thought not to affect protein synthesis in muscle cells. The anabolic effect of leucine occurs independently of insulin rise.[116] Effects of feeding depend on the type of protein, age of the consumer, and frequency of eating.[117–119] For elderly subjects, a pulse diet (where all protein is fed at once) is more efficient in stimulating muscle protein synthesis than a spread diet where protein is fed evenly throughout the day.[120] Most experts now interpret feeding effects on muscle size, in terms of the anabolic response to leucine. A great deal of the recently published research on the anabolic effects of EAA focused on the effect of leucine rather

* From the Milton S. Hershey Medical Center, Penn State University.

than BCAA as a whole[121–128] (see Section 3.5.6). However, it may be that undesirable effects of leucine can be avoided by using BCAA (Chapter 9).

3.5.3 Nutrient Signaling and Gene Interactions

Nutrients are described as bioactive if they moderate gene expression at the level of DNA transcription, mRNA translation, or protein/enzyme (de)activation (Figure 3.7). For instance, oil-soluble vitamins bind with cytoplasmic transcription factors that function as nutrient sensors. The ligated sensors then transfer to the nucleus where they interact with target genes having consensus binding sites. Other nutrient sensors are resident in the nucleus and are transferred outside following nutrient binding.[129] Approximately 50 cytoplasmic transcription factors have been described so far, including nutrient receptors for oil-soluble vitamins (e.g., the retinoic acid receptor [RAR], vitamin D receptor [VDR], unsaturated fatty acids sensor (peroxisome proliferator-activated receptor, PPAR)) and the cytoplasmic transcription factors for low molecular weight hormones (e.g., estrogen receptor [ER], glucocorticoids receptor [GR], and thyroid hormone receptor [TR]). The nutritional role of vitamin E as an antioxidant is relatively well known. Interestingly, vitamin E has been shown to moderate cell proliferation by inhibiting protein kinase C (PKC).[130] α-tocopherol regulates the expression of five major groups of genes related to (a) vitamin uptake and metabolism, (b) lipid metabolism and atherosclerosis, (c) biosynthesis of extracellular matrix components, (d) regulation of cell invasion and adhesion, and (e) cell proliferation.[131] Modulation of gene expression is considered one criterion for differentiating nutritional and bioactive roles of EAA. Current nutritional explanations for dietary protein health benefits do not take account of recent information showing that EAA are involved in regulation of gene expression.[132,133]

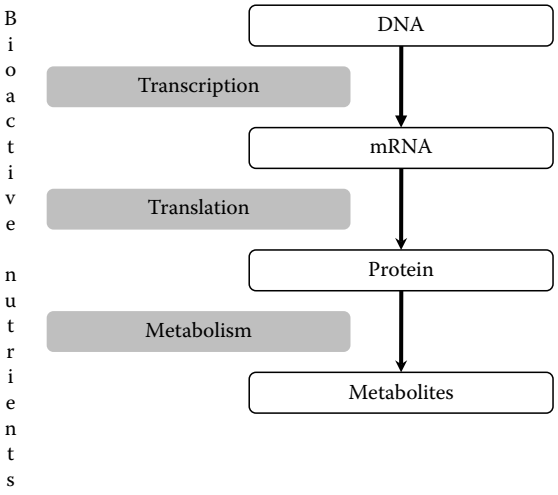


FIGURE 3.7 Effect of bioactive nutrients on gene expression. (Adapted from Davis, C.D. and Milner, J., *Mutat. Res.*, 551, 51, 2004.)

3.5.4 Receptor Activation by Bioactive Peptides

Nutrients with low membrane permeability act through membrane surface receptors or are conveyed inside cells by dedicated cell transporters. Endogenous peptide hormones (e.g., secretin, glucagon, and calcitonin) bind to G-protein-linked membrane receptors located on the cell surface leading to the activation of intracellular signals. The G-protein linked receptors belong to a large family of receptors, identified by their characteristic 7-transmembrane peptide segments. Many G-protein receptors remain in "orphan" status awaiting discovery of a natural ligand. Another important cell membrane receptor family is the tyrosine-kinase-linked receptor family, employed by growth factors, insulin, and cytokines. Some membrane receptors become transiently activated by association with gated ion channels. Cells also possess transporters for amino acids and peptides that may be involved in sensing and intracellular signaling.

Food-derived opioid peptides were some of the earliest bioactive peptides isolated outside of the human body. The painkilling effect of opiate drugs such as morphine has been known since the 1800s. The receptors for opiate drugs began to be characterized in the 1970s. Endogenous compounds with morphine-like activity (so-called endorphins) were first isolated in the mid-1970s. The four endorphins are brain peptides: β-endorphin, leu-enkephalin, met-enkephalin, and dynorphin. Like morphine, endorphins have analgesic effects and also increase feeding activity in rats.[135,*]

Investigations reported in 1978 showed that the products of casein and wheat gluten digestion by pepsin contain peptides that bind opioid receptors in vitro, monitored by their interference with naloxone-reversible inhibition of adenylate cyclase from homogenates of neuroblastoma × glioma hybrid cells. These so-called exorphins (exogenous compounds with morphine-like activity) could displace radiolabeled ligand (enkephalin ([3H] dihydromorphine and [3H-Tyr, D-Ala2]met-enkephalinamide)) from crude rat opioid receptor preparations.[136] Other food exorphins were discovered in the early 1980s, mainly from milk protein hydrolysis products including β-casomorphin (TyrProPheProGlyProIle) and morphiceptin[137] (see[138,139] for current developments in milk-derived bioactive peptides with opioid activity). In addition to the opiate-like exorphins, thyrotrophin-releasing hormone-like peptides and cyclic-hystidinyl-proline (cyclo-His.Pro) were found in some commercial high-protein supplements and protein digestions (Table 3.8). The study of bioactive peptides has grown enormously in the past 20–30 years.

3.5.5 Amino Acid Deprivation and Growth Retardation

The effects of EAA deprivation on human health is partly mediated by changes in gene expression (Figure 3.8). Initial experiments using yeast cells showed that elimination of a single amino acid from growth media could switch on genes coding for enzymes utilized for amino acid metabolism. The concentration of membrane transporters for amino acids also increases in response to amino acid starvation.[150–153]

* See the following link for a useful introduction to dynorphin and other opioid peptides and their effect on appetite: http://en.wikipedia.org/wiki/Dynorphin.

TABLE 3.8
Chronological Development of Dietary Bioactive Peptides

Year	Description/Comments
1979 Casomorphins	Opioid receptor active peptides (see text)
1980 Calcium phosphopeptides	Casein digest promotes calcium absorption[140]
1981 Thyrotrophin-releasing hormone	Alfalfa plant[141]
1982 ACE inhibitors	Trypsin digests of casein, hypotensive agents[142]
1984 Natriuretic peptide	Digests of cardiac muscle, muscle relaxant[143]
1984 Immunomodulatory peptide	Encrypted casein sequences[144]
1988 Antimicrobial peptides	Intact lactoferrin[145]
1991 Antimicrobial	Hydrolyzed lactoferrin fragment[146]
1992 Antithrombotic peptides	Milk peptides prevent blood coagulation[147]
1992	Cyclo His. Proline dipeptide[148,149]

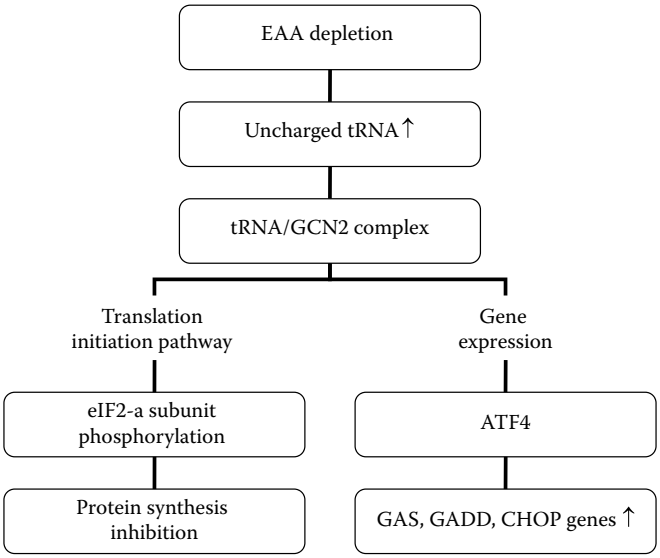


FIGURE 3.8 Depleted supplies of essential amino acids inhibit protein synthesis initiation and moderate the expression of stress-related genes (see text for details).

According to prevailing models, EAA limitation leads to rising concentration of free tRNA that moderate the activity of key enzymes involved in protein synthesis. Free tRNA binds a regulatory domain of the eukaryotic initiation factor 2-kinase (*eIF2* kinase).* The four enzymes belonging to this group play a vital role in the initiation of protein synthesis. The tRNA-activated eIF2 kinases catalyze the phosphorylation of α-subunit (Ser 51) of eIF2 and disables it from producing the eIF2α/tRNA-methionine complex required for protein synthesis initiation. Owing to the low specificity of

* Recent data suggest that eIF2 kinase is also a substrate for mTOR which is responsive to leucine.

eIF2 kinases for 20 different tRNA, these enzymes serve as indirect sensors for amino acid depletion resulting in the global inhibition of protein synthesis.

The eIF2 kinase also called GCN2 (general control non-derepressible-2) or eIF2α kinase 4 is involved in the detection of EAA depletion in mammalian cells. GCN2 is also implicated in the inhibition of protein synthesis arising from oxidative stress and exposure to UV radiation. Responses downstream of GCN2 are mediated by NFκβ activation.[154] Compared to wild-type mice GCN2 knockout, $GCN2^{-/-}$, mice are found to be more sensitive to leucine restriction as indicated by a more scruffy appearance, decreased number of offspring, greater loss of weight, increased general mortality due to nutrient restriction, less efficient downregulation of liver protein synthesis due to starvation, and increased muscle protein losses probably due to catabolism.[155]

Four different eIF2 kinases have been identified in mammalian cells that are involved in the inhibition of translational initiation in response to stresses; for example, PKR, HRI, PEK, and GCN2 (Table 3.9).* PKR moderates protein synthesis in response to viral infection. HRI is found in erythroid cells, where it controls protein synthesis in response to iron depletion. The third member of the eIf2 group is PEK, which responds to increased concentration of mis-folded proteins linked with endoplasmic reticulum stress. Finally, GCN2 is involved with signaling EAA depletion.[156,157]

EAA depletion has also been shown to moderate stress-related genes directly. Marten et al. classified three groups of genes in cultured liver cells that are induced (Class 1), repressed (Class 3), or unaffected (Class 2) by EAA depletion. Depletion of EAA decreased the expression of mRNA for serum albumin, transerythrin, transferrin, and urate oxidase, and was linked with activating transcription factor 4 (ATF4) pathway.[158] Apparently, the expression of ATF4 is upregulated by phosphorylated eIf2 binding to the open reading frame (ORF) in the 5′untranslated region of its mRNA. Newly produced ATF4 protein then binds to amino acid responsive elements (AARE) of DNA to induce target genes.[159] Another stress-related gene induced by EAA restriction is the growth arrest and DNA damage (GADD153) gene sometimes called the *C/EBP* homology protein (CHOP) gene. CHOP is thought to be important

TABLE 3.9
Eukaryotic Initiation Factor-2 Kinases Inhibit Protein Synthesis Initiation in Response to Stress

Enzyme Names	Stimulants
PKR	Viral infection
HRI (heme-regulated inhibitor or eIF2AK1)	Heme deprivation, oxidative stress, and heat stresses
PEK (pancreatic eIF2α kinase) or eIF2AK3	Mis-folded/unfolded proteins (endoplasmic stress)
GCN2 or eIF2α kinase 4	Amino acid depletion, UV irradiation
	Proteasome inhibition

* PEK = pancreatic eIF2α kinase; PKR = RNA-dependent protein kinase, HRI = haem-regulated inhibitor; GCN2 = eIF2 kinase GCN2 [general control non-derepressible-2. cf. Table 3.9 and Ref. [154].

for the remediation of cell damage or the promotion of programmed cell death. EAA deprivation also affects pathways related to protein breakdown. For instance, leucine deprivation has been shown to activate ubiquitin proteasome pathway as well as the process of autophagy. Starvation effects on muscle cells are described further in Chapter 4.

Finally, EAA restriction is also linked with increasing concentrations of insulin-like growth factor-1 binding protein (IGFBP-1). The growth-promoting effect of IGF-1 is normally moderated by serum IGFBP-1s (Chapter 9). Protein-free diets stimulate IGFBP-1 synthesis while inhibiting IGF-1 synthesis. On the other hand, single EAA restrictions seemed to have no effect of IGFBP-1 levels in contrast to results in vitro.[160] Anthony et al. found that EAA deprivation inhibits protein synthesis initiation in the muscles to a greater extent compared to the liver. GCN2 from peripheral tissue seem to be more sensitive than the corresponding enzymes in the liver. Finally, the eIf2 kinase stress pathway is implicated in the tendency of higher animals to reject foods deficient in EAA in preference for those high in EAA. In summary, current models of nutrient–gene interactions go a long way in explaining some of the dramatic effects of protein deprivation on muscle mass.

3.5.6 Increased EAA Availability and Gene Expression

Enhanced availability of EAA increases mRNA translation via a process that is mediated by eukaryotic initiation factor 4E (eIF4E).[161] Phosphorylated eIF4E binds to the capped end of mRNA allowing the formation of a complex with 40S ribosomal protein. EAA availability increases the phosphorylation and dissociation of eIF4E binding protein (eIF4E-BP) from eIF4E, thereby increasing the rate of mRNA translation.[162] The intracellular sensor for EAA availability is thought to be the enzyme *mammalian target of rapamycin* (mTOR), which catalyzes the phosphorylation eIF4E. The anabolic effects ascribed to leucine are mediated via mTOR,[128,163,*] which are shared by other agents that increase muscle cell size, in particular GH, IGF-1, and insulin (Chapter 8). As noted above, glutamine is believed to inhibit mTOR action in a manner that increases cell number rather than cell size.

3.5.7 Microarray Profiling of Dietary Protein–Gene Interactions

Expression profiling reveals that dietary protein intake or deprivation can moderate gene expression.[164–167,†] DNA microarray analysis showed that compared to a protein-free diet, consuming a 12% casein diet leads to substantive changes in the expression of ~281 genes in the rat liver with 184 and 97 being upregulated or downregulated,

* See Sections 5.5 and 9.1 for further discussion on the regulation of muscle protein synthesis by leucine and related EAA.

† The effect of dietary supplementation on gene expression has been monitored using real-time PCR, using gene knockout mice and DNA microarray technology.

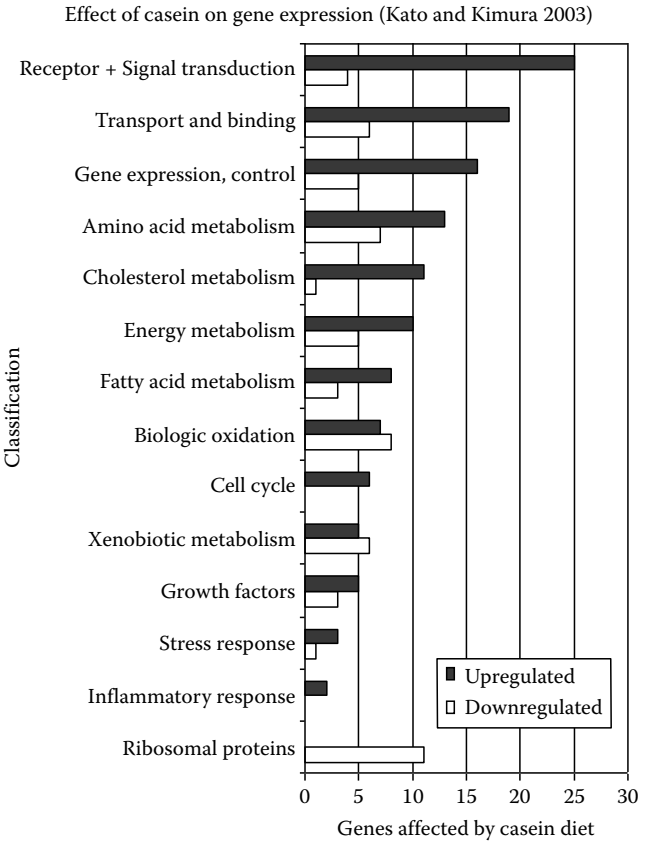


FIGURE 3.9 Effect of casein on rat liver gene expression. Investigations were performed using a commercial DNA microarray gene chip (Affymetrix Ltd., Santa Clara, CA.) that monitors 7000–8000 genes simultaneously. In addition to the results shown here, a further 51 and 34 unidentified genes were upregulated and downregulated by casein diet (results not shown). (From Kato, H. and Kimura, T., *J. Nutr.*, 133, 2073S, 2003.)

respectively (Figure 3.9). The consumption of gluten also had an impact on gene expression. Expression analysis also shows that there are characteristic changes in gene expression during muscle atrophy induced by food deprivation,[168] cachexia,[169] aging, disuse atrophy/bed rest,[170] and renal hemodialysis[171] (Chapter 5). These investigations may one day provide new insights on ways to develop novel dietary supplements for preventing unwanted weight loss.[172] Safdar et al. reported that creatine supplementation assists the retention of muscle mass in young adult subjects. The anabolic effects of creatinine were associated with increased expression of mRNA and protein associated with the regulation of cell hydration, signal transduction, cytoskeleton remodeling, protein synthesis, glycogen synthesis regulation, as well as muscle satellite cell proliferation and differentiation. Other aspects of muscle cell biology were affected, including genes for DNA replication and repair, RNA transcription control, and cell survival.[173]

3.6 TYPES OF DIETARY PROTEIN HEALTH EFFECTS

3.6.1 Types of Health Benefits

The health benefits associated with dietary proteins are only partially related to their nutritional role as building blocks for tissues and substrates for oxidation. In principle, the nonnutritive effects of dietary proteins (amino acids and peptides) are linked with receptor activation or nutrient–gene interactions. Bioactive proteins, peptides and amino acid (more simply, bioactive peptides) might also affect genes related to the host response to illness, for example, immune function, antibiosis, anti-inflammatory activity, and anabolic dysfunction.[174]

At least three types of health benefits can be recognized for dietary proteins some of which are unique to bioactive peptides. For convenience, we will designate "Type 1" benefits as effects that arise from meeting the RDA for dietary protein, taking into account changes during illness.[31,175,176] Type 1 benefits originate from the nutritive role related to the use of dietary EAA as building blocks for protein synthesis and as substrates for cell oxidation. About 50% of absorbed EAA are utilized by cells of the gut mucosa. Excess EAA enter the portal blood circulation and are transported to the liver for processing. EAA surviving first-pass metabolism in the liver (for oxidation, serum protein synthesis, etc.) are transferred to other tissues for metabolism. A small fraction of EAA is also utilized for production of nonprotein compounds. Branched chain amino acids are notable for their low utilization by the gut mucosa or liver. It is thought that approximately 50% of dietary leucine is utilized by the gut mucosa and liver and that the remainder reaches the muscle for metabolism. Type 1 health benefits can be cited to explain the effects of protein deficiency in otherwise healthy subjects.

Type 2 effects refer to health benefits for EAA that arise from their conversion to bioactive intermediates and brain hormones (cf. Appendix 3.A.2). For example, arginine is used in the production of NO, which is involved in the regulation of blood pressure.[177–179] Glycine, glutamine, and tyrosine are utilized for the synthesis of neurotransmitters. Finally, we refer to Type 3 benefits as responses requiring peptide interactions with specific cell receptors, enzyme targets, or transcription factors and that lead to modulation of gene expression (Section 3.5). Type 3 effects include amino acid stimulation of hormone production.[180–183]

3.6.2 Health Benefits and Non-Absorbed Proteins

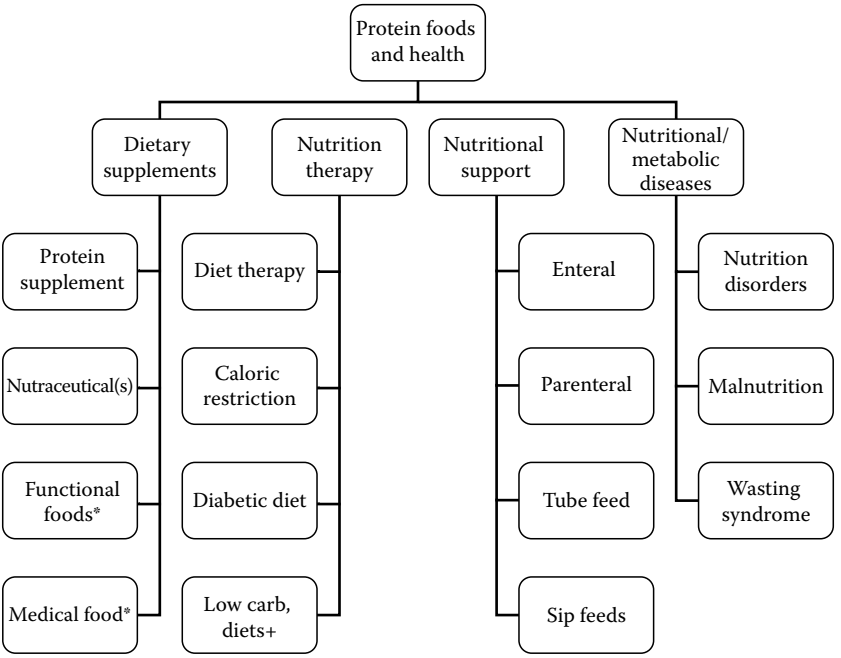
The health impact of non-absorbed bioactive peptides should not be ignored. The classic example of GI tract effects of bioactive peptides is illustrated by the soybean trypsin inhibitor (STI) and related proteins from raw legumes. Trypsin is secreted in the GI tract in response to sensory cues predicting food intake. The demand for trypsin is controlled by negative feedback loop involving the fragmentation of cholescystokinin by trypsin. CCK fragments (CCKf) generated within the gut inhibit further trypsin production. Interestingly, STI inactivation of gut trypsin leads to a drop in the concentration of CCKf, leading to the increased synthesis of trypsin. The continual loss of non-digestible trypsin–STI complex from the body protein has adverse effects on NBL, leading to wasting. Raw soybean fails to support growth

when fed to rats and livestock. The non-nutritional effects of STI occur solely within the GI tract. More generally, enterocytes lining the GI tract possess cell surface receptors that enable them to sample peptides and other products found in the GI lumen and thereby produce bioactive results. It is noteworthy that the effect of probiotic compounds on gut health does not require that such agents be absorbed into the bloodstream.

3.7 SUMMARY AND CONCLUSION

Undernutrition is a form of wasting attributed to deficiency in protein intake or the derangements of protein metabolism due to illness. The details of protein intake and requirements were outlined. The characteristics of tissue protein metabolism and its derangements were discussed. PEU occurs with a frequency of 30%–50% in hospitalized patients. Though the causes of PEU are not wholly understood, increasing protein intake alone is unlikely to resolve this problem. To maintain a state of positive nitrogen balance, patients should receive treatments to correct metabolic derangements arising from illness perhaps. The health benefits of dietary bioactive peptides range from normal nutritional effects obtained with "inert" dietary proteins to physiological responses more usually associated with drugs, hormones, and biopharmaceuticals.

APPENDICES



APPENDIX 3.A.1 Medical science subject headings (MeSH) for protein nutrition.

APPENDIX 3.A.2
Bioactive Amino Acids or Metabolites from Amino Acids

Bioactive Metabolite	Precursor Amino Acids	Functionality
Glutathione	Glutamate, cysteine, and glycine	Oxidant defense, immune modulation
Creatine	Glycine, arginine, and methionine	Muscle function
Taurine	Cysteine	Brain function
Nitric oxide	Arginine	Immune function, blood vessel tone
Carnitine	Lysine	Fat oxidation
Brain glutamate	Glutamate	Neurotransmitter
Brain glycine	Glycine	Neurotransmitter
Serotonin	Tryptophan	Satiety
Nicotinic acid	Tryptophan	Energy metabolism
Catecholamine	Tyrosine	Hormone
Melanine	Tyrosine	Pigmentation
Heme	Glycine	Oxygen transport
Bile acids	Glycine and taurine	Cholesterol metabolism
Nucleic acid bases	Glutamate, aspartate, and glycine	

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4 Protein Turnover and Economics within the Body

4.1 PROTEIN TURNOVER AND WASTING

4.1.1 Introduction

Muscle loss can be expected whenever the rate of muscle protein breakdown and utilization for energy exceeds the rate of protein synthesis. Methods have been developed that allow the measurement of the rates of protein synthesis, degradation, and oxidation simultaneously using amino acid tracers.[1,2,*] Improved understanding of factors that influence protein turnover is essential for developing nutritional therapies for the prevention of muscle loss. The turnover of body proteins and skeletal muscle protein (SMP) is discussed in this chapter. The biological significance of protein turnover is described in Section 4.1. Baseline values for whole body protein turnover are presented in Section 4.2. The contribution of different organs to whole body protein turnover is discussed in Section 4.3. Current research suggests that illness affects protein turnover (Section 4.4). Finally, the effect of bioactive peptides and related supplements on the whole body and SMP turnover is described in Section 4.5. Illness produces marked increases in the extent of protein synthesis, breakdown, and oxidation. More research is needed to ascertain whether particular interventions can increase protein synthesis or decrease muscle protein breakdown and oxidation, or both.

4.1.2 Biological Purpose of Protein Turnover

The total amount of protein degraded and resynthesized within the body each day exceeds the amount of protein consumed by a factor of fourfold to fivefold (Figure 4.1). It is thought that the recycling of body proteins requires energy; approximately 0.7 kcal/g protein is required per gram body protein recycled and hence the process must yield some survival benefits. Old and damaged proteins are identified, labeled, and degraded. The resulting amino acids are then employed as fuel while new amino acids from the diet are funneled for protein synthesis.

* Whereas nitrogen balance measurements tell us when there is net gain or loss of body proteins, there is usually no indication of factors leading to these losses (see Chapter 2). It is believed that protein turnover (i.e., the process of breakdown and resynthesis of body proteins that takes place in all organisms) may provide more important information about the causes of muscle loss.

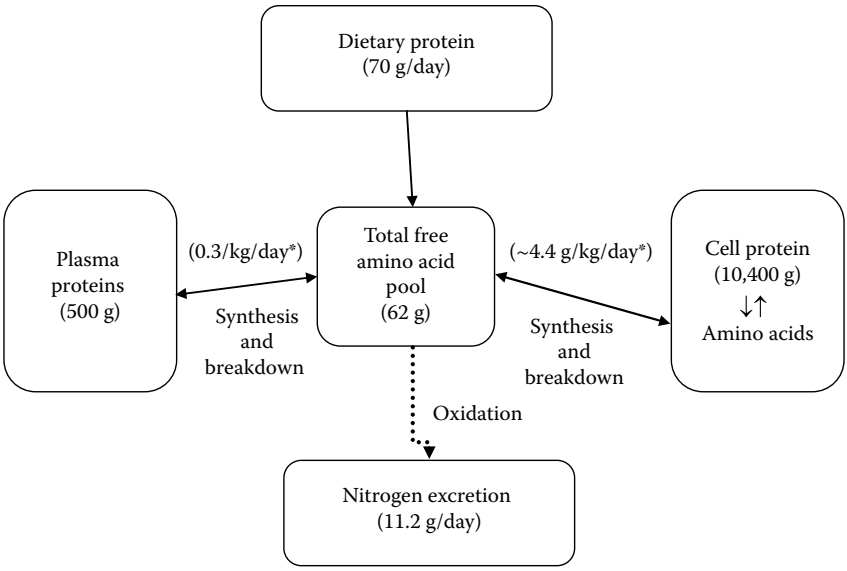

FIGURE 4.1 A three-compartment amino acid pool model for protein turnover. *Notice that total protein turnover is ~4.7 g/kg/day equivalent to 330 g/day. (Adapted from Mitch, W.E. and Goldberg, A.L., *N. Engl. J. Med.*, 335, 1897, 1996.)

Past research suggests that the rate of protein turnover is sensitive to a myriad of physiological factors including age, gender, and treatment with drugs; acute injury; and chronic disease. The process of turnover is essential for protein homeostasis where older, damaged proteins are degraded and replaced with new proteins. During illness, the host response to stress leads to increased turnover, and the mobilization of SMP stores that are used for serum (e.g., acute phase) protein synthesis by the liver and spleen. Increases in protein turnover may also lead to the inadvertent losses of amino acids via their oxidation to provide energy. Increases in the synthesis and degradation of body proteins may be part of the normal adaptation toward trauma, and exercise.[3] Protein turnover is implicated in organ remodeling.

4.1.3 Stable Isotope End Product and Precursor Flux

There are essentially two approaches assessing protein turnover involving (1) the historically older end product method and the (2) precursor method. The former approach uses a single administration of ^{15}N-glycine followed by mouth or intravenous injection, followed by a quantitative determination of end products and label excreted. In practice, the levels of urea, ammonia, and ^{15}N-label excreted are determined following a 24 h urine collection. The degree of protein flux can be calculated from the following relations:[4]

$$Q = ¢\frac{\text{Ex}}{e} \tag{4.1}$$

where

Q (mg/24 h/kg BW) = the total flux
e is the amount of label per unit amount of end product excreted (i.e., activity of label in the end product)
Ex is the total amount of end product excreted
¢ is the dose of label applied

The end-product method is considered straightforward and especially useful for free living subjects and studies involving large populations.[4,5]

The precursor method uses intravenous infusion of one or more stable isotope-labeled amino acids as tracers, followed by the detection of the same label in the plasma. ^{13}C-leucine is the most widely used tracer but other EAA are now being increasingly used. Phenylalanine, which is neither produced nor metabolized within skeletal muscle, is currently one of the most popular labels for SMP turnover. The tracer is applied via continuous infusion over several hours until the concentration of label in the plasma reaches a constant (steady-state) concentration. In practical terms, blood samples need to be collected intermittently over several hours and the appearance of labeled amino acid is determined until a steady-state concentration is reached. The leucine metabolite ^{13}C-α-ketocaproic acid may also be monitored to provide a measure of *intracellular* metabolism. Amino acid flux (Q; μmol/kg/h) or the rate of protein turnover (P_T; g/kg/day) is calculated from the steady-state concentration of label according to the relations below:*

$$Q = \varepsilon\left[\left(\frac{C_i}{C_P}\right) - 1\right] = \varepsilon\left[\frac{\nu_P}{\nu_i}\right] \tag{4.2′}$$

$$P_T(\text{g/kg/day}) = Q \cdot 24(\text{h/day}) * 625 \tag{4.2″}$$

In Equation 4.2′

ε is the isotope infusion rate (μmol/kg/h)
C_i is the initial concentration of isotope infused
C_P is the plateau concentration of isotope in the plasma
ν_i is the volume of isotope infused
ν_P is the total volume accessible to tracer amino acids

Under steady-state conditions, the rate of appearance (R_a) and disappearance (R_d) of labeled amino acids are equal ($R_a = R_d$), and the flux (Q) = $R_a = R_d$. The following relations are also thought to apply:

* Literature values for leucine flux are usually quoted in units of μmol (leucine) per kg (body weight) per hour (μmol/kg/h) or alternatively in terms of grams (g) of protein synthesized or degraded per kg body weight per day (g/kg/day). The two units can be interconverted assuming the human body composition is 8.2% leucine (or 625 μmol/g). A leucine flux of 143 (μmol/kg/h) translates (after division by 26.04) to 5.5 g/kg/day or 385 g/day for a 70 kg subject. In this chapter, values for leucine flux data will be given as μmol/kg/h.

$$R_a = B + In \quad (4.3')$$

$$R_d = S + Ox \quad (4.3'')$$

$$B = Q - In \quad (4.4')$$

$$S = Q - Ox \quad (4.4'')$$

$$NBL = S - B = In - Ox \quad (4.4''')$$

In Equations 4.3′ and 4.4′

Ox is the rate of amino acid oxidation to CO_2 and Urinary nitrogen
In is the dietary protein intake
B is the rate of tissue protein breakdown
S is amino acid removal rate due to tissue protein synthesis
NBL is the nitrogen balance*

Notice that the rate of protein breakdown can be calculated from the isotopic flux data combined with an independent measurement (In), whereas the rate of synthesis is determined from the flux and Ox. Amino acid oxidation can be assessed by collecting CO_2 exhaled by a subject followed by GC-MS analysis to determine the proportion of labeled $^{13}C–CO_2$. The rate of leucine oxidation is estimated from the rate of $^{13}CO_2$ release ($F^{13}CO_2$, μmol $^{13}CO_2$/kg/h). Another relevant index for nutritional support is the fractional rate of protein synthesis (Ks). The value for Ks is calculated from the amount of ^{13}C label bound to protein (C_b). Methods for protein turnover measurements have been reviewed.[6–8]

Data obtained using the end product and precursor method for estimating whole body protein turnover are significantly different for reasons yet to be established.[4,6,9] The end product method provides ~10% lower estimates of whole body protein synthesis in some circumstances compared with the precursor method. Interestingly also, the precursor method appears to be less sensitive to the effects of dietary protein restriction and repletion (see below). Results obtained by the precursor method appear to depend on the choice of EAA. The tendency these days is to measure turnover using two or more amino acid tracers in order to get a more representative picture of changes occurring in the muscles.[10,11]

4.1.4 Non-Tracer Methods for Estimation of Turnover

Urinary levels of 3-methyl histidine (3MH) can be used as a measure for the rates of muscle protein breakdown. The posttranslational modification of muscle histidine leads to the formation of 3MH that is released from muscle following protein degradation. 3MH is quantitatively excreted in the urine and may be used as an index

* "NBL is determined by amino acid flux as briefly outlined here. The major processes affecting R_d are expressed by equation 4.3". Additionally, $R_a = In + B + N$ though N (de novo synthesis of amino acids) is zero if a labelled essential amino acid is used as tracer. NBL reflects the difference between protein nitrogen intake and elimination, hence $NBL \approx In - Ox$ and also $NBL = (R_a - B) - (R_d - S) = (R_a + S) - (R_d + B)$. Such relations indicate that NBL will be positive where $R_a - B > R_d - S$ or where central amino acid pool is replenished not by tissue breakdown but by other means, e.g. dietary intake. In addition, the disappearance of amino acids from the central pool should occurs via tissue synthesis rather than via oxidation.

of protein breakdown.[12–14] Recent applications include the use of 3MH method to follow proteolysis arising from disuse atrophy[15,16] burns injury, obesity,[17] and Type 2 diabetes (T2D).[18] Unlike the determination of 1-methylhistidine, levels of which have been shown to increase with meat intake, 3MH is thought to be derived from endogenous muscle protein only.[19]

4.1.5 Protein Turnover Implications for Nutritional Support

In principle, information obtained from studies of whole body protein and SMP turnover should be invaluable for designing nutritional support. Protein intake is equivalent to zero in the post-absorptive state (In = 0) and therefore (1) the rate of appearance of isotopic label is directly proportional to the rate of whole body protein breakdown. An increase in whole body protein turnover may be taken as an indication of increased rate of catabolism, (2) from the standpoint of specific organ/or tissues, protein/amino acid intake can be negative or positive reflecting the direction of transfer of amino acid to or from the organ/tissue of interest, (3) Equation 4.4 shows that nitrogen balance is dependent on the rate of amino acid oxidation for fuel compared to intake. Moreover, the rate of non-oxidative disposal of ^{13}C-leucine (NOLD) is related to the rate of protein synthesis ($=R_d - Ox$), and, consequently, (4) amino acid oxidation competes with protein synthesis (Equation 4.3″) as routes for amino acid utilization. Data from SMP turnover are much readily interpreted using phenylalanine or lysine as tracers are neither produced nor metabolized (i.e., oxidized) within muscle tissue.

Some general interventions likely to assist in weight gain can be deduced based on the current models of protein turnover: (1) Increased dietary intake increases the net pool of amino acid subjected to flux, though this by itself will not guarantee a net increase in lean body mass. (2) The provision of substrate for protein synthesis (and oxidation) via dietary intake will spare endogenous amino acids. However, only a limited proportion of material for whole body protein flux comes from external sources. (3) The oxidative disposal of amino acids is the major route for material loss from the body (Figure 4.2).

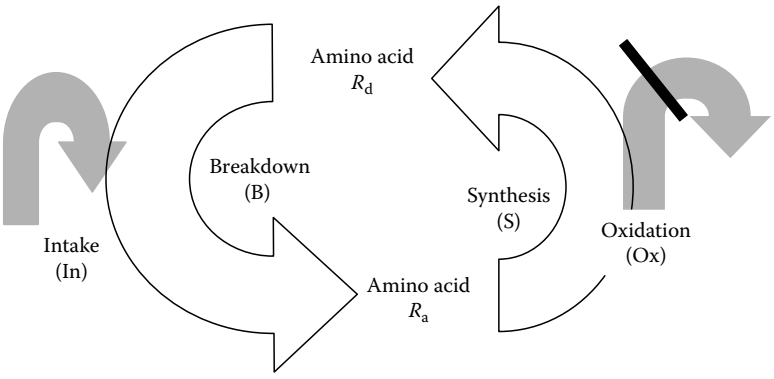

FIGURE 4.2 Schematic diagram showing interrelations between the different components of body protein flux, where R_a = rate of amino acid appearance (R_a = B + In) and R_d = rate of disappearance (R_d = S + Ox). See text for further details. The bar shows likely strategy for increasing weight gain.

Higher rates of amino acid oxidation (for energy) lead to a more negative nitrogen balance. The RDA for dietary protein assumes that there are adequate sources of (nonprotein) energy. Low dietary energy leads to increased requirement for dietary protein. The rate of amino acid oxidation increases with illness severity probably linked with changes in resting energy expenditure (see Section 3.4.1). Provided that ways could be found to deal with insulin resistance associated with many illnesses, providing extra dietary energy could have a "protein-sparing" effect. Strategies may be needed for reducing the body's reliance on amino acid oxidation during illness, perhaps by improvements in muscle insulin sensitivity (see Section 8.6.3).

4.2 BASELINE WHOLE BODY PROTEIN TURNOVER

4.2.1 Adults

The rate of whole body protein turnover for the typical adult is 103–143 μmol/kg/h (~385 g/day). The time necessary to break down and resynthesize differs for different proteins and different organs. Metabolically active proteins (mainly enzymes and regulatory proteins) undergo rapid turnover with a relatively short half-life of <10 min. By contrast, structural proteins in muscle and bones have a longer half-life. Alpha-crystalline protein within the eye lens apparently does not undergo turnover.

4.2.2 Gender and Pregnancy

Whole body protein turnover has been reported as both greater and lower in males compared to females. The results appear to depend on the methodology used by different investigators, the application of different tracers, and whether the measurement of isotopic enrichment is performed using blood plasma or muscle biopsy.[20] Short et al. reported no gender differences in whole body protein turnover after comparing 87 men and women aged 19–87 years old when the data were adjusted for differences in FFM; most of the data predicted 7%–10% higher whole body protein turnover in women compared to men. The main difference observed between the sexes was that leucine oxidation (expressed as the proportion of total flux) was 9% higher in men.[21,*] Morais et al. reported that whole body protein turnover was 20% higher in men compared to women. As noted above, the difference in protein turnover disappeared where results are expressed in terms of FFM.[22,†]

The above results are in contrast with the sex differences in whole body protein turnover reported by Henderson et al.[23] and by Smith et al.[24] The former study involving over 200 men and women (20–73 years old) showed that a diverse range of variables (age, sex, VO_2, and resting energy expenditure [REE]) had a significant association with the whole body protein turnover rate and the fractional rate of muscle protein synthesis. The rates of proteolysis (Phe-R_a), amino acid oxidation (Phe-transformation to tyrosine), protein synthesis (Phe-R_d), and the fractional

* The findings of Short et al.[21] concerning the effect of aging on whole body protein turnover are discussed in Section 4.4.2.

† There is also evidence that the rate of protein turnover per unit mass of FFM is the same for infants and adults.

rate of protein formation were all higher in women compared to men. Interestingly, despite the increased rate of protein synthesis, women did not show increased muscle accretion. The rate of protein turnover was unchanged or slightly decreased during early pregnancy. Leucine turnover was 143 ± 8 and 163 ± 26 μmol/kg/h in the first and third trimesters compared to 166 ± 35 μmol/kg/h for non-expectant females. There was a decline in the rate of amino acid oxidation during pregnancy showing increased tendency toward protein synthesis.[25]

4.3 REGIONAL PROTEIN TURNOVER

4.3.1 Splanchnic Bed Protein Kinetics

Significant amounts of dietary amino acids are processed within the splanchnic tissues, that is, liver, stomach, intestines, pancreas, and spleen.* Up to 50% of newly absorbed dietary amino acids are first incorporated into splanchnic bed proteins. Excess amino acids are then oxidized or transferred to the peripheral tissue (notably the muscles). Splanchnic proteins are later transferred to the portal circulation (Figure 4.3). The kinetics of splanchnic bed amino acid absorption and utilization is thought to have a profound effect on the efficiency of EAA transfer to the peripheral regions of the body.

Gelfand et al. suggest that the splanchnic tissues account for ~50% of whole body protein turnover.[26,†] This was one of the first studies to highlight the important role played by the splanchnic bed during protein metabolism. Hoerr et al. estimated that ~30% of EAA absorbed from lumen was utilized locally by gut tissue over a dietary protein intake range of 0.1–1.5 g/kg/day. Therefore, gut protein synthesis

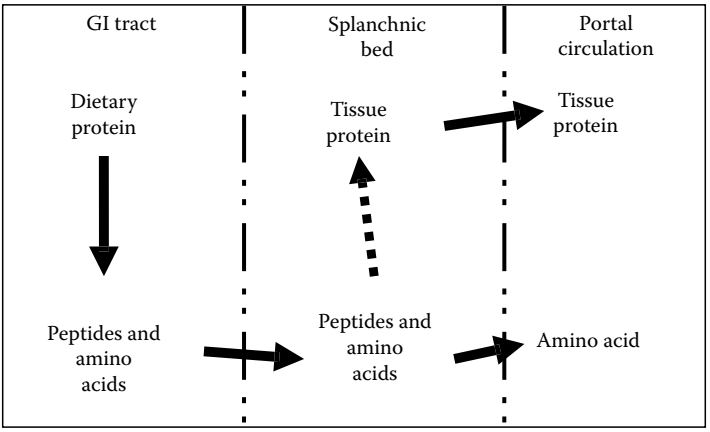

FIGURE 4.3 Effect of splanchnic bed (liver, stomach, intestines, pancreas, and spleen) protein synthesis (dotted line) on absorption of dietary proteins.

* Protein turnover within specific tissues and organs were determined by monitoring the flux of isotopic label across the artery/vein running to and from the organ of interest or by taking tissue biopsies.

† These investigators described the splanchnic tissues as portal drained viscera (PDV) including liver, stomach, intestines, pancreas, and spleen, i.e., tissue drained by the portal vein. Present estimates show the % of whole body protein turnover due to the splanchnic bed is ~25%–30%.

TABLE 4.1
Regional Protein Turnover within the Human Body

	Organ/Tissue		
Reaction	**Kidneys**	**Splanchnic Bed**	**Leg Skeletal Muscle**
Protein degradation (%)	11.4 ± 4.2	22.1 ± 6.7	35.8
Protein synthesis (%)	10.1 ± 4.5	26.8 ± 10	32.2
Oxidation (%)	25.6	17.9	48.6
Fractional synthesis rate (%)	42.0	12.0	1.5

Source: Tessari, P. et al. *Journal of Clinical Investigation,* 98, 1481, 1996.

could adapt to large variations in protein intake.[27] Biolo et al. compared EAA transport and incorporation into muscle versus gut tissue in an anesthetized dog and found that significantly more intracellular EAA was used for protein synthesis in the former tissue. By contrast, muscle intracellular EAA (from inward transport and protein degradation) was preferentially ($P < 0.05$) released into the bloodstream rather than incorporated into protein. The tendency for inward transport of EAA was greater for the gut tissue ($P < 0.05$) compared to muscle.[28] Such results are consistent with the net transfer of EAA from the muscle to the gut during the post-absorptive state.

Tessari et al. found that the kidneys, splanchnic bed, and leg skeletal muscles account for 70% of whole body protein turnover and 90% of protein oxidation (Table 4.1). Interestingly, the kidneys account for ~1% of lean mass, 11% whole body protein breakdown, 10% whole body protein synthesis, and 26% of amino acid oxidation.[29,30] The splanchnic bed accounts for 4%–6% of lean body mass and 20%–30% whole body protein turnover. Finally, muscle that contains nearly 70% of lean body mass accounts for ~30% of whole body protein turnover. The fractional rates of protein turnover were also enormously different in various organs with ~40% of kidney proteins turned over daily. The fractional synthesis rate was ~12% for splanchnic bed organs compared to 1.5%/day for SMP.

4.3.2 First-Pass Metabolism of Dietary EAA and Interorgan Effects

SMP accretion or loss is affected by changes in the splanchnic organs.[31] Boire et al. found that splanchnic bed protein turnover was similar in six young and six elderly subjects (mean ages: 22.7 years and 68.2 years, respectively) when results were expressed per kg lean body mass. Eating led to increased leucine flux and oxidation, decreased protein breakdown, and no changes in the rate of protein synthesis. The first-pass extraction of leucine was twofold higher (50% ± 11% vs. 23% ± 2%, $P < 0.05$) in the elderly subjects compared to young adults, resulting in a significant decline in the plasma levels of leucine with aging. It was feared that higher splanchnic bed utilization could lead to lower availability of EAA for peripheral tissue during aging.[32,*] The age-related increase in

* Consistent with this theory, a substantial proportion of EAA available from the diet are thought to be utilized by the splanchnic organs (e.g., liver) for serum protein and/or APP synthesis.

splanchnic extraction of EAA was confirmed by Volpi et al. using a different study design.[33] Following an oral dose containing a mixture of eight EAA (plus labeled phenylalanine as tracer), first-pass splanchnic extraction of phenylalanine was greater in the elderly compared to the young.* However, when the arterial concentrations of EAA were actually measured for young and elderly subjects, there were no significant differences. Apparently, EAA utilized by the splanchnic bed was re-released into the circulation. Therefore, differences in splanchnic first-pass extraction could not account for muscle loss observed during aging.† Much more interestingly, the study by Volpi et al. found that mixed oral EAA had anabolic effects on leg muscle protein synthesis—for both young and elderly subjects—but that the rate of SMP degradation was not affected by feeding. The 1999 publication discussed the anabolic role of EAA and alluded to possible effects on protein translational regulation. A further comparison between SMP synthesis rate for young and elder subjects given a single 15 g bolus of EAA showed the net of fractional synthesis rate (FSR) were similar but that the rate of label transfer was significantly slower in the elderly subjects compared to the young.[34] Data pertaining to SMP turnover responses to feeding are discussed further in Section 4.5.

4.4 PROTEIN TURNOVER DURING ILLNESS

Illness-related loss of muscle mass can be expected where the rate of protein breakdown exceeds the rate of protein synthesis. Another important condition for muscle wasting is that dietary amino acid intake is low compared to the rate of oxidation. Changes in the rates of protein synthesis, degradation, and oxidation observed during illness are discussed in this section. The majority of studies reported so far deal with changes in whole body protein indices (Table 4.2). The limited number of studies looking at SMP changes are highlighted wherever possible.

4.4.1 Preterm Babies and Children

Whole body protein turnover for premature babies is approximately twofold higher than the values for adults.[59,‡] The leucine tracer appearance rate (Leu-R_a) was 300–400 μmol/kg/h equivalent to 11.52–15.4 g/kg/day. Interestingly also, protein breakdown is not inhibited by feeding or amino acid supplementation in preterm babies unlike the response observed with full-term babies, children, or adults (see below). Parenteral and enteral nutrition was found to increase the values for Leu-R_d (protein synthesis) while Leu-Ox remained constant.[60] Zello et al. found that Leu-R_a = 332 ± 115 μmol/kg/h while Leu-Ox = 36.7 ± 16.6 μmol/kg/h for extremely low weight preterm infants over the dietary protein intake range of 1–3 g/kg/day. Nitrogen balance increased linearly with increasing protein intake reflecting the increases in rates

* The determination of splanchnic protein turnover employs two amino acid tracers administered intravenously and orally. This study employed L-[ring-^{2}H5] phenylalanine (intravenous tracer) and L-[ring-^{13}C6] phenylalanine (oral tracer).

† Later studies showed that the elderly were more resistant to the anabolic effect of EAA via a process analogous to insulin resistance (cf. Chapter 9).

‡ Preterm babies (infants delivered before the full pregnancy term of 39 weeks) are called premature babies by the British.

TABLE 4.2
A List of Some Conditions That Affect Whole Body Protein Turnover

Aging adults (elderly)[a]
HIV/AIDS infection[a]
Bed rest and steroid myopathy[35]
Burns injury[a]
Chronic kidney diseases[36,37]
Chronic obstructive pulmonary disease[38,39]
Cirrhosis of the liver[40–42]
Crohn's disease[43]
Cushing's syndrome[44]
Cystic fibrosis[45]
Diabetes[a]
Emphysema
Inflammatory conditions[46]
Obesity[47–50]
PEU/malnutrition[51,52]
Post-surgical trauma[53]
Preterm babies[a]
Sepsis and infection[54]
Sickle cell diseases[55,56]
Smoking and alcohol[57]
Thyroid disease
Trauma (skeletal trauma)[58]

[a] See text for description and citations.

of protein synthesis.[61] There is general agreement that early parenteral nutrition of preterm babies is beneficial, leading to improved rates of growth and other developmental benefits. Finally, there is evidence that the transient administration of EAA may lead to a more efficient stimulation of protein synthesis as well as depression of protein proteolysis (which is not affected by sustained amino acid infusion). This topic has been reviewed by Denne[62] and by Kalhan and Edmision.[63]

4.4.2 Aging Adults and Sarcopenia

A meta-analysis of 21 studies published in 2005 on the effect of aging on protein turnover found the overall outcome was inconclusive.[64] Approximately 60% of studies evaluated showed that whole body protein turnover declines with aging by about 20%–30%. However, 30% of the studies found no effect of aging on whole body protein turnover rate expressed per kg lean body mass. In all, 2 of the 21 studies found evidence of an increased protein turnover with aging and frail adults. It is generally agreed that the proportion of FFM decreases during aging while the proportion of fat mass increases from 62% to 74% between the ages of 28 and 71 years.[65,66] Current research findings

suggest that aging leads to a declining rate of basal protein turnover.[67,68] A declining rate of protein degradation cannot explain the observed decrease of muscle mass with increasing age. It has been suggested that differences in the rates of SMP turnover in the elderly and young are probably much smaller than previously reported.[69]

Several methodological difficulties were highlighted when comparing protein flux in young and elderly subjects.[69] Confounding factors include participants with a history of wasting conditions (cf. cardiac, pulmonary, liver, or kidney diseases; peripheral vascular disease; diabetes; deep vein thrombosis, etc.). Studies that require enforced bed rest will also provide false data since inactivity leads to muscle wasting. In the absence of large differences in protein turnover in the basal state, investigators have sought more subtle differences in aging and young muscle. It has been proposed that anabolic effects of dietary proteins and other agents may be more blunted in elderly subjects compared to the young.[70] The concept of anabolic dysfunction is discussed further in Chapter 9.

Short et al. demonstrated that EAA oxidation and NOLD (protein synthesis) both decline by 4%–5% per decade between the ages of 19 and 87 years ($P < 0.001$). FFM, which correlates with protein turnover rate, declined by 3% per decade ($P < 0.001$). Following 16 weeks of exercise training, the aerobic capacity improved 9% overall ($P < 0.01$), and mixed muscle protein synthesis increased 22% ($P < 0.05$). FFM, whole body protein turnover, and resting metabolic rate were unchanged. Short et al. noted difficulties with aging-related research including small sample size with insufficient statistical power. Comparison tended to be between two age groups (<35 years and >60 years) with no intermediate ages considered. This effect of protein nutrition on age-related wasting has been reviewed.[71,72] According to some recent studies reported in 2009, it is feasible that aging per se does not have an effect on whole body protein turnover, but that changes in protein metabolism are due to changes in other age-related attributes such as levels of fitness.[22,24]

4.4.3 HIV/AIDS Infection

Whole body protein turnover is significantly higher in HIV/AIDS patients compared to controls[73,74] though some reports find no difference.[75,76,*] Macallan et al. reported that the late stages of HIV/AIDS are associated with increased rates of whole body protein synthesis (111.6 ± 12.1 vs. 82.3 ± 9.2 μmol/kg/h) as well as elevated levels of whole body protein breakdown (129 ± 13 vs. 103 ± 10.1 μmol/kg/h) compared to controls. However, HIV infection did not impair anabolic response to feeding, that is, protein synthesis was stimulated to the same extent after feeding for HIV subjects and healthy controls. Interestingly, whole body protein turnover was not significantly correlated with REE, weight loss, or TNF-α concentration. Viral load was correlated to whole body protein turnover ($R = 0.87$, $P = 0.02$) and inversely correlated with CD4 cell count.[76] Hardin et al. showed that HIV-infected adults suffering weight loss ($n = 9$; 80%–88% normal body weight) showed increased whole body protein

* Studies showing no changes in whole body protein turnover tend to be those based on ^{15}N-Glycine end-product method, which is currently thought to be problematic and perhaps lacking theoretical validity.

turnover compared to controls. Insulin administration produced a lower degree of stimulation of protein synthesis in HIV patients compared to controls.[77]

Only a handful of studies of SMP turnover in HIV patients have been published. McNurlan et al. found that the rate of SMP synthesis was the same for HIV sufferers and for healthy controls. However, the rate of muscle protein breakdown was increased as measured by the rate of urinary excretion of 3MH. Growth hormone failed to stimulate SMP synthesis in symptomatic HIV sufferers, indicative of anabolic dysfunction.[77] Yarasheski et al. showed that HIV infection led to significantly higher whole body protein breakdown and whole body protein synthesis compared to controls. There was also increased amino acid oxidation and increased release of glutamine, probably derived from the muscles. The rate of SMP synthesis was reported to be lower in HIV patients compared to controls.[78] Breitkrueutz et al. demonstrated that HIV infection produces marked changes in the isotopic tracer flux across the leg muscle, indicative of enhanced proteolysis.[79]

4.4.4 Burns Patients

Burns patients had 83% higher SMP turnover and 50% increase in the SMP synthesis compared to controls.[80] The efficiency of amino acid transmembrane transport to muscle declined by 50%–63% whereas outward transfer increased by 40% (Phe) or 67% (Lys).* Burns injury leads to a disproportionate breakdown of muscle proteins and transfer of endogenous amino acids to other tissues as part of the catabolic response to injury. A meta-analysis by Hart et al.[81,†] showed that leg SMP breakdown increases with the total burned surface area of up to 40% and also with increasing resting energy metabolism. Subjects with larger initial muscle mass showed significantly higher muscle loss following burns injury. The rate of protein breakdown increased in those cases with septicemia. The above results confirm previous observations using animal models. Fang et al. found burns injury resulted in increased rates of SMP breakdown and inhibition of the rate of SMP synthesis. The glucocorticoid receptor antagonist RU38486 was found to block the rise in SMP proteolysis observed with burns injury. By contrast, RU38486 had no effect on the rate of protein synthesis.[82] Interestingly, more recent research suggests that glucocorticoids can increase proteolysis as well as inhibit protein synthesis but that the effects on rat and mice SMP is different.

4.4.5 Cancer Cachexia

Only a handful of studies have examined the effect of cancer on protein turnover.[83] Melville et al.[84] showed that whole body protein flux (degradation and synthesis) was elevated in patients with lung carcinoma (without metastasis or obvious cachexia) compared to controls (Table 4.3). However, there were no differences in the leucine balance, REE, or pattern of macronutrient utilization between cancer

* Data for n =18 patients with 66% surface burns, 14 days post injury.

† The study examined 151 different stable isotope studies involving 102 children and 21 adults.

TABLE 4.3
The Effect of Feeding on Whole Body Protein Turnover in Nine Lung Cancer Patients

	Cancer Patients		Control Patients		
Index/(μmol/kg/h)	**Mean**	**SD**	**Mean**	**SD**	**Notation**
Unfed state					
Flux	129.4	18.8	113	9.8	b
Oxidation	27	6.4	26.9	4.5	b
Synthesis	101.5	20.5	86.1	7.9	
Degradation	126.2	18.5	109.9	9.7	b
With feeding					
Flux	148.6	17.5	131.9	12.4	a, b
Oxidation	42.5	14.5	42.6	11.5	a
Synthesis	106.1	19.5	88.9	7.3	b
Degradation	58.6	12.4	42	14.3	a, b, c
Intake	90.1	14.2	89.6	15.4	

Source: Adapted from Melville et al., *Cancer Research*, 50, 1125, 1990.
Notation: a = mean value different between unfed and fed states ($P < 0.05$), b = significantly different between cancer and control patients, and c = no significant difference between cancer and control patients if single metastatic patient is excluded.

patients and controls. The rate of whole body protein breakdown was inhibited by feeding while the rate of whole body protein synthesis remained unchanged for cancer patients and controls. Overall, there were no major differences between cancer patients and controls that could explain weight loss. It should be noted that the cohort of patients studied were non-cachexic at the start of the study and therefore the magnitude of changes being measured may have been too small for accurate determination.

Dworzak found that the rates of whole body protein synthesis and breakdown for stomach cancer patients (87 ± 18.2 and 117.2 ± 10.4 μmol/kg/h) were not significantly different from values for healthy controls (113 ± 5.2 and 124 ± 5.2 μmol/kg/h), respectively. By comparison, SMP synthesis and breakdown was 2.3-fold lower or the same (respectively) for cancer patients compared to healthy controls.[85] Emery et al. also found that cachexic cancer patients had a 6.6-fold lower rate of SMP synthesis compared to healthy controls but the rate of SMP breakdown was similar.[86] Attard-Montalto et al. found that children with cancer (n = 8; ages 7.9–11 years) had a negative nitrogen balance due to a depressed rate of whole body protein synthesis (112 ± 10 μmol/kg/h) compared to breakdown (135 ± 13 μmol/kg/h). Chemotherapy increased the rate of whole body protein breakdown (156.2 ± 10 μmol/kg/h) with no effect on the rate of protein synthesis.[87] In summary, the limited data available suggest that SMP synthesis is depressed for cancer patients but that the rate of protein

breakdown is largely unaffected.* It has been suggested that cancer patients show anabolic dysfunction leading to decreased stimulation of protein synthesis by nutrient intake.[88] For another study of turnover in cancer, see Ref. [89]. More studies are needed into the effect of cancer on protein metabolism.

4.4.6 Chronic Renal Failure and Hemodialysis

The effect of chronic renal failure and hemodialysis on whole body protein and SMP turnover was reviewed by Lim et al.[90] Berkelhammer found the rates of whole body protein breakdown in renal hemodialysis patients was the same as in controls, measured via ^{13}C-leucine flux (103 ± 19 vs. 106 ±.19 μmol/kg/h), but leucine oxidation was increased by 43% compared to controls (20 ± 6 vs. 14 ± 4 μmol/kg/h, $P < 0.05$) and net protein synthesis was reduced ($P < 0.05$).[91] Other studies focusing on SMP turnover suggest that patients with end state renal disease (ESRD) without acidosis and maintaining adequate protein intake can maintain nitrogen balance. However, dialysis treatment per se leads to increased rates of breakdown coupled with increased rates of protein synthesis resulting in a net decrease in muscle accretion. There appears to be a net movement of amino acids from muscle to plasma induced by hemodialysis treatment.[92–94] The supplementation of amino acids to hemodialysis patients increased SMP synthesis as well as breakdown with no net increase in muscle accretion.[95]

4.4.7 Diabetes

Nair et al. showed that sufferers of Type 1 diabetes (T1D) exhibit increased rates of whole body protein synthesis and breakdown compared to lean controls.[96] However, T1D patients also show markedly more negative NBL owing to a disproportionately higher rate of breakdown compared to synthesis (Table 4.4). Treatment with

TABLE 4.4
Whole Body Protein Turnover in T1D Diabetic, Obese, and Lean Persons

Treatment Group	Breakdown (μmol/kg/h)	Synthesis (μmol/kg/h)	Protein Balance (μmol/kg/h)
Diabetes (TID)	151.2 ± 16.3	113.0 ± 13.1	(–) 38.3 ± 4.2
Obese	129.1 ± 8.12	105.1 ± 7.7	(–) 24.1 ± 2.1
Lean/controls	109.9 ± 8.8	90.6 ± 7.7	(–) 19.8 ± 5.1

Source: Nair, K.S. et al., *Diabetologia*, 25, 400, 1983; Pi-Sunyer, F.X., *Am. J. Clin. Nutr*., 72, 533S, 2000.
Original data reported as mg/kg LBM/h. Note that 241.9 mg/kg LBM/h = 151.2 μmol/kg/h.

* This is a surprising outcome, which is difficult to reconcile with the marked emphasis placed on proteolytic pathways for muscle wasting. See Sections 4.5 and 4.6.

insulin decreased protein breakdown but initial data suggested that there was no effect on the rates of whole body protein synthesis.[97–99] Pacy et al. showed that insulin administration decreased the rates of whole body protein synthesis, breakdown, and leucine oxidation. Apparently, insulin did not stimulate forearm SMP synthesis.[100] Nair reported that insulin treatment decreases protein synthesis within the splanchnic bed while stimulating SMP synthesis.[98] More recent investigation also indicates that insulin can stimulate protein synthesis provided that amino acid availability is maintained. The anabolic effects of insulin appear to be conditional upon having adequate amino acid supply in vivo.[101–104] Recent stable-isotope analysis of the effects of low, intermediate, and high doses of insulin in SMP metabolism suggests that intermediate doses are able to support increased synthesis and decrease proteolysis but that high doses induce amino acid limitations therefore reducing the anabolic effect.[105,*]

T2D is associated with defects in glucose metabolism but the effects on amino acid utilization remains controversial.[107–109] Staten et al.[110] found that the rates of ^{13}C-leucine flux, nitrogen flux, and amino acid oxidation were not different for T2D patients compared to age-matched nondiabetic controls. Though insulin treatment improved glucose uptake in T2D patients, there was no effect on whole body protein turnover. Halvatsiotis et al.[111] found that the basal rate of whole body protein was similar for T2D patients compared to nondiabetic controls. Eleven days insulin treatment had no effect on SMP turnover for T2D patients. Values for leucine, tyrosine, and phenylalanine rate of release (an index of proteolysis) was not significantly different for T2D patients and nondiabetic controls. There was also no difference in synthesis of muscle sarcoplasmic proteins or mitochondrial proteins.[111] Bell et al. found that whole body protein or SMP balance was not significantly different for T2D patients and normal controls. The rates of SMP breakdown measured via phenylalanine kinetics (56.4 ± 5.4 vs. 34.8 ± 1.2 μmol/kg/h) and synthesis (39.6 ± 1.8 vs. 22.4 ± 3.6 μmol/kg/h) were both elevated for T2D patients so that the net muscle loss was unaffected.[112]

Recent data from Canadian researchers suggest that the muscles of T2D patients undergo subtle changes to an extent that depends on the gender, degree of obesity, diet, etc. Pereira et al.[113] reported that baseline leucine kinetics for T2D patients and controls were not significantly different.[†] Sufferers of T2D show less increase in whole body leucine flux ($P = 0.016$) following insulin administration compared to healthy controls. There were sex differences in the insulin desensitization of muscle protein metabolism. In female T2D patients, there was a defect in protein breakdown inhibition by insulin but the protein synthesis increase was the same as for control subjects. In male T2D patients, it was the protein synthesis that was defective and protein breakdown decreased to the same extent as in control subjects following insulin

* These effects were observed in healthy subjects. The ability of insulin and amino acids to stimulate protein synthesis in diabetic subjects has been found to be impaired.

† This study using a metabolic clamp technique examined seven T2D subjects and aged and body composition–matched subjects. Investigators fixed blood insulin (570 pmol/L), glucose (5.5 mmol/L), and blood amino acid levels at predetermined levels and provided subjects with protein-controlled, iso-energetic diets for 7 days. They then determined the rates of uptake and appearance of glucose and leucine kinetics using labeled compounds.

treatment. Accordingly, it has been proposed that T2D affects muscle protein metabolism "indirectly" through changes in insulin resistance. Patients with T2D-associated insulin resistance may be more vulnerable to other wasting conditions.[114,*]

4.4.8 Sepsis

Animal models for sepsis have been developed using the intravenous injection of endotoxin/LPS or live bacteria.[115–117] Sepsis was also induced by cecal ligation and puncture technique.[118–121] The overall indications are that sepsis results in a differential effect on protein synthesis in different parts of the body. There was 50%–80% increase in the rate of protein synthesis in the rat liver, spleen, and gastrointestinal tract combined with a decreased rate of synthesis in the skeletal muscles. These differences produce net utilization of muscle amino acids for the synthesis of plasma proteins by the liver.[122,123]

Protein synthesis changes in sepsis have been examined by Vary and others from Penn State University (see Refs. [121 and 124] for review). In septic rats, protein synthesis in hind limb was 50% lower than controls whereas proteolysis was increased by 90%. Fast-twitch muscle is mainly affected compared to slow-twitch muscle.[124] There was an impairment of translational efficiency within the affected muscle with the critical defect occurring in the initiation stage of peptide synthesis rather than peptide elongation. Sepsis stimulates protein breakdown, as shown by measurements of 3MH release in urine.[125] An increase in proteolysis involves the stimulation of the ATP ubiquitin proteasome.[126] Sepsis has been shown to induce proteolysis via the ubiquitin proteasome.[127] As evidence, the administration of commercial proteasome inhibitors has been shown to reduce sepsis-induced wasting.[128]

4.4.9 Tuberculosis

Patients with TB have significantly lower average body weight (48.6 vs. 62 kg), decreased FFM (39.6 vs. 45.6 kg), and lower fat mass (6.2 vs. 12.6 kg). TB seems to produce almost equal losses of body protein and fat, though these occur predominantly from the limbs and trunk, respectively.[129–131] The effect of TB on protein turnover kinetics is qualitatively different from that discussed for HIV infection and other wasting conditions.[52,76] TB appears to have no effect or slightly reduces whole body protein turnover compared to controls (148 vs. 142 μmol/kg/h). By contrast, undernourished persons had significantly higher protein turnover compared to controls (163 vs. 142 μmol/kg/h) with similar BMI (18). Though TB is not accompanied by increased protein breakdown, there is a significantly higher rate of amino acid oxidation (Table 4.5). The available data suggest that patients with TB also exhibit a reduced anabolic response to feeding (Section 8.2).

* See Section 8.6.3 for further discussion of the effect of insulin resistance on muscle protein metabolism.

TABLE 4.5
The Effect of Tuberculosis and Undernutrition on Whole Protein Turnover

Treatment Group	WBP Turnover* (μmol/h)	AA Oxidation (μmol/h)	Protein Balance (μmol/h)
Undernourished	163 ± 9.4[(a)]	39	(–) 39.7
TB patients	148 ± 14[(b)]	47 ± 10.5	(–) 29.2
Control	143 ± 14.7[(b)]	37 ± 5.4	(–) 24.5

Source: Macallan, D.C. et al., *Clinical Science*, 94, 321, 1998.
* Different letters show significant differences ($P < 0.05$).

4.4.10 Anabolic Dysfunction Affecting Protein Turnover

Chronic illness leads to a blunted response to the anabolic effects of nutrients and hormones (Section 8.3.2) Cuthbertson et al.[132] found that leucine absorption was more efficient in older men compared to younger men, leading to twofold higher plasma and muscle levels—though the plasma-to-muscle ratio of EAA was the same in both groups. The basal rate of protein synthesis was also unchanged in older and young men (~1.34%/day). Leucine supplementation increased the fractional rate of protein synthesis in both elderly and young subjects but the degree of stimulation was about 40% depressed in older men. The data suggest daily muscle protein synthesis rates of about 58 and 45.6 g/day in young and old men, respectively, in the post-absorptive state, increasing to 196 and 108 g/day after the consumption of 10 g of dietary EAA. The deficit in protein synthesis rate in elderly muscle was estimated at 30 g/day. The possible causes of anabolic dysfunction and insulin resistance are described in Chapter 8. Some suggested changes in muscle protein regulation by anabolic signals are summarized in Table 4.6.

TABLE 4.6
The Effect of Aging on the Regulation of Muscle Protein Synthesis

Regulatory Feature	Aging Effect
Feeding-induced muscle protein synthesis	Impaired
Amino acid metabolism by visceral tissue and muscle proteins	Skewed toward visceral tissue with increased first-pass removal of EAA, less AA for peripheral
Sensitivity to leucine	Impaired
Levels of mTOR, p607SK, etc.	Reduced
Insulin, IGF stimulation of protein synthesis	Impaired, increased insulin resistance
Muscle recovery from starvation stress	Impaired
Glucocorticoids secretion	Increased, insulin antagonism enhanced

Source: Adapted from Waterlow, J.C., *Protein Turnover*, CABI Publishers, Cary, NC, 2006.

The findings of Koopman et al.[133] challenge the notion of anabolic dysfunction in aging muscle. The study considered the effect of light exercise following a protein leucine drink (6% whey protein [WP], 1% leucine) on muscle protein synthesis in eight young men (age 20 + 1 years) and elderly men (age 75.5 years old). Plasma insulin response following a carbohydrate drink supplemented with protein and leucine (CHO + Pro + Leu) drink was 50% greater than obtained for CHO drink alone. Baseline whole body protein synthesis was 25% lower in older muscle compared to young. Amino acid kinetic measurement showed that protein oxidation synthesis and breakdown rates were also lower in the elderly. The administration of the CHO + Leu + Pro drink produced 45% increase in the rate of protein synthesis, which was the same for elderly and young muscle. Interestingly, the current study did not support the concept of anabolic dysfunction advocated by others.

4.5 NUTRIENTS AND PROTEIN TURNOVER

Eating stimulates muscle protein building related to the stimulation of protein synthesis by EAA. Another reason for the increase in muscle building following a meal is increase in insulin secretion, which is stimulated by nutrients. The anabolic effect of EAA is independent of their function as building blocks for protein synthesis and/or energy substrates. The effect of dietary amino acid intake on protein turnover indices is discussed in this section.

4.5.1 Dietary Protein Intake and Whole Body Protein Turnover

Diurnal changes in the rates of protein synthesis, breakdown, and amino acid oxidation for energy correspond to meal times and periods in between. Muscle accretion occurs following a meal due to increased rates of protein synthesis. Amino acid oxidation also increases after meals due to their increased availability. During fasting, there is a decrease in protein balance ascribed to the heightened rates of protein breakdown and inhibition of synthesis.[134] However, opinions are divided on the effect of increasing dietary protein intake per se versus protein-energy intake on body protein accretion.

Garlick et al. noted that protein intake has a marked effect on whole body protein synthesis and that dietary energy has a relatively minor effect.[135] Other results indicate that a minimum intake of protein is needed for whole body protein synthesis, but that excess intake does not improve muscle accretion in humans. Interestingly, the effect of dietary protein quantity on whole body protein synthesis varies depending to the labeled tracer used. Measurements using orally administered ^{15}N-Glycine end product analysis tend to show increases in whole body protein synthesis with increasing dietary protein. Increasing dietary protein intake has been shown to improve whole body protein accretion by promoting whole body protein synthesis and depressing whole body protein breakdown.[136] In contrast, virtually all studies using the "gold standard" ^{13}C-leucine precursor method show no effect of excessive dietary protein intake on whole body protein synthesis. A discussion of the possible methodological differences and their possible impact on experimental outcomes is beyond the scope of this discussion. The interested reader should see the following reviews for expert opinions.[1,2,9] Despite these difficulties, there is ample evidence from protein turnover studies showing that the choice of dietary

protein (slow and fast proteins), the timing of administration feeding, and the administration of functional amino acids can all be moderated to improve muscle accretion.

Measurements of ^{13}C-leucine kinetics showed that whole body protein turnover, protein synthesis, and protein breakdown increases in proportion to dietary protein-energy intake in children recovering from malnutrition. By contrast, there was no significant dependence of any parameter on the net amount of (soy) protein fed.[137] The protein synthesis rate was ~threefold more sensitive (19.37 µmol/cal) compared to the rate of whole body protein breakdown (6.87 µmol/cal) to variations in dietary protein-energy intake. From the slope differences in Figure 4.4, the rate of whole body protein synthesis is expected to exceed the combined rates of protein breakdown and oxidation at a dietary intake of >6.25 cal/kg/day leading to muscle accretion. A minimum rate of protein flux (~150 µmol/kg BW/h) is necessary for a positive weight gain. In summary, dietary protein-energy intake affects the rates of whole body protein turnover in a predictable way. For children, there was a lack of sensitivity of whole body protein flux in response to increasing dietary (soy) protein intake.

^{13}C-leucine measurements also suggest there is no change in whole body protein turnover for human subjects exposed to increasing levels of dietary protein. Yang et al. found that ^{13}C-leucine flux was reduced by dietary protein restriction. However, there were no significant changes in the rate of protein turnover with increasing dietary protein intake.[138] Zello et al.[139] found that leucine and lysine fluxes in adults were not significantly affected by protein intake within the ranges of 0.6, 0.8, and 1 g/kg/day. Pacy et al.[140] concluded that average daily whole body protein synthesis and breakdown was

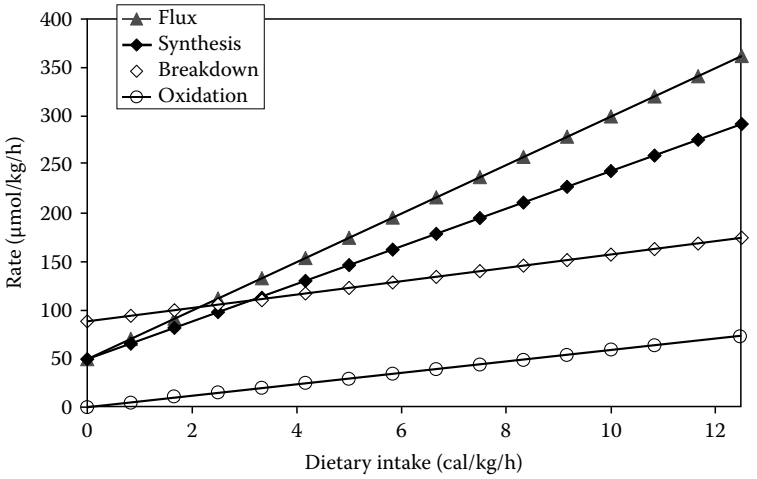

FIGURE 4.4 Effect of dietary protein-energy intake on whole body protein metabolism in malnourished children. Study involved 17 children fed differing levels of protein-energy (0.6, 1.2, and 5.2 g/kg/day) and different amounts of caloric intake (60–270 cal/kg/day or 2.5–11.25 cal/kg/day). Protein flux, protein synthesis, protein breakdown, and oxidation were measured by tracer method. Graphs are drawn according to the following equations of the line: Flux (y) = 24.998x + 49.476; synthesis (y) = 19.374x + 49.476; breakdown (y) = 6.8746x + 88.536; oxidation (y) = 5.6x. (Drawn using data from Golden, M. et al., *Am. J. Clin. Nutr.*, 30, 1345, 1977.)

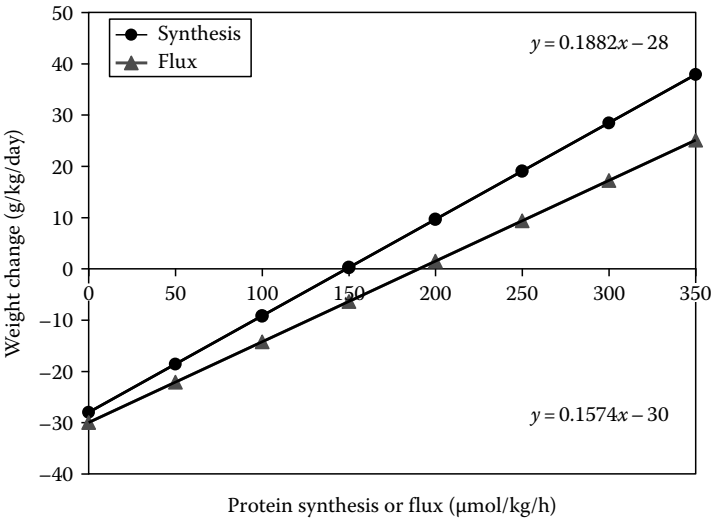

FIGURE 4.5 Relations between protein metabolism and weight gain in 17 children fed 0.6–5.2 g/kg/day and 60–270 cal/g/day. Rates for protein synthesis and whole body protein flux were measured using data from Goldman et al. (Drawn using data from Golden, M. et al., *Am. J. Clin. Nutr.*, 30, 1345, 1977.)

not affected by the amount (0.77–1.59 g/kg/day) of dietary protein consumed. Moreover, it was concluded that whole body protein measurements were unlikely to reflect changes in nutritional status. In summary, ^{13}C-leucine kinetics measurement using human subjects show no independent effect of protein quantity on whole body protein synthesis. The reason for these results is uncertain—but does not seem related to the unsuitability of ^{13}C-leucine as tracer. The magnitude of effects being measured by the ^{13}C-leucine tracer also seems to be sufficiently large, though we have not encountered any formal discussion of the limits of sensitivity for protein turnover studies in the literature. One possibility is that whole body protein turnover reflects two competing effects (of SMP vs. splanchnic organs), which thereby cancel out yielding a null response. Currently, there is no reliable explanation on why whole body protein measurements using ^{13}C seem to differ so remarkably from the results obtained using other tracers (Figure 4.5).

4.5.2 Skeletal Muscle Protein Turnover

4.5.2.1 Animal Studies

Well-fed and undernourished rats were found to grow at different rates, reflecting the effect of differing protein intake. For well-fed rats, there was a 22-fold increase in muscle mass arising from a fourfold rise in cell numbers (total DNA) and 5.2-fold increase in cell size (g protein/DNA). Muscle accretion was due to an almost equal contribution of cell hyperplasia and hypertrophy (Chapter 3). Underfed rats showed a markedly lower muscle cell number compared to well-fed rats particularly during the early parts of growth that was maintained for virtually all ages (Figure 4.6A through C). Muscle protein content per "unit" muscle cell was less affected by dietary restriction.[141]

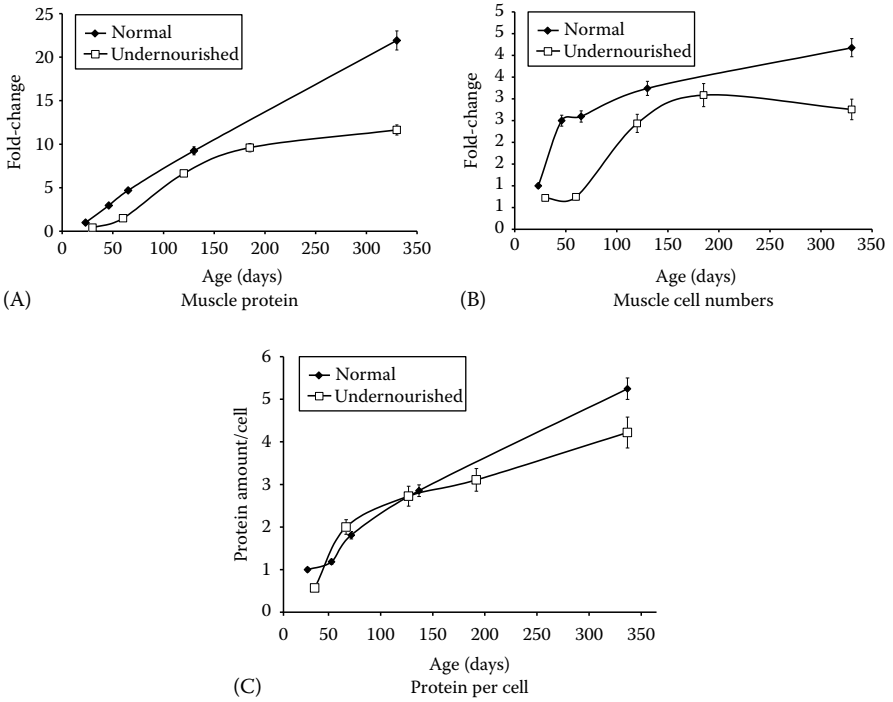

FIGURE 4.6 The effect of aging and diet on changes in (A) net muscle protein accretion, (B) muscle cell numbers, and (C) protein content/cell/increasing cross-sectional area of individual cells. Rats were fed on diet with 10% protein or 6.8% protein for 11 generations prior to the study reported in this figure. (Data from Milward, D.J. et al., *Biochem. J.*, 150, 235, 1975.)

SMP fractional rate of synthesis was higher compared to the fractional rate of breakdown accounting for growth/net muscle accretion in both underfed and well-fed rats. Both the fractional rates of synthesis and rates of breakdown were positively correlated with increasing growth rate. However, growth rate was more highly dependent on the rates of protein synthesis compared to rates of protein breakdown. It could be concluded that (1) rates of protein synthesis and protein breakdown decline with age and protein-energy restriction, (2) refeeding restores the rates of protein synthesis to rates observed in well-fed rats, (3) dietary restriction resulted in reduced rates of protein synthesis per unit mRNA—suggesting that the control was at the level of protein translation.

In summary, the levels of protein intake have profound effects on SMP turnover parameters in rats. *Since the amount of dietary protein consumed is small compared to net whole body protein turnover*, these growth effects can be ascribed to bioactive or "regulatory" effects as opposed to the nutritional function of consumed amino acids. The interpretation of data was complicated by confounding factors. When rats are fed a diet containing varying levels of casein (0, 4, 8, or 18% w/w total food), the low- protein diet is not eaten to the same extent as the high protein diets. Partial correlation analysis allowed the investigators to untangle interactions between multiple

variables leading to the conclusions that dietary protein (as opposed to dietary energy) had a major impact on muscle accretion and the rates of whole body protein turnover. Protein intake had a notable effect on the rate of protein breakdown with little effect on the rates of synthesis. By contrast, increased levels of plasma insulin both raised rates of synthesis and decreased the rates of breakdown.[142]

4.5.2.2 Effect of Nutrients on Skeletal Muscle Protein Turnover—Human Studies

Investigations by Rennie et al. from the University of Dundee, using ^{13}C-leucine and ^{15}N-phenylalanine labels, showed that the intravenous administration of a mixture of amino acids led to increases in leucine oxidation (+200%), protein synthesis (+24%–32%), and decreased SMP breakdown (8%–12%). Changes in SMP flux though weak (and not statistically significant) could account for the muscle accretion.[143,144] Tessari et al. found that a mixed meal (14% carbohydrates, 6.6% protein, 4% lipid, and vitamins) increased forearm SMP synthesis and breakdown by 26%–50% and 30%, respectively. The study, using ^{13}C-leucine and ^{15}N-phenylalanine tracers, also showed that the supply of amino acids to the arm muscles doubled after a meal and that leucine oxidation increased by 100% after feeding.[29,*]

The use of stable isotopes of phenylalanine and leucine to monitor SMP metabolism led to quite important breakthroughs by Wolfe and coworkers over the past decade.[33,145,146] The research program, while concerned mainly with age-related muscle loss, is relevant to a wider range of muscle wasting situations. The intravenous administration of a mixed amino acid feed improved leg SMP fractional synthesis rate in elderly subjects ~100% (from 1.1377 ± 0.1344%/day in the post-absorptive state to 2.256 ± 0.343%/day after feeding). The rate of protein breakdown was not statistically different after intravenous feeding.[145] Oral EAA was found to stimulate SMP synthesis in both young (30 years) or elderly subjects (72 years), to the same extent. In the unfed state, the fractional synthesis rate for leg SMP was the same for the young and elderly (1.0884 ± 0.8088 vs. 1.188 ± 0.2112%/day) increasing to 1.7023 ± 0.223 versus 2.287 ± 0.345%/day for the young and elderly, respectively. With oral feeding, the rate of SMP breakdown was significantly decreased ($P < 0.05$). The comparative effectiveness of intravenous versus oral feeding is not easy to establish owing to the different levels of amino acid fed as well as the route and frequency of feeding. However, by plotting the range of SMP flux responses obtained from oral administration versus intravenous administration, it may be suggested that the former route possesses at least 86% of the effect obtained by injection. The anabolic effect of amino acid administration was similar whether nutrients were administered by the enteral or the parenteral route.[33,146]

It is now well established (Chapter 9) that the anabolic actions of amino acids is due to EAA. Thus, the consumption of 18 g EAA could produce the same increase in SMP fractional synthesis as obtained by consuming 18 g EAA with 22 g of nonessential amino acids.[147] Moreover, leucine was found to be the major dietary anabolic

* The reader is directed to this publication and references cited therein for comprehensive discussion of effects of mixed meal on muscle accretion. This topic has received little attention subsequently (six citations as of 2009) compared to effects of proteins, amino acids, and leucine.

stimulant for SMP synthesis.[133] Rieu et al. showed that dietary supplementation with leucine alone increased SMP synthesis compared to the non-supplemented group (FSR = 1.992 ± 0.192 vs. 1.272 ± 0.216%/day), respectively, $P < 0.05$). These effects could be ascribed to leucine alone since this was the only amino acid that was not present in the control group.[148] Further investigations also suggested that the elderly subjects were less sensitive to the effects of leucine compared to younger subjects.[149]

4.6 SLOW AND FAST PROTEINS

4.6.1 Digestion and Absorption Kinetics

Protein digestion and absorption kinetics affect their utilization.[150,151] In general, dietary protein may be classified as "slow" or "fast" according to their rate of gastrointestinal transit, digestion, and/or absorption. For instance, WP or soybean protein may be classed as and considered fast proteins because of their relatively short transit times and faster rate of digestion within the GI tract compared to casein. This topic is important because fast and slow proteins show different levels of support for muscle accretion. Slow proteins are thought to be more able to support muscle accretion for athletes.[152,153] By contrast, recent research suggests that fast proteins may be more suited for increasing muscle buildup in older subjects (see below).

The fast–slow protein concept can be traced to results published in the mid-1990s by Sivlvane Mahe and other researchers from France.[154] This work showed that digestion characteristics of cow milk casein and the WP (beta-lactoglobulin) were remarkably different. After consuming a meal containing ^{13}-C-labeled beta-lactoglobulin or casein, ~78% and 38% of the meal-liquid phase arrived in the jejunum after 60 min, respectively (Table 4.7). The GI transit time was significantly greater for casein compared to WP. Casein was also readily degraded in the stomach to form small molecular weight peptides. In contrast, electrophoretic analysis showed that beta-lactoglobulin reached the upper small intestines in an undigested state. After a period of 4 h following a single-protein meal, 82.6% casein was absorbed compared to 44.7% of beta-lactoglobulin. However, rather confusingly, it is casein that is later designated as a slow protein.

TABLE 4.7
Differential Rates of Digestion of Fast (Whey) and Slow (Casein) Protein

Index	Casein	BLG/Whey P
Jejunum liquid flow, (mL/min)	6.32 ± 1.85	6.11 ± 2.2
% Meal, @ jejunum (60 min)	38	71
% Dietary N uptake, (240 min)	82.6 ± 9.5	44.7 ± 24.4
Exogenous N @ ileum	4.9	43.3

Source: Data from Mahe, S. et al., *American Journal of Nutrition*, 63, 546, 1996.

The digestibility values for casein and beta-lactoglobulin are the same in the longer term (Chapter 2). It is the time course or *kinetics* of digestion that is different. As indicated above, cow milk caseins are transferred more slowly through the stomach compared to WP. The slower GI transit of casein is attributed to their tendency to coagulate within the low pH environment of the stomach. However, the digestion of casein begins within the stomach, leading to peptide products that are gradually absorbed *in the stomach and jejunum*. By contrast, the fast-transit WP remains structurally intact during passage within the low pH of stomach. Beta-lactoglobulin is digested only after reaching the small intestines, followed by a relatively rapid absorption of WP breakdown products, and relatively sharp rise and fall in plasma amino acids (Table 4.7). The comparative analysis of the digestion and absorption rates of labeled soybean and milk protein showed that the former may be classed as fast protein.[155]

4.6.2 Effect of Fast Dietary Proteins on Protein Turnover

Investigations employing young adults showed that single meals containing WP can increase whole body muscle protein accretion (leucine balance) to a lesser extent than a meal containing casein. A WP diet led to a faster rate of absorption of amino acids (exogenous Leu-R_a), increased rate of proteolysis of body proteins (endogenous Leu-R_a), increased oxidation, and increased rates of protein synthesis. However, the casein meal inhibited the rate of whole body protein breakdown (endogenous Leu-R_a) by 34% compared to WP, which had no effect on the rate of protein breakdown.[156] In conclusion, the preceding observation showed that slow proteins (e.g., casein) had a more anabolic effect compared to fast protein (e.g., WP). The data summarized in Table 4.8 suggest that the benefit of slow proteins is associated with a number of independent effects on whole body protein synthesis, breakdown, and oxidation.

TABLE 4.8
The Effect of Slow (Casein) and Fast (Whey) Protein on Whole Body Protein Metabolism in Adult Humans Determined Using Amino Acid Tracer Method

Whole Body Protein Turnover Indices[a]	Casein	BLG (Whey P)
Total leucine-R_a (μmol/kg/h)	123 ± 19	252.6 ± 19.8
Exogenous-leucine-R_a[a] (μmol/kg/h)	~60	~180
Endogenous leucine-R_a[a] (μmol/kg/h)	64.8 ± 4.2 (34%↓)	82.8 ± 2.4 (0%)
Leucine-oxidation (μmol/kg/h)	21.6 ± 6.6 (123%↑)	90 ± 19.8 (212%↑)
NOLD (% increase)	31↑	68↑
Leucine balance ((μmol/kg) @420 min)	141 ± 96	11 ± 36

Source: Data from Boirie, Y. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 94, 14930, 1997.

[a] Maximum value @100 min.

NOLD = non-oxidative leucine disappearance, (↑↓) percent change compared to non-fed state. Study with 16 young adults (age 24.6 ± 4 years; BMI 21.9 ± 1.8 kg/m^2).

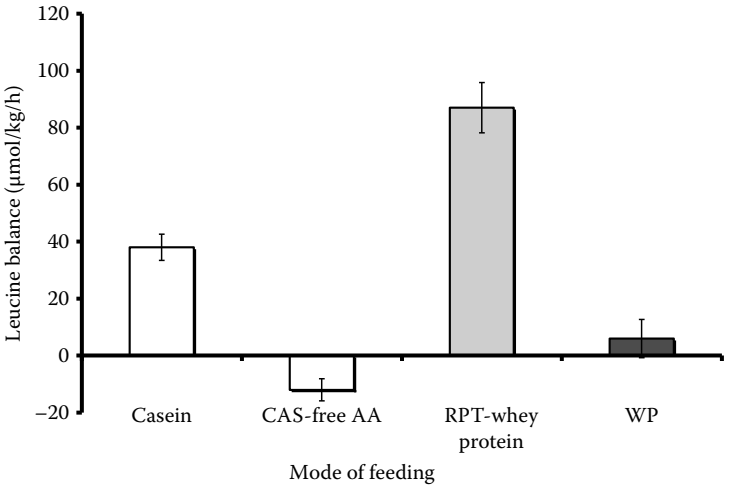

FIGURE 4.7 The effect of repeated WP (RPT WP) meals or casein meal, casein-amino acids, or a single WP meal on protein accretion (leucine balance). (Drawn from Dangin, M. et al., *Am. J. Physiol. Endocrinol. Metab.*, 280(2), E340, 2001.)

A further human study by the French group showed that casein protein meal produced grater muscle accretion compared to free amino acids equivalent to casein.[157] Interestingly, a single (30 g) WP meal was found to produce a negative nitrogen balance consistent with the notion of fast protein. On the other hand, repeated WP meals (total 30 g WP subdivided into 13 meals/7 h) produced a remarkable increase in the extent of protein accretion (Figure 4.7). In simple terms, repeated WP meals, taken at frequent intervals, lead to improved ability to restore body weight compared to a single meal of equal size.

The fast–slow proteins concept is supported by results obtained by studying the effect of feeding intact dietary proteins versus free amino acids on the rate of body protein synthesis. Daenzer et al.[158] showed that a diet containing intact casein stimulates whole body protein synthesis to a greater extent compared to diets containing free amino acids equivalent to casein. The free amino acids equivalent to casein diet produced higher levels of amino acid oxidation and nitrogen excretion compared to a diet containing intact protein. Analysis of plasma, gut, and liver concentrations of ^{13}C-leucine suggested that rapidly absorbed free amino acids results in a less efficient synthesis of whole body protein.[159] Consistent with the slow-is-good concept, starved rats fed WP oligopeptides (WP hydrolysates) showed greater nitrogen retention and increased body weight compared to animals fed a diet containing free amino acids equivalent to WP.[160] Apparently, intact protein and oligopeptides are preferable to free amino acids for the purpose of supplementation.

The effect of oligopeptides compared to intact proteins on protein synthesis rate has also been examined. Collin-Vidal et al. showed that the polymeric state of dietary protein affects the body's response in terms of protein turnover.[161] Nasogastric feeding using casein oligopeptides produced 12%–20% higher values for plasma leucine

concentration, turnover rate, oxidation, and non-oxidative disposal compared to a diet containing intact casein. However, the intact casein diet produced a 50% lower rate of whole body protein breakdown resulting in a more positive leucine balance. Ziegler et al.[162] found that 12 abdominal surgery patients given an enteral diet containing intact protein (66% casein plus 33% WP) or hydrolyzed protein (oligopeptides) showed marked differences in peripheral amino acid bioavailability. The blood levels of amino acids were elevated to higher levels (10%–30%) following the oligopeptides diet as compared to the intact protein diet. The former diet also produced a faster insulin stimulation that was highly correlated with the blood levels of leucine. The study did not cover changes in protein economy. In summary, given the limited number of studies, we may suppose that oligopeptides are at least as efficient in stimulating protein synthesis as free amino acids. Further research is needed specifically to compare oligopeptides with intact proteins in terms of their effects or suitability for special groups of consumers, for example, young versus elderly. It is not certain whether elemental, semi-elemental, or polymeric enteral formulas, containing free amino acids, oligopeptides, or intact protein, respectively, are more suited for particular types of applications.

4.6.3 Intrinsic versus Extrinsic Contributions to Fast and Slow Proteins

The efficiency of dietary protein utilization for muscle building is partly determined by the rate of digestion controlled by protein-related (intrinsic) factors as well as non-protein (extrinsic) factors (Table 4.9). Protein absorption varies inversely with their molecular weight: free EAA > oligopeptides > intact proteins. Evidence suggests that the efficiency of dietary EAA utilization for protein synthesis increases with the

TABLE 4.9
Considerations for Designating Dietary Proteins as Fast or Slow

Factors	Comment
• Size, composition, and frequency of protein meals	• Use frequent small protein meals
• Stomach transit time	• Physical state (e.g., casein coagulation) and gut mobility
• Intestinal transit time	• Same for most dietary proteins
• Enzymatic digestion	• Protein structure, protease subsite specificity
• Essential amino acid composition	• Nutritional characteristics, hormone stimulatory action
• Absorption, transport kinetics	• Specificity, and distribution of amino acid and peptide transporters
• Metabolism in the splanchnic bed	• Rate of EAA utilization and oxidation
• Release kinetics	• Release of EAA to the peripheral organs
• Liver metabolism	• Degree of first-pass utilization by liver

increasing extent of polymerization, for example, free EAA < oligopeptides < intact proteins < slow proteins. Compared to so-called fast proteins (e.g., WP and soybean protein), slow proteins such as casein exhibit an increased GI transit time, a decreased rate of digestion, a lower rate of intestinal absorption, lower rates of assimilation to form GI proteins, and slower re-release from the GI tract during the postprandial period. Luiking et al.[163] suggested that casein is nutritionally superior to soybean based on lower oxidation and greater splanchnic bed extraction of amino acids in the former case. By contrast, the rate of whole body protein breakdown (54.6–58.0 μmol/kg/h) and synthesis was not significantly different after consumption of soybean versus casein diet.

As indicated above, repeated feeding using WP led to postprandial kinetics expected for a slow protein. It follows that other technological strategies may be used to transform fast- to slow-proteins and vice versa. Lacroix et al. demonstrated that UHT treatment milk alters the plasma amino acid profiles for normal milk in a manner that leads to faster absorption of amino acid.[164] Smith et al. found that supplementation using commercial samples of casein and WP produced similar plasma amino acid profiles because the batch of casein employed had undergone degradation (presumably) during manufacture.[165]

4.6.4 Fast versus Slow Proteins for the Elderly and Young

There appears to be an age-related effect in the way that slow and fast dietary proteins affect body protein synthesis.[166–169] Slow proteins promote muscle protein synthesis in younger adults to a greater extent than fast proteins. By contrast, fast proteins were found to be more effective for increasing SMP accretion in the elderly. The different responses of young and elderly subjects to fast/slow proteins can be explained by the following: First, postprandial absorption of EAA has been shown be more rapid in the younger subjects, judging from the appearance of label in the blood plasma and muscle. On the other hand, splanchnic bed utilization of EAA is greater in aging adults resulting in a flatter response curve. Second, elderly subjects exhibit a blunted protein synthesis response to dietary EAA and a lower insulin stimulatory activity.[35,170] On the other hand, fast proteins and EAA have been shown to produce higher rises in plasma insulin levels. Accordingly, fast proteins that produce higher plasma concentrations of EAA will be able to stimulate protein synthesis in the elderly. Another important difference between fast and slow proteins appears to be related to their satiety characteristics; WP has been found to show more satiating effect compared to casein. Though the underlying modes of action have yet to be established, there is general agreement that "fast" proteins may be useful as adjuvant for the nutritional management of wasting diseases linked with wasting and enforced bed rest.[133,171]

4.6.5 Insulinotropic Action of Fast Proteins

Fast proteins appear to stimulate insulin release to a greater extent than slow dietary proteins though whether this effect has important consequences for muscle protein synthesis has yet to be determined. Calbert and MacLean[172] showed that test meals comprising 2.5% glucose and protein in various states of digestion increase insulin

production. However, the effect was 200%–300% greater with protein hydrolysates compared to intact proteins. The former test meals also produced faster gastric emptying, greater increases in plasma total amino acids and BCAA concentration. The insulinotropic action of WP is discussed further in Chapter 8.

4.7 SUMMARY AND CONCLUSIONS

The measurement of whole body and SMP turnover provides useful data related to the protein status of the body. Investigations using stable amino acid tracers provide data on the rates of protein synthesis, protein breakdown, and amino acid oxidation. Current data suggest that illness increases both rates of protein breakdown and synthesis, as well as the rates of amino acid oxidation. Improved understanding of these changes may offer novel ways of preventing muscle protein wasting.

APPENDIX 4.A.1
The Effect of Illness on the Rates of Protein Metabolism*

Condition	Protein Breakdown	Protein Synthesis	Oxidation
Preterm infants	↑	↑	?
Aging	↓	↓	↓
Burns injury	↑	↓	?
Cancer	↔	↓↑↑↓	↑
Chronic renal failure	↔	↓	↑
Diabetes	↑	↓↑	↔
Diabetes (T2D)	↑	±	
Feeding	↑	↑	↑
HIV/AIDS	↑	±	?
TB	↔	↔	↑

* Protein metabolism shows an increase (↑), decrease (↓), or no change (↔). Controversial responses (±).

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5 Major Processes for Muscle Gain and Loss

5.1 INTRODUCTION

Muscle loss contributes to the weight loss observed during illness. Therefore, it is important to consider factors that may contribute to changes in muscle accretion. Until recently, the numbers of fibers within different muscle groups were thought to be fixed prior to birth. Increases in muscle size, for example, arising due to resistance exercise, were ascribed to changes in cross-sectional area (CSA) for existing fibers.[1] According to this antiquated model, the formation of new fibers cannot occur after birth and the postnatal growth of muscle is controlled by events taking place within existing fibers. Unfortunately, the old model for muscle growth could not account for the enormous capacity for regeneration and adaptation to exercise observed in adult muscles. There are now alternative explanations for muscle growth that allow for the generation of new fibers from stem cells.[2,3]

The major cellular and molecular processes that contribute to muscle loss and accretion are reviewed in this chapter. The contribution of muscle satellite stem cell (MSSC) to muscle size increase is described in Section 5.1. The negative regulation of muscle cell proliferation by myostatin is discussed in Section 5.2. The contribution of muscle cell death to wasting is considered in Section 5.3. The role of the ubiquitin proteasome (UPS) in the breakdown of muscle protein is considered in Section 5.4. Intracellular signaling pathways involved in the promotion of muscle loss or muscle size increase are discussed in Sections 5.5 and 5.6, respectively.

5.1.1 Muscle Cells

Skeletal muscles contain two major groups of cells: muscle fibers and MSSC. Over 95% of the cells within muscle tissue consist of elongated fibers responsible for contraction. Muscle fibers are syncytial cells with large numbers of nuclei.* Though not divided by intervening cell membrane, each nucleus exerts influence over a defined mass of cytoplasm. Processes that lead to death or destruction of muscle cell nuclei alter the size of these so-called myonuclear domains.[4,5] Adult muscle fibers originate from fetal cells that undergo a series of programmed transformations within the first few months of conception (Figure 5.1). The developing fetal muscle cells eventually fuse together to form myotubes resembling adult muscle fibers. It is thought that myotube numbers increase until each acquires a surrounding basal membrane

* Syncytial cells are multinucleate mass cells with no dividing cytoplasm. With the exception of some syncytial viruses, there are few multinucleate cells within the body. Bone is sometimes classed as a syncytial mass similar to muscle.

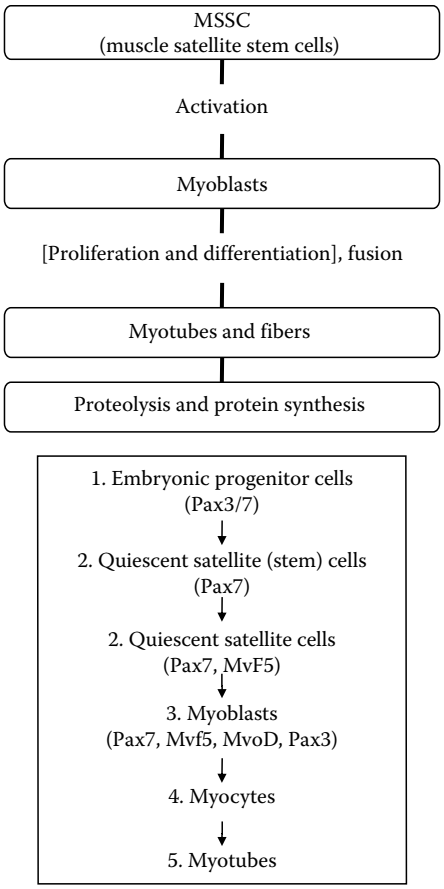


FIGURE 5.1 Developmental steps involved in transforming muscle stem cells to muscle fibers (myotubes). Genes expressed at each stage are shown as italicized. *Abbreviations*: Pax = paired box family of transcription factors. Myf5, MyoD, Mrf4 = genes specific to muscle cells. (Adapted from Bischoff, R. and Heintz, C., *Dev. Dyn*., 201, 41, 1994; Scrime, A. and Rudnicki, M.A., *Curr. Opin. Clin. Nutr. Metab. Care*, 9, 214, 2006; Sherwood, R.I. and Wagers, A.J., *Trends Mol. Med*., 12, 189, 2006; Kuang, S. and Rudnicki, M.A., *Trends Mol. Med*., 14, 82, 2008; Schultz, E. and McCormic, K.M., *Rev. Physiol. Biochem. Pharmacol*., 123, 213, 1994; Allen, R.E. et al., *Methods Cell Biol*., 52, 155, 1997; McFarland, D.C., *Poult. Sci*., 78, 747, 1999.)

during the later stages of fetal development. The differentiated myotubes are nondividing but undergo expansion or shrinkage in response to external stimuli.

Cells formerly known as muscle satellite cells make up 1%–5% of the total muscle mass. The satellite cells are normally found closely "hugging" muscle fibers but their function was largely unknown. Recent research has shown that muscle satellite cells exhibit characteristics associated with adult stem cells, including the ability to undergo unlimited proliferation and, under the right circumstances, to undergo differentiation to form new muscle fibers. MSSC also express specific markers associated

with adult stem cells found around the rest of the body.[6–9,*] Current research suggests that proliferating MSSC produce a population of cells capable of self-renewal. Some actively dividing MSSC also undergo migration, adhesion, and fusion with existing fibers, leading to muscle hypertrophy.[10] Such data indicate that the MSSC contribute to overall changes in muscle size. The MSSC remain quiescent (nondividing) under normal circumstances until activated to divide by stress, injury, or exercise.[11,12,†]

5.1.2 Muscle Stem Cell Proliferation

Maintaining MSSC numbers may have important consequences for muscle gain and loss. Ideally, ways should be found to promote MSSC proliferation and vitality in clinical situations in order to combat muscle wasting. As indicated above, MSSC can develop into entirely new muscle fibers.[13] On the other hand, decreases in MSSC numbers are thought to be associated with muscle loss due to aging, radiation therapy, and trauma (Table 5.1). MSSC may also be able to regenerate damaged muscle as well as contribute to increasing CSA.[14] MSSC have been proposed as treatment of muscular dystrophy and chronic heart failure.[15–17] Two reports from the NIH provide readable

TABLE 5.1
Activation and Inhibition of MSSC Proliferation

MSSC Activators	MSSC Inhibitors
Creatinine[29,30]	Aging[52,53]
Dietary amino acids[31]	Hind limb suspension[54]
Exercise[32,33]	Inflammation[55]
Glutathione[34–36]	Myostatin[56–58]
Growth factors[37–39]	NSAIDs (no detrimental effect reported)[59]
Growth hormone[40]	Obesity[60]
Insulin-like growth factor (IGF-1)[41–43]	Proteolysis-inducing factor[61]
Interleukin 15 (IL-15)	Radiation treatment[62]
Isoflavones[44]	Starvation[63]
Microphage activity[45]	Trauma[64]
NO and arginine[46]	Tumor necrosis factor-α (see text)
Pituitary hormones[47]	Undernutrition[65]
Post-hatching feeding (see text)	
Steroidal hormones (see testosterone)	
Testosterone[48–50]	
Thermal conditioning[51]	

* Muscle cells that retain the ability to divide are described by a variety of terms in the literature including muscle stem cell, satellite cell, muscle precursor cell, or C2C12 myoblast.

† Research indicates that the proportion of satellite cells in growing muscle may be higher than 5%. Antibodies for neural cell adhesion molecule (NCAM), a marker for satellite cells, were shown to label 30% and 14% of porcine skeletal muscle cells isolated from 1-week old and 7-week old animals, respectively.[11]

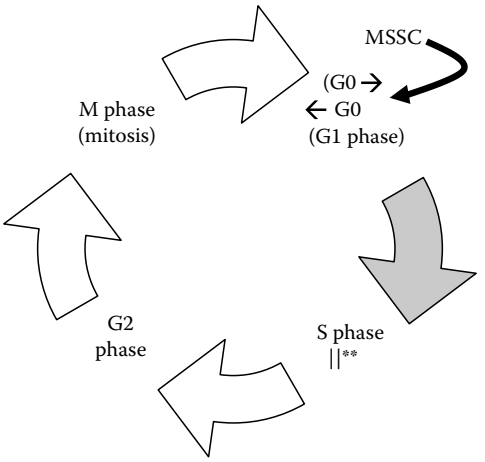


FIGURE 5.2 MSSC cycle events contributing to muscle growth. Nondividing MSSC in G0 phase enter into the G1 phase (preparation for DNA synthesis) and then the S phase (for DNA replication), followed by the G2 phase (preparation for mitosis or M phase), and then mitosis. **Shows restriction point, beyond which external signals are not needed. (Adapted from Shankland, S.J. and Wolf, G. *American Journal of Physiology Renal Physiology,* 278, F515, 2000.)

introductions to the field.* Several excellent reviews have appeared also on the topic of muscle stem cell therapy in general.[18–22] The rest of this discussion will focus on the relations between MSSC activity and muscle mass.[17,23–27]

MSSC exist in the nondividing G0 phase of the cell cycle (Figure 5.2). Transfer from the G0 to G1 phase occurs following treatment with hormones, growth factors, and also following injury. Hormonal activation probably occurs via G-protein membrane receptors (e.g., insulin and IGF-1). MSSC are also activated by exercise but the underlying pathways are not yet understood. Activated MSSC progress through the G1, S-phase, G2 phase, and M-phases of the cell cycle.† In general, the regulation of cell cycle events requires cyclin-dependent kinases (CDK1–CDK8) that catalyze the phosphorylation of regulatory proteins. CDK complex formation with cyclins (cyclin 1–8) is needed for enzymatic activity. Cell cycle progression is controlled by protein inhibitors (p21, p27, and p53) for cyclin–CDK complex formation leading to arrest at checkpoints 1 and 2. Obviously, the fundamental steps in the cell cycle are not unique to MSSC.[28–30]

Hypertrophy results from cell protein and RNA synthesis without DNA replication.[66] According to the basic principles of cell biology, withdrawal from G1/S transition is controlled by cyclin-CDK-mediated phosphorylation of E2F1-retinoblastoma protein complex to release E2F1 transcription factor that activates genes needed for G1/S transition leading to DNA synthesis and cell proliferation. Some of the target genes for E2F1 are involved in protein synthesis, organization of cell cytoskeleton, or mitochondrial organization. The expression of E2F1

* Stem cells: scientific progress and future research directions (http://stemcells.nih.gov/info/scireport/).
† Denoting a first gap (G1), DNA synthesis (S), second gap (G2), and mitosis phases respectively.

(and related proteins) may catalyze the reentry of quiescent cells in cell cycle. Though the underlying processes are not fully understood, growth factors, exercise, and injury are believed to stimulate the E2F1 and that these somehow moderate MSSC proliferation (and differentiation) in a manner that impacts favorably on muscle mass. Agents that inhibit MSSC proliferation tend to be those that promote muscle atrophy (Table 5.1).

Proliferating MSSC withdraw from the cell cycle before cell differentiation. The maturing myoblasts then fuse with existing myotubes leading to a net increase in muscle fiber cross-sectional area. Myoblast differentiation is controlled by several myogenic transcription factors (MyoD, Myf5, myogenin, and MRF4), which are activated by growth hormone *withdrawal*. Differentiation is also accompanied by the increased expression of CDK inhibitor p21. The general view is that inhibition of MSSC proliferation leads to muscle atrophy. For instance, myostatin is thought to inhibit MSSC proliferation thereby preventing muscle growth (Section 5.2). On the contrary, agents such as IGF-1, which increase MSSC proliferation, increase muscle size. What is not certain, however, is whether agents that delay muscle cell differentiation (e.g., some cytokines) necessarily inhibit muscle fiber hypertrophy or not.

5.1.3 Muscle Stem Cell Differentiation

Cell culture studies by Guttridge et al. demonstrated that TNF-α inhibits C2C12 expression of several biomarkers for muscle differentiation, that is, MyoD mRNA and MyoD protein.[67] The inhibitory effect of TNF-α on MSSC differentiation was mediated by NFκβ/p65. The action of TNF-α required the presence of IFNγ (but not IL-1β or IL-6). Fully differentiated C1C12 myotubes were not sensitive to the effects of TNF-α/IFNγ. These findings were corroborated by Langen et al. who found that TNF-α and IL-1β delay the differentiation of C2C12 myocytes by a NFκβ-dependent pathway. Actively, dividing myocytes were more sensitive to cytokines compared to C2C12 myotubes.[55] The TNF-α/NFκβ inhibition of myocyte differentiation involves the proteolysis of MyoD protein by proteasome pathway.[68] Note also that NFκβ activation has been linked with inflammation-related muscle wasting.[69,*]

TNF-α also enhances myotube proteolysis mediated by ROS. Li et al.[70] showed that TNF-α (1–3 mg/mL) lowers the concentration of myosin heavy chain fragment produced by differentiated C2C12 myotubes. However, the total number cell DNA (corresponding to nucleus number) and rate of protein synthesis was unaffected. TNF-α-stimulated ubiquitin conjugation activity linked with activation and transfer of NFκβ to the cell nucleus. TNF-α activity was enhanced by hydrogen peroxide treatment and inhibited by catalase. Cells transfected with a plasmid coding for proteasome insensitive (stable) inhibitory kinase kinase (IKK) did not show sensitivity to TNF-α. Current data suggests that the breakdown of differentiated muscle fiber under the influence of TNF-α requires IKK activation of NFκβ and increased expression of ubiquitin ligase.[71,72] Interestingly, TNF-α

* The role of nuclear factor kappa beta in muscle wasting is discussed in Section 5.5.2 and Section 6.2.3.

activation of NFκβ involves rising levels of cell ROS (Section 6.2.1), which offers considerable opportunity for therapeutic intervention using antioxidants.

5.1.4 Nutrient Effects on Muscle Stem Cell Growth

Restricted feeding inhibits the proliferation of MSSC isolated from 2 day old chicks, resulting in low muscle mass in adult birds.[20] By contrast, improving the nutrition of young chicks led to increased rates of MSSC proliferation and differentiation leading to increased muscle mass in adult hens.[31,73] Dietary EAA have been shown to improve MSSC activity and thereby stimulate muscle hypertrophy.[74,75] Relatively little work has appeared in the clinical nutrition literature concerning muscle stem cells. However, researches by animal scientists suggest that nutrient supply can enhance MSSC growth leading to improvements in muscle mass for agricultural livestock.[76]

5.2 MYOSTATIN

5.2.1 Double Muscling and Myostatin Mutations

Myostatin is a member of the transforming growth factor-beta (TGF-β) superfamily that includes bone morphogenesis factor, avinin, and ~1000 other proteins.[77,*] Mutations in the myostatin gene lead to the double muscling phenotype in Belgian blue and Piedmontese cattle (Figure 5.3). A normally functioning myostatin gene acts to inhibit muscle size increase. Reports suggest that Belgian blue cattle have increased muscle fiber numbers and 20%–25% increase in lean body mass compared to ordinary

FIGURE 5.3 A natural mutation in the myostatin gene leads to double muscling in Belgian blue cattle. (Wikipedia.com)

* Myostatin was discovered by Alexandra McPherron and Se-Jin Lee from the Johns Hopkins University School of Medicine (Baltimore).

cattle.[78–80,*] However, livestock with the double muscle phenotype have increased numbers of muscle fibers compared to normal breeds but fiber cross-sectional area remains normal. The Belgian blue and Piedmontese breeds have 20%–25% increase in lean body mass, decreased skin, adipose tissue, and bone mass.[79,80]

Low levels of myostatin occur in <29 days old embryo (presumably matching periods of most active muscle growth) but levels increase thereafter until late gestation. In adult animals, myostatin is found within skeletal muscles and also circulating in the serum (see below). Genetic analysis suggests that the level of expression of myostatin gene was the same in double muscle cattle compared to normal controls. However, in the former case, the myostatin gene showed mutations that are likely to produce defective proteins. Belgian blue cattle showed an 11 bp deletion in the myostatin gene leading to a loss of amino acid residues 275, 276, and 277, along with stop codon at AA287. In the double-muscled Piedmontese cattle, there was a single mutation leading to the substitution of cysteine 314 (314C) with tyrosine. In summary, myostatin functions in the negative control of muscle size. Natural mutations in the myostatin gene lead to enhanced muscle mass. Purposeful deletions of the myostatin gene have also been shown to cause double muscling in cattle.[81]

5.2.2 Myostatin Structure and Activity

The characteristics of myostatin resemble those of other TGF-β members including bone morphogenic peptide (BMP) and avinin.[82,83] The 12.5 kDa active form of myostatin (107 AA) is formed by the intracellular processing of a 47.5 kDa (375 AA) precursor protein that contains a signal sequence (AA1–23) joined to a 35 kDa propeptide (AA 24–266) and the 12.5 kDa active fragment (AA267–375). The primary structure of myostatin contains nine cysteine residues allowing the formation of four intramolecular disulfide bonds with one free cysteine residue spare. The net charge for the myostatin (p*I* = 6.4) shows it to be a neutral peptide (Table 5.2).

It is thought that the active form of myostatin exists as a disulfide-linked dimer. Myostatin occurs in the blood circulation bound with the myostatin pro-peptide. Hill et al. isolated myostatin complex from mice serum by affinity chromatography using a myostatin–antibody-coated support. Mass spectrometry and SDS-PAGE analysis suggested that 70% of myostatin was complexed with the pro-peptide and could be dissociated by acid treatment.[84,†] Circulating myostatin may also be bound by follistatin-related protein and a select number of circulating proteins, which reduces its bioactivity.[85]

5.2.3 Mode of Action of Myostatin

Myostatin has been shown to prevent muscle mass increase by inhibiting the proliferation of MSSC.[56,57,86,87] It is thought that myostatin induces cell cycle arrest probably by stimulating the expression of CDK inhibitors, p21 and p27.[88] Myostatin may

* The Internet is replete with remarkable pictures of double-muscled breeds of domesticated animals including racing dogs and sheep, many of which are derived from the article by Lee.[78]

† Myostatin concentrations in blood plasma were estimated as ~80 ng/mL for mice.

TABLE 5.2
Characteristics of Myostatin Mature Chain Fragment (Residues 267–375)

267 277S|* 288T| 295W| 305Y| 314C|**
DFGLDCD**EHS**|TESRCCRYPLT|VDF EAFGW|DWIIAPKRY|KANYCSGE**C**|EF
324H| 330 340 350
VFLQKYPH|THLVHQA N|PRGSAGPCC T|PTKM SPINM |LYFNGKEQII
|360 370 D|
|YGKIPAMVVD|RCGCS

Modification	Residue	No.	Type
Glycosylation	47	1	N-linked (GlcNAc…) Potential
Glycosylation	71	1	N-linked (GlcNAc…) Potential
Disulfide bond	281 ↔ 340		Intrachain
Disulfide bond	309 ↔ 372		Intrachain
Disulfide bond	313 ↔ 374		Intrachain
Disulfide bond	339		Interchain

Number of amino acids: 109
Molecular weight: 12407.2
Theoretical p*I*: 6.44
(Asp + Glu): 12
(Arg + Lys): 11

SwissProt accession O14793 (GDF8_HUMAN). *Sites with reported mutations in Belgium blue double muscle cattle (Res 275–277 deleted, Res 278 altered, Res 287 truncated). **Point mutation leading to Tyr/Cys substitution in mutation Piedmontese animals.

also inhibit MSSC differentiation by lowering the expression of myogenic genes (*MyoD*). There is some evidence that myostatin action is dependent on the mTOR pathway. Amirouche et al.[89] found that the overexpression of myostatin in rat muscle led to a decreased phosphorylation of components of the mTOR signaling pathway (Akt/mTOR/pS6k). Welle et al. reported that treating mice with myostatin antibody increased the phosphorylation of ribosomal protein S6 but not the Akt protein. The underlying pathways were not affected by rapamycin suggesting that this signaling did not involve mTORC1.[90]

5.2.4 Myostatin Role in Wasting Diseases

Gonzalez-Cadavid et al.[91] found a link between muscle wasting due to HIV infection and myostatin gene expression (Table 5.3). The team from the United States, Canada, and Sweden cloned the myostatin gene consisting of three exons and two introns from

TABLE 5.3
Plasma Serum Myostatin Levels in Healthy and HIV-Infected Men

	Healthy Men (n = 42)	HIV (n = 38)	HIV + WL (n = 23)
Myostatin (ng/mL)	290 ± 13	363 ± 21	500 ± 37
Age (years)	31.2 ± 1.9	42 ± 1.4	38.5 ± 1.4
BMI (kg/m^2)	24.9 ± 0.5	23.8 ± 0.7	20.1 ± 0.8
FFMI (kg/m^2)	21.0 ± 0.4	17.6 ± 0.4	15.5 ± 0.6
CD4+ (cell/mL)	—	224 ± 37	199 ± 49
CD8+ (cells/mL)	—	769 ± 75	367 ± 127
HIV RNA (no/mL)	—	23.4 (±11) × 10^3	172.6 (±104) × 10^3

Source: Adapted from Gonzalez-Cadavid, M.F. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 95, 14938, 1998. Values show mean ± SEM, BMI = body mass index, FFMI = fat-free mass index for HIV sufferers (HIV) or HIV with weight loss (HIV + WL).

human chromosome 2q33.2. Western blot analysis showed that a 26 kDa myostatin-like protein (double the expected molecular mass) was expressed in skeletal muscles and secreted into the blood plasma. In healthy men, the serum concentration of myostatin-like protein was 290 ± 13 ng/mL (range 140–490 ng/mL). Myostatin levels were significantly higher in HIV-infected subjects with overt (>10%) weight loss compared to those not showing weight loss. For the healthy and HIV-infected subjects taken together, myostatin-like protein concentrations were inversely related to FFM ($R = -0.33$; $P < 0.003$). Blood serum myostatin–related protein concentrations were not correlated with CD41 and CD81 T cell counts or plasma HIV-RNA copy number.

Reardon et al.[92] demonstrated that levels of myostatin in skeletal muscles increase during disuse atrophy. Myostatin concentrations were also negatively correlated with muscle fiber cross-sectional area. Levels of myostatin increase three- to fourfold within 24 h of burns injury (30% surface area). The rise in myostatin was prevented by the glucocorticoid receptor blocker, RU486. However, myostatin levels in burns patients were not related to TNF-α, sepsis, or challenge with endotoxin.[93] Myostatin is linked with the cachexic effect of dexamethasone.[94] Gene knockout mice lacking the myostatin gene were not susceptible to glucocorticoid-induced muscle wasting.[95] The involvement of myostatin in muscle wasting diseases was confirmed for various models of experimental cachexia[96–98] and also for wasting due to kidney diseases.[99] Zimmers et al. showed that injection of recombinant myostatin leads to symptoms of cachexia.[85] The treatment of adult mice with myostatin antibody led to increased muscle mass (see below). In summary, evidence from the past 10 years suggests that myostatin acts as an inhibitory agent that functions to actively prevent muscle size increase. Myostatin levels have been shown to increase in wasting diseases. There is considerable excitement, and also some alarm, that agents might be developed to switch off myostatin production, leading to muscle hypertrophy.

5.2.5 Myostatin Inhibition as Therapy for Muscle Wasting

An inactive form of myostatin is found in the serum bound to its own pro-protein fragment. Active myostatin can be dissociated from the pro-protein by acid treatment in vitro. The inhibition of myostatin activity has been proposed as a treatment for muscle atrophy and dystrophy,[100] including the use of DNA vaccine,[101] antisense iRNA,[102] myostatin-specific antibodies,[103] soluble myostatin receptors,[104] and myostatin-binding proteins.[105–109] The likelihood is that effective myostatin-based therapies for improving muscle mass are some years in the future.

5.3 MUSCLE CELL DEATH AND ATROPHY

5.3.1 Types of Cell Death

Muscle cells undergo three forms of cell death (Table 5.4): Type I (apoptosis), Type II (autophagy), and Type III (necrosis). Apoptosis and autophagy are examples of programmed cell death involving a series of ATP-requiring and potentially interruptible events as well as the expression of specific genes and proteins. Necrosis (Type III cell death) occurs via a relatively disorganized breakdown of cell mass.[110] By which of the three mechanisms of cell death muscle cells die also depends on the prevailing conditions, e.g., nutrient restriction, exposure to toxins, and/or oxidative stress. The type of cell death is also affected by their developmental stage (e.g., myoblasts vs. myotubes).

5.3.2 Muscle Apoptosis and Necrosis

Apoptosis can be distinguished from necrosis because the former is an ATP-requiring process.[111] Apoptosis is also characterized by cell shrinkage and the non-inflammatory removal of deceased cells. By contrast, muscle necrosis predominates under circumstances where there is reduced ATP availability. Necrosis leads to cell swelling and the disruptive release of cell contents followed by proinflammatory (macrophage) mediated clearing up of cell debris. The salient features of Type I–III cell death have been reviewed.[112–114]

Two apoptotic signaling pathways induce cell death by triggering caspases (thiol proteases found in cells). The extrinsic or death receptor pathway is activated by external signals and ligands including TNF-α (Figure 5.4). The extrinsic pathway involves reception of an intracellular/extracellular death signal, activation of initiator/effector caspases, followed by activation of DNases, endonucleases, and eventual increase in DNA fragmentation. Aside from the caspase-dependent processes described above, apoptosis may occur by a so-called mitochondria-dependent route or intrinsic pathway. The intrinsic pathway requires the transfer of apoptosis-related proteins (e.g., Bax) to the mitochondrial membrane where it causes the release of cytochrome *C* (Cyt *C*). Interactions between Cyt *C* and two other proteins (Apaf1 and caspase 8) then occurs, leading to the activation of caspase 3 and other caspases; for recent reviews, see Refs. [115–117] and Sections 5.3.3 and 5.3.4.

TABLE 5.4
Three Types of Muscle Cell Death (Type I–Type III)

Types of Cell Death	Indications, Processes
Apoptosis (Type I)	Cell morphological changes
	Membrane shrinkage and blebbing
	Fragmentation of nucleus
	Margination of chromatin
	No inflammation
	Degradation and fragmentation of DNA
	Membrane inversion exposes phosphatidylserine
	Activation of caspases such as caspase 3
	Cell ATP retained
	Phagocytosis by macrophages
Autophagy (Type II)	Mediated by lysosomal enzymes
	Large autophagy vacuoles
	Exteriorization of phosphatidylserine
	No inflammation
	Late DNA fragmentation if any
Necrosis (Type III)	Uncontrolled cell death
	Cell looses ATP or membrane pumps
	Cell and organelle swelling
	Proteins precipitated
	Inflammatory response
	Loss of membrane integrity
	No vesicle formation, complete lyses
	No energy requirement (passive process)
	Affects groups of contiguous cells
	Initiated by stress, e.g., complement attack, lytic viruses, hypothermia, hypoxia, ischemia, and metabolic poisons
	Phagocytosis by macrophages
	Shows inflammatory profile

Source: Adapted from Lockshin, R. and Zekeri, Z., *Int. J. Biochem. Cell Biol.*, 36, 2405, 2004.

Mitochondrial pathway:

DNA damage → p53 protein (+) → Bcl2 (?) → → Mitochondria → ROS/Cytochrome *C* release? → Caspase 3 → Cell lyses + DNA fragmentation

Death receptor pathway:

FAS ligand → (TNF-α receptors) → Caspase 8 → Caspase 3 → Cell lyses and DNA fragmentation

FIGURE 5.4 Summary of two major signaling pathways for apoptosis.

Techniques for studying apoptosis have not been widely applied to muscle cells. Electron microscopic observations may be carried out to determine cell shrinkage, chromatin condensation, membrane blebbing, etc. DNA fragmentation can be measured using gel electrophoresis, nick-end labeling, and, more recently, techniques such as the COMET assay. Changes in cell membrane permeability can be detected by staining with dyes including trypan blue or propidium iodide. The increase in caspase activity may be monitored by specific fluorescent substrates for direct assay or enzyme-linked immunoassay. Finally, changes in the expression of apoptosis-related proteins (Bcl2/Bax) have also been found to provide a useful index of apoptosis.[85,99–101]

5.3.3 Skeletal Muscle Wasting via Apoptosis

Skeletal muscle cell apoptosis was first reported around 1995.* Rat skeletal muscle myoblasts were found to show DNA fragmentation after exposure to hydrogen peroxide or nitric oxide. Abu-Shakra et al.[118] showed that differentiated skeletal muscle cells show cytoplasmic condensation and DNA fragmentation after exposure to steroids. Since high levels of cellular ATP were retained, it could be inferred that cell death involved apoptosis rather than necrosis. Studies employing myoblasts showed that cell cycle inhibitors prevent apoptosis. Myotubes have been shown to undergo apoptosis indicated by the characteristic cell changes.[119–122]

Current research suggests that apoptosis is implicated in muscle wasting[152–154] (Table 5.5). Dirks and Leeuwenburgh found that cell DNA fragmentation increased

TABLE 5.5
Muscle Apoptosis in the Development of Wasting Conditions

Apoptosis Triggers	Apoptosis Muscle Wasting
Acetaminophen	Aging[124–127]
Angiotensin II[123]	Burns injury[128,129]
Ethanol	Cancer cachexia[130,131]
Glucocorticoids	Chronic heart failure[132–137]
Growth factor withdrawal	COPD[138]
Hydrogen peroxide	Denervation wasting[139–143]
IGF-withdrawal	Disuse atrophy/Hind limb suspension[144]
Oxidative stress	Exercise[145]
Physical damage (UV, radiation)	Steroid myopathy[146,147]
Proteasome inhibitors	Muscular dystrophy[148,149]
Retinoic acid	Sepsis[150]
TNF-α	Duchene muscular dystrophy[151]

* Approximately 150 publications have appeared on the topic of skeletal muscle apoptosis over the past 12 years. Significantly more research work has been done on smooth muscle cell apoptosis and their possible involvement in the development of arterial diseases.

by 50% in older rats compared to younger animals but the levels of caspase were similar. In older rats, caspase 3 activities were closely correlated to Cyt *C* and DNA fragmentation.[124] Whitman et al.[155] found significant differences in the level of DNA fragmentation in muscle biopsies from young (21 year old) versus senescent (72 year old) humans. There were no significant differences in muscle types or cross-sectional area across the two age groups. The levels of caspase 3/7 activity and ubiquitin ligases (MuRF1 and MAFbx) gene expression were not significantly different in young and aging muscle. Such data suggest that though apoptosis may contribute to sarcopenia, the underlying pathways are not strongly caspase dependent. Chung and Ng[127] also found that muscle from senescing (29 month old) rats had significantly higher expression of mitochondria-related apoptotic proteins (Bcl-2, Bax, Apa1–1, and p53). The levels of caspase 12 and 7 were increased in aged muscles but pro-caspase 3/caspase 3 were not. The expression of several heat shock proteins were stimulated in the aged rat muscle. In summary, results from animal and human studies show that age-related muscle wasting (sarcopenia) involves apoptosis probably linked with increasing TNF-α levels. A list of other wasting conditions linked with muscle cell apoptosis is shown in Table 5.5.

5.3.4 Lysosome-Mediated Autophagy

Cell autophagy involves the destruction by auto-digestion of large organelles and long-lived molecules inside double membrane bound vesicles inside cells.[156] Recent research suggests that autophagy contributes to muscle loss particularly in response to amino acid deprivation, aging, and denervation muscle atrophy.[139,157,158] The process of autophagy can be broken down into four key steps: (1) induction, (2) formation of autophagosome, (3) autophagosome docking and fusion with lysosomes to produce an autophagolysosome, and (4) digestion of material within the autophagosome. Proteolytic enzymes (cathepsins) for degradative process are derived from lysosome. Several subtypes of autophagy have been described in addition to macro-autophagy (above). Chaperone-mediated autophagy involves a direct uptake of cellular material directly into a lysosome. The mechanisms of autophagy have been described[159–163] as have the possible involvement of autophagy in muscle health.[157,164,165] A diverse range of factors appear to be able to moderate muscle cell autophagy (Table 5.6).

It is thought that autophagy is regulated by mTOR along with a rapamycin-independent pathway probably involving forkhead box-O (Foxo) transcription factors (see below). Several autophagy-related gene (Atg) proteins are necessary for the regulation of autophagy. The binding of Atg proteins (structurally related to ubiquitin) to newly forming autophagic vesicles appears to be necessary to enable the capture of cellular components within autophagosomes. Atg6 protein, also called beclin1, forms a complex with P13K (III) and thereby activates this enzyme to produce its secondary messenger, P13P, which then activates AKT.[173] It has been suggested that the regulatory function of Atg proteins may be dependent on their state of phosphorylation catalyzed by mTOR under the influence of nutrients and amino acids.[174] Enhancers of autophagy appear to inhibit mTOR signaling.[168] Current evidence suggests that autophagy may be regulated also by a rapamycin-independent pathway.

TABLE 5.6
Autophagy Inhibitors and Activators

Autophagy Inhibitors	Autophagy Promoters
Leucine	Autophagy enhancers[166,167]
Branched chain amino acids	Everolimus[168]
Insulin	Ionositol[169]
IGF-1	Lithium
mTOR activators	Nutrient inhibition
	Radiation treatment[170]
	Rapamycin[170]
	Soya saponin[171]
	Trehalose[172]
	Leucine limitation
	Inositol-1,4,5-triphosphate (IP3)[169]
	Glucagon

Lysosomal autophagy is now considered a key element for muscle proteolysis. The effects of autophagy have been demonstrated most clearly in cell-based studies and also in some in vivo models.[175,176] Though autophagy is linked with muscle atrophy, it is not certain whether the prevention of autophagy is desirable or risk free. Impairment of autophagy is implicated in diverse diseases including aging, cancer, and neurodegenerative diseases. The enhancement of autophagy has been suggested for reducing malignancy, for treating Huntington's disease, and for improving resistance to microbial pathogens.[177–179] I found no evidence from the literature that nutrients such as the BCAA that inhibit autophagy pose a cancer risk.

Mammalian cells contain three members of the Foxo class of transcription factors (Foxo1, Foxo3, and Foxo4), which are involved in controlling the expression of genes involved in the regulation of apoptosis, cell cycle progression, DNA damage repair, oxidative stress, cell differentiation, and glucose metabolism (see Section 5.5.4 for recent reviews).

Recent investigation into the effect of amino acid deprivation on muscle protein breakdown in C2C12 myotubes suggests that proteolysis involves autophagy. Leucine deprivation accounted for 30%–40% protein loss observed with amino acid deprivation in general. By exposing myotubes to specific inhibitors it could be shown that muscle protein loss in starved C2C12 myotubes was due to autophagy rather than proteasome activity.[180,*] It has also been pointed out that such data overestimate the importance of autophagy in proteolysis because C2C12 myotubes contain a lower proportion of myofibrillar protein substrates for the UPS compared to adult muscle fibers. C2C12 myotubes expressing constitutively active Foxo transcription factor (ca-Foxo3) undergo muscle proteolysis by lysosomal and proteasome pathways by a ratio of 70%:20%.[181]

* Conclusions based on the use of rapamycin need to be revised, since mTORC1 is rapamycin sensitive but mTORC2 is not.

5.4 PROTEOLYSIS VIA UBIQUITIN PROTEASOME

5.4.1 Enzyme Systems for Muscle Wasting

TABLE 5.7
Muscle Proteolysis Occurs via Four Proteolytic Systems

ATP UPS
Ca^{2+}-dependent alkaline proteases
Lysosomal/autophagic proteases
Caspase-related apoptosis

Four proteolytic enzyme systems are usually implicated in muscle protein breakdown (Table 5.7) though the UPS is perhaps the most widely discussed.[182–184] UPS is activated during muscle wasting arising from stress and various catabolic states. The levels of mRNA for proteasome components are increased following burns injury, metabolic acidosis, and injection of LPS. Specific inhibitors of UPS have also been shown to inhibit muscle wasting in rats subjected to catabolic conditions.[185] The implantation of tumors in rat muscle activates UPS in nearby tissue. The involvement of UPS in muscle atrophy has been reviewed.[186–189]

5.4.2 Structure of the Ubiquitin-Proteasome

Ubiquitin is an 8.5 kDa (76 residue) protein that functions as a label for proteins destined for degradation. Multiple copies of ubiquitin are attached to the substrate protein via ATP-requiring reactions catalyzed by enzyme-1 (E1) during which the terminal cysteine group of ubiquitin is acetylated to form a thiol-ester. Activated ubiquitin is then passed to enzymes E2 and E3 and finally to the internal ε-NH2 group of a substrate protein. Ubiquitination serves as a signal for the recognition, unfolding, and degradation of substrate protein by UPS. Finally, ubiquitin is released from degraded peptide by E4 (ubiquitin C-terminal hydrolases). Peptide products from the UPS are further degraded to free amino acids by cellular peptidases. Some peptide products of the UPS become associated with the major histocompatibility complex-1 (MHC-1) molecule required for immune function.

The structure of 20S proteasome established 10 years ago shows a longitudinal assembly of four rings each comprising of seven (19–36 kDa) subunits; the structural formulas for the 20S unit can be represented as $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. At any time, each 20S particle is capped by two 19S particles that are thought to be needed for the recognition and unfolding of the ubiquitinated protein substrates before these can enter the central core of the UPS particle. Each 19S cap protein forms a pore with dimensions of 13 Å through which unfolded ubiquitinated protein substrate must pass before it can be degraded. The major protein components of the UPS are listed in Table 5.8. The UPS complex contains 19S and 700 kDa (20S) components in addition to ubiquitin, and four transfer enzymes (E1–E4) described above.[190]

Three proteolytic enzyme activities have been identified within the 20S proteasome particle based on substrate specificity. The UPS contains a chymotrypsin-like (P1′ = large hydrophobic AA), a trypsin-like (P1′ = basic amino acid), and a glutamyl peptidase (P1′ = acidic amino acid) activity. In all three instances, the enzyme active sites are formed from residues from adjacent proteasome β-subunits (see below). Substrate specificity is further determined by the different

TABLE 5.8
Components of Ubiquitin Proteasome System

Component	Molecular Weight (kDa)	Comments
Ubiquitin (Ub)	8.5	Label attached to old and damaged cell proteins prior to their degradation
Activating enzyme (E1)	110	Enzyme that catalyses acyl ester bond between Ub and E2 (below), two isoforms
Ubiquitin-conjugating enzymes (E2)	14–35	Carrier proteins that form a covalent complex with Ub before it is transferred to substrate protein, hundreds of different E2 have been identified
Ubiquitin protein ligases (E3)		Multi-subunit enzyme complex that transfers Ub to substrate proteins, E3 catalyzes the rate-limiting step for proteolysis
E3α	200	E3 with affinity for proteins with N-terminal basic or hydrophobic amino acids
E6-AP	100	p53 protein substrate
Destruction-box ligase	~1500	Targets cyclin and cell cycle regulators, promotes anaphase, end to mitosis
E3L	~550	Activity for actin, troponin T, and MyoD substrates
Ubiquitin C-terminal hydrolases *UCh class I*, *UCh class II/or ubiquitin-specific protease*	~30	Two classes catalyze release of ubiquitin from peptides. Net effect is (proteolytic) increase ubiquitin pool or anti-proteolytic (remove ubiquitin) for specific substrates

isoforms of ubiquitin ligase (E3) that appear to target different subgroups of ubiquitinated proteins. Ubiquitinated substrate proteins first interact with a 19S protein component located at one end of the proteolytic complex. The unfolded ubiquitinated protein then passes into the cylindrical 20S proteasome complex where it is degraded to oligopeptides containing 3–20 amino acids. It is thought that the UPS cannot degrade intact myofibrils until the native structure is first disrupted by other enzyme systems (possibly calpains and/or caspases) to release dissociated myosin and actin. The disruption of Z-disk proteins (nebulin, titin) by calpains may release muscle proteins from muscle sarcomere. Consistent with this view, Ca-chelators (inhibitors for calpains) have been shown to reduce the rate of proteolysis in septic rats.[191]

Ubiquitin ligases (E3) attach ubiquitin tags to protein substrates before their eventual degradation by the UPS. The reaction is the rate-limiting step for proteolysis and also determines substrate specificity, that is, the nature of protein hydrolyzed at any time. It is thought that cells contain over 1000 different E3s grouped into three broad categories: (a) HECT (for homology to E6-AP carboxyterminus) domain containing enzymes, (b) E3 containing a zinc-binding RING finger motif, (c) atrogin-1, which is highly expressed during muscle atrophy.[192]

5.4.3 Ubiquitin Proteasome and Muscle Wasting

UPS activity is upregulated by proinflammatory cytokines from diverse sources, for example, macrophages, injury, etc.,* and is also activated by glucocorticoids.[193–195] UPS is implicated in catabolic conditions that activate the immune and/or inflammatory response (burns injury, sepsis, cancer, etc.). However, the relations between UPS activity and muscle wasting processes appear to be highly complex. The UPS activity may promote muscle size increases under some circumstances. Research suggests that dexamethasone induces a catabolic response mediated by UPS in *differentiated* muscle fibers.[196] Therefore, UPS inhibitors reduce protein loss due to the dexamethasone treatment of fully differentiated myotubes. By contrast, investigations using actively dividing cells show that UPS activation supports cell cycle progression. UPS activation may also delay myocyte differentiation by degrading MyoD protein. The sorts of interactions outlined here may be behind some controversial reports in the literature surrounding the effect of macrophages and TNF-α on muscle generation (Figure 5.5). There may be circumstances where inflammatory conditions are not detrimental for muscle health (Section 5.4.4). Table 5.9 shows a list of compounds thought to activate or inhibit proteasome expression.

5.4.4 Cell Cycle Regulation and the Proteasome

Investigations using specific enzyme inhibitors showed that many short-lived regulatory proteins are substrates for UPS including cell cycle regulators, transcription factors, and tumor suppressor proteins.[197–199,†] UPS also moderates the concentration of

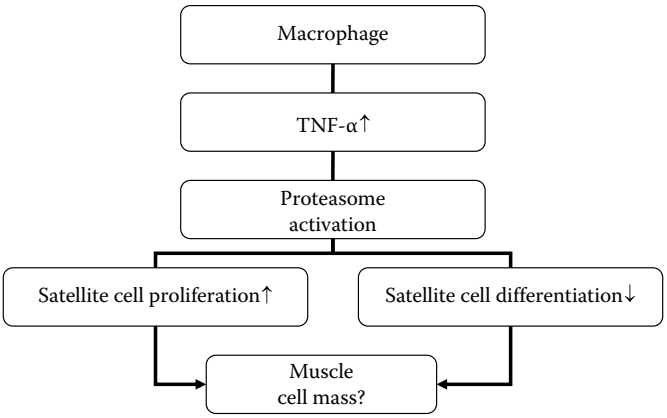


FIGURE 5.5 Paradoxical effect of macrophages, TNF-a, and proteasome activation on muscle development.

* The activation of ubiquitin proteasome by inflammatory signals, macrophages, etc., is expected to increase muscle cell proliferation on the one hand, but also increase muscle protein degradation for differentiated myotubes. There is some evidence that the effect of cytokines on muscle health is probably biphasic, being dependent on the time of exposure and/or concentrations of cytokines.

† Commercial proteasome inhibitors became widely available from 1994.

TABLE 5.9
Activators and Repressors of Proteasome Expression

Inducers	Repressors
Glucocorticoids	Insulin
Proinflammatory cytokines	Growth hormone
Increased oxidative stress	IGF

membrane receptors that affects responses to hormones with potential consequences on muscle atrophy.[200] Cell cycle progression has been shown to require UPS degradation of p53 protein leading to a fall in the concentration of the CDK inhibitors, p21 and p27. In contrast, high concentrations of p53 protein increase the expression of CDK inhibitors leading to cell cycle arrest.* p53 protein also induces the transcription of pro-apoptosis (Bax) genes upstream of the calpains while repressing the transcription of the anti-apoptosis (Bcl2) gene. Table 5.10 shows a listing of some regulatory proteins thought to be degraded by the proteasome.

TABLE 5.10
A List of Some Regulatory Proteins Thought to Be Degraded by the UPS

Protein Function	Examples/Comments
Cell cycle regulators/kinase subunit (+/−)	Cyclin A, Cyclin B, CDK inhibitors p27(kip1), p21(Cip1/Waf1)
Transcription factor/co-activator/ inhibitor	NFκβ/Iκβ, E2F/Dp1, p300
Cell surface receptor/membrane protein	E2R (steroid hormone receptor) EGFR (epidermal growth factor receptor) CFTR (cystic fibrosis transmembrane conductance regulator)
Tumor suppressor	p53 protein, β-Catenin
Proto-oncogene	c-Jun, c-Fos, c-Myc
O6-Methylguanine-DNA methyl, transferase (DNA repair protein)	MGMT
Enzymes	Receptor-associated protein kinases DNA topoisomerase Ornithine decarboxylase

Sources: Adapted from Chakravarthy, M.V. et al., *Int. J. Sport Nutr. Exercise Metab*., 11, S44, 2001; Machida, S. and Booth, F.W., *Proc. Nutr. Soc*., 63, 337, 2004; Rehfeld, C. et al., *J. Anim. Sci*., 83, S36, 2005; Merly, F. et al., *Muscle Nerve*, 22, 724, 1999.

* Regulation of p53 gene expression has been widely discussed in relation to the development of cancer. In non-transformed cells, DNA damage induces the p53 gene, resulting in cell cycle arrest and increased tendency for cell apoptosis. High concentrations of p53 protein prevent cell mitosis and promote apoptosis in damaged (but otherwise normal) cells. In many cells, the intact p53 gene product guards the rest of the genome from damage and mutations.

Proteasome depletion of p53 enhances cell proliferation. Experience from cancer cell biology suggests that mutations in the p53 tumor suppressor gene occur in approximately 40% of all human tumors. These mutations lead to alterations in p53 protein structure and stability, leading to increased degradation by the UPS. The resulting fall in p53 concentration accounts for higher rates of proliferation of cancer cells. In some forms of cancers, the structure of p53 protein remains unchanged but the steady state concentration of p53 is still reduced due to the increasing rate of degradation of this protein. A strain of human papilloma virus (HPV) that induces cervical cancer is thought to be a ubiquitin ligase (E3) variant that specifically catalyzes the ubiquitination of p53 protein degradation in infected cells. Other DNA tumors viruses code for rogue proteins that ultimately target p53 proteins for degradation. This narrative suggests that UPS inhibitors that prevent p53 degradation might be usefully employed in the treatment of some forms of cancer. By the same token, the effect of UPS inhibitors would be expected to decrease muscle cell division.

There is some evidence linking UPS inhibitors with muscle cell apoptosis.[201,*] Lopes et al.[202] found that proteasome inhibitors, PSI (CBZ-Ile-Glu (*O*-*t*-butyl) Ala-leucinal) or MG11 (CBZ-Leu-Leu-norvalinal), increased DNA fragmentation in proliferating Rat-1 and PC12 cell lines. Treatment with UPS inhibitors produced a rise in the intracellular concentration of p53 and p21 protein, which was abolished by the presence of calpain II inhibitor. Transfected cells that overexpress the p53 gene showed an increased apoptosis response. An et al.[203] found the induction of apoptosis correlated well with the activity of UPS inhibitors and their ability to induce p53 protein buildup within cells. A rising level of p21 inhibitor for cyclin-CDK complex, is another feature associated with apoptosis.

5.4.5 UPS and the Immune Response

UPS activation of IKK/NFκβ (Section 5.5.2) is an important aspect of the regulation of genes involved in the inflammatory response as well as adaptive immune function (Chapter 6). UPS is involved in antigen processing. Foreign proteins and microbes are degraded by the UPS and lysozomal systems to form peptide epitopes and antigens. These bind with MHC-1 and then migrate to the surface of T cells. Both self- and nonself peptides are processed to MHC-1 peptide complexes. Faulty processing by the UPS could induce autoimmune response to otherwise normal proteins. Inhibitors of UPS have been shown to reduce antigen processing.[204,205]

5.5 FURTHER SIGNALING PATHWAYS FOR MUSCLE ATROPHY

Wasting conditions such as AIDS, aging, bed rest, cancer, muscle denervation, glucocorticoids treatment, injury, joint immobilization, renal failure, and sepsis appear to involve the same terminal pathways for muscle loss. Key intracellular signaling pathways implicated in the control of muscle wasting are outlined below.

* Though many of these studies focus on cancer, the results may be relevant to muscle wasting.

5.5.1 Skeletal Muscle Differentiation Program

As noted previously, skeletal muscle differentiation (SMD) is controlled by five so-called myogenic transcription factors expressed in myocytes (Figure 5.1). Guttridge et al. demonstrated that exposure of C2C12 myocytes (model for dividing muscle stem cells) to TNF-α blocks the expression of SMD-related genes as indicated by the following: (1) decreased levels of MyoD mRNA and MyoD proteins within cells, (2) reduced concentrations of CDK inhibitor (p21), and (3) decreased levels of myosin heavy chain indicator for differentiation. The effect of TNF-α was mediated by NFκβ transcription factor. Guttridge et al. also reported that cells engineered to express *MyoD* gene constitutively were insensitive to the effects of added TNF-α.[67] Other research confirms that TNF-α affects actively dividing muscle myocytes more than fully differentiated myotubes. The majority of studies published so far employ the C2C12 muscle cell line.[206,207] The preceding results suggest that TNF-α delays cell differentiation via the inhibition of *MyoD* expression, though the relation to muscle size is not easily predictable. The repair of damaged or injured muscle tissue requires the activation of MSSC and downregulation of *MyoD*. However, failure to express *MyoD* will eventually impair the formation of myotubes.[208]

5.5.2 Nuclear Factor Kappa Beta and Muscle Wasting

Many forms of muscle loss mediated by proinflammatory cytokines require the activation of transcription factor, NFκβ.[55,68–72,209–212] The transfer of NFκβ to the cell nucleus is regulated by IKK, which is thought to be sensitive to TNF-α-induced increase of intracellular reactive oxygen species. NFκβ activation by TNF-α can be demonstrated by the direct electrophoretic analysis of NFκβ/DNA binding or via the luciferase reporter assay. Sensitivity to TNF-α appears to be more pronounced for proliferating myocytes compared to myotubes (Section 5.1.3). Figure 5.6 shows a possible pathway leading from NFκβ activation to UPS activation. The role of NFκβ in the inflammation and muscle wasting is discussed further in Section 6.2.1.

5.5.3 MuRF and Atrogin-1 Gene Expression

Two muscle-specific genes for ubiquitin ligases are consistently activated in animal models for atrophy. Atrogin-1 and MuRF1 code for ubiquitin (E3) ligases.[213–216,*] cDNA microarray profiling of 16,392 genes showed that 10% undergo changes in expression in rat cachexia models for renal failure, cancer, diabetes, and fasting.[217] Marked changes were found in a set of genes linked with protein degradation (upregulated), nonprotein nitrogen metabolism (upregulated), and genes controlling extracellular matrix components (Table 5.11). Genes related to energy (ATP) generation were consistently downregulated with the notable exception of glutamine synthetase. A large number of UPS components in addition to MAFbx and MuRF1 genes were unregulated, but others (e.g., E1, E2, and some E3s) were not affected.

* Consistent with this view, atrogin-1 and MuRF1 gene knockout mice were 36%–56% more resistant to denervation atrophy.

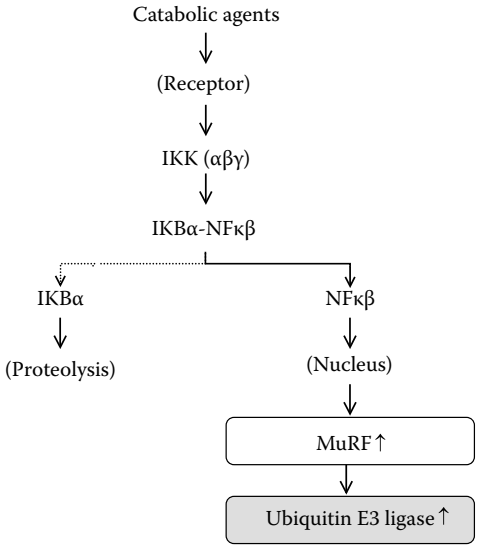


FIGURE 5.6 Signaling pathways leading to muscle atrophy in fully developed myotubes; NFκβ-mediated activation of MuRF1 gene under the influence of TNF-α, and endotoxin and other catabolic agents. (Adapted from Mozdziak, P.E. et al., *Poult. Sci*., 81, 1703, 2002; Oksbjerg, N. et al., *Domestic Anim. Endocrinol*., 27, 219, 2004; McPherron, A.C. et al., *Nature*, 387, 83, 1997; Lee, S.-J., *Trends Genet*., 23, 475, 2007; Kambadur, R. et al., *Genome Res*., 7, 910, 1997; McPherron, A.C. and Lee, S.-J., *Proc. Natl. Acad. Sci. U.S.A*., 94, 12457, 1997; Grobet, L. et al., *Nat. Genet*., 17, 71, 1997.)

The expression of genes for Foxo1 transcription factor thought to be involved in muscle cell apoptosis was increased (Section 5.5.4).

MuRF1 and atrogin-1 gene expression is *increased* in several experimental models for sepsis, for example, endotoxin administration or cecal ligation and puncture.[218] Changes in gene expression were more pronounced in fast-twitch (gastrocnemius) muscle compared to slow-twitch (e.g., heart and soleus) muscles. In the case of sepsis, there was also a rise in plasma levels of TNF-α and corticosterone. IGF-1 treatment reduced MAFbx/atrogin-1 expression but MuRF1 gene expression was not affected. Apparently, atrogin-1 is more important than MuRF1 gene during muscle wasting. Sepsis-induced expression of atrogin-1 mRNA was not prevented by leucine, which may therefore have more of an impact on the anabolic phase for muscle control. By contrast, IGF1 clearly shows an effect on atrogin-1 expression probably acting via Foxo transcription factors. These results show that MAFbx/atrogin-1 and/or MuRF1 genes are implicated in the wasting effect produced by glucocorticoids such as dexamethasone on the one hand and the anabolic effect of IGF-1.[219,220]

5.5.4 AKT/Foxo/Atrogin-1 Pathway and Proteolysis

The PI3K/AKT/Foxo/atrogin-1 pathway has emerged as a major intracellular signaling route for controlling muscle loss in response to low levels of nutrients and anabolic hormones.[181,221–225] Sandri et al. showed that amino acid deprivation

TABLE 5.11
A Listing of Groups of Gene Upregulated during Muscle Atrophy in Rat Models for Renal Failure, Cancer, and Diabetes and Fasting

Gene Group (Response)[a]	Genes Affected
Protein degradation (↑)	Ubiquitin (B,C), ribosomal protein S27a, atrogin-1/MAFbx (E3), MuRF1 (E3), ubiquitin-conjugating (E1) enzyme, and 20S proteasome subunits (α_1, α_5, β_3, and β_4)
	19S proteasome subunits (ATPase, non-ATPase)
	Cathepsin L
ATP production and substrate metabolism (↓)	Glycolysis enzymes, ATP related, glutamine synthetase (+)
Nonprotein nitrogen metabolism (↑)	Purine nucleotide metabolism
	Spermidine N1-acetyltransferase
Extracellular matrix components (↓)	Collagen Type Iα1, Collagen Type IIIα1, Collagen Type Vα2, and Collagen Type XVα1, fibrillin, fibronectin
	Osteoclast-specific factor
Transcription (↑)	Nuclear factor, activating transcription factor
	Foxo1, TG interaction factor
	Enhancer of zest homolog 1
Translational control (↑)	Eukaryotic translation initiation factor 4A, isoform 2
	Eukaryotic translation initiation factor 4 binding protein 1
	Eukaryotic translation initiation factor 4 gamma 3
	Nucleolin, ribosomal protein L1, RNase helicase–related protein
Metallothionein and proteins for oxidative stress (↑)	Metallothionein (1, 1L, 1LB), thioredoxin like
Genes involved in muscle growth and differentiation (↓↑)	IGF-1, IGF1-BP (+)

Sources: Adapted from Jagoe, R.T. et al., *FASEB J.*, 16, 1697, 2002; Wray, C.J. et al., *Int. J. Biochem. Cell Biol.*, 35, 698, 2003; Lecker, S.H. et al., *FASEB J.*, 18, 39, 2004.

[a] Genes are upregulated (↑) or downregulated (↓) by 1.5–5-fold (analysis by DNA microarray assay).

or the treatment of C2C12 myotubes with dexamethasone increased atrogin-1 mRNA expression 2.5-fold and reduced fiber diameter by 40%–60%. Levels of phosphorylated AKT and Foxo decreased in cells exposed to steroid. Tests involving IGF-1, expression of constitutively active (dephosphorylated) Foxo within muscle cells, and work using adult mice confirmed that pathway for atrophy shown in Figure 5.7.[226]

The Foxo transcription factor activates genes for muscle atrophy including some that produce cell cycle arrest and inhibit protein synthesis. ROS and stress conditions increase the expression of Foxo (Table 5.12). In contrast, anabolic agents

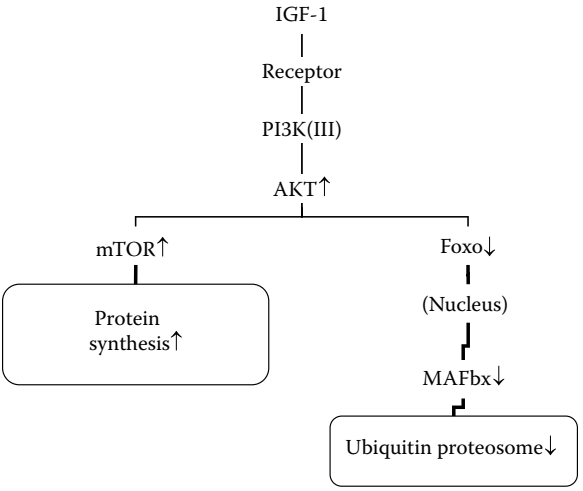


FIGURE 5.7 Muscle hypertrophy/atrophy signaling related to IGF-1 and AKT/mTOR pathways. Foxo pathway is activated under conditions of reduced anabolic signaling by IGF-1 and nutrients. (Adapted from Mozdziak, P.E. et al., *Poult. Sci*., 81, 1703, 2002; Oksbjerg, N. et al., *Domestic Anim. Endocrinol*., 27, 219, 2004; McPherron, A.C. et al., *Nature*, 387, 83, 1997; Lee, S.-J., *Trends Genet*., 23, 475, 2007; Kambadur, R. et al., *Genome Res*., 7, 910, 1997; McPherron, A.C. and Lee, S.-J., *Proc. Natl. Acad. Sci. U.S.A*., 94, 12457, 1997; Grobet, L. et al., *Nat. Genet*., 17, 71, 1997.)

TABLE 5.12
Foxo Transcription Factors and Muscle Atrophy

	Foxo-Dependent Cellular Processes
Insulin (−)	Apoptosis
IGF-1 (−)	Atrophy
Nutrient (−)	Autophagy
Oxidative stress (+)	Cell cycle arrest
Specific genes activated	Gluconeogenesis
Cell cycle inhibitors p27, p21	Neuropeptides secretion
Caveolin-1	Stress resistance
MURF/Ring gene (E3 ligase)	Immune function
MaFBx/Atrogin-1(E3 ligase)	Aging

inhibit Foxo signaling. Both Foxo and mTOR are downstream of AKT (PKB). The stimulation of AKT by external stimuli leads to Foxo phosphorylation at serine/threonine residue (Ser-256, Thr-24, and Ser-319), thereby decreasing its DNA-binding activity.* mTOR should normally be switched on under circumstances where Foxo signaling is switched off.

* Foxo may also be controlled by other kinases including SGK (serum and glucocorticoids regulated kinases), IKK and CDK2.

The deactivation of Foxo transcriptional activity stimulates cell proliferation and growth in response to nutrient availability, high insulin, or IGF-1 levels. Foxo activity is also partly controlled by the UPS. The attachment of multiple ubiquitin proteins to Foxo1 and Foxo3a leads to their degradation by the proteasome but the attachment of single ubiquitin molecule to Foxo4 facilitates its nuclear localization and augments its transcriptional activity. ROS and stress increase the expression of Foxo transcription factors by activating JNK.[227–232] A great deal of research has shown a link between the expression of Foxo protein and muscle wasting. Foxo transcription factor is also important in the control of autophagy, which is important to muscle wasting.[233] In summary, two signaling pathways involving NFκβ and Foxo (Figures 5.6 and 5.7) are thought to be involved in the regulation of muscle atrophy. An increased understanding of the events leading to muscle loss may lead to better therapies for the prevention of cachexia.[234,235]

5.5.5 Oxidative Stress and Muscle Wasting

Chronic exposure to ROS may induce muscle atrophy through redox-sensitive transcription factors, most notably NFκβ and Foxo[236–239] (cf. Section 6.2.1). Examples of ROS found in cells include the superoxide radical (O_2^*), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^*). Nitrogen-based ROS include nitric oxide (NO) and the peroxynitrile radical (NO_3^*), formed by the reaction of NO + O_2^*. The oxidation of sulfhydryl (SH) leads to the thyl radical (S^*), disulfide (S–S), sulfenic, and sulfonic acid species. A major source of ROS is mitochondrial respiration (O_2^*) and oxidase-catalyzed reactions (H_2O_2). The activation of NFκβ by ROS stimulates the UPS. Antioxidant networking compounds (vitamin E, ascorbic acid, α-lipoic acid, and thioredoxin) that deactivate NFκβ may be useful in the prevention of muscle wasting.[212]

5.5.6 Angiotensin-Related Muscle Wasting

High levels of angiotensin (ANG) and angiotensin-converting enzyme (ACE) are found in skeletal muscle (Figure 5.8). Early reports 10 years ago, showing that angiotensin II (ANGII) can stimulate muscle proteolysis, in addition to possible effects on muscle blood flow, received scant attention.[240,241] Cultured C2C12 myocytes and myoblasts show increased rates of protein breakdown when exposed to ANGII.[242] Western blot analysis indicates that ANGII stimulates the expression of UPS subunits. The proteolytic effect of ANGII was duplicated by treatment with ANGI, presumably after this was converted to ANGII by muscle ACE. Treatment with the angiotensin converting enzyme inhibitor (ACEI) imaprilat™ prevented muscle proteolysis induced by exposing cultured C2C12 myoblast to ANGI.[242] Tisdale and coworkers showed that ANGI (0.05–2.5 μM) can reduce the rate of protein *synthesis* by cultured murine myocytes by 40%–50%.[243] The effect could be ameliorated using the ACEI imaprilat, suggesting that the conversion of ANGI to ANGII was essential for the cachexic effect. Directly treating cultured cells with ANGII (0.1–5 μM) also inhibited the rate of protein synthesis. The cachexic effect of ANGI/ANGII was abolished by co-incubating

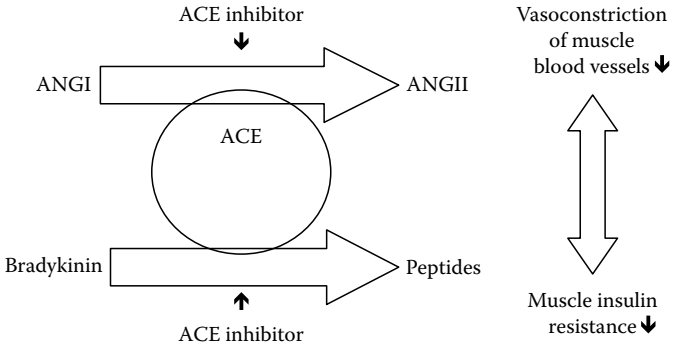


FIGURE 5.8 Schematic diagram showing the effect of angiotensin converting enzyme (ACE) on muscle vasoconstriction and insulin resistance. (Adapted from Henriksen, E.J. and Jacob, S., *Diabetes Obes. Metab*., 5, 214, 2003.)

cells with IGF-1. These studies are important as they indicate that ANGII wasting could be partially addressed using ACEI.

Animal studies by Mitch and coworkers have shown that ANGII induces muscle wasting in rats with symptoms similar to cachexia.[244] In young rats, ANGII treatment leads to growth inhibition. ANG treatment produces weight loss in aged rats compared to pair-fed controls. Postmortem analysis shows that the ANGII treatment enhances muscle proteolysis via a process that was not sensitive to lysosomal protease inhibitors, or inhibitors for Ca^{2+}-dependent proteases. It has been suggested that ANGII treatment increases the expression of UPS.* ANG treatment also affected the IGF-1 synthesis: levels of mRNA for IGF-1, IGF-BP3, and IGF-BP5.

Evidence from human observational studies suggests that ACEI, normally used for controlling high blood pressure, could have beneficial effects on muscle health. It has been suggested that ANGII and proteolytic breakdown products of bradykinin, which are both formed via the action of ACE, increase muscle insulin resistance and decrease muscle blood flow.[245] It was proposed that T2D patients may benefit from therapy using ACEI, normally used for the treatment of hypertension.[246] According to one model, the effects of ACEI are linked with sparing of bradykinin peptide degradation, leading to an increase in NO production, which moderates insulin sensitivity. Since insulin resistance is widely associated with chronic wasting conditions as well as T2D (Chapter 9), there is a distinct possibility that the ACEI may be useful for treating muscle-wasting conditions and perhaps improving performance. Aging-related loss of muscle function may also be amenable to ACEI therapy.[247,248]

5.6 MAMMALIAN TARGET OF RAPAMYCIN AND HYPERTROPHY

Muscle size gain arises from increases in cross-sectional area of existing muscle fibers or increased fiber number. Exercise and nutrient intake also stimulate MSSC activity (Section 5.1). Muscle fiber cross-sectional area is dependent on the balance between the processes of protein synthesis, degradation, and amino

* Mitch et al. are the same group famed for their work on the ubiquitin proteasome.

acid oxidation for fuel (Chapter 4). The role of the mammalian target of rapamycin (mTOR), which functions as a nutrient sensor involved in the regulation of muscle hypertrophy, is briefly described in this section. Intracellular signaling pathways for muscle hypertrophy have been recently reviewed by Glass[249–251] and other investigators.[234,252–254]

5.6.1 mTOR Function as a Nutrient Sensor

mTOR, isolated in 1995 by Sabers et al.,[255] plays a pivotal role in the regulation of muscle mass. As a measure of the degree of interest in this topic, over 3852 publications dealing with various aspects of mTOR biology were published between 1995 and 2010. Key investigations using rapamycin (a specific inhibitor) showed that mTOR functions as a nexus for the regulation of muscle mass by amino acids and insulin.[256–258] mTOR also catalyzes the phosphorylation of insulin receptor protein and thereby contributes to insulin resistance mediated by high nutrient availability.[259,260] Cell death under nutrient replete conditions is also dependent on mTOR activity.[261] Kimball et al. showed that the anabolic effects of branched chain amino acid (BCAA) operate via the mTOR pathway.[262] Recent investigations now indicate that other amino acids (arginine) and some leucine metabolites (alpha-keto-isocaproic acid) may also activate the mTOR.[263–265] The characteristics of mTOR as a "nutrient sensor" have been reviewed.[266,267] The involvement of mTOR in hormone sensing is discussed in detail in Chapter 9.

5.6.2 Two mTOR Complexes and Their Function

There appears to be two mTOR complexes inside cells, designated mTORC1 and mTORC2. Whereas mTORC1 is activated by nutrients as well as growth factor, mTORC2 is only activated by growth factors. The gene for TOR codes for three subunits forming a serine/threonine kinase. The structure of mTORC1 is formed by the catalytic mTOR subunit bound to a regulatory associated protein of mTOR (RAPTOR), which serves as a binding site for rapamycin. A second regulatory subunit for mTOR called mLst8/GβL or the mammalian ortholog of the yeast Lst8p (LST8) was initially thought to be involved in sensing BCAA; the latest data suggest that BCCA interact with cellular GTPase linked with mTOR function.[268] mTORC2 contains the two subunits combined with rapamycin-independent regulatory subunits rapamycin-insensitive companion of mTOR (RICTOR). mTORC2 may be involved in mediating the anabolic effects of growth factor related to cell proliferation, metabolism, cytoskeleton changes, and cell survival.[269–273] Recent research suggests that mTORC2 can phosphorylate AKT (not shown in Figure 5.7). Details of mTOR signaling are shown in Appendix 5.A.1.

Both mTORC1 and mTORC2 are part of a kinase cascade that includes ribosomal protein S6 Kinase (p70S6 kinase) and elongation factor kinase (eEF2K). Other downstream effectors or substrates for mTOR1 include elongation factor binding protein (4EBP1), eukaryotic initiation factor-4 (eIF4E), and GSK2 (Table 5.13). The mTOR substrate, 4EBPI (also called PHAS-1), functions as

TABLE 5.13
Significance of mTOR Signaling

Upstream effectors of mTOR	Branched chain amino acids
	Growth hormone
	Insulin
	Insulin-like growth factor
Cell process regulated via mTOR	Autophagy
	Cell cycle progression
	Energy sensing
	Insulin resistance
	Nutrient signaling
	Ribosomal biogenesis
	Transcriptional control
	Translational control
Protein substrates of mTOR	Elongation factor binding protein (EF-BP1)/PHAS1
	Eukaryotic elongation factor 2 kinase (eEF2K)
	Hypoxia inducible factorα(HIFα)
	Insulin receptor protein (IS1)
	Ribosomal protein S6 Kinase 1 (S6K1)
	p21 Cyclin-dependent kinase inhibitors
	p27 Cyclin-dependent kinase inhibitors
	STAT3
	Glycogen synthase
	Cytoplasmic linker protein 170 (CLIP-170)
	Protein phosphatase 2A
	PKC
Inhibitors for mTOR	*Low molecular weight activators for mTOR*
Rapamycin	Leucine
Rapamycin analogues	Hydroxy methyl butyrate
	Valporic acid
	Melatonin
	Isocaproic acid
	Melatonin
	Clenbuterol
	Zn^{2+}

an inhibitor for eukaryotic initiation factor-4 (eIF4E) required for protein synthesis initiation. The phosphorylation of 4EBPI (catalyzed by mTOR) decreases its binding affinity for elF4E thereby increasing protein synthesis translation initiation. In a parallel pathway, mTOR activates S6K1 that phosphorylates the ribosomal protein S6. Together, these effects of mTOR stimulate protein translation. Recent research suggests that mTOR is involved in other diverse processes related to the control of muscle size including exercise, and cell hydration and autophagy (cf. Section 5.3.4, Figure 5.7 and Table 5.13).

5.6.3 Regulation of Protein Synthesis by mTOR

Muscle size is influenced by food deprivation, hormones, muscular activity, compensation growth, muscle denervation, and postoperative trauma (for major reviews over the past decade cf.[266,274–281]). Factors that favor muscle growth generally enhance protein synthesis. Li and Jefferson showed that leucine, isoleucine, and valine stimulate protein synthesis by 25%–50% and inhibit protein degradation by a maximum of 30% using at rat hemi corpus skeletal muscle preparation.[282,*] Since iso-caloric amounts of glucose and palmitate did not stimulate muscle buildup, it could be reasoned that the effect of leucine was not due to the utilization of this amino acid as an energy source. Further careful studies revealed that leucine stimulates peptide-chain initiation and that the control of protein synthesis is a major route for promoting muscle size increase (Chapter 8).

5.7 SUMMARY AND CONCLUSIONS

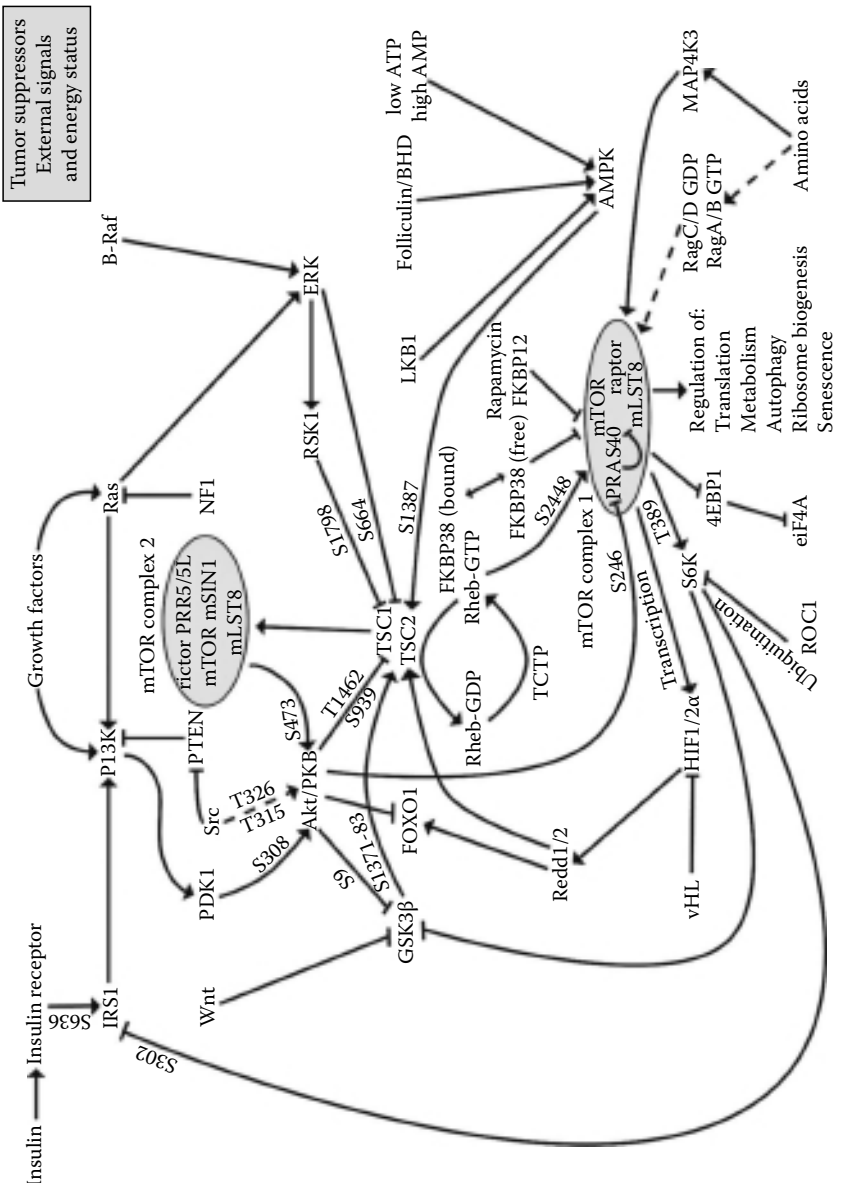
Muscle loss is associated with diverse wasting conditions including cancer,[283–288] heart failure,[289–293] chronic obstructive pulmonary disease (COPD),[294–297] renal failure,[298] HIV/AIDS,[299–304] sepsis,[305–307] rheumatoid arthritis,[308] Type 2 diabetes, and aging.[309] There is some uncertainty regarding the underlying differences between cancer-cachexia, burns-cachexia, and aging-cachexia. It seems likely that many wasting conditions begin differently but share the same end pathways leading to increased muscle protein breakdown and oxidation as well as reduced rates of synthesis. Many wasting conditions are preceded by a rise in tissue proinflammatory cytokines (e.g., TNF-α and IL-6) that produce a rise in intracellular levels of ROS. Improving understanding of the factors that control muscle loss or growth may lead to better approaches for maintaining lean body mass in the sick and frail.

APPENDICES

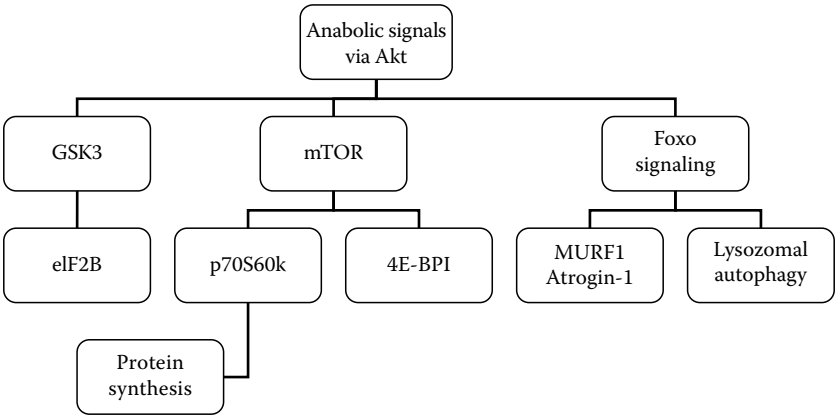
APPENDIX 5.A.1 DETAILS OF mTOR SIGNALING

Notes: Intracellular signaling via the AKT/mTOR/p70S6K pathway. Diagram shows signaling from insulin, insulin-like growth factor 1 (INS/IGF-1), amino acids, and hypoxia. *Abbreviations*: phosphoinositol-3-phosphate kinase (PI3K), protein kinase B (AKT), tuberous sclerosis complex 1 and 2 (Tsc1 and 2), mammalian target of rapamycin (mTOR—also called FRAP or RAFT-1), elongation factor-4 binding protein (4EBP—also known as PHAS-1), and 70S ribosomal protein (p70S6K). (With permission from Charles C. Betz, University of Basel, Switzerland.)

* The hemicorpus preparation is essentially a dissected and perfused rat model that offers a more complex system for the study of protein synthesis compared to muscle homogenate or excised diaphragm.


Tumor suppressors
External signals
and energy status
Insulin
Insulin receptor
S636
IRS1
S302
P13K
PDK1
Wnt
PTEN
Src
T326
T315
S308
Akt/PKB
S9
S1371-83
GSK3β
FOXO1
Redd1/2
vHL
HIF1/2α
Transcription
S6K
T389
4EBP1
eiF4A
Translation
Growth factors
Ras
NF1
mTOR complex 2
rictor PRR5/5L
mTOR mSIN1
mLST8
S473
T1462
S939
TSC1
TSC2
S1798
S664
RSK1
ERK
B-Raf
S1387
LKB1
FKBP38 (bound)
Rheb-GTP
Rheb-GDP
TCTP
FKBP38 (free) FKBP12
Rapamycin
S2448
mTOR complex 1
S246
PRAS40
mTOR
raptor
mLST8
Regulation of:
Translation
Metabolism
Autophagy
Ribosome biogenesis
Senescence
low ATP
high AMP
Folliculin/BHD
AMPK
RagC/D GDP
RagA/B GTP
MAP4K3
Amino acids
Ubiquitination
ROC1

APPENDIX 5.A.2 ANABOLIC AND CATABOLIC SIGNALING IN MUSCLE



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6 Inflammation and Innate Immune Response

6.1 TYPES OF INFLAMMATION

6.1.1 Introduction

Inflammation is a contributing factor to the muscle loss observed during cancer, chronic heart failure, chronic renal diseases, HIV/AIDS, and bacterial infections.[1–4] This chapter is concerned with anti-inflammatory bioactive peptides. Major kinds of inflammatory responses to illness are discussed in Section 6.1. Intracellular signaling leading to inflammation is reviewed in Section 6.2. Naturally occurring and synthetic peptides having anti-inflammatory activity are described in Section 6.3. We describe in vivo studies and human clinical trials involving anti-inflammatory peptides in Section 6.4.

A selection of conditions linked with inflammation and weight loss are listed in Table 6.1.* Causes of acute inflammation include physical trauma, exposure to noxious chemical agents, and UV irradiation; surgery may also produce weight loss, though the underlying mechanisms are not well understood.[5] Regardless of the causes, a state of inflammation is most often associated with anorexia, insulin resistance, and muscle wasting (Figure 6.1). Therefore, anti-inflammatory peptides and protein could find widespread uses for nutritional support. For convenience, this chapter will address anti-inflammatory responses moderated within host cells. Therapies that involve binding interactions with pathogenic bacteria or bacteria-derived products are discussed in Chapter 7 under the heading of antimicrobial peptides.

6.1.2 Acute and Chronic Inflammation

Inflammation (also known as the innate immune response) should be differentiated from the adaptive immune reaction that leads to antibody formation. Signs of acute inflammation include redness and a sensation of heat in the affected area, quickly followed by swelling and a sensation of pain. The physiological changes can be traced to alterations in vascular permeability, recruitment of inflammatory cells to the affected area, and the production of proinflammatory mediators linked with symptoms of pain and itchiness.

* Descriptions of each disease can be found in *The Merck Manuals & Medical Library* available from http://www.merck.com/mmhe/index.html or the Centre of Disease Control (CDC) Web site http://www.cdc.gov/nccdphp/dnpa/obesity/index.html

TABLE 6.1
Inflammatory Conditions Linked with Muscle Wasting

Disease	Inflammation	Wasting Prevalence %
Aging	↑	100
Alzheimer's	↑	100
Cancer	↑	50–80
Chronic heart failure	↑	10–16
Chronic renal failure	↑↑	100
COPD	↑↑	100
Crohn's disease	↑↑	In young
HIV	↑	47
IBD	↑	?
Muscle dystrophies	↑	100
Obesity	↑	?
Rheumatoid arthritis	↑↑	60
Sepsis	↑↑↑	100
Type 2 diabetes	↑	In elderly

Source: Adapted from Moyland, J.S. and Reid, M.B., *Muscle Nerve*, 35, 411, 2007.

Abbreviations: COPD, chronic obstructive pulmonary disease; IBD, inflammatory bowel diseases.

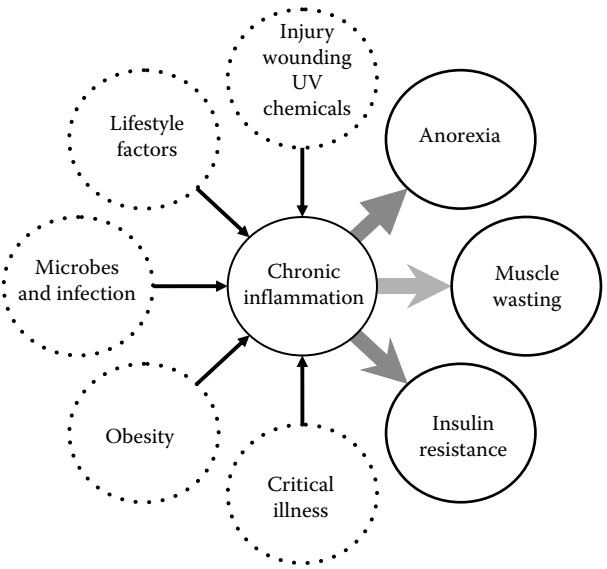

FIGURE 6.1 A schematic diagram showing the role of inflammation in human health. The causes of inflammation are discussed in the text.

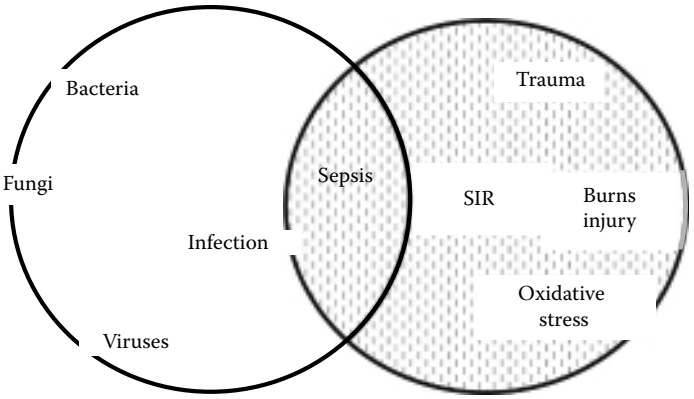

FIGURE 6.2 Interrelationships between infection, sepsis, and SIR. (Adapted from Bone RC. et al., *Chest*, 112, 235, 1997.)

We may differentiate also between "infection inflammation" due to microbial pathogens and "sterile inflammation" arising from physical injury or trauma (Figure 6.2). Bacterial infections lead to the condition of *sepsis* defined by the American College of Chest Physicians as a systemic inflammatory response (SIR) caused by the infection of the normally sterile host tissue by microorganisms.[6] The presence of viable bacteria in the bloodstream is referred to as septicemia. Sepsis may lead very quickly to a series of changes within the body culminating in organ failure and death. Acute, systemic inflammation is a key element of the sepsis.[7] However, many therapeutic strategies intended for patients from the ICU may be useful (in a modified form) for more chronic inflammatory conditions.[8] Sterile and infectious inflammation appear to share underlying pathways with implications for anti-inflammatory and antiseptic therapy (see Chapter 7).

In the aftermath of injury, endothelial cells lining the blood vessels near the affected area* increase the production of proinflammatory cytokines; the other important phenomena include recruitment and chemotaxis of immune cells, generation of reactive oxygen species (ROS) by phagocytes, and increased expression of enzymes phospholipase A2 (PLA2), 5-lipoxygenase (5-LOX), and cyclooxygenase-2 (COX2) involved in eicosanoid production. The expression of several inflammation-related transcription factors, for example, the nuclear factor kappa beta (NFκβ), also increase.[9] Phagocytes attracted to the site of injury or infection neutralize the infectious pathogens. Inflammatory cells also undergo so-called rolling motions, adherence, and migration through the vascular walls toward the damaged area and/or invading bacteria. Vasodilatation leads to increased blood flow into the affected area accounting for swelling. A rise in vascular permeability leads to increased permeation of fluid into the interstitial cell layers and ingression of lymphocytes into the damaged tissue area. Histamine, prostaglandins, and interleukins produced by macrophages

* White blood cells (leukocytes) involved in inflammatory response include the polymorphonuclear granulocytes (neutrophils, eosinophils, and basophils). These cells with multi-lobed nuclei possess granules containing defense peptides. Mononuclear (single nucleus) white blood cells like macrophages with phagocytic role are also important in the inflammatory response.

TABLE 6.2
Inflammatory and Immune Mediators

Compound	Function
Histamine	Histamine (H1) receptor[a] produces vasodilatation, increased vascular permeability, contraction of other smooth muscles, increased gastric secretion
Eicosanoids	
Prostaglandins	Increased vasodilatation, contraction of bronchial smooth muscles
Leukotrienes	Increased vasodilatation, cell adherence and chemotaxis of polymorphonuclear cells, contraction of bronchial muscle
Platelet activating factor	Platelet aggregation, vasodilatation, increased vascular permeability, chemotaxis, and activation of leukocytes,
Bradykinin	Increased vasodilatation, increased vascular permeability, stimulation of pain nerve endings, induces cough
Nitric oxide	Proinflammatory, increases vasodilatation, increases vascular permeability
Cytokines	Proinflammatory (TNF-α and IL-1) or anti-inflammatory (IL-4, IL-10, and IL-13)

[a] Three other histamine receptors produce other effects—including increased gastric secretion (H2) and inhibition of neurotransmitters (H3).

and mast cells increase vascular permeability and attract phagocytes and lymphocytes to the site of infection. Mediators involved in the inflammatory response are listed in Table 6.2.

The proinflammatory response to pathogenic microorganisms is initiated by host cells binding to specific pattern molecules possessed only by the invading organisms, resulting in the activation of the former (Chapter 7). It is feasible that host cells possess pattern recognition receptors (PRR) capable of detecting necrotic and dying cell fragments.[10] Suggestions that heat shock protein (HSP70) may be an endogenous ligand released by damaged host cells and detected by inflammatory cells remain controversial because materials used for testing this hypothesis proved to be contaminated with extraneous LPS.[11,12]

6.1.3 Infection Inflammation

Evidence that bacterial infections are a contributory factor for localized inflammation comes from studies using mice with defective PRR system for detecting pathogenic bacteria. In the C57BL/10ScCr mice strain, the entire Toll-like receptor 4 (TLR4) gene is deleted from chromosome 4, so these animals lack a functioning receptor for gram-negative bacteria. Another strain of mice (designated C3H/HeJ) carries a point mutation in the *lps* gene coding the LPS-binding protein (LPB). The mutation results in substitution of proline residue by histidine. Both mice strains show endotoxin resistance,[13–16] decreased resistance to diet-induced obesity,[17,18] and resistance to inflammatory diseases such as atherosclerosis,[19–21] IBD,[22–24] myocardial injury response to LPS,[25] and a host of other conditions including diet-induced obesity (Table 6.3).

TABLE 6.3
Effect of Toll-Like Receptor (TLR) Deficiency on Inflammatory Responses

Disease State	Relative Occurrence in TR4(−/−) Mice[a]
Acne vulgaris	↓[26]
Alcohol-induced liver damage	↓
Arthritis	↓[27]
Atherosclerosis susceptibility	↓
Autoimmune conditions	↓
Chronic inflammation	↓
Diet-induced obesity (diet induced)	↓
Endotoxin response	↓
Fat-induced insulin resistance[b]	↓
Infection-induced proteolysis	↓[28]
Inflammatory bowel disease (IBD)	↓
Leukocyte–endothelial cell interactions	↓
Macrophage phagocytosis	↓[29]
Mortality from LPS-induced shock	↓
Muscle cytokines expression with LPS	↓[30]
Myocardial injury response to LPS	↓
Neutrophil-induced chemotaxis	↓
Obesity (see diet-induced obesity)	↓
Organ reperfusion injury	↓
Susceptibility to pathogens	↑
Thrombosis	↓
Transplant rejection	↓
Tumor immune-surveillance	↓

[a] Some cited references are not based on toll-like receptor deficient mice but nevertheless discuss the probable role of innate immunity in the development of the indicated disease.

[b] The association between bacterial infection, inflammation, and insulin function has been poorly examined. But it could be predicted that antimicrobial, anti-inflammatory agents would be expected to enhance insulin sensitivity.

6.1.4 Obesity-Related Inflammation

Forsythe et al. considered the relationship between obesity-related inflammation (ORIM), weight loss (due to dieting, physical exercise, and surgical intervention), and levels of circulating biomarkers for inflammation (C-reactive protein [CRP], TNF-α, IL-6, and leptin). The meta-analysis involving 66 human trials examined obese (BMI > 30) but otherwise healthy subjects and did not cover the "low end" of the BMI scale pertaining to cachexic patients.[31] Obesity is linked with increased risk of cardiovascular disease, Type 2 diabetes (T2D), hypertension, increased levels of blood cholesterol (i.e., metabolic syndrome), and cancer.[32–38] Obese men and women are 2.13 and 6.2 times more likely to have high plasma CRP levels, respectively,

compared with age-matched lean subjects.* Plasma CRP is positively correlated with waist-to-hip ratio even after excluding the effect of smoking, diabetes, cardiovascular disease, and a host of inflammatory conditions. A study of non-Hispanic whites, African Americans, and Mexican Americans showed that the association between CRP and obesity persists independently of age or ethnicity.[33] Forouhi et al. found an association between serum CRP and indices of visceral obesity (waist size and BMI) in 113 Southern Asian men and women of Indian, Pakistani, Bangladeshi, and Sri Lankan descent.[36]

White adipose tissue (WAT) is believed to be the immediate contributor to ORIM.[32] Obese WAT cells, which are enlarged as compared to non-obese WAT, release proinflammatory mediators (leptin and TNF-α) that are associated with insulin resistance.[39–42] Obese WAT also contain greater numbers of infiltrating macrophages compared to fat tissue of lean subjects.[43] It is feasible that proinflammatory cytokines associated with obese WAT are actually produced from infiltrating macrophages.[44] At any rate, high levels of IL-6 produced by WAT signal the liver to produce CRP and other APP (Table 6.4). The inflammatory mediator TNF-α from WAT is linked with increasing insulin resistance in obese subjects.[40–43,45]

Though ORIM could be linked with muscle wasting, evidence supporting this assertion is limited.† For any given amount of fat mass, plasma leptin concentrations increase with increasing TNF-α and other proinflammatory cytokines.[46–48] Leptin receptors are widely distributed in various tissues; those located in the CNS are linked with the decrease in food intake observed during illness (Chapter 9). Leptin receptors expressed by immune cells (T lymphocytes and macrophages) appear to stimulate TNF-α production perhaps explaining the link between obesity and chronic inflammation.[49–51,‡] Inflammation-related loss of muscle mass has been cited as the basis of "sarcopenic obesity," which refers to the tendency toward decreasing muscle mass and increasing adiposity during aging.[52] Cesari et al. reported a positive correlation between serum CRP and IL-6 with percent body fat and BMI for aging adults (n = 286; mean age = 66.0 years). Also interestingly, serum inflammatory biomarkers were independently correlated with the loss of appendicular lean mass.[53] More research work is needed to understand sarcopenic obesity.[54,55] The topic may also be of some interest in relation to smooth muscle function and cardiovascular health in obese subjects.

Obese WAT and infiltrating macrophages produce proinflammatory cytokines that lead to the stimulation of lipolysis. The rise in saturated free fatty acids (FFA)

* Levels of CRP were reported by Visser et al. as elevated (>0.22 mg/dL), or raised (>1 mg/dL) in 27.6% or 6.7%, respectively, of 16,616 apparently healthy subjects (men and nonpregnant women; ≥17 years).

† Two apparently contradictory effects of TNF-α are reported in lean versus obese subjects, respectively. Lean subjects (with leptin sensitivity) exhibit restricted food intake (anorexia) following TNF-α and leptin stimulation (Chapter 9). TNF-α originates from activated inflammatory cells (endothelial cells, macrophages) for lean subjects as opposed to adipose tissue. In contrast, obese subjects exhibit decreased CNS sensitivity to TNF-α and leptin, and these agents do not inhibit food intake in the overweight. It is feasible that leptin and TNF-α resistance in muscle tissues of obese subjects could explain the lack of cachexia in these circumstances.

‡ Adipose-tissue-derived cytokines (adipokines) are currently the focus of studies linking obesity, inflammation, and insulin resistance.

TABLE 6.4
Adipose-Tissue-Derived Hormones—Adipocytokines

Hormones	Effects/Comments
Adiponectin	Levels increase with declining WAT
	Insulin sensitivity (muscle)↑
	FFA oxidation (muscle fibers)↑
	TNF-α expression and activity↓
	Macrophage activation↓
Leptin	Levels increase with WAT
	Food intake↓
	Energy expenditure (CNS)
	Insulin sensitivity↑
	TNF-α production by immune cells↑
Acylation-stimulating protein	
Proinflammatory cytokines, TNF-α, IL-6	Multiple catabolic effects
	Muscle wasting↑
	Acute phase proteins↑
	Insulin resistance↑
Other	Monocyte chemotactic protein 1, inducible nitric oxide synthase, transforming growth factor, plasminogen activator inhibitor Type-1, tissue factor

Source: Summarized from Stenholm, S. et al., *Curr. Opin. Clin. Nutr. Metab. Care*, 11, 693, 2008.

may stimulate macrophage TLR4 leading to further increases in proinflammatory cytokines. The resulting positive-feedback loop means that the production of cytokines leads to lipolysis products that further stimulate cytokine production. As support for this model, Suganami et al.[17] found that coculture of macrophages and adipocytes produced greater amounts of proinflammatory cytokines and increased NFκβ signaling compared to the controls where macrophages or adipocytes were cultured on their own. Recent research shows that the activation of NFκβ in cultured muscle cells by FFA increases with chain length.[56]

6.1.5 Chronic Inflammation, Illness, and Lifestyle Factors

Chronic illness is associated with increased plasma CRP that is a well-known biomarker for inflammation.[57–61] High concentrations of CRP (>11 mg/dL) are reported for conditions comprising the metabolic syndrome: obesity, raised blood triglycerides, elevated serum cholesterol, and T2D.[62] Table 6.3 shows a list of the inflammatory biomarkers associated with T2D.[63] Alcohol consumption, overeating, and physical inactivity contribute to the development of chronic inflammatory state.

Dietary alcohol is thought to increase the permeability of the stomach lining to gut- derived endotoxins, which then stimulate liver Kupffer cells to produce proinflammatory cytokines.[64–66] TLR4 mRNA was hardly detectable in Kupffer

cells before exposure to injurious agents, but levels increased after 2 weeks exposure to CCl_4, peaking at 4–6 weeks before declining.[67,68] Injury due to CCl_4 produced changes in morphology, inflammation, fibrosis, and necrosis of liver cells. Marked changes in protein metabolism and muscle wasting occur with liver cirrhosis.[69]

Exercise may reduce the risk of inflammatory diseases by affecting TLR signaling. McFarlin et al. found that the expression of TLR was 124% lower after exercise in postmenopausal women (65–80 years old; $n = 10$) compared to nonexercising controls. However, there was no difference in plasma IL-6 or TNF-α levels detected using ELISA, or in the cytokine mRNA analyzed by real-time polymerase chain reaction (RT-PCR). Stratification into high TLR and low TLR groups showed that the former had 305% greater TNF-α production, 209% greater IL-1b, and 167% more IL-6 production compared to the low TLR receptor group. By contrast, hormone therapy in trained postmenopausal women had no significant effect on TLR expression or related indices of inflammatory status. Further studies comparing young and elderly subjects indicated that the benefits of physical activity were independent of age. When a sample of young and elderly were subjected to an exercise program for 12 weeks, maximum oxygen consumption capacity increased by an average of 10.4% compared to physically inactive controls. LPS stimulated IL-6 production; cell surface CD14 and TLR4 expression were lower in the trained compared to untrained subjects. However, TLR2 levels were not changed.[70–73] In principle, changes in tissue TLR expression due to aging, diet, alcohol, and exercise could provide a means by which lifestyle factors moderate inflammatory capacity.[74,75]

Finally, there are indications that TLR may play a role in obesity-related diseases. Studies of TLR4-deficient mice (C3H/HeJ) show that these are more resistant to dietary fat-induced obesity and insulin resistance.[76–79] Kim et al. demonstrated that mice fed a high-fat diet showed increased expression of inflammatory markers including IKK and blockade of insulin signaling. By contrast, a high-fat diet produced less inflammatory response with mice that are deficient in TLR4 function.[80] TLR is also implicated in the activation of macrophages by oxidized LDL[81] (Table 6.3). We found no indication in the literature that bacterial infections lead to decreased insulin sensitivity except for cases of sepsis (Chapter 8). The relation between hygiene and metabolic diseases is worth further study (cf. Section 7.2.3).

6.2 PROINFLAMMATORY SIGNALING

6.2.1 Proinflammatory Cytokines

TNF-α is involved in weight loss linked with cancer, sepsis, inflammation, and COPD.[45,82–84] Proinflammatory cytokines accelerate lipolysis, increase muscle protein breakdown, and inhibit protein synthesis. Other effects associated with TNF-α include anorexia and other so-called illness behaviors. Proinflammatory cytokines activate leptin production in adipose tissue and eventually affect neuropeptide production in the CNS, leading to the inhibition of feeding behavior (Chapter 9). IL-6 is thought to regulate the synthesis of APP in the liver.[85]

The cytokine superfamily includes five to six subgroups: interleukins,* chemokines, interferons (IFN), colony-stimulating factors (CSF), growth factors, and TNF. These bioactive peptides can stimulate producer cells (autocrine function) or other cells (paracrine stimulation). TNF-α, IL-1, and IL-6 have proinflammatory action whereas IL-4 and IL-10 have anti-inflammatory activity. The cachexic effect of TNF-α is mediated by the transcription factor NFκβ and involves tyrosine kinase-linked membrane receptor-1 (TNFR1) linked with the Jak/Stat pathway. Elements of TNF-α signaling are summarized as: TNF → TNFR1 → mitochondria (ROS↑) → (ubiquitin proteasome activity↑ →) NFκβ → ubiquitin proteasome → protein loss. TNF-α binding to its receptor stimulates mitochondrial ROS formation and activation of the ubiquitin proteasome and NFκβ (Section 6.2.3). In accordance with this model, levels of ubiquitin proteasome were elevated by the administration of TNF-α as well as IL-6.[86–89]

In vivo studies have shown IL-1 and TNF-α induce loss of lean body mass.[83] The injection of low concentrations of TNF-α into rats reproduces symptoms of cachexia. The wasting response is blocked by antibodies for TNF-α receptor. Third, exposure to low concentrations of TNF-α increases protein breakdown in cultured muscle cells, and fourth, tumor implantation into rodents induces an increase in TNF-α production.[90] Past studies suggest that TNF-α (previously called cachectin) induces cachexia in animal models. The half-life of TNF-α is thought to be about 30 min within the circulation, and high concentrations of this agent have been shown to correlate with increased insulin resistance as well as altered peripheral blood flow. The involvement of TNF-α in muscle wasting has been extensively reviewed over the past decade.[3,91–99] Some of the major effects of proinflammatory (PIM) cytokines are summarized in Figure 6.3, and Tables 6.5 and 6.6.

6.2.2 Transcription Factors for Inflammatory Signaling

NFκβ is involved in the regulation of genes important for the host response to stress[101] and a variety of inflammatory states including AIDS, asthma, atherosclerosis, rheumatoid arthritis, diabetes, osteoporosis, obesity, IBD, heart disease, and reperfusion injury.[102–106] NFκβ contains protein subunits NFκβ1 (p50 dimer), NFκβ2 (p52 dimer), RelA (p65), RelB (p68), c-Rel (p75), and v-Rel. The two NFκβ subunits are derived from single larger proteins by proteolytic processing involving the ubiquitin proteasome. NFκβ (p50/p65) is the most common NFκβ. Inactive NFκβ is found in the cell cytoplasm in association with an inhibitory protein (Iκβ) that masks the DNA-binding domain of NFκβ. The phosphorylation of Iκβ and its degradation by UPS releases NFκβ, which translocates into the nucleus.[107,108] It is thought that NFκβ binds to promoter sites for expression of proinflammatory genes. Subunits of NFκβ bind to sequences of the DNA promoter regions of affected genes containing the base sequence GGGRNNYYCC, where R = purine, Y = pyrimidine, and N = any base.

NFκβ is activated by external and internal agents resulting in its transfer from the cytoplasm to the cell nucleus.[107,110–113] The classical pathway for NFκβ

* Interleukin (IL) describes a subgroup of cytokines produced by leukocytes to stimulate other leukocytes.

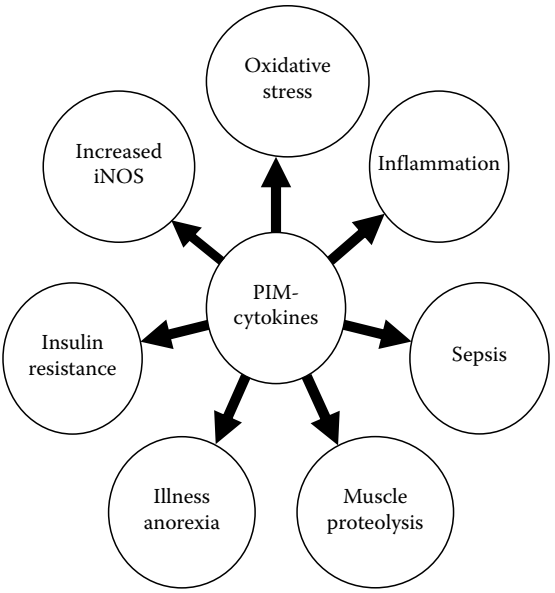

FIGURE 6.3 Effect of PIM cytokines on muscle wasting and related processes.

TABLE 6.5
Effect of Cytokines on Processes Contributing to Cachexia

	TNF-α	IL-1	IL-6	IFN-γ	LIF
Lipid synthesis	↓	↓	—	↓	↓
Lipolysis	↓	↓	↑	↓	↑
Lipase activity	↓	↓	↓	↓	↓
Protein synthesis	↓	↓	—	↓	—
Muscle proteolysis	↑	↑		—	—
APP synthesis	↑	↑		—	↑
Food intake	↓	↓	↓	↓	↓
Body weight	↓	↓	↓	↓	↓
Body temperature	↑	↑			

Source: Adapted from Yeh, S.S. and Schuster, M.W., *Am. J. Clin. Nutr*., 70, 183, 1999.

Note: LIF, Leukemia inhibitory factor; APP, Acute phase protein.

activation is initiated by TNF-α and LPS binding to their respective receptors, followed by activation of inhibitory kinase kinases (IKK) that ultimately stimulate NFκβ (p50/rel A). IKK controls the transfer of NFκβ from the cytoplasm to the nucleus, leading to the transcription of proinflammatory genes. IKK have two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ, NEMO).

TABLE 6.6
Effect of Selected Cytokines on Innate Immune System

Inflammatory Interleukins	Producer Cells	Actions
Interleukin-1 (IL-1)	Macrophages, endothelial cells, B cells, and fibroblast cells	Inflammatory responses; edema; production of prostaglandins, corticosteroids, and IL-2; leukocytes growth; induces fever and shivering
Interleukin-6 (IL-6)	Macrophages, T cells	Production of APP in the liver, differentiation of B cells into IgG producing cells, stimulates leukocytes
Interleukin-8 (IL-8)	Macrophages, endothelial cells, and most cells of the body	Proinflammatory, stimulates immune cells migration into tissues, inducer of chemotaxis for neutrophils
Interleukin-9 (IL-9)	?	Proinflammatory, increases T cell growth, upregulates Th1 responses by inhibiting T cell apoptosis
Anti-inflammatory and other		
Interleukin-4 (IL-4)	Helper T cells	Decreases proinflammatory cytokines (IL-1 and TNF-α) production
Interleukin-10 (IL-10)	T cells, B cells, monocytes, and macrophages	Inhibits IFN-γ production, stimulates antigen presentation, IL-1, IL-6, TNF-α production by macrophages, B cell activation
Interleukin-16 (IL-16)		Adhesion molecule and activator for T cells, plays role in asthma and autoimmune diseases

Source: Adapted from the multiple sclerosis Web site: The role of interleukins: http://www.mult-sclerosis.org/interleukins.html (accessed April 2007).

Activation of NFκβ via the "classical" pathway follows receptor tyrosine kinase activation, and phosphorylation of IKKγ at a serine or threonine residue. IKK then phosphorylates the inhibitory protein (Iκβ), causing this to dissociate from NFκβ. The newly released Iκβ is ubiquitinated and then degraded via the proteasome pathway, freeing NFκβ for transfer to the nucleus. The alternative pathway for NFκβ (p100/Rel B) activation involves the reaction of IKKαβ directly with ROS. Cell receptor binding by LPS or TNF-α is thought to activate ROS release by mitochondria, though there are other sources of oxidative stress.[114] IKKαβ may serve as one of the many redox-sensitive agents in cells that link rising ROS levels with inflammation (see Section 6.2.3).

NFκβ has been shown to affect the expression of genes for proinflammatory cytokines, cell surface receptors, APP, COX2, and inducible nitric oxide synthetase (iNOS) as well as several adhesion molecules. NFκβ enhances anti-apoptosis signaling and may be important for increasing proliferation of cancer cells.[115] Of particular interest also are the effects of NFκβ activation on processes affecting muscle accretion, for example, muscle stem cell proliferation, differentiation, cell apoptosis, and proteasome activation (Chapter 5). The characteristics of NFκβ are summarized in Table 6.7.

TABLE 6.7
Characteristics of NFκβ Related to Inflammation

External activators of NFκβ

- Lipopolysaccharide
- Heat shock
- Osmolarity
- UV light, etc.
- Phorbol ester,
- ROS
- Long chain fatty acids

Inhibitors

- Steroidal drugs
- Nonsteroidal drugs (aspirin)
- Antioxidants
- Protease inhibitors
- Cellular thiol
- Protein kinase inhibitors
- Phytochemicals

Internal activators of NFκβ

- Chemokines
- EGF
- TNF-α

Selected genes with NFκβ *binding sequences*

Cytokines and growth factors	TNF-α, IL-1, IL-2, IL-6, IL-12, CSF, granulocyte colony-stimulating factor, interferon B, and vascular endothelial factor
Chemokines	IL-8 growth-related oncogene (a, b, Y)
	Regulated upon activation, normal T cell expressed and secreted (RANTES)
	Monocyte chemoattractant protein 1
Cell adhesion molecules	E-selectin, intercellular cell adhesion molecule 1, vascular cell adhesion molecule
Enzymes	Matrix metalloprotease-inducible nitric oxide synthetase (iNOS)
	COX2, phospholipase A2
Immunoproteins	Tissue factor, IL receptor 2, major histocompatibility complex I
APP	C-reactive protein

Inhibitors of NFκβ have potential applications as anti-inflammatory agents. Conventional NFκβ inhibitors include steroidal drugs, nonsteroidal anti-inflammatory drugs (NSAID), as well as a variety of dietary phytochemicals. Antioxidants may break essential disulfide bonds between residues of IKKαβ, thereby generating an anti-inflammatory response. Dietary antioxidants that do not affect IKKαβ directly, can also produce an anti-inflammatory action via increased levels of glutathione (GSH). Modification of IKK-essential cysteine by GSH has been shown to counteract the proinflammatory actions of TNF-α.[116–118] The effect of NFκβ activation on muscle wasting is discussed in Chapter 9.

Activator protein-1 (AP-1) is a transcription factor formed from two subunits (c-Jun and c-Fos). AP-1 is activated by three kinases (P38, ERK, and JNK) via phosphorylation, followed by nuclear translocation, and AP-1 binding to DNA response elements that induce the transcription of inflammatory and immune genes. It is thought that anti-inflammatory drugs such as glucocorticoids (GC) induce mitogen-activated protein kinase (MAPK) phosphatase-I, which indirectly decreases

activation of AP-1 by limiting phosphorylation of the complex. The AP-1 are important as the focus of proinflammatory signaling related to cancer.

Nuclear receptor factor 2 (Nrf2) regulates the expression of genes for detoxifying enzymes and antioxidant enzymes under the control of antioxidant responsive element (ARE). Inactive Nrf2 is covalently bonded to an inhibitor subunit, keap 1 (Kelch-like ECH-associated protein 1) via a disulfide link. Reducing compounds break the cysteine–cysteine bond and dissociate Nrf2 from keap 1, resulting in the transfer of Nrf2 from the cytoplasm to the nucleus. Several plant phytochemicals with anti-inflammatory action are believed to moderate Nrf2 transcription activity leading to increased expression of key antioxidant enzymes (GSH peroxidise and GSH reductase) and detoxification enzymes, for example, GSH transferase and glutamyl-cysteine synthetase.[119–123]

6.2.3 Redox-Sensitive Inflammatory Transcription Factors

NFκβ is one of several redox-sensitive transcription factors now recognized in cells (Table 6.8) that serves to link inflammation with oxidative stress and muscle wasting.[124–128,*] The molecular detectors for ROS upstream of NFκβ are nuclear-inducible kinases (NIK) typified by IKKαβ. As mentioned elsewhere, IKKαβ is part of regulatory kinase cascade (NKKK → NIK → substrate Iκβ) along with NIK phosphatases (NIKP), which ultimately control NFκβ activity. NKKK (corresponding to protein tyrosine kinase) as well as NKK (cf. IKK) are thought to possess essential reduced cysteine residues required for activity. Oxidation of the key cysteine residues to form cystine-disulfide bonding by ROS inactivates NKI phosphatase thereby increasing the state of phosphorylation of NIK and thereby *activating* IKK and hence NFκβ.

The DNA-binding domains of NFκβ (p65/p60) subunits may also contain essential cysteine residues that need to be in the reduced state and that are modifiable by

TABLE 6.8
Redox-Sensitive Anti- and Proinflammatory Transcription Factors

Name	Genes Affected/Comments
Nrf2–Keap 1 complex	Nrf2 binds to ARE to upregulate antioxidant enzymes and detoxification enzymes
NFκβ	Proinflammatory transcription factor, inflammatory cytokines, chemokines, COX-2, inducible nitric oxide synthase (iNOS), LOX-2, and cell adhesion molecules. anti-apoptosis genes, cell proliferation
Activator protein-1 (c-Fos, c-Jun)	Stimulated by proinflammatory signals, e.g., growth factors, oxidative stress, and tumor promoters
FOXO family of transcription factors	—

* ROS including superoxide radical, hydrogen peroxide, and the hydroxyl radical may damage cellular components by direct chemical actions.

redox compounds. This feature could explain why exogenous reducing compounds are found to activate rather than inactivate NFκβ. From a host defense viewpoint, activation of NFκβ upregulates a group of adaptive enzymes including superoxide dismutase (SOD-2). Activation of NFκβ may also lead to non-adaptive changes such as septic shock.[114,126,128–131]

There are indications that NFκβ can be inhibited by high intracellular concentrations of GSH, thioredoxin, or α-lipoic acid.[127,132–134] However, results have not always been consistent perhaps because of crossover effects at low and high concentrations of cellular thiol. There are only a limited number of methods for increasing intracellular GSH levels. Indirect strategies for changing cellular levels of GSH involve the administration of buthionine sulfoximine (BSO), an inhibitor for GSH synthetase. Dietary glutamine has been shown to stimulate cellular GSH synthesis and thereby moderate the redox and inflammatory status (Section 6.3.9). GSH pro-drugs are available that promote GSH synthesis. Dietary phytochemicals may deplete cellular GSH owing to their excretion as GSH-conjugates from cells. On the other hand, some phytochemicals induce the expression of Phase II enzymes such as GSH synthetase. A full discussion of the anti-inflammatory activity of plant phytochemicals and its relation to intracellular GSH is beyond the scope of this discussion. The key point is that moderation of the NFκβ pathway might be a useful therapy for addressing muscle wasting.[135–137]

6.2.4 MAP Kinases, Phosphatases, and Inflammation

MAPK regulates the proinflammatory AP-1. In its turn, MAPK is activated by the so-called Type 3 tyrosine kinase-linked receptors. TNF-α and ROS also stimulate the MAPK and thereby induce an inflammatory response.[138–140] MAPK signaling involves a 3-tier arrangement of enzymes: MAPK3/MAPKKK/MEKK → MAPK2/MAPKK/MEK → MAPK1 linked to several nuclear transcription factors including AP-1 (Table 6.9). The MAPKs include the stress-activated protein kinases (SAPK), namely, c-Jun NH_2-terminal kinase (JNK), p38 kinase, and extracellular signal regulated kinase (ERK). ERK1/2 is linked with responses to growth factors (e.g., insulin, insulin-like growth factor [IGF], epidermal growth factor [EGF], platelet-derived growth factor [PDGF], and fibroblast growth factor [FGF]), which all operate through classic G-protein linked receptors. These growth factors generally inhibit cell apoptosis and increase cell proliferation. On other hand, stimulation of p38 and JNK1/2 is associated with proinflammatory pathways.* Four p38 isoforms (α, β, δ, and γ) support the inflammatory and immune responses due to their ability to stimulate TNF-α and IL-1 production. Upstream signals for p38 activation include TNF-α, LPS, cytokines (IL-1, -2, -7, -17, and -18), tumor growth factor beta (TGFβ), heat stress, osmolarity, UV irradiation, ROS, and hypoxia. The role of MAPK in the inflammatory response has been reviewed.[138–140]

* Alternative nomenclature is shown for the three tiers of kinases forming the MAPK signaling. MAPK1 is nearest to the cellular response while MAPK3 is at the receptor end of the chain.

TABLE 6.9
Summary of MAPK Pathways

Ligands Sensed→	Growth Factors	Cytokines, Chemicals, and UV Stress	Cytokines, Chemicals, and UV Stress
MAP3K	Raf-1	Tak1/MTK1	MEKKS
↓			
MAP2K	MEK1/2	MKK3/6	MKK4/7
↓			
MAPK	ERK1/2	P38	JNK1/2
↓			
NTF	Elk1	AP1 (c-Jun and c-Fos)	AP1 (c-Jun and c-Fos) MEF, ATF-2
↓			
Cell cycle targets	ETS, p27	ETS	E2F1
Kinases targets	Increased cell proliferation	Inflammatory and immune responses	Inflammatory and immune responses

Abbreviations: c-Jun amino-terminal kinases (JNKs); MEF, myocyte enhance factor; API. activator protein 1; ATF-2, activating transcription factor 2.

Column 1 shows the three-tier arrangement of kinases ending with the activation of specific nuclear transcription factors (NTFs). ETS are a family of transcription factors comprising at least 12 members (cf. http://en.wikipedia.org/wiki/ETS_transcription_factor_family).

The substrates for p38 are thought to include activating transcription factor 2 (ATF2), SRF accessory protein 1 (Sap1), CHOP, p53, myocyte enhancer factor 2A (MEF2A), myocyte enhancer factor 2C (MEF2C), CAAT-enhancer-binding protein β (C/EBPβ), nuclear factor of activated T cells p (NFATp), and signal transducers and activators of transcription (STAT4). One notable biological consequence of p38 stimulation is increased production of TNF-α and IL-1, leading to increased synthesis of chemo-attractants and adhesion molecules. Recent p38 studies have emphasized the unique role of this enzyme in inflammatory conditions such as bone osteolysis. p38 signaling has also been implicated in the control of T cell differentiation, where this serves to shift the Th1/Th2 balance toward the Th1 autoimmune response and/or chronic inflammatory conditions.[141,142] Blocking MAPK activation has been considered as therapeutic strategy for achieving anti-inflammatory and anticancer therapy.[143] Some biological consequences of MAPK signaling are listed in Table 6.10.

6.2.5 Renin–Angiotensin System and Inflammation

Hypertension is often associated with atherosclerosis and cardiovascular disease suggesting that the renin–angiotensin system (RAS) may be proinflammatory.[144–148] According to the textbook description of the RAS system and how this affects blood pressure (Figure 6.4), angiotensinogen is converted to angiotensin I (ANGI) and then

TABLE 6.10
Biological Consequences of MAPK Signaling

Apoptosis—from nutrient or growth factor withdrawal
Cell cycle arrest at G1/S phase
Brain development
Cardiomyocyte development and hypertrophy
Ischemic perfusion injury from ROS production by perfused cells
T cell maturation
JNK promotes Th(1) cytokine profile and protects against T cell apoptosis

Proinflammatory role
P38 stimulates IFγ production
Stabilizes mRlNA for inflammatory cytokines (IL-6 and IL-8) and COX2
Promotes Th1 response (and inflammatory, autoimmune diseases)
Inhibitors for regulatory kinases upstream from p38 may be important as novel anti-inflammatory agents though lack of specificity leads to toxicity

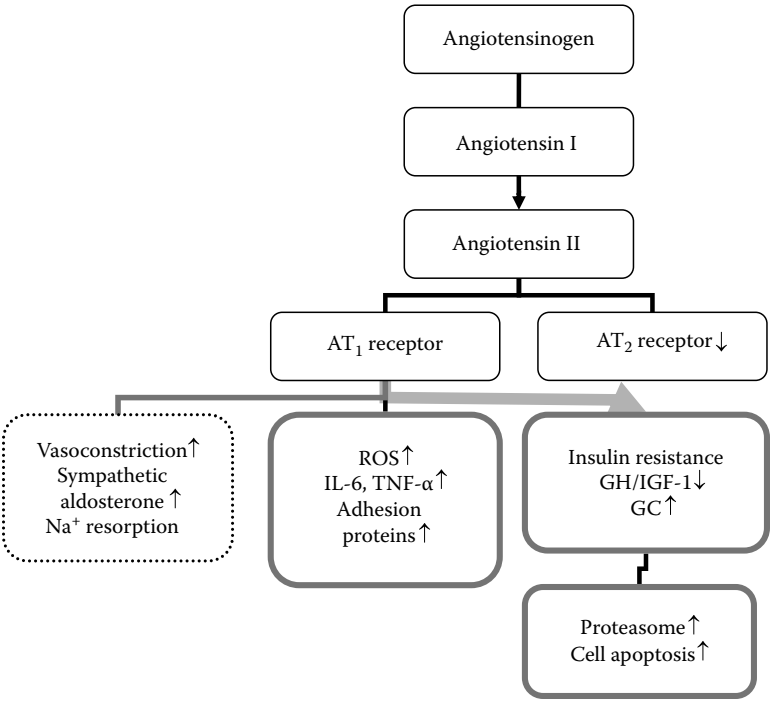

FIGURE 6.4 Involvement of the RAS in proinflammatory process. Abbreviations: ROS, Reactive oxygen species; IL-6, Interleukin-6; UPS, ubiquitin proteasome. Other elements are as defined in the text. Adhesion proteins affected by ANGII include endothelial selectin (E-selectin), the intracellular adhesion molecule (ICAM-1), and vascular adhesion molecules (VCAM 1).

angiotensin II (ANGII) by renin and angiotensin-converting enzyme (ACE; dipeptidyl carboxpeptidase, EC 3.4.15.1). The last reaction leads to ANGII (FPHIYVR), which expresses bioactivity via the ANGII receptor, subtypes 1 (AT_1) and 2 (AT_2). The well-known effects of ANGII binding to smooth muscle AT_1 is that there is a rise in intracellular Ca^{2+} levels, muscle contraction, constriction of blood vessels and capillaries, and a rise in blood pressure. ANGII is also involved in the control of Na^+ and fluid balance, which is presumably expressed via AT_1 molecules located in the kidney tubules.

Hypertension can be treated using angiotensin-converting enzyme inhibitors (ACEI) and ANGII receptor blockers (ARBs) that inhibit ANGII function. ACEI and ARBs are also considered particularly useful for the treatment of cardiac failure, ischemic heart disease, and for treating cases of diabetic nephropathy and renal failure.[149]

Recent research suggests that ACEI might also be useful as anti-inflammatory or antidiabetic agents for treating T2D and inflammation-related muscle wasting.* In support of this view, ANGII has been shown to stimulate virtually all stages of the inflammatory response, for example, vascular permeability, migration of inflammatory cells, and expression of adhesion proteins. ANGII has also been reported to increase ROS formation, to stimulate the formation of adhesion molecules, iNOS and COX (Figure 6.4). The proinflammatory effect due to RAS is apparently mediated by NFκβ activation and increasing cellular ROS. Activation of NFκβ may involve ANGII as well as ANGIV formed by ANGII degradation by endogenous aminopeptidases.[150,151] Current investigations of ANGII tend to focus on two areas: First, activation of RAS may explain the well-documented correlation between hypertension and markers of inflammation.[152] Second, there is the possibility that established ACEI may be adapted for treating chronic inflammatory conditions (cf. Section 6.3.2).

6.3 ANTI-INFLAMMATORY BIOACTIVE PEPTIDES AND SUPPLEMENTS

6.3.1 Adiponectin

Adiponectin is a 30 kDa peptide hormone produced by WAT, though the extent of secretion increases with decreasing fat cell size. Adiponectin has been found to increase insulin sensitivity and fatty acid oxidation in muscle cells. Animal studies show that adiponectin is able to reverse insulin resistance and induce fat loss in obese models. At the other end of the BMI range, elevated levels of adiponectin have been reported for several cachexic conditions leading to suggestions that adiponectin may be considered a marker for wasting; in catabolic conditions, elevated adiponectin is linked with increased rates of mortality. The effect of adiponectin on muscle biology is uncertain. Adiponectin and proteolytic fragments have anti-inflammatory properties, being able to reduce LPS-induced production of TNF-α.[66] Inhibition of myostatin leading to increased muscle mass was found to be associated with increasing

* The effect of the RAS system on insulin resistance and muscle health is discussed in Section 5.5.6.

levels of serum adiponectin and increased fatty acid oxidation in muscle. The effect of adiponectin levels on muscle mass is likely to be complex. Adiponectin is thought to function through AMPK activation; on the other hand, this same enzyme is activated by rising levels of intracellular c-AMP leading to increased rates of proteolysis.[153] Past research suggests that AMPK antagonizes the mTOR pathway and is liable to depress the anabolic effects of amino acids.[154]

6.3.2 Angiotensin-Converting Enzyme Inhibitor Peptides

Conventional ACEI and ARBs have been examined for their role as anti-inflammatory agents.[146,155,156,*] ACEI use may also be beneficial because ANGII can directly stimulate muscle wasting.[157] Administration of LPS increases the plasma concentration of ANGII as well as TNF-α. Consequently, ACEI treatment was shown to protect the lungs of LPS-treated rats from inflammation, edema, and tissue damage. Pretreatment with the ACEI enalapril was found to inhibit LPS-induced rise in ANGII and tissue damage due to sepsis.[158] Animal studies show ACEI ameliorates LPS-induced liver damage[159] and dextran sodium sulfate (DSS) induced colitis.[160]

A handful of RCT have examined the effect of ACEI on inflammatory indices for human subjects with mixed results.[†] Schieffer et al. found that coronary artery disease patients treated for 3 months with enalapril (20 mg/day) or the ARB irbesartan (300 mg/day) showed improvements in inflammatory status. Patients receiving ACEI (n = 22) or ARB (n = 21) showed increased serum IL-10 levels and reduced matrix metallo-protease expression. Serum levels of high sensitivity CRP (hsCRP) and IL-6 levels were also lower following ARB treatment but not ACEI treatment. Both ARB and ACEI were found to have anti-inflammatory effects in heart disease patients though the former was more effective.[161] A 2005 randomized clinical trial from Argentina showed that ACEI therapy could reduce the levels of several inflammatory biomarkers in patients at risk of cardiovascular disease. The study involving 77 patients showed that 6 month treatment with ramipril produced a fall in the hsCRP levels (2.7 mg/L vs. 1.7 mg/L; P = 0.0009). The benefits of ACEI treatment were observed with highly at risk groups (hsCRP = 5 mg/L) whereas there was little effect on patients with low or moderate hsCRP (1–3 mg/L).[162] Another RCT reported from Greece showed that 6 month therapy with perindopril led to declines in serum levels of TFNα, IL-6, and total peroxides in T2D patients.[163]

Not all RCT have shown a positive effect of ACEI on inflammation. A 2008 placebo-controlled RCT found that levels of ACEI and ARBs sufficient to reduce hypertension produced no significant changes in plasma hsCRP.[164] Another placebo-controlled double-blind investigation called the "trial of angiotensin-converting enzyme inhibition and novel cardiovascular risk factors" or TRAIN found that ACEI had no effect on biomarkers for inflammation markers (hsCRP, IL-6, VCAM, endothelin-1).[165] To explain these data, the trial authors noted that the state of activation of RAS in the study population was not recorded, but ACEI treatment did produce a highly

* Many of the studies described refer to pharmacological ACEI drugs. Relatively little research has appeared on the anti-inflammatory activity of ACEI.

† Currently, over 350 RCT have been published on the effect of ACEI on blood pressure.

significant drop in levels of ANGII concentrations ($P < 0.002$). Second, ANG can be activated by other enzymes other than ACE leading to so-called ACEI escape. This well-documented phenomenon implies that the effect of ANGII on health can be evaluated precisely only if one uses ACEI together with ARBs. The third feature of the 2009 study is the particular type of ACEI examined; clearly, data obtained for the drug fosinopril may not be applicable to different types of ACEI. The final issue that may affect the study outcome is the study population itself, which was at high risk of cardiovascular diseases.[165] ACE gene polymorphism may also affect how different groups respond to ACEI.

Food-derived peptide ACEI can be produced from virtually all major commodity groups (milk, eggs, wheat, soybean, etc.) by limited protease action. In vivo tests using spontaneously hypertensive rats have shown that food ACEI can apparently be absorbed from the GI tract resulting in significant falls in blood pressure.[166] Though the food ACEI peptides are <12 amino acid residues in size, some behave as "pro-drugs" so that prior digestion leads to a more potent material. The structural requirement for ACEI activity seems to be fairly flexible with quite a diverse range of sequences being able to act as competitive or noncompetitive inhibitors of ANGII formation.[167] Technological aspects of food-derived ACEI peptides have been reviewed.[168–172]

Several RCT of food ACEI have been reported to examine their effects on blood pressure. A meta-analysis by Pripp Hugo (Oslo, Norway) examined most of the human RCT published in English language sources up to and including 2006 ($n = 15$ trials, 826 participants). The available evidence show that food ACEI could reduce systolic blood pressure (SBP) and diastolic blood pressure (DBP) by −5.13 mmHg (95% CI: −7.12,−3.14) and −2.42 mmHg (95% CI: −3.82,−1.03). The majority of 15 human RCT employed ingredients containing the tripeptides Val-Pro-Pro (VPP) or IsoLeu-Pro-Pro (IPP) as the active ingredient. One RCT using protein hydrolysates (without active ACEI) showed negative results indicating that the hypotensive effect was due to bioactive peptides.[173] Another meta-analysis of RCT focusing entirely on milk-derived ACEI ($n = 12$ trials, 623 participants) also found an average change of SBP and DPB of −4.8 mmHg (95% CI: −3.7 to −6.0) and 2.2 mmHg (95% CI: −1.3 to −3.1). The effects were confined to mildly hypertensive subjects.[174] These results should be balanced against data from two recent RCT from the Netherlands, which suggest that milk-derived ACEI or so-called lactotripeptides produce no effect on blood pressure.[175,176] In summary, there is substantive evidence for the role of food ACEI in the control of blood pressure. However, more work is needed to determine the limits of such therapy between different human populations. More investigations are required into the effect of food-derived ACEI on inflammatory indices.

6.3.3 Antimicrobial Peptide Endotoxin Antagonists

Protein and bioactive peptides which function as endotoxin antagonists include bactericidal/permeability increasing protein (BPI),[177–179] cationic antimicrobial peptide (CAP18),[180] and lactoferrin (Lf)/lactoferricin.[181–185] These proteins possess anti-inflammatory as well as bacteria-killing activity (Chapter 7). The endotoxin antagonists are positively charged (cationic) peptides, proteins or synthetic polymers that block the harmful effect of endotoxin by electrostatic binding,[186–188] thereby interfering with

LPS binding to the LPS-binding protein CD14 or the subsequent interaction of this with the extracellular domain of TLR.[189] Larrick et al. showed that human CAP18 also had endotoxin-binding activity,[190] possibly accounting for the protective effect of human CAP18 against endotoxin shock in the pig.[191] Premixing LPS with 0.1 or 1.0 μg CAP18 prior to IV administration increased the survival rate in mice from 36% (control) to 50% or 80%, respectively. Protection was also achieved when CAP18 (residues 106–135) was injected immediately before LPS. High doses (4.4 mg/kg body weight) of CAP18 increased survival rates in bacteremic mice from 6% to 75%. In these trials, the number of live bacteria in the circulation did not differ for control or treated mice. It could be concluded that CAP18 (109–135)[192] functions by neutralizing LPS. In vitro studies showed that treatment of cultured cells with CAP18 (109–135) inhibits LPS-induced production of TNF-α. An interesting and rather unexpected use of endotoxin antagonist is exemplified by use of these agents in medical devices during extracorporeal treatment of inflammatory conditions. The antimicrobial peptide polymyxin B has been immobilized onto polystyrene solid support for removing circulating LPS from a patient's circulation.[193–195] Though intended initially for seriously ill patients, there are also signs that extracorporeal detoxifying devices are beginning to be used for general "blood cleaning." Other endotoxin antagonists currently in the pipeline include E5564,[196,197] Eritoran™,[198] and the sushi peptides.[199]

6.3.4 Cytokine Antibodies

Anti-inflammatory agents have been suggested for the treatment of muscle wasting conditions. In principle, a range of anti-inflammatory bioactive peptides and protein supplements (BPPS) are available that help to moderate cytokine concentrations. Inhibitors for cytokine synthesis, cytokine-binding antibodies, soluble cytokine receptors, and anti-inflammatory cytokines have been proposed as strategies for reducing chronic or acute inflammation. Rheumatoid arthritis (a well-known inflammatory condition) involving increased protein degradation has been shown to respond to therapeutic antibodies designed to target cytokines.[200–204]

6.3.5 Ghrelin and Growth Hormone Secretagogues

Ghrelin shows anti-inflammatory activity acting via the growth hormone secretagogue receptor 1-a (GHS-R1a) expressed by a veriety cells, including macrophages, T cells, neutrophils. Exposure of cultured T cells and macrophages to ghrelin decreases proinflammatory cytokine (IL-1, IL-6, TNF-α) formation in response to LPS.[205,206] Ghrelin also inhibits cytokine (IL-8) production by human endothelial cells and the activation of NFκβ induced by TNF-α.[207] Inflammatory cells also produce ghrelin under the influence of the exogenous hormone. The anti-inflammatory action of ghrelin is thought to be independent of the effects on food intake.[208] Synthetic growth hormone-release peptides (GHR-P6, GHR-P6, etc.), that function as ghrelin agonists, can be expected to show anti-inflammatory activity mediated through the GHS-R. The characteristics of ghrelin agonists are discussed further in Chapter 9.

In vivo studies with rodent models for inflammatory diseases show that ghrelin (and ghrelin agonists) reduce serum levels of proinflammatory cytokines, improve

TABLE 6.11
Rodent Models for Inflammatory Diseases Treatable with Ghrelin

LPS endotoxemia, sepsis[210]
Mice, colitis[211]
Ischemia–reperfusion injury[212,213]
LPS-induced chronic arthritis[214]
Pancreatitis induced, pulmonary, and renal injury[215]
Muscle catabolism, thermal injury[216]
Post-surgery cachexia[217]

weight gain, and avoid muscle wasting—to a greater degree than expected from changes in food intake (Table 6.11). Figure 6.5 shows a schematic diagram for the anti-inflammatory action of ghrelin and related agonists that act directly on inflammatory cells and other tissues possessing GHS-R (see Section 9.5). The model seeks to represent the effect of endogenous ghrelin in health, which may be counterintuitively obvious. It has been shown that some wasting conditions result in elevated levels of serum ghrelin—perhaps due to an "adaptive" response to the effect of wasting.[209] In view of this, it seems odd that further administration of ghrelin should improve food intake in the way that is observed in practice.

Low molecular weight peptides GHS or so-called growth hormone release peptides (GHRP) are available (Chapter 8, Section 8.4.1) that mimic the action of ghrelin by binding to GHS-R1a, leading to increased GH release. In fact, synthetic GHRP were the only known ligands for GHS-R1a prior to the discovery of ghrelin in 1994 by so-called reverse-pharmacology. Recently, the study of GHRP has been overtaken by interest in ghrelin—the natural GHS and promoter of food intake. So far, very few investigations have appeared on the anti-inflammatory effects of GHRP.

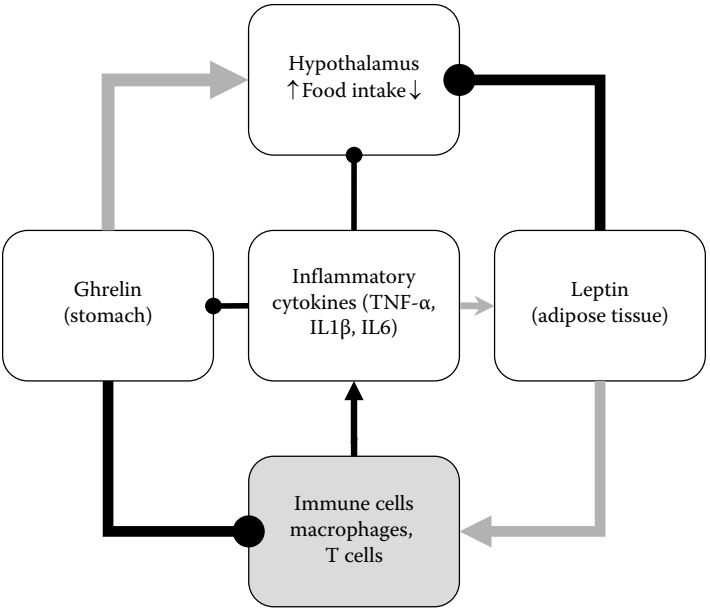

FIGURE 6.5 Schematic model for the anti-inflammatory action of ghrelin on immune cells. (From Dixit, V.D. et al., *J. Clin. Invest.*, 114, 57, 2004.)

6.3.6 Glucocorticoid-Inducible Peptides

The GC such as dexamethasone are important anti-inflammatory drugs with undesirable side-effects including a tendency to produce muscle wasting and to depress the immune function. The anti-inflammatory and other effects of GC are summarized in Figure 6.6 and discussed below. Dexamethasone and other synthetic GC increase skeletal muscle protein loss in animal models.[218,219] Experiments using the selective glucocorticoid-receptor blocker RU-485 suggests that cortisol is involved in muscle wasting[220] related to metabolic acidosis,[221,222] burns injury,[223,*] physical trauma, fasting, and sepsis.[224–226] By contrast, cancer cachexia was not linked with GC secretion.[227] The GC stimulate protein breakdown probably by enhancing the expression of multiple pathways, for example, lysosomal autophagy, Ca^{2+}-dependent calpains and, most important of all, ubiquitin proteasome system.[228,229] GC administration increases the expression of muscle-specific genes atrogin-1 and MURF1 that code for ubiquitin ligases. Lang et al. demonstrated that burns injury (40% total burns surface area) increased atrogin-1 and MURF1 mRNA in rat gastrocnemius muscles (fast twitch, Type II fibers) by 3.1-fold and 8-fold, respectively, while polyUB

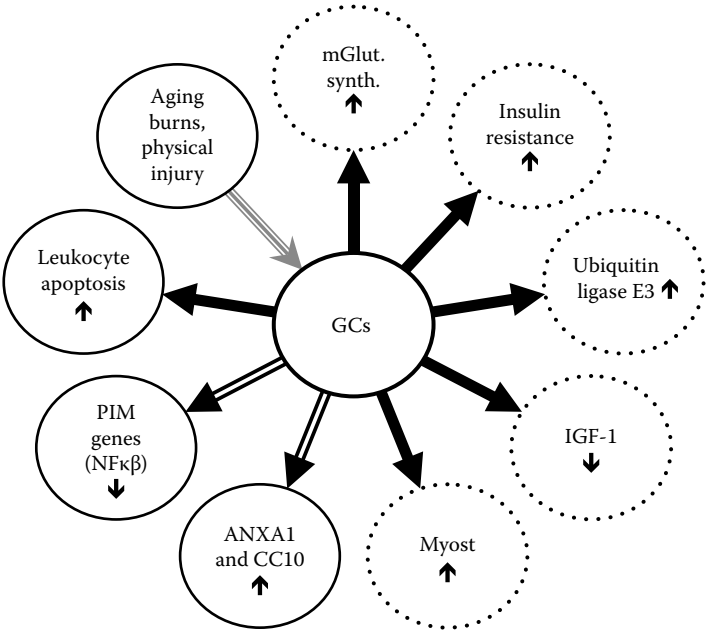

FIGURE 6.6 Effect of GC on inflammation (solid circles) and muscle wasting (dotted circles). Myost = myostatin, mGlut.Synth. = muscle glutamate synthetase. ANXA1 = annexin-1, CC10 = Clara cell 10 kDa protein.

* There are surprisingly few studies on the possible effect of GC in burns-related protein wasting. Burns injury is associated with two- to threefold increased levels of naturally occurring glucocorticoids (cortisone) in rats. But there is disagreement on the consequence for GC on burns victims. See studies by Fang et al. and Lang et al. (2007) discussed in this section.

mRNA increased 2-fold.* Heart and soleus muscle (slow twitch, Type I fibers) did not show changes in MURF1 and atrogin-1 gene expression. IGF-1 reversed the burns-induced changes in MURF1 and atrogin-1 expression. The effects of burns injury on expression of atrophy genes were not affected by GC antagonist RU-486,[230] in contradiction with previous reports of Fang et al. who found evidence that GC was involved in burns-induced proteolysis.[233]

GC have a catabolic effect linked also with increased expression of myostatin[231,232] that functions as a negative regulator of muscle stem cell proliferation and differentiation (Chapter 4). GC have also been found to depress the production of IGF-1 and to stimulate the synthesis and release of glutamine from muscle.[233,234] Recent evidence suggests that GC affects muscle wasting, but that the detailed mechanism may differ according to muscle type (fast twitch Type II > slow twitch Type I), with age of model animals (old > young); furthermore, some reports noted a different response between mice and rats that are the main models for studying GC action.[235,236]

GC inhibit protein synthesis in vivo partly via their effects on anabolic hormones and nutrients. The administration of dexamethasone led to increased insulin resistance and blunted muscle sensitivity to leucine and the other branched-chain amino acids.[237,238] The effects of GC are linked with inhibition of protein translation initiation moderated by the state of phosphorylation of 4EBP.[239,240] As discussed elsewhere, hormone and nutrient effects on translational initiation is regulated by Akt/mTOR/p70S6K. Dexamethasone treatment interferes with the phosphorylation of Akt and p70S6K while promoting the state of phosphorylation of GSK3-β.[241] However, absolute concentrations of Akt, p70S6K, or GSK-3β proteins did not change following dexamethasone treatment.[242] Akt/mTOR/p70S6K is a key pathway moderated by insulin/IGF-1 and branched-chain amino acids. The catabolic effects of GC can be combated by anabolic steroids and β-adrenergic receptor antagonists, for example, clenbuterol.†

GC produce anti-inflammatory activity by inducing the expression of a series of endogenous peptides.[243] GC bind to the cytoplasmic glucocorticoid receptor (GR), which results in the transfer of the activated receptor to the nucleus, which moderates the expression of a diverse range of genes including some of which are linked with gluconeogenesis, protein degradation, and fat metabolism. GC also inhibit the production of inflammatory mediators, for example, proinflammatory cytokines, prostaglandins, leukotrienes, platelet activating factor (PAF) and ROS. GC treatment decreases migration of leukocytes to sites of inflammation, decreases cell expression of adhesion molecules, and lowers the attachment of inflammatory cells to endothelia. A noted disadvantage of GC is their ability to reduce the immune response due to their tendency to stimulate leukocyte apoptosis. GC also produce muscle wasting.[244]

6.3.6.1 Lipocortin or Annexin-1

The 37 kDa protein annexin-1 (ANXA1) is produced by diverse tissues but is not found in significant amounts in the plasma. Expression of ANXA1 increases following GC administration. The structure of ANXA1 consists of 375 AA consisting of a conserved C-terminal joined to the core region consisting of 4 × 70 amino

* Note that MURF1 and atrogin-1 expression is mediated by the FOX transcription factor (Chapter 4).
† Clenbuterol is a controlled substance.

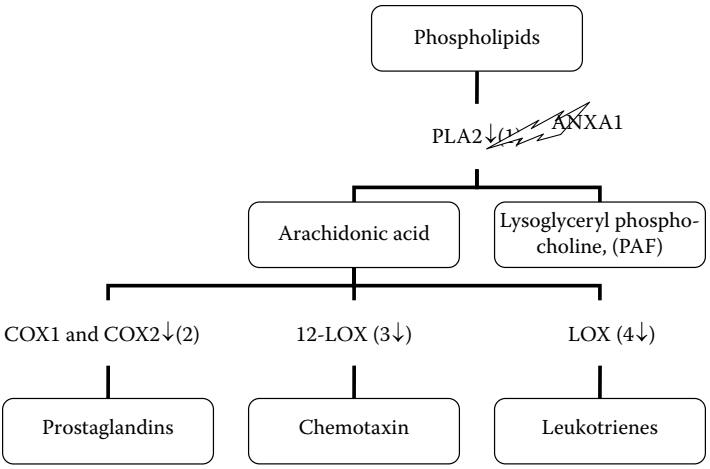

FIGURE 6.7 Transformation of phospholipids to inflammatory mediators including prostaglandins, chemotaxin, and leukotrienes. Arrow (↓) show enzymatic targets for anti-inflammatory agents. Inhibition of phospholipase A2 (PLA2) by glucocorticoid-inducible peptides (annexin1/lipocortin-1 and Clara cell 10 peptide (CC10) is major route for moderating inflammation; COX-1 and 2 are targets for non-steroidal anti-inflammatory drug (NSAID). Lipoxygenase (LOX) may also be inhibited.

acid repeats. The N-terminal sequence, which is the site of the anti-inflammatory activity, contains various amino acids that are subject to posttranslational modification by phosphorylation, glycosylation, and acetylation.[245] ANXA1 binds to lipid membrane in a Ca^{2+}-dependent fashion.[246,247] The inhibition of PLA2 by ANXA1 is also an efficient mechanism for reducing the formation of inflammatory mediators (Figure 6.7).* The bioactivity of ANXA1 resides in the (Ac 2–26) N-terminus which retains the anti-inflammatory activity of the parent protein. GC have been shown to moderate inflammatory cells (neutrophils, leukocytes, etc.), probably by increasing the expression of ANXA1/LC-1. In vitro studies also show that ANXA1 inhibits eicosanoid production, and induces PMN cell apoptosis, as well decreasing the synthesis of cellular adhesion proteins.[248–251]

6.3.6.2 Uteroglobin or Clara Cell 10 Protein

Uteroglobin or Clara cell 10 protein (CC10) is an anti-inflammatory protein thought to be stimulated by GC administration.[252,253] The 10 kDa protein is produced by endothelial cells and secreted into body fluids.† Levels are highest in seminal fluid and bronchial fluid. The structure of CC10 consists of homodimers. Each subunit

* The anti-inflammatory action of GC is mediated by two groups of endogenous peptides: annexin 1A (ANXA1)/lipocortin-1 and an uteroglobin (see Section 6.3.6.2). These peptides inhibit phospholipase A2 (PLA2), a key enzyme for releasing arachidonic acid from membrane phospholipids. Chemical mediators derived from arachidonic acid (prostaglandins, leukotrienes, and chemotaxins) orchestrate elements of inflammatory response, for example, vasodilatation, cell chemotaxis, etc.

† Clara cell 10 peptide is also called uteroglobin or secretoglobin 1A1. The size of Clara 10 protein appears to be 16 kDa by SDS PAGE.

TABLE 6.12
Anti-Inflammatory Activity of Annexin-1 and Clara Cell 10 Peptides

Inhibition of inflammation-related enzymes
- Phospholipase A2↓[a]
- COX1 and 2 expression↓
- Inducible nitric oxide synthetase expression (iNOS)↓
- Transglutaminase activity↓

Inflammatory cells migration, extravasations
- Leukocyte chemotaxis↓
- Leukocyte apoptosis↑
- Neutrophil adhesion↓
- LPS activation of macrophages↓

In vivo effects↓
- PMN trafficking and adherence↓
- Histamine release↓
- Cytokine formation↓
- Acute and chronic inflammation↓
- Pain↓

Hormone effects
- CRH/ GC secretion↓
- Insulin secretion↑
- TSH↓

Other
- Interferon-γ production↓
- MAPK phosphatase 1 expression↑

Sources: Parente, L. and Solito, E., *Inflammation Research*, 53, 125, 2004. Lim, L.H.K. and Pervaiz, S., *FASEB J*., 21, 968, 2007.

[a] Some PLA2 forms not affected.

comprising 70 amino acid contains a helical secondary structure and two disulfide bonds. CC10 has a central cavity for binding small hydrophobic ligands including retinol. The biological activity of CC10 is uncertain, but the protein has been shown to have many of the anti-inflammatory activities described for ANXA1.[254] The anti-inflammatory activity of ANXA1 and CC10 is summarized in Table 6.12.

6.3.6.3 Antiflammins

The antiflammins (AFs) refers to several small peptides (~9 amino acids) produced by proteolytic digestion of ANXA1 and CC10. The AFs possess the anti-inflammatory properties of the parent proteins including limited ability to inhibit PLA2, reduction of inflammatory cell chemotaxis, inhibition of macrophage activation, prevention of neutrophil adhesion to endothelial cells, etc. The structures of the nine representative antiflammins are shown in Table 6.13.

6.3.7 Map Kinase Inhibitors

The MAP kinases (in particular p38) are involved in the inflammatory response to LPS and a wide range of stressors. In addition, the NFκβ pathway often works in concert with other transcription factors (e.g., AP-1) that is controlled by MAP

TABLE 6.13
Sequences for Some Antiflammins Peptides

Name	Amino Acid Sequence	Source
AF1	MQMKKVLDS MetGlnMetLysLysValLeuAspSer	Rabbit UG
AF2	HDMNKVLDL HisAspMetAsnLysValLeuAspLeu	Annexin 1
AF2a	HDANKVLDL HisAspAlaAsnLysValLeuAspLeu	Annexin 1
AF2n	HD(nL)NKVLDL HisAsp(nLeu)AsnLysValLeuAspLeu	Annexin 1
AF2ns	HD(nL)NKVLDS HisAsp(nLeu)AsnLysValLeuAspSer	Annexin 1
AF3	SHLRKVFDK SerHisLeuArgLysValPheAspLys	Annexin V
Af4	LRKVFDK LeuArgLysValPheAspLys	Annexin V
AF5	AQLKKLVDT AlaGlnLeuLysLysLeuValAspThr	Human UG

Source: Adapted from Mukherjee, A.B. et al., *Endocr. Rev.*, 28, 707, 2007.
Abbreviations: Af, antiflammins; nL, norleucine.

kinase signaling. Not surprisingly, inhibitors for MAP kinase have been found to exhibit anti-inflammatory action. p38 is implicated in the development of rheumatoid arthritis,[255] COPD,[256] and IBD.[257] Inhibitors for p38 MAP kinase have shown promise in prevention of inflammatory conditions, though the current batch of compounds are largely nonspecific and show problems of toxicity.[258] Interestingly, the MAP kinase system has been considered a therapeutic target using dietary phytochemicals. So far, few MAPK inhibitors based on peptides have been developed. In principle, these are possible based on peptide substrate specificity; for example, phosphorylation of tyrosine or threonine residues two residues away from proline.

6.3.8 Melanocortin Peptides and KPV*

The melanocortin peptides (α-, β-, δ-, and γ-MSH and ACTH) are thought to have anti-inflammatory and antipyretic activity in addition to effects on sexual behavior, memory retention, and food intake.[259–264,†] The anti-inflammatory activity is partly attributable to a core tetra peptide sequence (HisPheArgTrp), corresponding to MSH residues 6–9 (MSH6–9) as well as the C-terminal tripeptide (Lys-Pro-Val or KVP) sequence corresponding to α-MSH residues 11–14

* KPV is a tripeptide Lys. Pro. Val.
† See Chapter 9, Section 9.3 for more information on the melanocortin.

TABLE 6.14
Amino Acid Sequence of Anti-Inflammatory Melanocortin Peptides

Peptide	Sequence
ACTH	SYSMEHFRWGKPVKKRRPVKVYPNGAEDESAEAFPLEF0
	SerTyrSer[MetGluHisPheArgTrpGly]LysProValLysLysArgArgProValLysValTyrPr oAsnGlyAlaGluAspGluSerAlaGluAla PheProLeuGluPhe (38)
ACTH4–10	MEHFRWG
	MetGluHisPheArgTrpGly (7)
α-MSH 6–9	HFRW
	HisPheArgTrp (4)
α-MSH	Ac-SYSMEHFRWGKPV
	Ac-SerTyrSerMetGluHisPheArgTrpGlyLysProVal (13)
β-MSH	AEKKDEGPYRMEHFRWGSPPKD
	AlaGluLysLysAspGluGlyProTyrArgMetGluHisPheArgTrpGlySerProProLysAsp (21)
α-MSH 11–13	KPV
	LysProVal (3)
Melanotan I	Ac-SYSnLEHFRWGKPV
	Ac-SerTyrSernLeuGluHisPheArgTrpGlyLysProVal
Melanotan II	Ac-nL-c[XH.dFRWK]-NH2
	Ac-nLeu[Xaa.dHisPheArgTrpLys]-NH2

Abbreviations: nL, norleucine; dF, D-phenylalanine.

(MSH11–14). The anti-inflammatory effects of ACTH (4–10), MSH(6–9), and MSH (11–14) are due to ligand binding to melanocortin receptor subtype -1 and/or -3 (MCR1 and MCR3) expressed by inflammatory cells, though the relative importance of these elements continues to be debated.[265,266] Since MCR1 is associated with pigment formation in melanocytes, there is likely to be some overlap between the skin tanning and anti-inflammatory effects of α-MSH (Chapter 9). Table 6.14 lists the amino acid sequences of melanocortin peptides.

Lipton et al. showed that α-MSH and the C-terminal tripeptide KVP had anti-inflammatory activity toward chemically induced contact dermatitis, LPS-induced sepsis, adult respiratory distress syndrome, and arthritis.[267,268] Interestingly, plasma levels of MSH were found to be elevated in the various inflammatory conditions including HIV/AIDS,[269,270] sepsis,[271,272] and congestive heart failure[273] and in hemodialysis patients. As noted previously, increases in endogenous peptides (here plasma MSH) during the stress response may be a sign of an adaptive response to inflammation.[274] Tests in various animal models for inflammation showed that injection of α-MSH in the CNS or peripheral tissues produced a marked decline in the level of inflammation.

The anti-inflammatory action of α-MSH is normally explained in terms of binding with MCR1/MCR3 in the CNS followed by a localized anti-inflammatory response (in the brain) or transmission of nerve signals via the adrenergic system to the periphery (Table 6.15). In support of this idea, it has been shown that ICV injection of α-MSH leads to anti-inflammatory effects in peripheral tissue, provided

TABLE 6.15
Anti-Inflammatory Action of α-MSH Peptides

Inflammatory Status	Causes
Acute inflammation	Induced by histamine, carrageenan, picryl chloride, inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α), LPS administration
Chronic inflammation	Arthritic conditions
	Inflammatory bowel diseases
	Myocardial infarction
Systemic inflammation[a]	Sepsis
	Endotoxemia
	Experimental respiratory distress syndrome

Peripheral actions of MSH peptides

- Cytokines production↓
- Nitric oxide synthesis↓
- Prostaglandin synthesis↓
- IL-10 synthesis
- Neutrophil migration↓
- Macrophage activation↓

Mechanism of α-MSH anti-inflammation

1. Inhibits cytokine production by peripheral host cells
2. Inhibits MRCs in the CNS
3. Inhibits CNS inflammation by local action of the peptides
4. Antimicrobial activity
5. Inhibits cytokine receptor binding

[a] Typically induced by LPS injection.

the spinal cord remains intact. Second, the α-MSH receptor (MCR1) is found in melanocyte (pigment) cells as well as various inflammatory cells, for example, macrophages, neutrophils, T cells, and dendritic cells. In addition, α-MSH administered by IV injection can produce an anti-inflammatory action directly on peripheral tissue. Third, α-MSH activates cells transfected with the MCR1/3 gene leading to a rise in intracellular c-AMP, activation of protein kinase A, and inhibition of NFκβ transfer from the cell cytoplasm to the nucleus.[275,276] The anti-inflammatory effect of KPV was also ascribed to competitive inhibition of the cytokine IL-1β receptor. Research by Getting et al. indicates that MCR-3 is important for the anti-inflammatory activity of γ-MSH, ACTH (10–39), and selective MSH antagonists such as melanotan II (MTII). It was recently suggested also that KPV produces anti-inflammatory activity in mice that lack a functional MCR1. Macrophages pretreated with MCR3/4 inhibitor SHU911 were also sensitive to the effects of KPV. These observations tend to emphasize the role of KPV as inhibitor for IL-1β activity. Currently, the general consensus appears to be that MCR1 and MCR3 are targets for melanocortin anti-inflammatory activity.

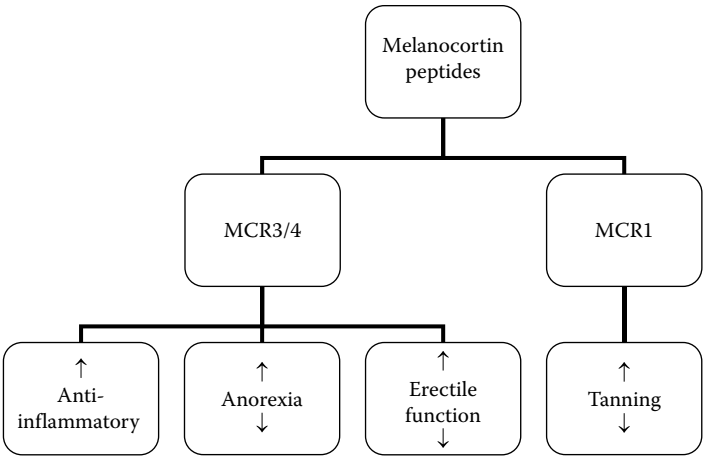

FIGURE 6.8 A summary of bioactivity associated with melanocortin peptides and the receptor subtype MRC-13. Receptor agonists (↑) such as α-MSH, MTII show anti-inflammatory, anorexic, tanning, and erectile functioning. MCR antagonists (↓), for example, AgRP, SHU119 are anti-anorexic and anti-tanning. See text for details.

Based on the preceding discussion, it may be envisaged that melanocortin peptides could be useful for controlling ORIM via their inhibitory effect on food intake. As discussed above, compounds that function as MCR4 agonists (e.g., α-MSH, KVP, and MSH4–6) produce anorexic activity. On the other hand, activity of MCR3 is linked with anti-inflammatory activity. The melanocortin peptides that function as MRC4 antagonists (e.g., SHU119 and AgRP) have appetite-promoting behavior. In the case of anorexic-inflammatory patients, the "win-win" situation would be to identify melanocortin peptides that have anti-inflammatory activity but also lack the anorectic effect[277] (Figure 6.8).

6.3.9 Glutamine and Glutamine Dipeptide

Glutamine is considered a good example of so-called immunonutrients with the ability to moderate both the innate and adaptive immune systems. Glutamine is currently used as an immunonutrient for enteral and parenteral nutrition support of critically ill patients.[278–280] Though normally the most abundant amino acid within the body, glutamine may be conditionally essential during times of illness.

A range of bioactive functions have been proposed for glutamine and glutamine peptide (see Chapter 3 and Table 6.16) supported by in vitro studies and experiments using small animals. Glutamine stimulates the

TABLE 6.16
Immunomodulating Action of Glutamine

Action on cell proliferation
Intestinal epithelial cells↑[293]
Microphage function↑[294]
Leukocytes↑
T cell activation↑[295]
Improved gut mucosal health and barrier function
Bacterial translocation↓[296]
Experimental enterocolitis↓[297]
Enhancement or tissue glutathione supply
Liver glutathione↑[298]
Gut glutathione↑[299]
Plasma glutathione↑[300]

proliferation of immune cells, improves mucosal gut barrier function, and enhances tissue GSH supply. Glutamine may enhance the proliferation of immune cells of T lymphocyte group, for example, cytotoxic T cells and natural killer cells.[281–283] In addition, glutamine exerts anti-inflammatory activity via its role as substrate for inflammatory cells[284,285] and/or cells of the gut mucosa. Improvements in gut barrier function due to glutamine supplementation may reduce uptake of endotoxin from the GI tract resulting in reduced SIR.[286] Recent animal studies also suggest that glutamine can reduce LPS-induced sepsis and endotoxemia[287–289] as well as intestinal inflammation.[290–292]

One of the most compelling explanations for the anti-inflammatory effect of glutamine is the ability to downregulate NFκβ activation.[301–303] Glutamate has also been shown to reduce TNF-α and proinflammatory cytokine release by intestinal cells exposed to LPS in vitro.[304] Observations by Zhang et al. suggest glutamine supplementation moderates cell antioxidant status leading to changes in anti-inflammatory status. Cultured alveolar epithelia exhibit increased activation of NFκβ, increased TNF-α production, and depletion of intracellular GSH levels after exposure to LPS. However, pretreating epithelial cells with glutamine led to increases in their intracellular GSH concentration and a reduction in the proinflammatory response to LPS. The anti-inflammatory effect of glutamine could be abolished by pretreating cultured cells with BSO, which is an inhibitor for GSH synthetase.[305] Observations from human studies also suggest that glutamine supplementation can restore tissue GSH levels, which are severely depressed following surgery.[306] Glutamine inhibits mTOR signaling leading to increases in cell numbers at the expense of increasing size.[307] Potential applications of glutamine and glutamine dipeptide are discussed in Section 6.4.

Alanine-glutamine dipeptide (L-AlaGln) introduced by Furst and colleagues in 1984 may well be the first purposefully designed synthetic peptide for nutritional support. The dipeptide originally produced by reverse hydrolysis using immobilized plant proteases was manufactured to correct apparent deficiencies of glutamine as a nutrient, for example, poor water solubility and instability toward heat sterilization. The water-solubility of L-alanyl-L-glutamine (AG) is markedly higher than that of glutamine (586 g/L vs. 35 g/L), enabling a more efficient administration in a concentrated form. AG (as with other peptides) is more efficiently absorbed compared to free amino acids. AG has undergone extensive animal trials as well as human clinical trials that have shown it to be safe and well tolerated.[308,309]

6.3.10 Food Proteins and Supplements

Food-derived peptides and protein supplements have not been subjected to extensive testing for their anti-inflammatory activity. Prgomet et al. showed that Lf and lactoferricin increased the expression of proinflammatory cytokines (IL-1B, IL-6) and anti-inflammatory cytokine (IL-10) by LPS-treated bovine blood cells. There was no change in the expression of NFκβ.[310] Udenigwe et al. demonstrated that flaxseed bioactive peptide inhibits NO production by LPS-treated macrophages.[311] Dia et al. also using the LPS-treated Raw 264.4 macrophage model for inflammation found that soybean lunasin inhibits the production of NO, PG2, as well as the COX2 pathway.[312] In spite of the apparent simplicity of cell-based assays,

the basis for anti-inflammatory activity in this system remains uncertain. It may be that anti-inflammatory peptides function mainly as endotoxin antagonists (Section 6.3.3).

Moderation of cell adhesion proteins represents another important mechanism of anti-inflammatory action. Investigations using cultured CaCo-2 cells suggest probiotic lactic acid bacteria produce anti-inflammatory activity reducing the adhesion of pathogenic microorganisms to the intestinal lining. Botes et al. reported that conventional anti-inflammatory drugs inhibited the adhesion of probiotic bacteria to the CaCo-2 monolayer. On the other hand, protease-sensitive products from the probiotic strains (not the probiotic cells themselves) could inhibit the adherence of *Lysteria monocytogenes* to the CaCo-2 layer.[313] These interactions could alter CaCo-2 monolayer fundamentally leading to reduced recruitment and transmigration of neutrophiles.[314]

6.4 IN VIVO APPLICATIONS AND CONTROLLED TRIALS

6.4.1 Proof for Anti-Inflammatory Action

Preclinical investigations have shown that anti-inflammatory peptides could be useful for nutritional support (see previous section). In addition, some human RCT have been published that confirm that nutrient supplementation may improve outcomes including a reduced length of stay in hospital, decreased mortality and reduced post-treatment complications (Section 2.4.1). At the time of writing, the overwhelming impression from human RCT is that benefits of anti-inflammatory peptides demonstrated in simple systems have been overestimated. The controversy surrounding immunonutrients are briefly illustrated using glutamine as case study.[315,316] A check of the PubMed search engine and the Cochrane database identified approximately 14 meta-analysis dealing with the health benefits of glutamine in critically ill patients.* The majority of studies (Table 6.17) suggest that glutamine supplementation had no real benefit. On the other hand, there have been some memorable single human RCT that have shown that glutamine supplementation can result in improved outcomes. For example, the well-known study by Griffiths showed that glutamine PN reduced mortality and cost of treatment of ICU patients.[317]

The disparity between preclinical studies (highly favorable) and clinical trial data (weak or absent benefits) is puzzling leading to quite polarized views of glutamine supplementation; the issues are nicely illustrated by two reviews.[327,328] A possible explanation for the prevailing results is that the human RCT may be measuring endpoints that are far removed from the inflammatory response. Another interesting feature is that a more positive picture emerges of the efficacy of glutamine supplementation if one considers alanyl-glutamine (AlaGln) dipeptide. A meta-analysis of 10 human RCT of by Jiang et al. suggests that AlaGln supplementation may reduce the length of hospital stay by 3.5 days and also the risk of postoperative infections by >50% in surgical patients.[329,330]

* Search date, June 2009, over 60 human clinical trials are listed in PUBMED dealing with the health effects of glutamine.

TABLE 6.17
Meta-Analysis and Systematic Reviews of Glutamate Use

Meta-Analyses	Trials (*n*)[a]	Endpoints	Conclusion
2001—Critical illness	22 (2419)	BC, SM	NB[318,]
2005—Preterm infants	6 (2300)	NC, LOS, BC, ND	NB[319]
2006—Critical illness		LOS, SM	NB
2006—Burns care	?	?	PB[320]
2007—Infant GI disease	2 (100)	SM, BC	NB[321]
2007—Crohn's disease	1 (?)	Remission	NB[322]
2008—Acute pancreatitis	3 (?)	BC, SM, LOS	NB[323]
2008—Critical illness	12 (3013)	SM, LOS, BC	NB[324]
2008—Preterm infants	7 (2365)	NC, LOS, BC, ND	NB[325]
2009—Marrow transplant	21 (>300)	BC, LOS, SM	PB[326]

[a] Number of trials examined (number of patients in parenthesis).

Abbreviations: NB, no benefit; PB, possible benefit. *Endpoints*: BC, blood culture or invasive infections; LOS, length of hospital stay; SM, survival and mortality; NcE, necrotizing encolitis; ND, neural development.

6.4.2 Inflammatory Bowel Disease, Colitis, and Mucosal Injury

In vivo experiments using models for animal inflammatory bowel disease indicate that glutamine supplementation improves outcomes. The benefits were ascribed to ability of glutamine to down regulate NFκβ expression.[302,303] Administration of glutamine was found to reduce the severity of IBD induced using 2, 4, 6 tri-nitrobenzoic acid (TNBS)[331] or DSS.[332] Despite the results from animal model studies, results with human trials reported so far have been disappointing.

A variety of melanocortin peptides have been evaluated in vivo for applications in IBD. Recent investigations by Konnengiesser et al. showed that KPV (10 μg/day by oral administration) could improve recovery in two models of IBD. Mice treated with dextran sulfate (2.5% in drinking water) developed IBD with ~30% loss of body weight, inflamed gut and crypt damage. Orally administered KPV improved the rate of recovery from IBD as indicated by more rapid weight gain and reduced gut inflammation in treated rodents. The potential for applying melanocortin peptides in the treatment of IBD is corroborated by studies using rat IBD induced using TNBS. A single administration of α-MSH intraperitoneally 60 min before exposure to TBNS reduced the extent of colonic damage in rats.[333] Further investigations suggest that IBD is more serious in MCR1 gene knockout mice. Mice lacking an effective MCR1 showed reduced sensitivity to the effects of KPV though these mice showed reduced lethality due IBD. KPV treatment was also effective in cell-induced IBD. Injecting mice with a specialized cell strain (CD4-CD45Rbhi) led to IBD with associated weight loss. The administration of KPV (0.1 μg; alternative days) could ameliorate the weight loss.[334] Dalmasso et al. demonstrated that orally administered KPV could alleviate the symptoms of IBD induced by DSS and TNBS, including

weight loss and histological signs of tissue damage. Interestingly, the effects of KPV were interpreted in terms not requiring MCR1 function. Transport studies using cultured CaCo2 cells suggest that orally administered KPV was transported by intestinal cells followed by the inhibition of NFκβ and MAPK signaling.[335]

An alternative approach for treating IBD extends previous work in the area of probiotics. Yoon et al. showed that lactic acid probiotic bacteria, modified to express a-MSH reduce the symptoms of IBD. Mice given live bacteria orally showed increased weight gain, decreased colitis score, and improved survival rate following IBD induced using DSS. Levels of anti-inflammatory cytokine (IL-4 and IL-10) were significantly increased from the gut tissue (+2 μg/μL CON A).[336] We have not identified human trials of the potential effectiveness of α-MSH for IBD.

Anti-secretory factor protein (ASFP) is a 41 kDa peptide discovered in 1996. ASFP is produced from the pituitary gland and appears to inhibit fluid release from the intestines in response to cholera toxin.[337,338] The peptide has also been demonstrated to have anti-inflammatory activity in experimental models of IBD.[339] Rodents injected with ASFP were protected from the effects of IBD. The mechanism of anti-secretory factor protein action is uncertain. However, bioactivity of ASFP resides in the short peptide sequence (residue 65–42; (I)VCHSKTR; IleValCysHisSerLysThr) encrypted within the full protein.[340] ASFP from chicken egg yolk has been shown to provide protection from IBD when administered orally. Indeed human clinical trials suggest egg-yolk containing AF may be beneficial for IBD.[341] A proprietary medical food has also been developed that contains ASFP for treatment of childhood diarrhea.[342]

Cortistatin-14 (CST-14) is a small cyclic peptide isolated from the CNS in 1996 that shows strong homology with the cyclic somatostatin (SST). Both peptides inhibit growth hormone release but there appears to be differences in the site of production. Whereas SST is produced in the CNS, CST-14 is produced in the CNS and also by cell of the immune system (lymphocytes, monocytes, macrophages, and dendritic cells). It is thought that both SST and CST-14 bind to the 5-SST receptors discovered so far. In addition, CST-14 has also been shown to act as ghrelin antagonist owing to ability to bind to GHS-R.[343–346] Recent investigations mainly by Gonzalez-Rey et al. suggest that CST-14 has anti-inflammatory activity linked with an ability to inhibit the release of proinflammatory mediators (TNF-α, IL-6, NO, MMP) from cultured cells and to induce the synthesis of IL-10.[343] Further investigations showed that CSN-14 offers significant protection in experimental colitis. Administration of CNT-14 at ~12 h before TNBS (6% in 50% ethanol) resulted in significantly lower weight loss, lower bowel inflammation and lower mortality compared to untreated controls. The benefits of CST-14 treatment were comparable to other common therapies for inflammation.[347] CST-14 treatment could induce weight gain in IBD mice despite the possibility that this agent may interfere with GH secretion or ghrelin bioactivity, for reviews see references[344–346].

Animal trials by Ushida et al. suggested that alpha-lactalbumin (a major whey protein from human milk) has anti-inflammatory activity toward gastrointestinal ulcerations induced with a mixture of 60% ethanol containing 0.1 M HCl. Compared to a distilled water control, a single oral administration of milk protein (500 mg/kg BW) reduced the degree of intestinal damage caused by acidified

ethanol. Several indices for gut inflammation (e.g., GI levels of pepsinogen, IL-1 β, mucosal myeloperoxidase, and mucosal TBARS) were found to be lower in the protein supplemented animals suggesting that milk alpha-lactalbumin could protect the gut from GI damage by ethanol.[348] Peptide anti-inflammatory activity has also been demonstrated using the dextran sodium sulfate (DSS)- induced colitis model for inflammation. Lee et al. reported that egg white and hen egg lysozyme inhibits DSS induced colitis in rodents and pigs.[349,350] The effects of protein supplementation were multifarious including a decreased expression of proinflammatory cytokines (TNF-α, IL-6, IL-1 beta, IFN-γ, IL-8, and IL-17), decreased intestinal permeability, and reduced intestinal damage.

Currently there are insufficient numbers of human trials of food derived dietary protein on inflammatory markers. Ozawa et al. reported that the levels of transforming growth factor-beta (TGFβ) found in pasteurized milk were sufficient to protect against inflammatory bowel disease in mice. TGFβ from milk was also shown to be bioavailable in humans though (clearly) this agent could act directly on cells in the GI tract.[351] Initial studies suggest also that soybean lunasin, previously demonstrated to have anti-inflammatory activity in vitro, may be bioavailable since this could be detected in the blood plasma of subjects who consumed soybean products.[352] So far, only one human trial has been reported looking at the effect of food protein consumption on inflammatory parameters.[353] As indicated above, though food derived ACEI have been shown to produce significant declines in SBP and DSP, no studies have appeared on the effect of these agents to match studies using pharmacological ACEI drugs.

6.4.3 Systemic Inflammatory Response and Sepsis

SIR can be induced by agents that increase gut permeability to bacterial endotoxin (see below). As a consequence, attempts have been made to moderate gut barrier function using a range of supplements as a means of reducing SIR. A double-blind, randomized, controlled trial by Wishmeyer et al. showed that IV administration of glutamine can produce anti-inflammatory response in burns victims (n = 31 patients; 25%–90% total burns surface area).[354,*] The treatment group showed reductions in the number positive blood cultures for gram-negative bacteria (8% vs. 43%), decreased antibiotic usage, reduced mortality (one vs. four patients), increased serum pre-albumin and transferrin (i.e., better nutritional status), and a decrease in the serum CRP levels. However, there were no differences in the length of hospital stay. The study is important, being one of the few to directly monitor the effect of glutamine supplementation on inflammatory indices. The anti-inflammatory action of glutamine was ascribed to improvements in gut barrier function though no direct measurements of intestinal permeability were made. Conjero et al. reported glutamine supplementation produced a significant reduction in the infections in patients suffering from SIR, though there were no significant changes in the gut permeability.[355] van den Berg et al. found that glutamine supplemented

* The treatment groups were given glutamine by IV infusion of 0.57 g/kg BW/day over 24 h. The control group received an isonitrogenous solution containing essential amino acids (amino 0.57 g/kg BW/day) but lacking glutamine. Both groups were also receiving standard enteral nutrition.

enteral diet (0.3 g/kg BW/day) had no significant effect on the rate of decrease of gut permeability observed for low birth weight infants fed a normal enteral diet.[356]

Animal trials by Rajora et al. showed that mice treated with LPS along with α-MSH (50 μg, ×2 daily injection) showed improved weight gain and reduced levels of blood in their feces compared to mice treated with LPS alone. Effects could be partly ascribed to inhibition of TNF-α production by gut tissue under the influence of concanavalin A. α-MSH treatment was also found to inhibit NO production by gut cells.[357] Chiao et al. reported that α-MSH protects against LPS-induced liver damage in mice. When female mice were treated with LPS or heat-inactivated *Corynebacterium parvum* followed by another bout of LPS with or without 50 μg a-MSH, the melanocortin treatment offered remarkable protection against liver damage. A-MSH treated mice showed decreased serum nitrate levels indicative of NO synthetase inhibition. α-MSH reduced neutrophile infiltration into the liver. These effects were interpreted in terms of α-MSH activity at the MRC1 expressed by hepatic macrophages.[358] Investigations in endotoxemic mice showed that CNS administration of a-MSH prevented or ameliorated several inflammatory changes and protected the liver and lungs from damage.[359] San et al. showed that α-MSH (20 μg/rat; IP-twice daily) provides protection from LPS-induced intestinal injury.[360] This investigation also suggested that the effects of α-MSH treatment reduced neutrophil infiltration inhibited NO production. Hopeful that these studies are, much more work is obviously warranted. Many of the studies are of short duration and so we cannot be sure that α-MSH treatment.

No human intervention studies have been conducted to examine the effect of α-MSH on sepsis. In vitro evidence suggests that melanocortin agonist may be useful in the management of sepsis. Catania et al. showed that administration of LPS to human subjects increased blood levels of α-MSH by two- to fourfolds in proportion with increasing levels of fever. Whole blood α-MSH was negatively correlated to levels of TNF-α.[272] A prospective study of 21 sepsis patients showed that their plasma concentrations for α-MSH was lower compared to healthy controls. Levels of α-MSH increased in the proportion of patients that survived during therapy but not in patients that later died. Addition of exogenous α-MSH to whole blood samples inhibited TNF-α production in response to LPS. Gatti et al. found that the α-MSH C-terminal tripeptide dimmer (CKPV2) inhibits TNF-α production in LPS-treated human peripheral blood mononuclear cells (PBMC). CKPV2 was comparable with other anti-inflammatory MSH peptides, for example, KPV and [Nle4-dPhe7]-alpha-MSH (NDP-alpha-MSH). CKPV was found to be effective in vivo when tested against rats injected with LPS.[361] The preceding studies suggest that α-MSH could be useful for sepsis therapy.[271] Several melanocortin-related peptides (e.g., melanotan II) have undergone Phase I trials related to their tanning affect in human subjects though these have yet to reach approved drug status.[362]

6.4.4 Respiratory Distress Syndrome, Lung Injury and Related

The antiflammins (Section 6.3.6) have been considered for therapy in lung inflammation. Normal changes in the concentrations of CC10 may be implicated in lung inflammation and allergic diseases.[254] Levels of CC10 are also downregulated in the lungs of premature infants, during cystic fibrosis,[363,364] pulmonary fibrosis,[365] and lung

injury due to viral infection.[366] A commercialized recombinant human CC10 (rhCC10) expressed in *Escherichia coli* cells has been developed by a pharmaceutical company.* Trials using rhCC10 has shown improvements in lung health.[367] Miller et al. showed that rhCC10 could reduce lung injury induced by aspiration of saline in young rabbits. The study showed that rhCC10 could be administered either via the intratracheal route or by IV injection, though the former was more effective judging from the concentrations of the peptide detected in the lung fluids and plasma. Interestingly, rhCC10 was more rapidly excreted via the urine following IV administration.[368,369] Shashikant et al. showed that rhCC10 addition to lung surfactant decreased lung inflammation associated with respiratory distress syndrome modeled in preterm lambs. Moderate doses of rhCC10 (1.5 mg/kg) were more effective in reducing levels of inflammatory mediators (IL-6, IL-8, TNF-α, and manganese peroxidase) in lung fluid compared to low or high doses (0.5 or 5.0 mg/kg). Lung compliance and ventilatory pressure were also decreased following treatment with rhCC10. The beneficial effects of CC10 treatment on lung health were ascribed to the anti-inflammatory actions as well as the ability to protect lung surfactant degradation by secreted phospholipase A2.[370] Angert et al. found that rhCC10 could reduce the inflammatory symptoms following the aspiration of particulate meconium in newborn piglets. Treatment with rhCC10 reduced TNF-α levels by 60%, but PLA2 activity was unchanged compared to non-treated controls.[367]

Levine et al. have reported the only human trial so far using rhCC10. The Phase I multicenter, placebo-controlled RCT to assess the safety of rhCC10 involved 22 preterm infants with BW between 700 or 1300 g. Intratracheal administration of rhCC10 (1.5 and 5 mg/kg) produced an anti-inflammatory effect in human preterm infants similar to results obtained for immature piglets. rhCC10 treatment decreased total lung neutrophil count, reduced neutrophil chemotaxis, and reduced IL-6 production. The time-course of rhCC10 clearance was also similar to results observed in piglets; bioactive peptide was wholly cleared from the bloodstream by 48 h. The trials revealed no adverse effects or major safety issues.[371] In summary, current research suggests that prospects of developing adjuvant therapy for lung inflammation conditions using CC10 seems favorable.

Kodama et al. found that ghrelin may be useful in the treatment of respiratory disease, helping to alleviate inflammatory cachexia.[372,†] According to the human study from Japan, cachexic patients (n = 7, 3 women; age 62–80 years) were treated with ghrelin (2 μg/kg; 20 mL over 30 min) for 3 weeks. Compared to pretreated patients, IV ghrelin administration led to improvements in food intake and increased net body-mass. There were improvements in biochemical indices of nutritional status (total plasma protein, albumin, and plasma transferrin). Measures of plasma inflammatory markers decreased but blood inflammatory cells (neutrophils, lymphocytes and WBC) were unchanged. Analysis of lung fluid showed decreased levels of proinflammatory cytokines (IL-8, TNF-α, myeloperoxidase) in all patients following ghrelin treatment but the number of bacteria in the lung fluid remained unaltered.[372] The beneficial effects of ghrelin treatment for inflammatory lung disease are comparable to its effects reported for other wasting conditions, for example, cancer, cachexia, and chronic renal failure,

* Claragen Inc., 387 Technology Drive, College Park, MD 20742, USA.

† See treatment of heart failure—by stimulating GH secretion (Chapter 9), ghrelin therapy for cancer cachexia—moderation of food intake (Chapter 9).

TABLE 6.18
Effect of Ghrelin Treatment on Patients with Chronic Respiratory Infection[a]

Index	Pretreatment	Posttreatment	Significance (*P*-Value)
Body mass			
Food intake (g)	7.1 ± 2.2	8.9 ± 1.9	<0.005
Body weight (kg)	39.4 ± 9.7	41.8 ± 9.9	<0.005
BMI	15.8 ± 2.6	16.8 ± 2.7	<0.005
Fat-free mass (kg)	32.4 ± 7/1	34.5 ± 11	ns
Fat mass (%)	13.2 ± 6	13.3 ± 6.3	ns
Nutritional status			
Serum total protein (g/dL)	6.9 ± 0.6	7.4 ± 0.8	<0.02
Albumin (d/dL)	3.7 ± 0.3	3.9 ± 0.4	<0.05
Transferrin	208 ± 36	238 ± 47	<0.01
RBP	1.67 + 0.64	1.96 + 0.81	<0.05
Total cholesterol (mg/dL)	164 + 20	173 + 27	ns
Blood inflammatory markers			ns
CRP (mg/dL)	1.67 ± 1.20	1.1.0	<0.01
WBC (μL)	6686 ± 1170	6386 ± 1139	ns
Neutrophils (μL)	4083 ± 1293	3758 ± 796	ns
Lymphocyte (μL)	1919 ± 518	1820 ± 616	ns
sICAM-1 (ng/mL)	521 ± 230	371 ± 287	<0.06
Hormonal changes			
GH (ng/mL)	0.8 + 0.5	2.0 + 1.5	ns
IGF (ng/mL)	103 + 55	117 + 63	<0.05
Leptin (ng/mL)	2.22 + 2.14	2.05 + 1/78	ns
Noradrenaline	721 + 380	487 + 197	<0.05

Source: Summarized from Kodama, T. et al., *Pulm. Pharmacol. Ther*., 21, 774, 2008.
Abbreviations: CRP, C-reactive protein; WBC, white blood cell count; sICAM-1, soluble intracellular adhesion molecule-Type-1; RBP, retinol-binding protein.

which focused on food intake rather than overt inflammatory indices (see Sections 9.6.1 through 9.6.3). Apparently, ghrelin may be considered a multifunctional bioactive peptide able to induce anti-inflammatory response via receptors in the appropriate frontline cells whereas effects in the CNS lead to changes in food intake. Table 6.18 shows the effect of ghrelin treatment on patients with chronic respiratory infection.

6.4.5 Rheumatoid Arthritis

Lipocortin-1 (ANXA1) has been shown to have anti-inflammatory effects in experimental models of arthritis. Joint inflammation was induced using intra-articular injections of carrageenan or methylated bovine serum albumin. Dexamethasone could

alleviate inflammation but *not* when animals were pretreated with monoclonal antibodies specific for lipocortin-1. In addition, gene knockout mice unable to produce lipocortin-developed more severe arthritic symptoms following the administration of joint irritants. The administration of exogenous lipocortin by IP injection or direct intra-articular injection was also demonstrated to alleviate joint inflammation.[373–375] In all, it could be concluded that ANXA1 may show real promise in the treatment of arthritis. Other peptides have also been shown to have the potential for alleviating arthritis including fibronectin fragments,[376] vasoactive intestinal peptide (VIP), and conventional ACEI,[377] though much more work is needed in this area.

6.5 SUMMARY AND CONCLUSIONS

Inflammation is considered a major contributory factor to muscle wasting. The inflammatory response is a key element of the host responses to injury and chronic illness. Elevated proinflammatory cytokines are thought to have a direct effect on tissue protein losses associated with wasting. This chapter provides a background to inflammation. Several promising groups of bioactive peptides that have shown some efficacy for the treatment of inflammatory conditions were discussed, including AlaGln dipeptide, melanocortin peptide (HisPheArgTrp), and the endotoxin antagonists. The role of ghrelin, hexarelin, and other growth hormone secretagogues as anti-inflammatory agents is exciting, but more work is needed to fully evaluate these agents.[215,378] Further areas for research were identified, particularly the potential use of food-derived ACEI to combat inflammation-related conditions remains relatively underexplored. Finally, space restrictions led to the exclusion of several other promising anti-inflammatory peptides. Studies using the rodent TNBS model for IBD suggest that intestinal glucagon-like peptide 2 (GLP2) may possess anti-inflammatory activity via its ability to stimulate gut cell proliferation and because it inhibits intestinal production of inflammatory cytokines.[379,380] Trefoil (5–6 kDa) peptide produced by the goblet cells of the GI tract has been found to offer protection against TNBS induced IBD via a process involving the inhibition of TNF-α, TLR4, and NFκβ production and activity. Trefoil peptide treatment led to improvements in body weight.[381]

APPENDIX 6.A.1
Inflammatory Biomarkers Linked with Type 2 Diabetes

APP	α-1 acid glycoprotein, CRP, serum amyloid A protein, heptaglobin, fibrinogen
Circulating cytokines	IL-6, IL-10, IL-18, TNF–α, soluble TNF–α receptors 1 and 2, MIF, MCP-1, RANTES
Indicators of immune cell activation	sICAM-1, VICAM-1, E-selectin, P-selecting, soluble CD40 ligand
Immune cell	Increased leukocyte count

Source: Adapted from Kolb, H. and Mandrup-Poulsen, T., *Diabetologia*, 48, 1038, 2005.

Abbreviations: E-selectin, endothelial selectin; ICAM-1, intracellular adhesion molecule-1, VCAM-1, vascular adhesion molecules; iNOS, inducible nitric oxide synthetase.

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7 Infection and Sepsis

7.1 INTRODUCTION

7.1.1 Bacterial Infections, Sepsis, and Weight Loss

Infection is a well-known cause for weight loss. The presence of microbes in the normally sterile tissues leads to sepsis, that is, an inflammatory response induced by circulating bacteria or products of bacterial lyses.[1] Septicemia refers to the occurrence of viable bacteria in the bloodstream. The potential for applying bioactive antimicrobial peptides (AMPs) for nutrition and health is discussed in Chapter 7. Section 7.1 considers the background to sepsis and its relation to weight loss. Host cell receptors for detecting invading pathogenic bacteria are discussed in Section 7.2. The major groupings of host AMPs are described in Section 7.3. The functions of AMPs are described in Section 7.4. Current in vivo studies and human clinical trial data for AMPs are surveyed in Section 7.5.

Severe sepsis may be accompanied by septic shock, uncontrolled blood clotting, and multiple organ failure followed by death in 20%–30% of cases. The characteristic features of sepsis are summarized in Table 7.1. The systemic inflammatory response (SIR) associated with sepsis is linked with rising levels of proinflammatory cytokines (TNF-α, INF-γ, IL-2, IL-6, and IL-8) followed by compensatory anti-inflammatory response (CAR) that leads to falling levels of inflammatory mediators.[2] Current treatments for sepsis include using conventional antibiotics, fluid loading, and vasoactive drugs to correct hypotension.[3] It is noteworthy that conventional antibiotics have limited ability to neutralize bacterial endotoxins from the circulation. Endotoxemia and sepsis lead to unwanted weight loss and therefore we expect AMPs and antiseptic protein supplements could be useful for nutritional support to help maintain weight gain.*

The symptoms of sepsis are due to physiological derangements including defects in blood coagulation, cellular dysfunction exemplified by lymphocyte apoptosis, neutrophil hyperactivity, and endothelial dysfunction. There is also a loss of glycemic control due to increasing insulin resistance.[4] According to current models for sepsis, microbe-derived endotoxins stimulate tissue macrophages to secrete proinflammatory cytokines that lead directly to multiple organ failure at some distance from the site of infection. Aside from microbial infection, SIR also arises due to

* The term antimicrobial refers to compounds that kill or prevent the growth of microorganisms including bacteria, viruses, and fungi. Antiseptics (from Greek αντί - anti, "against" + σηπτικός - septikos, "putrefactive") are antimicrobial substances that are applied to living tissue/skin to reduce the possibility of infection, sepsis, or putrefaction—Wikipedia.com; we use antisepsis in the more restricted sense of agents that not only kill bacteria but also combat the inflammatory consequences of infection. In principle, some antiseptic peptides could be lacking antimicrobial properties. Accordingly, not all AMPs have antiseptic properties.

TABLE 7.1
The Characteristic Features of Sepsis and Bacteremia

Term	Standard Definition
Infection	Invasion of normally sterile host tissue by microorganisms leading to inflammatory response
Bacteremia	Presence of viable microorganisms in the blood
SIR	Systemic inflammatory response with body temperature >38°C or <36°C, heart rate >90 beats/min, breathing rate >20 per min, white blood cell count (>12,000 mm^3 < 4,000 mm^3)
Sepsis	SIR due to infection
Severe sepsis	Sepsis combined with organ dysfunction, reduced blood flow (hypoperfusion) and abnormally low blood pressure (hypotension)
Septic shock	Sepsis induced hypotension

Source: Bone, R.C. et al., *Chest*, 101, 1644, 1992. Bone, R.C., Grodzin, C.J., and Balk, R.A., *Chest*, 112, 235, 1997.

burns injury, trauma, and pancreatitis. Noninfectious causes of inflammation are described in Chapter 6.

Chronic sepsis is linked with muscle loss partly attributed to anorexia linked with rising serum leptin and IL-1.[5–12] Sepsis is also associated with the expression of mRNA for the ubiquitin proteasome subunits.[13–15] In animal studies, the injection of bacterial LPS was found to stimulate atrogin-1 and MuRF gene expression in a dose- and time-dependent manner within the gastrocnemius muscles but not the heart muscle. The changes of atrogin-1 gene expression could be prevented by IGF-1 but this harmone had no effect on sepsis-induced MuRF1 expression. Atrogin-1 and MuRF1 expression was not sensitive to leucine treatment during sepsis. Exogenous TNF-α increased atrogin-1 and MuRF1 in gastrocnemius, but pretreatment of septic rats with TNF-binding protein inhibitors did not prevent increased expression of atrogin-1 and MuRF1 mRNA. Exogenous dexamethasone increased atrogin-1 and MuRF1 expression, but the glucocorticoid receptor antagonist RU-486 failed to decrease the sepsis-induced increase in atrogin-1 and MuRF1. To summarize, sepsis induces an increase in muscle atrogin-1 and MuRF1 mRNA, which is both glucocorticoid- and TNF-independent and is unresponsive to leucine. The characteristics of muscle breakdown during sepsis have been reviewed.[16–18] Proteasome inhibitors have been shown to interfere with the loss of lean body mass during mild sepsis (Figure 7.1).[19]

7.1.2 Host Antimicrobial Peptides for Innate Defense

AMPs are important for the host innate immune response to infection.[20–24,*] Cationic host defense peptides occur in virtually all groups of organisms examined.[25] AMPs produced by mucosal cells defend the various surfaces within the oral cavity, alimentary canal, respiratory system, urinogenital tract, and the outer skin surface. Most of

* The acute phase response to infection includes inflammation (Chapter 6).

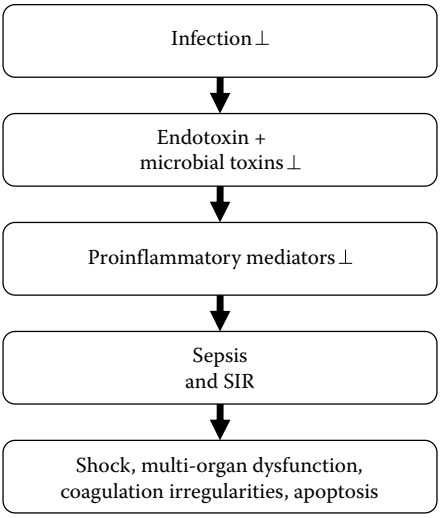

FIGURE 7.1 A paradigm for sepsis showing possible sites for intervention (⊥). (Adapted from Bone, R.C. et al., *Chest*, 112, 235, 1997.)

the host AMPs studied so far are small peptides (<100 amino acids), bearing a strong overall net positive charge. However, some larger proteins, for example, lysozyme and lactoferrin, also demonstrate antibacterial action as intact molecules or after partial digestion to produce bioactive fragments.[26,27,*] The AMPs, show broad spectrum activity against both gram-positive and gram-negative bacteria. Unlike conventional antibiotics, it appears that AMPs do not elicit antibiotic resistance. A recent PubMed/Medline search using key words "AMPs" yields over 9000 records plus 300 reviews. Approximately 900 papers and 50 reviews appeared within the past 5 years alone; the following publications provide a representative sample of the general research into AMPs over the past 10 years.[28–40]

7.1.3 Antibiotics and Muscle Mass

Livestock kept under hygienic conditions have been shown to have improved feed conversion compared to those kept under unhygienic conditions. Antibiotics enhance the growth performance of agricultural livestock because less diseased animals spend less of their body's resources fighting infection.[41,42] Klasing and Calvert reported that chickens injected with LPS increased the genesis of leukocytes and antibodies by 100% and 6%, respectively while lysine incorporation into the APP fraction increased from nil to ~33% of total daily consumption. The quantity of total daily lysine utilization diverted to the immune response rose from 1.17% to 6.71% of the total daily intake.[43] A further indirect effect of increased infection is the rise in cytokines. Symptoms such as thermogenesis and shivering are attributable to a rise in proinflammatory cytokines. Reduced food intake, anorexia, and changes in the

* The term "antimicrobial peptide" is used for small as well as large proteins employed in host defense without regard for their molecular weight.

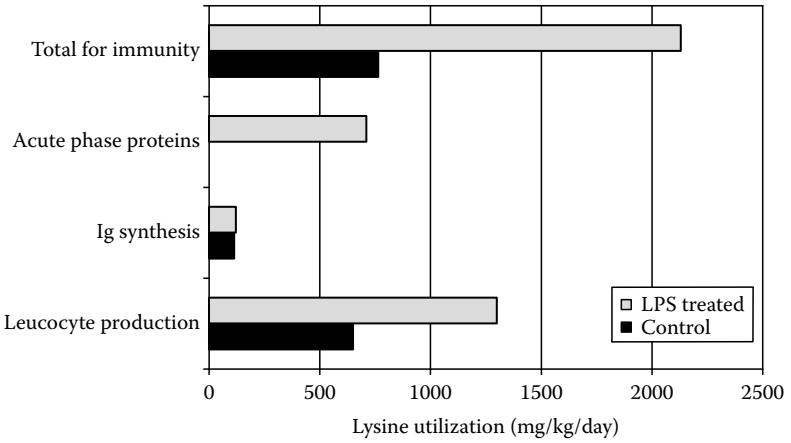

FIGURE 7.2 Utilization of lysine for maintenance of the immune system and during an immune response in chickens. (Drawn from data reported by Klasing, K.C. and Calvert, C.C., The care and feeding of the immune system: An analysis of lysine needs. In *Proceedings of the VIIIth International Symposium on Protein Metabolism and Nutrition*, Aberdeen, U.K., September 1–4, 1999. Wageningen Press, Wageningen, the Netherlands, pp. 253–264.)

nutrient absorption efficiency may all contribute to the growth deficiency allied to the response to disease (Figure 7.2).

7.2 PATHOGEN RECOGNITION AND INTRACELLULAR SIGNALING

7.2.1 Toll-Like Receptors

Cell sensors for detecting invading pathogens were not discovered until 1998. Host cell pattern recognition receptors (PRR) bind with specific elements (so-called pathogen-associated molecular patterns, PAMPS) found only on the invading microorganisms (Table 7.2). Pathogens approaching a host cell are detected by a toll-like receptor (TLR) mounted on the external cell membrane whereas intracellular pathogens and viruses are detected by the nucleotide-binding oligomerization domain (NOD) proteins. PRR binding to pathogens leads to increased expression of agents that attract host defense cells to the site of infection. The activation of host PRR also initiates the production of co-stimulatory peptides that help to stimulate the immune system. The characteristics of TLR and NOD and their function as PRR are briefly reviewed in the following section. Elements of the innate immune response to microbial pathogens are summarized in Table 7.3.

Buetler et al. discovered the TLRs by sequencing a mutated gene from the C3H/HEJ strain of mice that have increased resistance to diet-induced obesity. The C3H/HEJ phenotype was tracked down to a gene that codes for a protein possessing homology to the IL1 *receptor* and also bearing a high degree of resemblance to a protein from the fruit fly termed toll-protein. Mature fruit flies carrying the mutated toll gene show increased susceptibility to fungal infections. Subsequent research led to evidence that TLRs are the major receptors for host recognition of invading

TABLE 7.2
Host Pattern Recognition Receptors and Their Ligands

PRR	Ligand(s)	Adapter(s)
TLR1	Gram-positive: triacyl lipoproteins	MyD88 MAL
TLR2	Peptidoglycan from Gram-positive bacteria, lipoteichoic acids; diacyl-lipoproteins; fungi; viral glycoprotein	MyD88 MAL
TLR3	Viral double-stranded RNA poly I:C	TRIF
TLR4	Lipopolysaccharide (LPS) mainly from Gram-negative bacteria, viral glycoprotein	MyD88 MAL
	Fragments of hyaluronic acid (sHA); polysaccharide fragments of heparin sulfate; Fibrinogen; Heat-shock proteins (Hsp)60 and Hsp70; extra domain A of fibronectin (EDA)	TRIF TRAM
TLR5	Bacterial flagellin	MyD88
TLR6	Diacyl lipoproteins from Gram-positive bacteria	MyD88 MAL
TLR7	Small synthetic compounds; single-stranded viral RNA	MyD88
TLR8	Small synthetic compounds; single-stranded RNA	MyD88
TLR9	Unmethylated deoxycytidyl-deoxyguanosine dinucleotides (CpG) DNA	MyD88
TLR10	Unknown	Unknown
TLR11	Profilin	MyD88
TLR12	Unknown	Unknown
TLR13	Unknown	Unknown
NOD1	D-Glucose-meso-diaminopimelate; Gram-negative peptidoglycan motif	
NOD2	Muramyl dipeptide from peptidoglycan	

Source: Compiled from Brightbil, H.D. et al., *Science*, 285, 732, 1999; Poltorak, A. et al., *Science*, 282, 2085, 1998; Yang, R.B. et al., *Nature*, 395, 284, 1998; Anon, Toll-like receptors. An interview with Dr Bruce Buetler. http://esi-topics.com/tlr/interviews/BruceBeutler.html, accessed Nov. 2007; Akira, S. et al., *Nat. Immunol*., 2, 675, 2001; Takeda, K. et al., *Annu. Rev. Immunol*., 21, 335, 2003; Aderem, A. and Ulevitch, R.J., *Nature*, 406, 782, 2000.

pathogens. The literature covering TLR includes >8000 original papers and over 600 published in just 10 years. Key reviews include the following papers.[45–51] and others cited in the following sections. Eleven TLR (vs. 13 TLR in mice) have so far been identified within the human genome though only the first 10 have defined functions. TLR have varied specificity for different pathogen-associated molecular patterns (PAMP) (Table 7.2). The activation of different subsets of TLR may provide information concerning the nature of invading pathogens.

7.2.1.1 TLR in Peripheral Tissues and Muscles

Prior to 2001, most of the publications dealing with TLR focused on their role in front-line cells (macrophages, dendritic cells, neutrophils, endothelial cells, mucosal cells, and antigen presenting cells (Table 7.3)), which are the first to encounter invading pathogens. Clearly, such cells are an essential part of the innate immune/inflammatory

TABLE 7.3
Elements of Innate Immune System

Frontline Cells	PAMS	PRR	Intracellular Signaling	Innate Immune Response
Macrophages	Liposaccharide (LPS)⊥	LPS binding protein CD14	MyD88	Antimicrobial peptides
Neutrophils	Lipoprotein	Toll-like receptors (TLR)	IRAK	Chemotaxis
Dendritic cells	Peptidoglycan (PGN)	NOD1 and NOD2	TRAF	Cytokines
Endothelial cells (blood vessels)	Lipoteichoic acid	B2 Integrins (CD11/CD18)	P38	Phagocytosis
Epithelial cells (oral and skin)	Unmethylated CpG DNA	C-type lectins	MKK/JNK	RNS
Mucosal cells (gut, nasal cavity)	Lipoarabionoglycan	Macrophage scavenger receptor	IKK/NFκβ	ROS
Antigen presenting cells (APC)	Single or double stranded viral RNA Flagellin monomer Necrotic cells	Complement receptor (CR2/CD21)		Vasodilatation

Source: Summarized from Zhang, G. and Gosh, S., *J. Clin. Invest*., 107, 13, 2001.
Note: Cell components limited to gram-negative bacteria (⊥) and/or both.

response at mucosal surfaces, arteries, gut, and other sites.[52] It has become evident that TLR also occur within a variety of tissues and organs, including gut-associated lymphoid tissue[53] as well as muscle cells.[53–56] Cultured C2C12 muscle myoblasts express mRNA for TLR1–7 (not TLR8 and 9) in response to exposure to LPS. TLR ligands induce proinflammatory cytokines production in cultured muscle cells similarly to effects observed in vivo.[52] The increase in IL-6 mRNA (but not TNF-α) was inhibited by the well-known anti-inflammatory agent, dexamethasone.

Though LPS increases plasma cytokines, evidence for the direct involvement of tissue TLR is scarce. Lang et al.[57] showed that the intraperitoneal injection of LPS increased the expression of mRNA for TNF-α and IL-1 by 10-fold and 80-fold, respectively. Furthermore, mRNA for TLR4 and TLR2 were directly detected in muscle though only the latter increased by only ~50%. Nashimura and Naito[58] demonstrated the presence of mRNA for TLR1–10 in a wide variety of fetal human tissue. Their analysis showed TLR-related genes including those for ICAM1, CD14, MyD88, LY96, TRIF, TICAM2, TIRAP, CD83, SOCS1, TNFAIP3, TOLLIP, IRAK1, IRAK2, IRAK4, and TRAF6 in the adrenal glands, heart, kidneys, lungs, prostate, large/small intestines, trachea, and skeletal muscles. The implications are that most tissues possess TLR and the appropriate signaling pathway to respond directly to microbial and viral infection resulting in increased production of proinflammatory cytokines.

7.2.1.2 Lipopolysaccharide Signaling via TLR4

Investigations using fruit fly as model suggest the host cell interaction with LPS upregulates up to 230 genes coding for aspects of innate immune response, including recognition, phagocytosis, coagulation, melanization, synthesis of AMPs, production of reactive oxygen species, and regulation of iron metabolism.[59] Intravenous injection of LPS induces the symptoms of sepsis. LPS first binds to a intermediate protein (CD14) and the complex then binds to TLR4 resulting in diverse responses including the expression of endogenous AMPs, TNF-α, IL-1, IL-6, and IL-8, NO, superoxide radicals, and lipid intermediates.[60–62] A further 170 genes were repressed by microbial infection whereas the function of many LPS-activated genes have not been identified.[63] Another major downstream event from TLR4 is the activation of NFκβ, which is implicated in the innate immune response[64,65] (Chapter 6).

LPS binding to cells was studied by several investigators prior to the discovery of TLR. The dissociation constant for LPS binding to Chinese hamster ovary cells was ~2.8×10^{-8} M with a maximum of 3.5 million ligands per cell. Monoclonal antibodies for CD14 or LPS-binding protein blocked LPS binding implying that both proteins were vital for endotoxin signaling. Further investigations using ^{35}S-labeled LBP and H^3-LPS showed that the formation of an LPS-LBP-mCD14 ternary complex was required for the activation of NFκβ. Antibodies for LBP and CD14 prevented NFκβ activation. Following LPS signal transduction, the ligands were internalized by a process independent of LPS signaling.[66,67]

TLR is required for LPS signaling. The LPS-binding protein complexes directly with CD14 and then the two-way associated proteins bind to TLR4. Downstream signaling is thought to follow dimerization of TLR4 and the recruitment of the intracellular linker-protein, MyD88. The protein adapter recruits several intracellular kinases including interleukin-1 receptor activated kinase (IRAK). The ensuing intracellular signaling eventually results in the stimulation of two independent protein kinase cascades linked with major proinflammatory transcription factors: the mitogen activated protein (MAP) kinase (MKK) cascade upstream of activator protein 1 (AP1) and the IKβ kinase (IKK) system that activates NFκβ.

Studies using partially purified IKK suggest that the activation of NFκβ could be inhibited by synthetic peptides possessing sequences similar to those residues in the NFκβ molecule that are phosphorylated by IKK.[64] The effect of sepsis on NFκβ was discussed by Gang et al.[68] TLR4 activation is linked with increasing NFκβ expression leading to the synthesis of cytokines (TNF-α, IL-1, IL-6, and IL-12), chemotaxis factors (IL-8, RANTES, and MIP-1α, MIP-1β), and co stimulatory factors (CD40, CD80, and CD86). The signaling pathway for TLR can be found at the following Kyoto Encyclopedia of Genes and Genomes Web site.* Details of TLR receptor signaling have been reviewed.[50,69–72]

7.2.1.3 Peptidoglycan Signaling via TLR2

Peptidoglycan from gram-positive microbial cell walls activates TLR1, TLR2, and TLR6. TLR2 in particular is believed to lead to two effects: (a) stimulation of

* http://www.genome.jp/kegg/pathway/hsa/hsa04620.html; copyright restrictions do not allow reproduction here.

proinflammatory cytokine production via the RAC1/PI3K/Akt/NFκβ pathway and (b) induction of cell apoptosis. Evidence has also been presented that TLR9 functions in the detection of bacterial (unmethylated) DNA.[73]

7.2.1.4 Phagocytosis and TLR Function

TLRs though primarily linked with innate immunity and the detection of extracellular pathogens, also affect phagocytosis that is a key element of antigen-dependent adaptive immunity. Briefly, antigen-processing cells (e.g., dendritic cells and macrophages) respond to foreign cells by phagocytosis. The digested cell fragments are combined with the major histocompatibility complex and presented to naïve T-cells, which then differentiate into Th(1) or Th(2) cells. Research has shown that APC with defective TLR2/TLR4 signaling show impaired phagocytosis.[70,74]

7.2.2 NOD Intracellular Receptors for Pathogens

The cytoplasmic PRR involved in host cell detection of intracellular pathogens are called nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1 and NOD2) though genes for up to 23 NOD-like receptor (NLR) proteins have been reported.[71,75–78] It is thought that NOD1 and NOD2 participate in the innate immune response via the detection of peptidoglycans (muramyl dipeptide) from the cell walls of both gram-negative and gram-positive bacteria. The structure of NLR consists of three functionally distinct domains: (1) C-terminal leucine-rich domain required for binding AMPs, (2) a NACTH (neuronal apoptosis inhibitor protein, CIITA, HET-E, and TP1) domain, and (3) N-terminal caspases activating and recruitment domain (CARD). While NOD1 contains one CARD element, NOD2 possesses two. CARD propagates downstream signaling by forming complexes/recruiting other CARD containing proteins and ultimately stimulates cell apoptosis. The activation of NOD1 (γ-Glu DAP ligand) and NOD2 (muramyl dipeptide ligand) is believed to lead to an inflammatory response involving the serine/threonine kinase RIP2/RICK activation of NFκβ via the following chains of events leading to the increased expression of proinflammatory genes: NOD → RIP2/RICK → IKKγ → (IKB) → NFKB. NOD signaling also impacts on cell apoptosis via the activation of caspases. RIP2/RICK* contains a CARD domain. So-called inflammatory apoptosis affecting macrophages and other immune cells may exacerbate the effects of conditions such as sepsis (Figure 7.3).[71,75–78]

7.2.3 Toll Polymorphism and the Hygiene Hypothesis

Polymorphisms affecting TLR function has been considered by some researchers as a possible basis for differences in susceptibility of different subjects to endotoxins and the effects of sepsis.[79] Human populations show two common single nucleotide polymorphism (SNP) affecting the TLR4 (Asp299Gly and Thr399Ile) with an incidence rate of 8%–14% and 0.3%.[80] Chen et al. reported 16 other SNPs with a frequency of >5% in the population.[81] Kolek et al. found that the Asp299Gly SNP in

* RIP2 is also referred to in some texts as RICK, CARDIAK, CCK, and Ripk2.

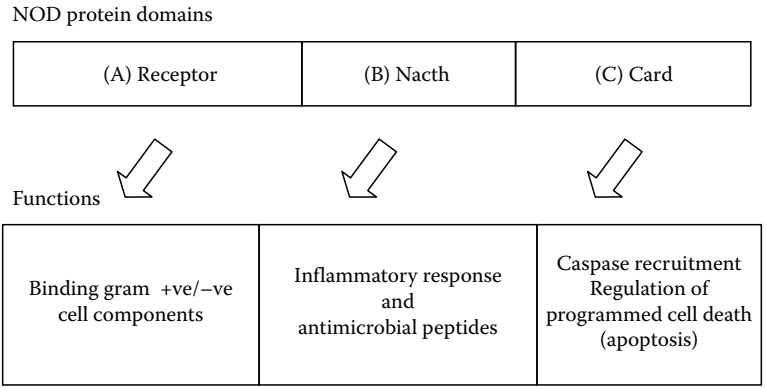

FIGURE 7.3 Functional domains for nucleotide-binding oligomerization domain-containing proteins (NOD) proteins.

TLR4 was associated with decreased risk of coronary arterial diseases.[82] Hamann et al. reported that TLR2 polymorphism may be associated with *decreased* risk of coronary artery narrowing (stenosis) and slightly reduced risk of atherosclerosis.[83]

According to the hygiene hypothesis, infectious agents (bacteria, viruses, fungi, and parasites) may give rise to inflammatory conditions. For instance, impaired TLR function or excessive TLR signaling may support lung disease.[84,85] Blocking TLR function using therapeutic agents (by interrupting intracellular signaling or removing the microbial ligands for TLR) may be an important strategy for improving health. However, impairment of TLR function does not only lead to beneficial effects. It has been suggested that some SNP may increase the risk of prostate cancer.[86] Loss-of-function mutations affecting TLR genes in mice provide some of the clearest evidence of the effect of innate immune response in illness. As discussed elsewhere, TLR-deficient mice show increased resistance to bacterial endotoxin as well as diet-induced obesity (Chapter 6).

7.3 HOST ANTIMICROBIAL PEPTIDES

7.3.1 General Properties

Host AMPs are broad spectrum antibiotic agents produced by a wide variety of organisms for self-defense.* Gene-encoded AMPs are major components of innate immunity against invading pathogenic bacteria. Examples of host AMPs include bactericidal peptides, amphibian dermaseptins, as well as defensins and cathelicidins produced by mammals. About 700 different AMPs had been reported prior to 2004, with an equal number reported between 2005 and 2008 (Table 7.4). Host AMPs are generally between 20 and 100 amino acids in length and positively charged or amphipathic due to the high content of basic and hydrophobic amino acids. The range of AMPs is very diverse, with many other AMPs not falling neatly within the α-helix or β-sheet groups. Braff et al. emphasized functional classification taking into

* A listing to host endogenous antimicrobial peptides will be found in Appendix 7.A.1.

TABLE 7.4
Number Distribution of Host Antimicrobial Peptides

Host Antimicrobial Peptides by Species	Structural Types	Structural Classes
Insect species (56)	Linear peptides (370)	α-Helical (297)
Plant species (58)	S-S bridged (380)	β-Defensins (90)
Frog/toad/other amphibian species (41)	Peptides with linear and S-S bridged domains (67)	α-Defensins (55)
	Macrocyclic backbone (8)	Arthropod (insect) defensins (54)
Mammalian species (30)	Proteins >100 residues (45)	Plant defensins (58)
		Other plant defense peptides (22)
Arachnid (spider/scorpion/tick) species (17)	Dimeric peptides (8)	Proline-rich peptides (44)
Fish species (14)		
Nematode/annelid worm species (5)		
Chelicerate (horseshoe crab, etc.) species (4)		
Crustacean (crab, shrimp) species (3)		
Gastropod (3)		
Moulds (3)		
Bird species (3)		
Protozoa 2…		
Tunicate species (2)		
Bivalve species (2)		

Source: Adapted from Web sites: http://www.bbcm.univ.trieste.it/~tossi/pag5.htm; http://www.bbcm.units.it/~antimic/tools.html; http://defensins.bii.a-star.edu.sg/; http://www.copewithcytokines.org/cope.cgi?key=Alpha%2dDefensin%2d1; http://www.cryst.bbk.ac.uk/peptaibol/introduction.htm.

account the ability of a significant number of AMPs to exhibit secondary bioactivity. Some well-known chemokines, neuropeptides, and protease inhibitors have also been shown to possess antimicrobial activity. Finally, it is noteworthy that virtually all higher organisms produce endogenous antimicrobial peptides, ranging from invertebrates (mainly insects and crustaceans),[28] plants,[87,88] amphibians,[89,90] birds, and mammals.[91] The general characteristics of AMPs and their potential application in therapy have been reviewed.[31,40,92–98]

7.3.2 Defensins and Cathelicidins

7.3.2.1 Defensins

Two major classes of AMPs are distinguishable based on their secondary structure: the defensins (~8 kDa) possess a beta-structure and the cathelicidins (~16.5 kDa) have essentially a α-helical secondary structure (Table 7.5). Defensins are further

TABLE 7.5
Classification and Sources of Human Endogenous-Antimicrobial Peptides

Classification	Subfamily	Abbrev.	Secondary Structure	Source	Bioactivity
Defensin	↑	NHP1	β-Sheet	Neutrophils	Antimicrobial
	α	NHP2	β-Sheet	Neutrophils	Induces IL-8 production
	↓	NHP3	β-Sheet	Macrophages	Inhibits glucocorticoid production
		NHP4	β-Sheet		
		HD5	β-Sheet	Paneth cells	Chemotaxis, defense response to bacterium, immune response
		HD6	β-Sheet	Paneth cells, reproductive tissue	
Defensin	↑	HBD1	β-Sheet	Epithelial cells	Antimicrobial chemotaxis of dendritic cells, CD4, CD45, and CD8 T cells
	β	HBD2	β-Sheet	Keratinocytes	
	↓	HBD3	β-Sheet		
	↑		α-Helix	Neutrophils	Antimicrobial chemotaxis of phagocytes and T cells
	Cathelicidin				
Cathelicidin	LL-37				
	↓		α-Helix	Keratinocytes	Endotoxin antagonists
			α-Helix	Epithelial cells	

HNP = human neutrophil peptide, HD = human defensin, HBD = human beta defensin.

subdivided into α-defensin or β-defensin depending on their disulfide bonding pattern (see below). A variety of human α-defensins (HD1–4) are produced by neutrophils, monocytes, or macrophages. A further source of human α-defensin (HD5 and HD6) is the Paneth cells located within crypts adjacent to microvilli; these are part of the four different cell types including enterocytes, goblet cells, and enteroendocrine cells that produce brush border peptidases, mucus, and peptide hormones, respectively.[99] As noted above, α-defensins are characterized by a distinctive set of three disulfide bonding between cysteine residues 1–6, 2–4, 3–5. The β-defensins have disulfide bonding pattern involving cysteine residues 1–5, 2–4, 3–6. The disulfide links may be necessary for activity. It is thought that genes coding for defensins contain three exons, with exon 1, 2, and 3 coding for an untranslated sequence of DNA, pre-protein region, and mature protein, respectively. The AMPs are typically produced as inactive pre-proteins and are later activated by limited digestion probably by matrix metallo-protease-7 (MMP7).[100,101]

7.3.2.2 Cathelicidin or hCAP18

Cathelicidins are AMPs found in mammals though only one example appears in humans (Appendix 7.A.2). Human cationic antimicrobial protein (hCAP) is associated with neutrophils, testis, and bone marrow cells. Recent evidence suggests that cathelicidins are also formed by epithelial cells, skin keratinocytes, monocytes,

TABLE 7.6
Selected Bioactive Peptide Sequences Encrypted within Cathelicidins

Progetrin (131–148)[a]	RGGRLCYCRR RFCVCVGRG
Indolicidin (131–143)[b]	ILPWKWPWWP WRR]G
LL37 (134–170)[c]	LGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
Fall-39 (132–170)[c]	ALLGDFFR KSKEKIGKEF KRIVQRIKDF LRNLVPRTES

[a] Pig AMP—UniProtKB accession number P32194.
[b] Bovine AMP Cathelicidin-4, UniProtKB accession number P33046.
[c] Human Cathelicidin AMPs, UniProtKB accession number P49913.

NK cells, B cells, and mast cells.[32,102,103] hCAP18 has a molecular mass of 18 kDa. The degradation of hCAP18 by elastase generates a 37 amino acid peptide fragment (LL-37) with the primary sequence shown in Table 7.6. A second AMP sequence designated FALL39 is encrypted within the structure of hCAP18. The UniProtKB database lists one structure for human cathelicidin (accession no. P49913), though this shows 90% homology with similar proteins from various apes: gorilla, gibbons, orangutans, etc. The structures of over 100 cathelicidins have been listed, ranging from 200 amino acids to ~170 residues. In most cases, the N-terminal ~40 AA appear to be responsible for the antimicrobial action.*

Cathelicidins have been isolated from rabbit (rabbit CAP18), pig (protegrin 39), and other higher organisms. As noted for the AMPs in general, hCAP18 has multiple functions. Aside from the ability to kill bacteria, AMPs also bind to bacterial endotoxin. hCAP also interacts with inflammatory cells. Binding to mast cell induces these to release chemicals from their granules. LL-37 may also induce angiogenesis or the formation of new blood vessels, which may be useful in the promotion of wound healing. Tests by Turner et al. demonstrated that LL-37 has antibacterial activity against a host of bacteria with a minimum inhibitor concentration (MIC) <10 μg/mL); susceptible microbes include *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and vancomycin-resistant *Enterococci*. Antibacterial activity occurred even in media that contained up to 100 mM NaCl.[104] Other studies suggest that *Mycobacterium tuberculosis* growth is inhibited in the presence of cathelicidins, suggesting that these agents might find applications in the treatment of TB.[105] A synthetic analogue of the porcine cathelicidin (protegrin) is available under the commercial name Iseganan HCl®.[106] Clinical trials for Iseganan are discussed below.

7.3.3 Dermaseptins and Frog Peptides

Mor et al. isolated AMPs from a South American tree frog species, *Phyllomedusa saugagi*, in 1991. Frog dermaseptins (DS), which contain 27–34 amino acids

* The interested reader may refer to the UniProt Web site for the latest update on cathelicidins http://www.uniprot.org/uniprot/?query = Cathelicidin&offset = 50&sort = protein + names&desc = no

TABLE 7.7
Sequences for Dermaseptins from South American Tree Frog

Dermaseptin-1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
Dermaseptin-2	ALWFTMLKKLGTMALHAGKAALGAAANTISQGTQ
Dermaseptin-3	ALWKNMLKGIGKLAGKAALGAVKKLVGAES
Dermaseptin-4	ALWMTLLKKVLKAAAKALNAVLVGANA
Dermaseptin-5	GLWSKIKTAGKSVAKAAAKAAVKAVTNAV

Source: Adapted from "dermaseptins" at the Horst Ibelgaufts' COPE: Cytokines and Cells Online Pathfinder Encyclopedia, http://www.copewithcytokines.de/cope.cgi?key=dermaseptins (accessed June 2008).

(Table 7.7), are effective against a broad spectrum of microorganisms.* The antimicrobial activity of DS is associated with 18 amino acids from the N-terminal region. Subsequent research showed that DS1 and DS2 also possess antiviral activity. In addition, DS appear to stimulate polymorphonuclear (PMN) cells microcidal activity by increasing the cellular production of ROS and myeloperoxidase. Dermaseptin has undergone extensive development since 1991, including the development of truncated versions with modified specificity. The selectivity of dermaseptin (fragments) could be modified by acylation. As far as could be determined, at the time of writing, there are no published human trials using dermaseptin.

7.3.4 Bactericidal/Permeability-Increasing Protein

Bactericidal/permeability-increasing protein or BPI is a 50 kDa protein produced by PMN cells or neutrophils. BPI acts against live gram-negative bacteria and binds endotoxin or LPS (see Appendix 7.A.3). Research using a 23 kDa human recombinant BPI fragment (rhBPI23) showed that this has both LPS-binding activity and bactericidal activity. By contrast, the 30-kDa C-terminal is able to bind LPS but is lacking bactericidal activity.[107,108] A fascinating comparative study of BPI and LBP suggests that the C-terminal section of both proteins may be closely related. The C-terminals of LBP and BPI have significant affinity for LPS, which they tend to direct to TLR of host cells and leukocytes, respectively. In addition, rhBPI(23) affinity for endotoxin probably exceeds the binding affinity with LBP, explaining the former peptide's ability to block the proinflammatory action of LPS.[109] There is also evidence that BPI inhibits angiogenesis.[93,110,111] The commercial exploitation of BPI is currently being explored via rhBPI under the trade name Opebacan/Nueprex®. Suggested areas for application for Nueprex include the treatment for endotoxemia/sepsis,[112] meningococcal sepsis, and trauma.[113]

* A search conducted at the ExPASy (Expert Protein Analysis System) server lists over 119 structures for dermaseptin from a variety of tree frog species.

7.3.5 Lactoferrin, Lactoferricin, and Talactoferrin®

Lactoferrin (Lf) constitutes 1%–2% of the whey protein fraction from bovine milk.[114] Though found in other body fluids (tears, nasal fluid, and blood plasma), milk seems to be the most accessible source for ingredient Lf.* High Lf concentrations occur also in fecal matter, where it is considered a biomarker for inflammatory bowel conditions. Lf belongs to the transferrin family of proteins that share 50%–80% sequence homology. Other closely related iron-binding proteins include serum transferrin and ovotransferrin from egg. Lactoferrin functions as an AMP partly because of its high affinity for metal ions. Lf binds to iron with 300-fold greater affinity compared to transferrin or ovotransferin. Moreover, Lf-Fe complex is stable over a wide pH > 3 unlike other proteins. The other important feature of Lf that distinguishes it from other iron-binding proteins is the relatively high isoelectric point (pI # 8–9) that shows it to be much more negatively charged. The major natural function of lactoferrin is iron binding, transport, and uptake.[115]

Aside for its role in Fe-binding and transport, Lf also behaves as an antioxidant, anti-inflammatory agent and wide spectrum antimicrobial protein (effective against bacteria, fungi, and viruses). The compendium of functions attributed to Lf has grown recently to include the ability to stimulate cell proliferation, hematopoiesis, as well as moderating the innate immune system. Lf is an endotoxin antagonist. Lf may also have beneficial effects on bone growth.[116] The discovery of cell receptors for lactoferrin suggests that this ligand could elicit diverse functions in different tissues.[117] Interestingly, Lf contains a series of encrypted micro-peptide sequences possessing the bioactivity of parent molecule. Thus, the N-terminal region of Lf contains lactoferricin[118] and lactoferrampin,[119] which are released by pepsin digestion. Lactoferrin structure and function have been extensively reviewed.[118,120–128,†]

Applications of Lf in the nutrition and food industries are well established. Thus, Lf has been added to infant formulas since the late 1980s owing to its ability to improve GI tract health in children.[129] At the time of writing, ~24 RCT of lactoferrin use with human subjects had been documented, mainly related to application in the treatment of *Helicobacter pylori* induced peptic ulcers (~7 trials), infant diarrhea (~2 trials), iron adsorption (2 trials), wound healing (~2 trials), treatment of postoperative patients, oral hygiene, and the treatment of hepatitis C (2 trials).[‡] Pepsin hydrolysis of lactoferrin forms a 25-mer encrypted peptide fragment termed lactoferricin B (LfnB). The latter, comprising amino acid residues 36–60, is highly basic and possesses the antimicrobial properties of Lf.[118]

Human lactoferrin has been cloned and expressed in diverse vehicles, including aspergillus,[130] cow's milk,[131] rice crop,[132,133] chicken eggs,[134] for large-scale production of recombinant human lactoferrin (rhLF) for nutritional and health applications related to antimicrobial activity, improving GI tract health, iron absorption, and cancer therapy (see below). Talactoferrin, which is proprietary rhLF developed by Agennix LTD (Houston, Texas, United States), is currently undergoing clinical trials

* The structural characteristics of lactoferrin are summarized in Appendix 7.A.4.

† See http://www.copewithcytokines.de/cope.cgi?key = Lactoferrin, for a short summary of lactoferrin functions. Appendix 7.A.3 shows some key structural characteristics for Lf.

‡ PubMed search shows 98 randomized trials involving lactoferrin (April 2008).

for cancer treatment, wound healing, and the treatment of sepsis.[135] It is noteworthy that Talactoferrin received Orphan Drug designation from FDA in 2006, allowing the use of rhLf in medical foods (Chapter 1). According to corporate literature, Agennix has established a 35,000 L cGMP fermentation facility for the commercial scale production of rhLf.* A representative sample of RCTs for Talactoferrin is discussed in Section 7.5.

7.4 FUNCTIONS OF ANTIMICROBIAL PEPTIDES

7.4.1 Antibacterial Activity

AMPs exhibit bactericidal activity due to direct interaction with the microbial cell membrane. Positively charged AMPs bind to negatively charged sites on bacterial membranes. Anionic sites that occur mainly within the plasma membrane of pathogens include LPS, phosphatidylglycerol, and phosphotidyl-ethanolamine. Cholesterol and phosphatidylcholine that occur in mammalian plasma membrane are believed to hinder AMP binding. Several models have been proposed to describe events subsequent to binding that lead to membrane pore formation and lyses: the aggregate or toroidal model, the barrel-starve model, and the carpet model. The aggregate or toroidal model involves the formation of an array of AMPs molecules inside the bacterial lipid membrane in the same orientation as phospholipids. By contrast, the barrel-starve model has aggregates of AMPs form perpendicular to the membrane surface giving rise to a hydrophobic core and hydrophilic-facing protein chains.[136] No matter the exact geometric arrangements involved, antibacterial action requires direct interaction with the bacterial membrane. In addition to membrane disruption, some AMPs may also transfer into cells where they then produce an intracellular effect such as mRNA synthesis, decreased protein synthesis, and impaired protein folding.[137,138] Models for AMPs binding to microbial pathogens have been reviewed.[139–142]

7.4.2 Anticancer Activity of AMPs

A number of AMPs have been shown to have toxicity toward cancer cells whereas they are relatively harmless toward normal host cells.[143] The anticancer effect of AMPs is not wholly understood. It has been proposed that AMPs kill cancer cells via membrane lyses, that is, via the same process that leads to bacterial killing. It is thought that AMP toxicity toward malignant cells arises due to the higher negative charge, lower cholesterol, and higher glycoprotein content of cancer cell membranes compared to normal cells.[144] A higher phosphatidylserine density on the cancer cells may also act as a focus of AMP activity.[145] Other global differences between cancer cells and normal cells may contribute to the greater susceptibility of the former to AMPs; for example, resistance to apoptosis, increased cell proliferation, tendency for adhesion, invasiveness, and metastasis, as well as the tendency to promote angiogenesis.[146] On the other hand, AMPs may produce antitumor activity partly due to

* See http://www.agennix.com/contentpages/patents.htm.

TABLE 7.8
Functions of Mammalian Endogenous Antimicrobial Peptides

Antimicrobial
Antiviral (anti-HIV1)
Augment cytokine production (IL-5, IL-6, IL-10, and IFNγ)
Block LPS binding to LBP
Chemotactic for naive resting T cells, CD8 T cells, immature dendritic cells
Complement activation
Degranulation of mast cells
Enhance antigen induced immune responses
Immunoadjuvant effects in mice
Increase epithelial cell proliferation
Inhibit glucocorticoid production
Promote phagocytosis of microbes
Promote antigen induced splenocyte proliferation
Recruitment of inflammatory cells
Repair antimicrobial
Stimulate production/release of pro- and anti-inflammatory agents

Source: Adapted from Elsbach, P., *J. Clin. Invest*., 111, 1643, 2003.

their ability to function as inhibitors for tumor proteases and/or protein tyrosine kinases.[147] Finally, AMPs can also moderate host immune response (Table 7.8), which may impact on the development of tumors. Though the mechanisms of AMP anticancer activity are not yet fully understood, considerable progress is being made to apply these agents for the treatment of a diverse range of tumors (see below). Table 7.8 shows a listing of some of the functions associated with mammalian AMPs.

Wang and Ng showed that lima beans and also the ground bean contain a 7 kDa antifungal peptide with an N-terminal homology with α-defensin, which inhibits the proliferation of breast cell (MCF-7) and leukemia cells. In both cases, there was no cytotoxity toward normal cells.[148,149] Lehman et al. demonstrated that magainin was selectively cytotoxic toward human bladder cancer cell lines. The concentration of AMP required for 50% inhibition (IC50 value) was 198 or 75.2 μM, using a colorimetric assay or LDH release as index of cytotoxity. Magainin was nontoxic for ordinary fibroblasts.[150] Wang et al. showed that a synthetic AMP, polybia-MPI, derived from wasp peptide could inactivate leukemic K562 cells lines but was non-cytotoxic against ordinary leukocytes. Polybia-MPI showed the same level of effectiveness against a drug- resistant K562 strain as compared to the parental nonresistant strain. Scanning electron micrographs for treated and non-treated cells showed the former underwent enormous changes in morphology probably as a result of lytic action of AMP. Flow cytometry analysis using fluorescence-labeled annexin-V suggested that the cancer cells had an unsymmetrical distribution of negative sites on the outer membrane surface, thus explaining the higher binding of AMP.[151] In summary, in vitro analysis suggests that some AMPs may have selective cytotoxity toward malignant cells whereas non-transformed cells are free from attack. The numbers of cell

types examined so far are limited as is the number of different AMPs. Some AMPs attack cancer as well as normal cells whereas other AMPs do not. Undoubtedly, more research is needed to resolve such questions. Human trials showing that AMPs may have some efficacy in clinical situations are presented in Section 7.5 and summarized in Appendix 7.A.4.

7.5 IN VIVO APPLICATIONS AND HUMAN-TRIALS OF AMPs

7.5.1 General Considerations

A variety of applications have been suggested for AMPs including treatments for pneumonia, eczema, sexually transmitted viral diseases, cystic fibrosis, respiratory infections, hepatitis, and bone diseases. AMPs may also be effective in promoting wound healing and immune health.[31,93–99,*] Evidence for AMP use in clinical settings is discussed within the section. Some suggested advantages and disadvantages of AMP use compared to conventional antibiotics are summarized in Table 7.9.[152] The broad spectrum activity of AMPs is noteworthy with effects not only confined to bacterial but also viruses, fungi, and some parasites. The AMPs work rapidly compared to conventional drugs and thereby lessen the chance of the development of resistance. Interestingly, peptide antibiotics can also work synergistically to improve

TABLE 7.9
A Comparison of Conventional Antibiotics with Cationic Antimicrobial Peptides

Property	Conventional Antibiotics	Cationic Antimicrobial Peptides
Spectrum of activity	Bacterial infections (often selective)	Bacterial, fungal and viral infections; septicemia; anti-inflammatory
Uptake	Specific mechanisms	Non-specific based on charge. Self-promoted uptake
Targets	Limited	Multiple targets in any given cell
Resistance mechanism	Reduced uptake, increased efflux, chemical medication, enzymatic degradation	Impermeable outer membrane, proteolytic degradation, peptide efflux transporters
Additional activities	Na	Anti-endotoxin and/or innate immunity enhancing
Pharmacokinetics	Varied	Short serum half-life from proteolytic degradation
Toxicology	Generally safe	Safe topical application; systemic toxicity possible
Manufacturing costs	inexpensive (e.g., $0.8/g)	Expensive ($50–400/g)

Source: Adapted from Marr, A.K. et al., *Curr. Opin. Pharmacol.*, 6, 468, 2006.

* See Appendix 7.A.5 for a list of current clinical trials involving some commercial antimicrobial peptides

the efficacy and to reduce the required inhibitory concentration of classical drugs. The major disadvantages of the AMPs are their incompatibility with high ionic strength caused by the screening effect of salt ions binding to their positively charged sites. The AMPs also exhibit low stability toward serum peptidases and proteolytic enzymes found in the GI tract and intestinal brush border.

7.5.2 Burns Injury, Wound Healing

The expression of endogenous AMPs is reduced following burns injury and other forms of wounding.[153–155] Since AMPs are needed for defense and clearance of invading pathogens, the provision of exogenous AMPs could help with healing.[156] AMPs may also help to facilitate angiogenesis (formation of new capillary vessels) at the site of injury—but there are indications that not all AMPs possess this activity.[157] Chronic wounds that are infected with drug-resistant pathogens (e.g., *Pseudomonas*, *Streptococci*, and *Staphylococcus*) appear to be a particular concern. Results from animal studies and one human RCT suggest that topical applications of Talactoferrin gels (1%–8.5% active ingredient) may increase wound healing, monitored as ¾ decrease in initial wound size.[158] As indicated above, the mode of action of AMPs is only partly due to the killing effect on bacteria. It has been suggested that Talactoferrin may also enhance wound healing partly as a consequence of its *proinflammatory* activity, which helps to reverse localized deficiencies in the innate immune response affecting the wounded tissues. Common indices of inflammation were increased following AMP treatment compared to controls.[159]

7.5.3 Cancer Therapy

A limited number of human trials to examine the use of AMPs for treating cancer have been completed and some are ongoing. Hayes et al. reported a Phase I/II trial for Talactoferrin, which indicated that high doses of this protein (1.5–9 g/kg/day) produced little or no adverse effects when given orally. After consuming rhLf in a buffered sweetened drink, rhLf was completed digested though no peptide fragments could be detected in the blood plasma or urine. Subjects showed an increase in serum levels of IL-18 after the administration of Talactoferrin. There were reported decreases in tumor growth from 33% per month to 9.9% per month. According to data at hand, it could be inferred that rhLf probably works at the GI tract level.[160] Jonash et al. examined the effectiveness of Talactoferrin treatment of renal cell carcinoma.[161] The trial employed 44 volunteers (age 62 years, 28 males) most of whom had undergone nephrectomy. Oral Talactoferrin (1.5 g/kg; twice per day) produced minor GI tract symptoms: abdominal distension (14%), flatulence (10%), diarrhea (14%), and constipation (7%). The primary outcome, so-called disease progression free survival (PFS), was higher for treatment volunteers compared to historic placebo controls (56% vs. 20%, $p < 0.001$). The mechanisms of Lf anticancer effect are uncertain at present. It remains to be determined whether Lf is absorbed from the gut or whether such absorption is actually necessary for bioactivity. According to Wolf et al., oral Lf may exert its effect directly by inhibiting cancer cell growth. In addition, Lf appears to upregulate the function of immune cells leading to the suggestion that the anticancer effect is due to infiltrating PMN cells.[162]

7.5.4 Infant Diarrhea

In vitro tests have shown that lactoferrin is effective against a range of microorganisms that cause diarrhea (Section 7.3.5). Zavaleta et al.[163,164] demonstrated that Lf can reduce the extent of diarrhea in children as during rehydration therapy. The RCT employing 140 young infants (5–33 months old) suffering from diarrhea examined the effectiveness of rehydration therapy by comparing days of diarrhea in three treatment groups treated with: normal oral rehydration solution, a rice-based rehydration solution (RORS), or RORS with added lysozyme and lactoferrin. The RORS + lactoferrin/lysozyme group had significantly shorter episodes of diarrhea (3.67 days vs. 5.21 days, $p = 0.05$) compared to the other two groups. The antidiarrheal effect of Lf is partly explained by its bactericidal properties. Lf also functions as an endotoxin antagonist that blocks the action of LPS on gut cells.[165]

King et al.[166] found that Lf supplementation of bottle-fed healthy infants (85%–90% ethnic blacks; 0–4 weeks old and >2000 g BW) produced no significant changes in the incidence of diarrhea between control and treatment groups given 102 mg Lf/L vs. 850 mg Lf/L feed, respectively. There was also no significant difference in the other symptoms of illness. However, the Lf-supplemented group showed significantly lower respiratory tract infection, improved hematocrit values, and a general (but insignificant) trend toward increased weight again. Ochoa et al.[167] found that the incidence of diarrhea was not changed in healthy weaned infants (13–36 months old, $n = 26$) supplemented with 0.5g Lf/day, 6 days per week for 9 months. This pilot study showed there were significant reductions in the rate of colonization with microbial pathogens (*Giardia* spp.). Anthropometric measurements showed that the Lf treatment group achieved a greater increase in height-for-age score but that there were no differences in body weight.

7.5.5 *Helicobacter pylori*–Related Ulcers

There is a growing desire to reduce our dependence on classical antibiotics.* In a trial involving 150 dyspeptic patients, researchers from the University of Parma found that Lf supplementation increased the rate of *H. pylori* eradication by 92%–96%, compared with 71%–72% increased clearance with antibiotics.[168–171] A recent study also comparing triple antibiotic therapy with the same treatment plus lactoferrin and a probiotic found that supplementation led to improved eradication of *H. pylori* infection in (95/105) patients compared to (73/96) patients subjected to conventional antibiotic therapy.[172] There is indirect clinical evidence for the involvement of other AMPs in the prevention of *H. pylori* infections. For instance, Isomoto et al. found that the concentration of human neutrophil protein HN1-3 in gastric juice was higher for patients with *H. pylori*–mediated gastritis compared to normal controls. The concentration of HNP1-3 also decreased following successful treatment. The data suggests that α-defensins may be important for a part of the response to *H. pylori*.[173] Despite early promising results, other clinical trials suggest

* Peptic ulcers arising from microbial infection may be treated using a triple or quadruple combination of conventional antibiotics (rabeprozole, amoxicillin, clarithromycin, and tinidazole).

that the benefits of lactoferrin administration may be modest or insignificant when compared with a standard triple antibiotic treatment.

7.5.6 Hepatitis C Treatment

AMPs have shown efficacy against hepatitis virus in vitro. However, human studies have given only mixed results. Thus, the initial study of 11 hepatitis patients indicated that those with a low titer of virus could benefit from treatment with Lf but that patients with high serum levels of virus could not. Recent larger scale studies concluded that orally administered Lf has no significant effects on outcome of treatment.[174–176]

7.5.7 Oral Mucositis

Ulceration and inflammation of the oral mucosa accompanies radiotherapy to treat head and neck cancer. The development of oral mucositis (OM) is believed to involve colonization of oral cavity by microbial pathogens. In vitro tests showed that Iseganan or IB-367 has minimal inhibitor concentrations of 0.13–64 μg/mL against common aerobic gram-positive organisms (*Streptococcus* spp.) and MIC of 0.06–8 μg/mL against gram-negative pathogens (*Klebsiella*, *Serratia*, *Escherichia*, and *Pseudomonas*) from the oral cavity.[177] IB-367 could reduce the oral population of pathogens 1000-fold. Consistent with its broad spectrum activity, speed of action, and potency in reducing bacterial numbers, it was inferred that IB-367 was at least as effective as conventional antibiotics for oral mucositis treatment. A recent Phase I/II clinical trial failed to find support for this hypothesis and these studies have been abandoned. There is no clear indication or reason for the failure of AMP therapy, except to suggest that the involvement of microbes in OM may have been overestimated.[178–180]

7.5.8 Ventilator-Associated Pneumonia

Ventilator-associated pneumonia (VAP) occurs when bacteria are sucked up from the upper GI tract by mechanical ventilators and redistributed via the mouth into the airways. The incidence of VAP is estimated at 9%–27%, with mortality rates of up to 50%. Studies dating from the 1990s first suggested that pneumonia could be treated using AMPs (e.g., polymyxin) to decontaminate the oral cavity. A recent meta-analysis by Chan et al. noted that 7/11 of randomized clinical trials showed that oral decontamination using non-absorbed antibiotics (including AMPs) could reduce the incidence of VAP. However, there was no change in mortality, duration of ventilator support, or the length of stay in intensive care units.[181] The possibility of applying peptide antiseptic agents to combat VAP was examined by Kollef et al. The RCT using 709 mechanically ventilated intensive care unit patients showed that those treated with Iseganan (n = 282) showed the same levels of WAP incidence as the placebo-treated subjects (16% vs. 20%, respectively). The mortality rate at 14 days was also not significantly different between the treatment and placebo

groups (22% vs. 18.2%). Clearly, the treatment with Iseganan had no significant improvements in VAP[182] and there is clearly a need for further studies.[183]

7.5.9 Sepsis and Endotoxemia

Researchers from the University of Ancona (Italy) have shown that peptide AMPs from diverse sources may be useful in the treatment sepsis. The AMPs were considered in combination with classical beta-lactam antibiotics for the treatment of experimental sepsis induced by LPS injection or cecal ligation and puncture. Investigations indicated that peptide AMPs (~1 mg/kg) could synergize the antimicrobial effect of beta-lactams, possibly because of the endotoxin-binding activity of the former.[184] As noted earlier, classical antibiotics are limited in their application for sepsis, owing to their tendency to release LPS following the lyses of bacterial cell walls. Tests suggest that the bovine AMP,[185] pexiganan,[186] and temporin L frog peptide,[187] may all potentiate the antiseptic effect of classical antibiotics (Figure 7.4).

Investigations using rat model for sepsis, induced by surgical operation and bile duct ligations followed by LPS injection, suggest that the progestin analogue (IB-367) could be useful for the treatment of endotoxemia. In the experiments summarized in Figure 7.4, sham-operated and bile duct-ligated specimens showed a large rise in serum endotoxemia after injection of LPS. By contrast, treatment with

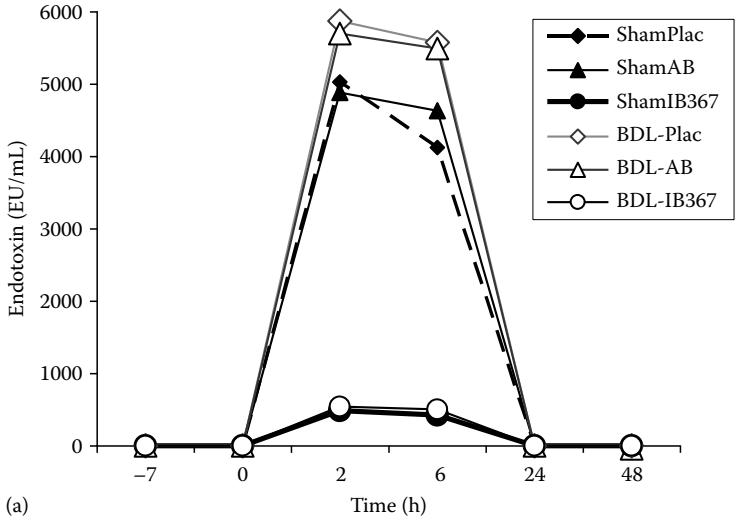

FIGURE 7.4 Effect of peptide antibiotic (IB367) on serum endotoxin in a rat model of sepsis. Top panel shows endotoxin profile after LPS administration. Lower panel shows final serum endotoxin level at 48 h. Treatments are: sham operated and placebo treated (ShamPlac), Sham operated + antibiotic treatment (ShamAB), sham operated + IB367 treated. BDL = rats undergoing bile duct ligation. (Original data from Giacometti, A. et al., *Guts*, 52, 874, 2003; Levy, O., *Expert Opin. Invest. Drugs*, 11, 159, 2002.)

(*continued*)

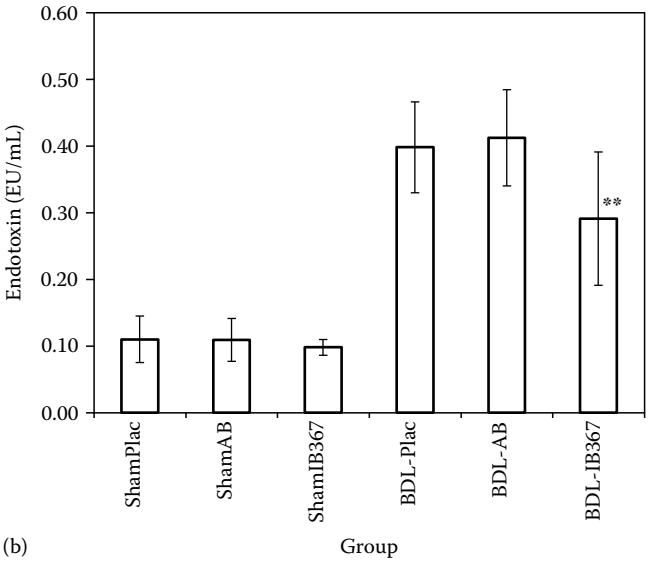

FIGURE 7.4 (continued)

IB-367 prevented endotoxemia to a great extent. The AMP treatment also resulted in a reduction in endotoxin lethality from 55% to 60% for the placebo- or antibiotic-treated groups to lethality of 10% for the AMP-treated group.[188] Other peptide antimicrobials that have also been shown to have favorable effects on sepsis include cathelicidin peptide LL-77[189] and polymyxin B.[190–193] As noted in the previous chapter, the application of polymyxin columns is arguably one of the most successful of the new peptide AMP-based therapies for sepsis.[194]

7.6 SUMMARY AND CONCLUSIONS

We have shown that infection by disease-causing microbes leads to a host response related to inflammation. Intensive care for acute sepsis patients in order to reduce the risk of death is a major area of concern for health professionals. Infection-related inflammation can also lead to wasting and "undernutrition." Therefore, antimicrobial peptides and antiseptic agents might be usefully applied for improving nutritional health. The evidence from preclinical or laboratory tests show that some AMPs have bioactivity toward common disease-causing microbes and some anticancer activity. In vivo tests and clinical trial data show some promise but the level of proof of concept is missing. On the other hand, AMPs such as lactoferrin that have a history of long-term applications in the food industry have been discovered to have novel characteristics that may be exploited for nutritional and health applications. The huge number of novel AMPs from diverse sources may be examined in the future for their efficacy for the treatment of gastrointestinal ailments that are common in the elderly or other groups at risk from wasting diseases.

APPENDICES

APPENDIX 7.A.1
List of Selected Endogenous Antimicrobial Peptides

AMPs from Resident Cells	AMP/Proteinase Inhibitors
Cathelicidins	hCAP18/LL-37 pro-sequence (cathelicidin-like domain)
α-Defensins	Secretory leukocyte proteinase inhibitor (SLPI)/ Antileukoprotease
Bactericidal/permeability-increasing protein (BPI)	Elafin/skin-derived antileukoprotease (SKALP)
Lactoferrin	P-cystatin a
Lysozyme	Cystatin C
RNase 7	
AMPs from infiltrating cells	*AMP/chemokines*
Cathelicidins	Psoriasin
α-Defensin	Monokine induced by IFN-γ (MIG/CXCL9)
Lactoferrin	IFN-γ-inducible protein of 10 kDa (IP-10/CXCL10)
Granulysin	IFN-γ-inducible T cell a chemoattractant (I-TAC/CXCL11)
Perforin	
Eosinophil cationic protein (ECP)/RNase 3	
Eosinophil-derived neurotoxin (EDN)/RNase 2	
Regulated upon activation, normal T cells expressed and secreted (RANTES)	
Platelet factor 4 (PF-4)	
Connective tissue activating peptide 3 (CTAP-3)	*AMP-neuropeptides*
Platelet basic protein	α-Melanocyte stimulating hormone (α-MSH)
Thymosin b-4 (Tb-4)	Substance P
Fibrinopeptide B	Bradykinin
Fibrinopeptide A (FP-A)	Neurotensin
	Vasostatin-1 and chromofungin (chromogranin A)
	Secretolytin (chromogranin B)
	Enkelytin and peptide B (proenkephalin A)
	Ubiquitin
	Neuropeptide Y
	Polypeptide YY
	Adrenomedullin

Adapted from Braff, M.H. et al., *Journal of Investigative Dermatology*, 125, 9, 2005. Jenssen, H., Hamill, P., and Hancock, R.E.W., *Cellular and Molecular Life Sciences*, 64, 922, 2007.

APPENDIX 7.A.2
Characteristics of Human Cathelicidin

Protein name	Cathelicidin antimicrobial peptide; UniProtKB /Swiss-Prot entry P49913
Synonyms	18 kDa cationic antimicrobial protein, CAP-18, hCAP-18
Encrypted peptides	Antibacterial proteins LL-37, and FALL-39 (FALL-39 peptide antibiotic)

Function

Binds to bacterial lipopolysaccharide (LPS)
Antibacterial activity
Chemotaxis
Degranulation of mast cells
Regulation of immune-related genes
Angiogenic activity
Wound repair
Epidermal permeability?
Growth factor in lung cells?
Found in atherosclerosis plaques

Features

Key	AA residues		Length	Description
SIGNAL	1	30	30	Potential
PROPEP	31	131	101	
CHAIN	132	170	39	Antibacterial protein FALL-39[a]
CHAIN	134	70	37	Antibacterial protein LL-37[a]
MOD_RES	31	31		Pyrrolidone carboxylic acid (by similarity)
DISULFID	86	97		By similarity
DISULFID	108	125		By similarity

Sequence information

Cathelicidin, size 170 AA for unprocessed pre-peptide, molecular weight: 19.301 kDa

[a] Antimicrobial cathelicidin domain, reference summarized from UniProtKB/Swiss-Prot entry P49913 (http://www.uniprot.org/).

APPENDIX 7.A.3
Characteristics of Bactericidal/Permeability-Increasing Protein (BPI)

Protein name	Bactericidal/permeability-increasing protein
Synonym	BPI

Function: Cytotoxic toward gram-negative bacteria

Cell location: Cytoplasmic granule membrane, membrane-associated in PMN granules

Tissue distribution: Restricted to cells of the myeloid series

Domain: The N-terminal is possibly exposed to the interior of the granule, whereas the C-terminal portion may be embedded in the membrane. N-terminal may be cleaved and released for antimicrobial action

APPENDIX 7.A.3 (continued)
Characteristics of Bactericidal/Permeability-Increasing Protein (BPI)

Key	From To	Length	
SIGNAL	1–26	26	
CHAIN	27–482	456	Bactericidal/permeability-increasing protein
REGION	235–240	6	Cleavage sites for elastase (potential)
CARBOHYD	62, 303, 375, 389, 463	N-linked (GlcNAc…)	

Length: 482 AA for pre-protein, molecular weight: 53.442 kDa

Source: Antimicrobial cathelicidin domain, summarized from UniProtKB/Swiss-Prot entry P17213 (http://www.uniprot.org/).

APPENDIX 7.A.4
Characteristics of Bovine Lactoferrin

Protein name	Lactotransferrin/lactoferrin
Synonyms	EC 3.4.21, lactoferrin
Encrypted peptide	Lactoferricin B
Protein existence	1: Evidence at protein level

Function : Binds two $Fe(3^{+})$ ions with bicarbonate counter ion

Antimicrobial activity against *E. coli* and *P. aeruginosa* (lactoferrin)

Serine protease (S60 domain 1) cuts at -ArgSerArgArg|- and -ArgArgSerArg-|-rich regions, inhibited by PMSF and Pefabloc

KM = 50 µM for Z-PheArg-aminomethylcoumarin; optimum pH is 7.5; optimum temperature is 25°C

Features

Key	Description	
SIGNAL	1–19	Description
CHAIN	20–708	Lactotransferrin
PEPTIDE	36–60	Lactoferricin B
DOMAIN	25–352	Peptidase S60 1
DOMAIN	364–693	Peptidase S60 2
ACT_SITE	92–92	By similarity
ACT_SITE	278–278	Nucleophile (by similarity)
METAL	79, 11, 211, 272,	Iron 1
METAL	414, 452, 545, 614,	Iron 2
Carbonates	136, 142, 143, 478, 482, 484, 485	

(*continued*)

APPENDIX 7.A.4 (continued)
Characteristics of Bovine Lactoferrin

CARBOHYD 252—N-linked (GlcNAc) [GlycoSuiteDB
CARBOHYD 387—N-linked (GlcNAc
CARBOHYD 495—N-linked (GlcNAc [GlycoSuiteDB]
CARBOHYD 564—[GlycoSuiteDB]
DISULFIDES 28 ↔ 64; 38 ↔ 55; 134 ↔ 217; 176 ↔ 192; 189 ↔ 200;
250 ↔ 264; 367 ↔ 399; 377 ↔ 390; 424 ↔ 703; 444 ↔ 666; 476 ↔ 551
500 ↔ 694; 510 ↔ 524; 521 ↔ 534; 592 ↔ 606; 644 ↔ 649
Sequence information
Length: 708 AA for pre-protein, molecular weight: 78.05 kDa

APPENDIX 7.A.5
Clinical Trials of Antimicrobial Peptides

Antimicrobial Peptide	Commercial Name, Sponsoring Company	Applications
Lactoferricin/lactoferrin recombinant fragment	Talactoferrin alfa®; Agennix Inc., Houston, Texas	Cancer, wound healing, pneumonia, diarrhea
Bactericidal/permeability increasing protein (BPI), recombinant fragment	Neuprex® (obebacan), XOMA (US) Ltd., Berkeley, California	Transplantation-related complications
Indolicidin	Omiganan® (MX-226), MIGENIX Inc. Vancouver, British Columbia, Canada	Wound healing Catheter infections[a]
Magainin, frog peptide Recombinant fragment	Pexiganan®; Access Pharmaceuticals Inc. & MacroChem Corp., Dallas, Texas	Mild diabetic-food infections
Polymyxin Colistin (polymyxin E)	Various	Sepsis, diarrhea
Protegrin, analogue of LL-37	Iseganan HCl®	Oral mucositis, ventilator-associated pneumonia

Note: See text in Section 7.5 for details.

[a] Information from corporate literature, http://www.migenix.com/prod_226.html.

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8 Anabolic Dysfunction

8.1 INTRODUCTION

A variety of hormones and nutrients promote muscle growth though this anabolic drive may be compromised by illness, injury, or aging. Anabolic dysfunction arises from resistance to the effects of anabolic hormones (e.g., growth hormone and insulin) and nutrients during ill health. There may also be a shift in the secretion of catabolic vs. anabolic hormones during illness so as to favor weight loss. Bioactive peptides for combating weight loss due to anabolic dysfunction are described in this chapter. Changes in levels of hormones that contribute to anabolic dysfunction are summarized in Section 8.1. The effects of insulin, growth hormone, and IGF-1 in promoting muscle gain are discussed in Sections 8.2 and 8.3; this is followed by a discussion of the synthetic bioactive peptides that promote growth hormone secretion in Section 8.4. Ghrelin, the first natural growth hormone secretagogue, is discussed in Section 8.5. Finally, in vivo applications and human clinical trial data related to the use of bioactive peptides to promote muscle growth are reviewed in Section 8.6.

8.1.1 Anabolic–Catabolic Imbalance

It is well established that levels of anabolic hormones decline as a result of illness and stress, while catabolic hormones increase in concentration (Table 8.1). The resulting anabolic–catabolic imbalance can lead to serious consequences (Section 1.2.4) for cardiovascular disease patients.[1,2] As discussed elsewhere, proinflammatory cytokines are also implicated in the weight loss response to illness. The evidence suggests that clinicians may intervene in some of the processes leading to anabolic dysfunction using a range of bioactive peptides.[3–6]

8.1.2 Anabolic Dysfunction Allied to Nutrient and Hormone Resistance

Leucine stimulates muscle growth (Chapter 3, Section 3.5.3) but this effect is impaired in the muscles from aging rat.[8,9] The muscles of aging humans are also more resistant to the anabolic effects of leucine; Guillet et al. found that the baseline rate of muscle protein synthesis was lower in 72 year old men compared to younger (~25 year old) men.[10] Protein synthesis was stimulated by leucine administration to a greater extent in young men compared to the elderly. Apparently, aging muscles contain lower concentrations of Akt/mTOR enzymes required to stimulate protein synthesis.

Cuthbetson et al.[11] found that the basal rate of muscle protein synthesis not different for elderly and younger men. However, muscle from aging men was less

TABLE 8.1
A List of Hormones and Other Factors That Increase Muscle Gain or Loss

Factor	Protein Synthesis	Protein Breakdown	Net Effect
Hormones			
Insulin	↑	↓	Anabolic
Insulin-like growth factor (IGF)	↑	↓	Anabolic
Growth hormone (GH)	↑	↔	Anabolic
β-adrenergic agents	↑	↓	Anabolic
Glucagon[a]	↓	↔	Catabolic
Glucocorticoids (GC)[a]	↔	↑	Catabolic
Adrenaline[a]	?	?	Catabolic
Thyroid hormone (excess)	↑	↑	Catabolic
Food substrates			
Glucose	↔	↓	Anabolic
Ketone bodies	↔	↓	Anabolic
Glutamine	↑	↓	Anabolic
Arginine	↑	↓	Anabolic
Leucine	↑	↓	Anabolic
Branched-chain amino acids	↑	↓	Anabolic
Inflammatory mediators			
Cytokines	↓	↑	Catabolic
Prostaglandins	↓	↑	Catabolic
Proteolysis inducing factor	↓	↑	Catabolic
Ciliary neurotrophic factor	↓	↑	Catabolic

Source: Adapted from Baracos, V.E., *Nutrition*, 16, 1015, 2000.

[a] Stress hormones = glucagon, glucocorticoids, and adrenaline.

stimulated by essential amino acid infusion. Levels of mTOR/PKC, p70S6k, and EIF2B were reduced by 50%, 50%, and 30%, respectively, in muscle biopsy taken from elderly subjects compared to younger men (~26 years). The catalytic efficiency of mTOR/PKC was also lower by 35% in elderly muscles, judging from the rate of substrate phosphorylation after nutrient stimulation. The preceding studies were performed under the conditions of insulin and glucose "clamp" and hence the findings could be interpreted without reference to changing insulin levels. Other investigators have also reported age-related changes in the sensitivity of muscle to anabolic amino acids.[12–18]

Muscle sensitivity to anabolic hormones may be impaired though the concentration of hormones in the bloodstream remains high. Insulin resistance is a well-known

TABLE 8.2
Selected Conditions Associated with Anabolic Dysfunction

Aging associated insulin resistance[a]
Alcohol[a]
Cancer[23,24]
Chronic heart failure[a]
Chronic kidney diseases[25]
Diabetes[19]
Gender[26]
Sepsis[27,28,a]
Tuberculosis[a]
Type 2 diabetes[29,a]
Bed rest and immobilization[30]
Obesity[31]

[a] See text for citations.

feature of T2D where otherwise normal levels of this hormone fail to promote glucose uptake by the muscles. Current research suggests also that insulin is less able to prevent protein breakdown in the elderly.[19,*] Growth hormone resistance may also contribute to wasting conditions (Section 8.3.4). An early example of anabolic dysfunction or "anabolic block" was reported in connection with tuberculosis. Though sufferers of TB had the same fasting protein turnover rate as healthy subjects, the former have a weakened "growth" response to feeding and increased likelihood for muscle wasting.[20] Exposure to alcohol has also been shown to reduce muscle sensitivity to leucine.[21] Lang et al. also noted that sepsis induces a state of growth hormone and leucine resistance resulting in a reduced ability to stimulate protein synthesis.[22]

There is reason to believe that resistance to anabolic hormones and essential amino acids could be a general feature of the host response to illness. Under such circumstances, muscle wasting would be expected to develop as a result of deficits in protein synthesis over a period of time. The extent to which anabolic dysfunction may be related to growth hormone, insulin, and essential amino acids is not entirely clear at present. In principle, there could be a singular "anabolic resistance" arising from increasing proinflammatory status observed during illness (Table 8.2). If so, then targeting the inflammatory state (Chapter 7) would be expected to improve hormone sensitivity. Insulin and growth hormone resistance are discussed further in Sections 8.2.3, 8.2.4, and Section 8.3.4.

8.1.3 Protein Synthesis and Breakdown during Illness

Millward et al.[32] suggested that muscle wasting arises mainly from deficits of protein synthesis rather than from increases in protein breakdown.[†] Chemically induced diabetes, removal of the pituitary, and starvation were shown to lead to 20%–80% decreases in the rate of protein synthesis, whereas the rate of protein breakdown decreased in all cases. The exception was glucocorticoid treatment, which produced a clear increase in the rate of protein breakdown.[32,‡] More modern studies of protein turnover (Chapter 4) suggest that muscle loss can arise during illness from either a decreased rate of protein

* See Section 8.2.

[†] The authors also reasoned that the release of BCAA from muscle tissue during stress made it unlikely that these same amino acids are involved in the regulation of muscle protein synthesis. This paradox can be resolved by the fact that leucine stimulates muscle protein synthesis but not if the other EAA are limiting.

[‡] In any event, there was little information available about the processes leading to protein breakdown in the 1970s. In general, attempts to avoid muscle wasting tended to focus on methods for improving muscle protein synthesis.

TABLE 8.3
Effect of Various Conditions on the Rates of Protein Metabolism[a]

Condition	Protein Breakdown	Protein Synthesis	Oxidation
Preterm infants	↑	↑	?
Aging	↓	↓	↓
Burns injury	↑	↓	?
Cancer	↔	↓↓	↑
Chronic renal failure	↔	↓	↑
Diabetes	↑	±	↔
Diabetes (T2D)	↑	±	
Feeding	↑	↑	↑
HIV/AIDS	↑	±	?
TB	↔	↔	↑

[a] Summarized from Section 4.4. Protein break down or synthesis can show an increase (↑), decrease (↓), severe decrease (↓↓), or no change (↔) during illness. Controversial responses (±), No data (?).

synthesis and/or from an increased rate of protein breakdown (Table 8.3). Moreover, muscle loss may occur even if the rates of protein synthesis and/or breakdown are both increased, so long as the rate of breakdown exceeds the rate of synthesis; of course, these arguments do not take account of changes in the rate of amino acid oxidation (Chapter 4). Much more insidious perhaps, it has been found that deficits in muscle accretion may occur due to a decreasing sensitivity of muscles to anabolic stimulus.

8.2 INSULIN AND MUSCLE PROTEIN METABOLISM

Patients suffering from Type 1 diabetes (T1D) show generally lower muscle mass and higher waste-to hip ratio compared to controls.[33] Low blood insulin levels in T1D patients is thought to account for the generally higher rates of protein breakdown. Early studies suggested (erroneously as it turned out) that T1D does not adversely affect the rate of tissue protein synthesis.[34–36] It is now agreed that insulin therapy can help prevent muscle wasting associated with untreated T1D.[37,38] Evidence from animal studies suggest that insulin stimulates the rate of protein synthesis where dietary amino acid supply is adequate.[39,40] Insulin therapy alone produces only a modest increase in protein synthesis compared with treatments using insulin plus amino acid. The general view is that insulin functions as permissive hormone, low levels of which are required for the anabolic effect of amino acids.[41–43]

The effect of insulin levels on the muscles of T2D patients remains a controversial topic. Insulin levels in T2D patients are similar to those found in

healthy controls. However, it is also well established that T2D patients show insulin resistance. Past research suggests that insulin treatment may improve glucose status in T2D, but that there is no effect on muscle protein accretion.[44] Leucine, phenylalanine, and tyrosine fluxes were found to be similar for T2D and weight-matched controls. Non-treated T2D patients had similar blood profiles for hormones (C-peptides, cortisol, epinephrine, glucagon, free and total IGF, and IGF-BP3), with exception of lower growth hormone ($P = 0.045$) and IGF-2 ($P = 0.025$) levels compared to controls. In one small study, treatment of T2D patients (4 males, 4 females: age 58 years) with insulin for 11 days produced no significant changes in muscle protein turnover compared to weight-matched controls. There was a slight decrease in leucine flux in T2D but no changes in rates of leucine incorporation into a variety of muscle proteins within mitochondrial or cell cytoplasm. Up until 10 years ago, the consensus was that glucose metabolism in T2D is sensitive to exogenous insulin compared to protein metabolism.[45–47] More recent data on the effect on insulin resistance on muscle protein balance is reviewed in Section 8.2.3.

8.2.1 Insulin Signaling

Insulin signaling is briefly discussed in this section and the relation with insulin resistance briefly outlined.[48–50] Hormone binding to the insulin receptor sets off a self-phosphorylation process catalyzed by the receptor-linked tyrosine kinase. The activated receptor modifies specific tyrosine residues of insulin receptor substrate (IRS). Activated IRS then recruits so-called SH domain proteins, one of which is a regulatory subunit of phosphoinositol phosphate 3 kinase (PI3K), which catalyzes the formation of PIP3 (3,4,5, phosphoinositol). It is thought that PIP3 acts as a secondary messenger to promote the three major effects of insulin treatment (Figure 8.1): (a) Increased glucose uptake. PIP3 stimulates protein kinase C (PKC) that is required for the transfer of glucose transporter protein (GLU4) from cell vesicles to the outer cell membrane. (b) Insulin shift of fatty acid metabolism from lipolysis toward increased synthesis. PIP3 stimulates FA synthesis. (c) Insulin increases protein synthesis. PIP activates the Akt/mTOR cascade (Section 5.6) leading to the stimulation of protein translation.[51,*]

Insulin can also reduce the rate of protein breakdown via the Akt-mediated deactivation of the Foxo transcription factor (Figure 8.1; and cf. Section 5.5.4). Binding Foxo protein to the appropriate DNA segments activates genes related to muscle breakdown, cell cycle arrest, and apoptosis. Foxo is activated by the absence of insulin, IGF-1, and by low nutrient availability.[52] In contrast, insulin and IGF-1 signaling lead to Foxo inhibition and mTOR stimulation, which are both events that are expected to support muscle growth. Low levels of IGF-1 stimulate muscle atrophy "by default" due to decreased activation of PI3K/Akt.[53–55] It is also thought that Foxo is involved in insulin resistance; according to this model, high levels of insulin lead

* A detailed diagram of the insulin receptor signaling can be found at the Kegg Web site at http://www.genome.jp/kegg/kegg2.html

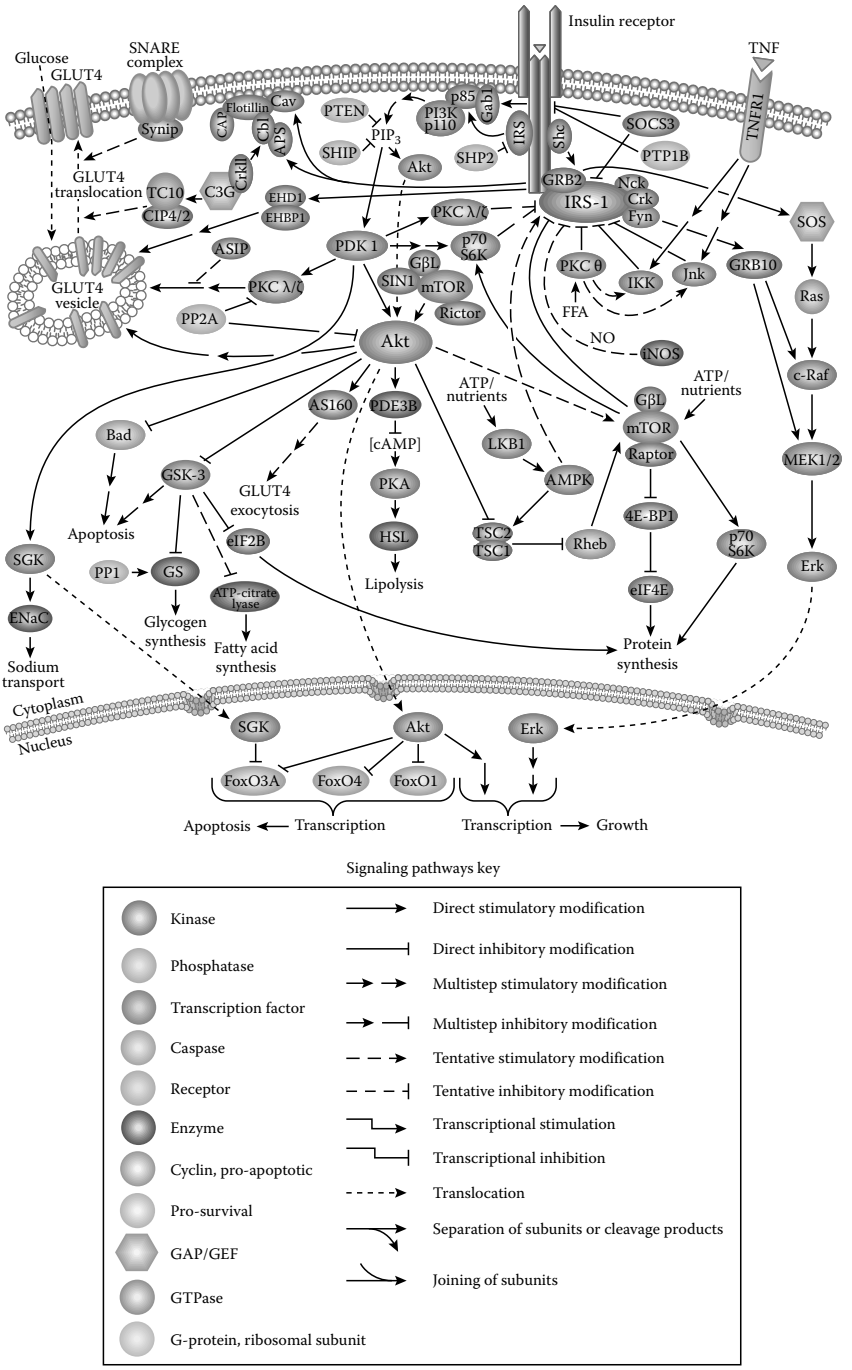

FIGURE 8.1 Insulin receptor signaling pathway. (Courtesy of Cell Signaling Technology, Inc., Danvers, MA, www.cellsignal.com. With permission.)

to the inhibitory phosphorylation of Foxo, which reduces the expression of insulin receptor, leading to decreased insulin signaling.[56,*]

8.2.2 Insulin Resistance of Glucose Metabolism

Patients suffering from T2D show insulin resistance characterized by normal insulin levels combined with elevated blood glucose or hyperglycemia.[57] Intensive care patients have also been shown to have a high incidence of insulin resistance and hyperglycemia related with the increasing severity of their illness.[58] High blood glucose is associated with a range of adverse effects in ICU patients including a higher rate infection, increased length of stay in hospitals, and significant rises in mortality. Other conditions associated with insulin resistance include, HIV/AIDS,[59–61] hepatitis C infection,[62] cystic fibrosis,[63] obesity,[64] burns injury,[65,66] surgery,[67] critical illness,[68–70] and aging.[71] It is thought that inflammation provides a possible link between these diverse illnesses and insulin resistance.[72–75] Accordingly, some very well-known anti-inflammatory agents such as aspirin have can improve insulin sensitivity.[76] Several treatments currently under development for improving insulin sensitivity may also be useful for avoiding muscle loss, for example, physical activity[16] and the use of chromium compounds.[77,78] So far anti-inflammatory peptides (Chapter 6) have yet to be tested for their effect on insulin resistance.

According to current models, insulin resistance results from the aberrant phosphorylation of serine residues of IRS1 and ISR2 from muscle and liver (respectively) in a manner that interferes with their activation by insulin signaling (Figure 8.2). IRS is deactivated by kinases (IKK, JNK, PKCθ, and mTOR), which are stimulated by dietary fatty acids, amino acids, or proinflammatory cytokines. For instance, excess dietary amino acids appear to induce insulin resistance via mTOR/S6K1 kinases leading to ISR1 phosphorylation at Ser 636 and Ser 639.[79,80] As support for this view, rapamycin inhibitor for mTOR improves insulin sensitivity.[81,82] In obese mice, ISR1 phosphorylation at Ser 1101 was linked with muscle insulin resistance mediated by high-fat foods and diets containing high amounts of amino acids. Mice carrying mutations in ISR1 serine residue were less affected by high-fat-diet-induced insulin resistance.[83,84]

Insulin resistance of glucose transport has been demonstrated in response to excess dietary lipids, carbohydrates, and amino acids.[83,85–87] Treating cultured cells with amino acids results in decreasing uptake of the non-metabolized glucose analogue, 2-deoxy-glucose (H^3-glucose) by 30%–55%, compared to cells not exposed to amino acids.[88] Selected amino acids were found to induce insulin resistance (e.g., Leu, His, Met, Cys, Thr, and Tyr) while others (Ile, Val, Arg, Lys, Phe, Tyr, and Gln) had no effect. The induction of insulin resistance by amino acids requires mTOR/p70S6k modification of key serine residues for IRS-1 as discussed above. Somehow we need to reconcile the effect of excess amino acids on insulin resistance with the effect of these agents on muscle building.

* See Sections 5.5.4 and 6.5 for an extensive discussion of Foxo transcription factors and their role in muscle atrophy. Foxo transcription factors control the expression of over 300 genes including atrogin-1 and 4EBI.

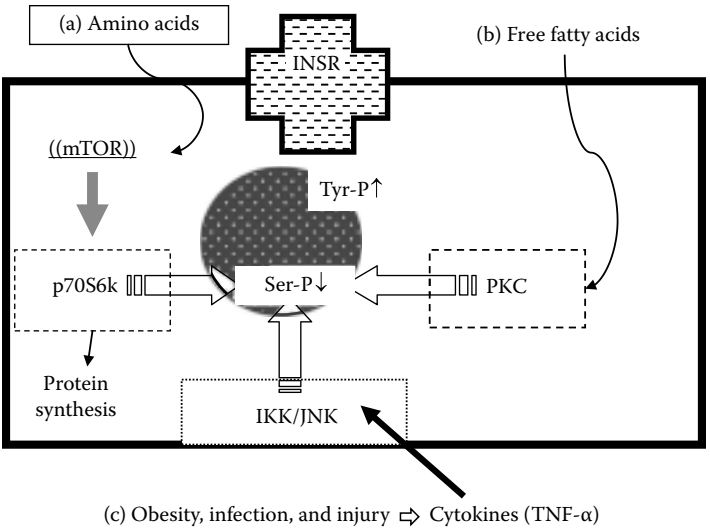

FIGURE 8.2 Inhibition of insulin receptor (INSR) signaling by (a) amino acids, (b) fatty acids, and (c) tumor necrosis factor alpha (TNF-α), leading to insulin resistance. Normal receptor function requires phosphorylation of insulin receptor substrate (IRS-1) at a tyrosine residue. Insulin resistance results from phosphorylation at serine residue of IRS catalyzed by IKK, JNK, and p70S6k.

8.2.3 Insulin Resistance of Muscle Protein Metabolism

As noted previously, the effect of insulin resistance on muscle protein metabolism in T2D patients is controversial.[89,*] There is growing evidence that insulin resistance contributes to muscle wasting. Diabetic (db/db) mice show insulin resistance along with markedly decreased muscle mass and elevated blood insulin levels (19.9 vs. 1.31 ng/mL), elevated blood glucose (408 vs. 124 mg/dL), and increased body weight (41.2 vs. 24.9 g) compared to normal mice.[90] Muscle atrophy in db/db mice was ascribed to protein breakdown (28% increase cf. normal mice). Furthermore, muscles from db/db mice had increased proteasome chymotrypsin-like peptidase activity ($P < 0.05$; $n = 6$) and a higher concentration of the 14 kDa actin fragment produced by the caspase-3 breakdown of muscle protein. There were also increased glucocorticoid levels in db/db mice that may account for the increased levels of proteolysis. The muscles of db/db mice also weighed significantly lesser than their wild-type counterparts, and showed increased intracellular proteolysis mediated by caspases and ubiquitin proteasome enzymatic activities. Finally, db/db mice showed abnormalities in the insulin signaling pathways characterized by increased phosphorylation of Ser307 of IRS1 (previous section) and decreased activity of PI3K/Akt system. Treating mice with insulin sensitizers (e.g., rosiglitazone) improves muscle accretion. The derangements in muscle protein metabolism for db/db mice were interpreted in terms of elevated TNF-α, IL-6, and glucocorticoid levels.[89]

* See Section 4.4.6 for further discussions.

Chevellier et al. reported fairly incontrovertible evidence linking insulin resistance for glucose disposal and insulin resistance of muscle protein flux by comparing the effects of artificially elevated insulin (clamp) on young vs. aging subjects,[31] lean vs. obese subjects,[64] or males vs. females.[26] In general, the *relative* increases in glucose uptake between the different groups in response to rising insulin levels were closely matched by changes in muscle protein synthesis. Insulin was also found to produce decreased levels of stimulation of glucose uptake and amino acid disappearance in the elderly, obese, or male subjects consistent with higher levels of insulin resistance.

To summarize, glucose intolerance leads to subtle changes in muscle response to insulin depending on gender, level of obesity, and dietary composition.[91] Obese-T2D patients were found to have higher muscle protein turnover and decreased net protein accretion compared to lean subjects. According to one analysis, 40% and 60% of the variation in protein metabolism in T2D patients could be ascribed to insulin resistance related to fat mass and fat free (muscle) mass, respectively.[92] Pupim et al.[93] found that hemodialysis patients suffering from T2D had significantly higher rates of muscle protein breakdown compared to non-diabetic hemodialysis patients (137 ± 27 vs. 75 ± 25 μg/min/100 mL leg volume). By comparison, the rate of skeletal muscle protein synthesis was the same for diabetic and non-diabetic hemodialysis patients accounting for the more negative nitrogen balance (–59 + 4 vs. –9 + 6 μg/min/100 mL leg volume) in the former group. Reed et al. showed that HIV+ T2D patients have increased glucose intolerance as compared to HIV+ patients, as expected. In addition, the former group showed increased levels of whole body protein breakdown despite a twofold higher plasma insulin concentration. Both HIV+ and HIV+ T2D patients responded to further exogenous insulin.[60]

8.2.4 Insulinotropic Bioactive Peptides

Agents that stimulate insulin secretion could be useful for inducing muscle growth. Insulin secretagogues are agents, other than glucose, that stimulate insulin production. Glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are naturally occurring insulin secretagogues or incretins that have received a great deal of attention recently for therapeutic application in T2D.[94,95] Theoretically, insulin secretagogues could be useful for the maintenance of muscle mass and the prevention of muscle wasting.* Protein foods stimulate insulin secretion in a manner that is unrelated to their carbohydrate content. The insulinotropic action of dietary protein has been studied in relation to dietary management of T2D[96] and the possible use of high-protein diets for weight management.[97] Furthermore, dietary amino acids and proteins are known to stimulate muscle building in a manner related to the rise in plasma insulin response.

It is well established that the insulin response to carbohydrate foods is related to their glycemic index (GIn) or ability to increase plasma glucose.[98] However, only ~28%

* Whether the satiating effect of insulin secretagogues, useful in the prevention of obesity, is incompatible with their use for nutritional therapy for muscle wasting diseases will be considered at the end of this section.

of variation in insulin secretion is related to GIn while ~10% variation in the insulin response is related to protein and fat content. High GIn foods produce a more rapid/higher increase in blood glucose and insulin levels compared to low GIn foods.[99] It has been suggested also that high GIn foods are associated with greater risk of cardiovascular diseases, obesity, and cancer though these effects may be important in individuals with BMI > 23.[100] Low GIn foods may have health benefits associated with the ability to induce prolong tissue "insulinization," sustained reduction in circulating free fatty acid concentrations, decreased counter-regulatory hormone (e.g., glucagon) secretion, reduced blood cholesterol, and increased circulating HDL.

According to a critical review by Py-Sunyer,[101] the GIn (and by implication the insulinotropic effect) of different foods is affected by the physical state of the food (e.g., particle size), processing methods, preparation techniques, and interactions with other dietary macronutrients (e.g., protein and fat). Factors that affect gastric emptying rate also moderate the apparent GI. Relations between GI and diseases such as obesity and insulin resistance are also thought to be controversial. Overall, the effect of different foodstuffs on insulin secretion is poorly understood and an estimated 78% variation of the insulinotropic effect of different foods is currently unaccounted for.

The effect of different dietary protein amounts and type on insulin secretion has received considerable attention. Krezowski et al.[102] showed that ingestion of 50 g protein (cooked lean hamburger) with 50 g glucose stimulated insulin secretion by an extra 28% compared to the effect of glucose alone. The effect of protein and glucose were additive. Protein consumption stimulated glucagon secretion but glucose did not, and consequently the combination of glucose and protein delayed glucagon increase. Gannon et al. showed that feeding T2D patients with 50 g glucose in addition to 25 g of various proteins lead to increases in insulin secretion according to protein type with the largest increases being obtained with cottage cheese and least with egg protein[103]; the insulinotropic effects of different protein foods can be ranked per 1000 kJ servings as: beef > fish > lentils > cheese > beans.[98] Van Loon et al. showed that the insulin response to dietary amino acids, oligopeptides, and proteins (consumed with glucose) was related to the combination of leucine, phenylalanine, and arginine content.[104]

Recent investigations suggest that whey protein (WP) is more insulinotropic than casein, possibly related to its rate of digestion. Calbert et al.[105] showed that supplementation of test meals with milk protein, pea, soybean, or WP hydrolysates led to higher insulin responses for hydrolysates that produced faster rises in plasma absorption of amino acids. Nilsson et al.[106] described the insulinotropic effect of reconstituted milk, whey, cod, and wheat gluten in terms of the rate of release of insulinotropic amino acids (BCAA and lysine) and stimulation of GIP. Nilsson et al.[107] found that a mixture of leucine, isoleucine, valine, and threonine could evoke the same insulin response observed with WP supplementation. However, unlike WP, the BCAA did not lead to changes in plasma GIP or GLP-1 concentration. Tessary et al.[108] accounted for the enhanced insulinotropic effect of WP compared to casein in terms of fast–slow protein concept; apparently WP being a fast protein induced an increase in insulin response. The study showed also that supplementation using WP (or free amino acids equivalent to casein) produced an elevated insulin response related to increasing plasma leucine that

was not observed to the same extent with intact casein. Clasessens et al.[109] noted that supplementation of flavored carbohydrate-free drinks with soybean protein hydrolysates produced a lower insulin response compared to intact soybean protein isolate (SPI) but that there was no difference between the insulinotropic effects of intact versus hydrolyzed WP; the differences in insulin response between soy protein hydrolysates and intact proteins disappeared at high doses of protein. For different types of proteins tested, increasing protein intakes resulted in greater glucagon and insulin response, though the former was more prominent. Aspects of this result is contrary to data reported recently by Power et al. who found that WP hydrolysates have a 45% greater insulinotropic effect compared to intact WP.[110] In conclusion, there is substantial evidence that dietary proteins and amino acids stimulate insulin production. The insulinotropic effect appears to be related to the content of BCAA and other insulinotropic amino acids (arginine and threonine). WPs also enhance levels of circulating GIP, which functions as an incretin, though the underlying mechanism is uncertain. One intriguing possibility suggested recently is that whey-derived peptides may function as competitive inhibitors for dipeptidyl peptidase IV (DPPIV), thereby preventing the inactivation of serum GIP; more research is needed to determine if this mode of action can be found in mice and humans.[111,112] Little direct work has been done on the effect of insulinotropic agents on muscle protein synthesis. Another area requiring further research is the possible effects of satiety-promoting action of dietary WPs on their anabolic action. For patients suffering from muscle wasting and anorexia, it would be crucial that the supplementation with dietary proteins does not exacerbate the tendency toward under-eating (see Chapter 9).

8.3 GROWTH HORMONE AND IGF-1

Investigations from the 1920s showed that alkaline extracts from pituitary could stimulate growth in rats. Early evidence that growth hormone treatment reduces carcass fat was reported in the 1930s[113] (Figure 8.3). Growth hormone treated rats showed a higher weight gain by 23% (net 67 g) of which 13.5 g was protein. Crude growth hormone preparations could increase muscle mass in rats by 47%. Purified growth hormone was first prepared by Professor Cho Hao Li from the Department of Biochemistry, the University of California at Berkeley, who reported its full sequence in 1970. Growth hormone was cloned, leading the widespread availability of recombinant growth hormone (rhGH) from 1985.[114]

Growth hormone and IGF-1 are the main hormones involved in control of growth.[115] Growth hormone is produced in the anterior pituitary gland under the influence of growth hormone releasing hormone (GHRH). On the other hand, growth hormone is under negative regulation by somatostatin produced from the hypothalamus. According to the somatomedin hypothesis, the biological effects of growth hormone are mediated by IGF-1 produced by the liver. The theory has been modified recently to allow for the direct effect of growth hormone on body tissues though growth hormone and IGF-1 action are still interlinked.[116–118] The so-called growth hormone-IGF axis has therapeutic potential related to treatment of catabolic conditions.

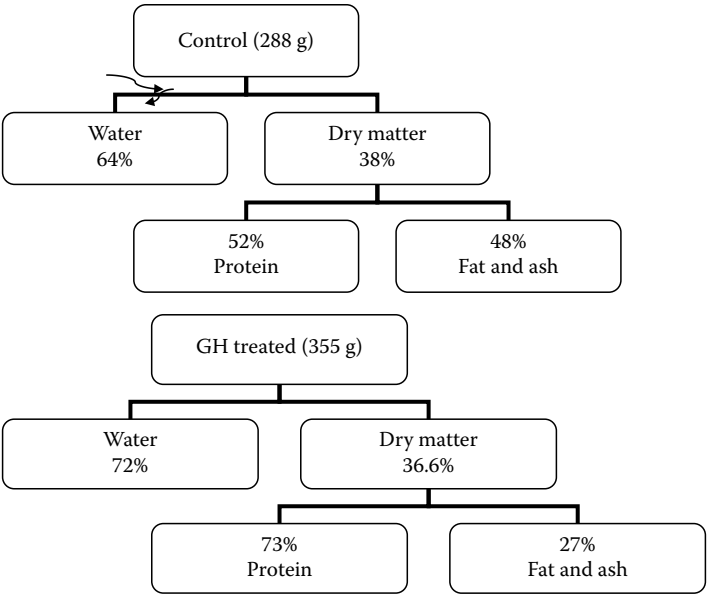

FIGURE 8.3 Effect of growth hormone treatment on the body composition of rats. Top and bottom panels show response in the control and treatment groups. (From Jenkins, R.C. and Ross, R.J., *Baillieres Clin. Endocrinol. Metab.*, 12, 315, 1998.)

8.3.1 Properties of Growth Hormone and IGF-1

Growth hormone is a single polypeptide (191 amino acids) with 2 interchain S–S bonds. The synthesis of growth hormone occurs within somatotroph cells that form about 50% of the pituitary mass. Growth hormone is initially stored in the form of polymeric granules and released at a rate of 500–875 μg/day. The pattern of release pulsates during the day with the highest levels observed after meals, exercise, or during deep sleep. The release of growth hormone is stimulated by fasting, estrogen treatment, insulin treatment, and also by amino acids, particularly arginine. Inhibitors for growth hormone production include high blood glucose, high serum fatty acids, obesity, high levels of glucocorticoids, and also late pregnancy. Growth hormone is cleared from the plasma with a half-life of about 20–30 min.[124–126]

8.3.2 Growth Hormone Receptor Signal Transduction

Liver cells respond to growth hormone stimulation by producing IGF-1. High concentrations of growth hormone receptor (GHR) occur within liver. Lower concentrations of GHR also occur in the cells of muscle, kidney, bone, and mammary gland. Binding of growth hormone to GHR leads to receptor dimerization and recruitment of the intracellular kinase, JAK2. Activation of JAK leads to further intracellular signaling including activation of IRS-1, MAP kinases, PKC, Ras-linked GTPAses, and the signal transducer and activation for transcription (STAT1, STAT3, and STAT5) proteins. STAT then relocates to the nucleus where it activates genes related to cell

cycle control. Most notably, growth hormone stimulation increases IGF-1 production at the liver. The GHR appears to be switched off by a process of endocytosis wherein the receptor is gathered over so-called coated pits and then internalized into cells where it is ultimately digested. GHR regulation is also linked with a family of proteins, suppressors of cytokine signaling (SOCS), apparently involved in growth hormone synthesis. SOCS gene knockout mice exhibit gigantism. SOCS inhibit growth hormone signaling by binding to JAK2 or the GHR or by facilitating proteasome breakdown of internalized GHR.[119]

8.3.3 Biological Effects of Growth Hormone/IGF-1 Axis

The effects growth hormone are thought to be mediated by IGF-1 (cf. Section 8.3.1). IGF-1 binds to a dedicated IGF-1 receptor as well as the insulin receptor. However, the degree to which each type of receptor contributes to growth effect of IGF is uncertain. The IGF system comprises three proteins (IGF-1, IGF-2, and IGF-3) and six binding proteins (IGFBP1–6). Circulating IGF-1 is bound to IGFBP3, which extends its half-life in the blood but also limits its bioavailability. The effects of growth hormone/IGF-1 in mammals are well known (Table 8.4). Defects in synthesis lead to growth hormone deficiency associated with dwarfism whereas excess production leads to gigantism in humans. The administration of recombinant porcine growth hormone or bovine growth hormone to cattle (100 μg/kg/day) increased livestock production efficiency from 13% to 30%. Growth hormone–treated animals show decreased fat accretion (80%), increased lean protein accretion (70%), and decreased food intake by about 10%–15%. (see Etherton 2004 for a recent review). The anabolic effects of growth hormone treatment in animals has been replicated in humans.* The hormone has been shown to produce a diverse range of effects leading to therapeutic applications in the clinical area.

8.3.4 Growth Hormone Resistance

Tissue sensitivity to growth hormone is reduced during infection, injury, and a variety of chronic conditions including cancer,[120] heart failure,[121,122] kidney failure,[123,124] HIV/AIDS,[125] sepsis,[22] and critical illness.[126] Indeed, most wasting diseases appear to be linked with growth hormone resistance.† Plasma concentrations of growth hormone are found to be normal or elevated whereas the concentration of IGF-1 is lower than expected. In principle, growth hormone resistance arises from three effects: (a) reduced numbers of growth hormone receptors in the liver and peripheral tissue. Levels of GHR, assayed indirectly in terms of plasma concentration of soluble growth hormone serum protein, decreases with some diseases. (b) Overexpression of IGFBP3. Circulating IGF-1 partitions between free and bound forms. Expression of IGFBP3 may enhance the fraction of IGF bound, leading to decreased bioavailability. On the contrary, a decrease in IGFBP3 could reduce the serum lifetime of IGF-1. (c) Post receptor signaling defects. Growth hormone/IGF-1 signaling involve the JAK/STAT pathway.

* GH has been consistently studied over the past 90-years leading to a vast and diverse literature. A recent ISI web of sciences search using "growth hormone" in the title field produced 31,500 publications and 820 reviews. A PubMed search showed 723 clinical trials related to GH.

† Thus, GH resistance shows parallels with insulin resistance in terms of causes and possible consequences.

TABLE 8.4
Multiple Effects of Growth Hormone Treatment in Farm Animals

Skeletal muscle (*growth*) *accretion*
↑ Protein synthesis
↑ Amino acid and glucose uptake
↑ Efficiency of lysine utilization
↑ Muscle fiber length
↑ Muscle fiber diameter
↓ Protein degradation
↑ Total body nitrogen

Bone (*growth*)
↑ Mineral accretion
↑ Tissue growth
↑ Chondrocyte proliferation
↑ Osteoblast proliferation
↑ Type 1 collagen synthesis

Intestine
↑ Absorption of Ca, P
↑ Vit. D3 stimulation of Ca uptake
↑ Calcium-binding protein

Liver
↑ Glucose output
↓ Insulin inhibition of gluconeogenesis

Mammary tissue (*lactation*)
↑ Synthesis of milk
↑ Uptake of nutrients for milk synthesis
↑ Activity per Secretory cell
↑ Maintenance of Secretory cells
↑ Blood flow for milk synthesis

Adipose tissue and fat mass
↓ Glucose uptake and oxidation
↓ Lipid synthesis
↑ Basal lipolysis negative energy balance
↓ Insulin stimulation of glucose metabolism
↓ Insulin stimulation of lipid synthesis
↑ Catecholamine-stimulated lipolysis
↓ Ability of insulin to inhibit lipolysis
↓ Glucose transporter-4 translocation (?)
↓ Transcription of fatty acid synthase gene
↓ Adipocyte anabolism
↑ IGF-1 mRNA abundance

Systemic effects ↑IGF-1 and IGFBP3
↓ IGFBP2
↑ Acid-labile subunit
↓ Amino acid oxidation
↓ Blood urea nitrogen
↓ Glucose clearance
↓ Glucose oxidation
↓ Response to insulin tolerance test
↑ Free fatty acid oxidation
↑ Cardiac output consistent with increases in milk output (lactation)
↑ Enhanced immune response

Immune modulation
↑ B cell proliferation
↑ T cell proliferation
↑ Natural killer cells
↑ Macrophage activity
↑ Immunoglobin production

Source: Adapted from Etherton, T.D., *J. Anim. Sci*., 82, E239, 2004. With permission.
Note: Increases in parameters designated by ↑.

Bjarnasson et al.[127] found that surgery produces ~100% decrease in muscle IGF-1 gene expression while serum IGF-1 concentration (derived from the liver) remains unchanged. There was also no change in GHR gene expression or circulating concentrations of the soluble GHR protein following surgery. Patients treated with daily subcutaneous injections of growth hormone (and parenteral nutrition) prior to surgery did not loose muscle IGF-1 gene expression. Of particular notice is the suggestion that growth hormone resistance in the liver and muscle may not occur simultaneously. Lang et al. suggested that growth hormone resistance in the liver was mediated by proinflammatory cytokines and the defective phosphorylation of the JAK/STAT5 pathway. In contrast, muscle growth hormone resistance was likely to involve the MAPK/JUNK pathway.[128]

8.4 GROWTH HORMONE SECRETAGOGUES

8.4.1 Growth Hormone Release Peptides

Agents that increase growth hormone release may have application for promoting muscle mass. The first growth hormone release peptides (GHRP) were developed in the US during the 1980s.[129–131] GHRP are synthetic growth hormone secretagogues (GHS) that are compounds that stimulate growth hormone production. Perhaps the best known synthetic GHS is the hexa-peptide GHRP-6, though others have been developed as summarized in Table 8.5 and in the following reviews.[132–137]

GHRP can be administered intravenously by subcutaneous (SC) injection, by intranasal administration, or orally.[138]* Enterally administered GHRP-6 was found to have bioactivity of 0.7% compared with the same of dose of peptide applied by injection. Intra-duodenal administration was more efficient than gastric delivery. Monkeys were highly sensitive to GHRP-6, with an oral dose of 0.3 mg/kg able to elicit growth hormone release. The oral bioavailability of GHRP-6 was about 1% in monkeys, suggesting that noninvasive applications were a possibility. Intranasal administration of GHRP-6 in conscious dogs (0.25–0.5 mg/kg) resulted in a bioavailability of 34.445% compared to 100% for intravenous (IV) injection. The peak plasma concentration for GHRP-6 ranged from 11 ± 4.8 to 28.6 ± 8 μg/L.[139] GHRP-6 (0.253 μg/kg (IV)) produced a concentration-dependent increase in plasma growth hormone levels. The efficacy of GHRP in humans was independent of gender but affected by age. Growth hormone secretion in response to GHRP appears at birth, increases and peaks at puberty, and decreases with further aging. By contrast, the growth hormone response to natural GHRH declines from birth exponentially till old age.[134]

* GHRP-6 was administered by gavage (rat), intragastric administration, or intraduodenal application (dog and monkey). According to patent literature, GHRPs are suitable in various dosage forms including capsules, tablets, pills, powder, and granules containing solids compounded with inert materials (e.g., starch, sucrose, lactose, and a buffering agent); this is in fact the text book description that is applicable to classical pharmaceutical drugs. It was further suggested that GHRP-6 could be applied for oral use in the form of emulsions, solutions, syrups and elixirs, or suspensions in water with additives as wetting agents, emulsifying agents, sweetening and flavorings (see U.S. patent 4,410,513, 5,663,146, and references cited therein).

TABLE 8.5
Chronological Development of Major Growth Hormone Release Peptides

Date[a]	Structure	Name
1977	TyrDTrpGlyPheMetNH_2	(DTrp[2])MetEKNH_2
1981	TyrDTrpAlaTrpDPheNH_2	
1984	HisDTrpAlaTrpDPheNH_2	
1984	HisDTrpAlaTrpDPheLysNH_2	GHRP-6 (SK&F 110679)
1991	AlaHisDβNalAlaTrpDPheLysNH_2	GHRP-1
1992	HisD2MeTrpAlaTrpDPheLysNH_2	Hexarelin (EP23905)
1993	DAlaDβNalAlaTrpDPheLysNH_2	GHRP-2
1998	AibHisD2NalDPheLysNH_2	Ipamorelin,
	AlaHisd2methylTrpAlaTrpdPheLysNH_2	Alohexarelin

Source: Adapted from Camanni, F. et al., *Front. Neuroendocrinol*., 19, 47, 1998. With permission.

[a] Date entering public domain literature.

8.4.2 Hexarelin and Alexamorelin

Hexarelin was developed from GHRP-6 in 1992 by substitution of D-tryptophan residue of GHRP-6 with 2-methyl tryptophan. According to researchers from the University of Milan, hexarelin has improved stability, potency, and reduced toxicity compared to GHRP-6.[140,141] Clinical trials using healthy humans (6 males and 6 females; age ~25 years) showed that hexarelin can be administered via the oral (OR), IV injection, and SC injection routes with results as summarized in Figure 8.4. Depending on the route of administration, the following different doses of hexarelin can produce broadly similar profiles in humans: 2040 mg/kg (OR), ~20 μg/kg (IV), and 12 μg/kg (SC) injection.[142] The characteristics of hexaralin have been reviewed.[134–136,143–148] Example trial data is briefly discussed below.

Frenkel et al. showed that hexarelin administration for 7 days improves biomarkers for growth in short-statured children, for example, serum IGF-1 and alkaline phosphatase concentrations increased.[149] Loch et al. also reported that hexarelin (dose 2 μg/kg) stimulated growth hormone secretion in 45 short-statured children. However, growth hormone release was not improved for idiopathic growth hormone deficiency arising from anatomical lesions between the pituitary and hypothalamic glands.[150] Laron et al. also reported that IV administration of hexarelin (60 μg/kg; × 2 per day) for 8 months increased serum IGF-1 by 50% and increased linear growth rate from 5.3 (±0.8) to 8.8 (±1.7) cm/year.[151] Hexarelin treatment also decreased skin fold thickness and improved serum phosphate and serum alkaline phosphatase levels.

Eating reduces the growth hormone response to hexarelin.[152–154] Current results show that fasting may increase the growth hormone response to GHRP. Oral glucose and lipid supplements have been shown to reduce the growth hormone response to hexarelin and GHRH, though the effect is greater for the latter. The growth hormone

response was greater in fasted compared to fed dogs following hexarelin and GHRH treatment, though the difference was less for hexarelin. In a study of 12 fasted men and women (age 23–29 years), hexarelin produced a greater growth hormone response compared to results obtained with GHRH (3010 ± 695 vs. 1339 ± 281 µg/L/120 min). Following feeding, the relative responses were 1523 ± 121 vs. 309 ± 61. In other

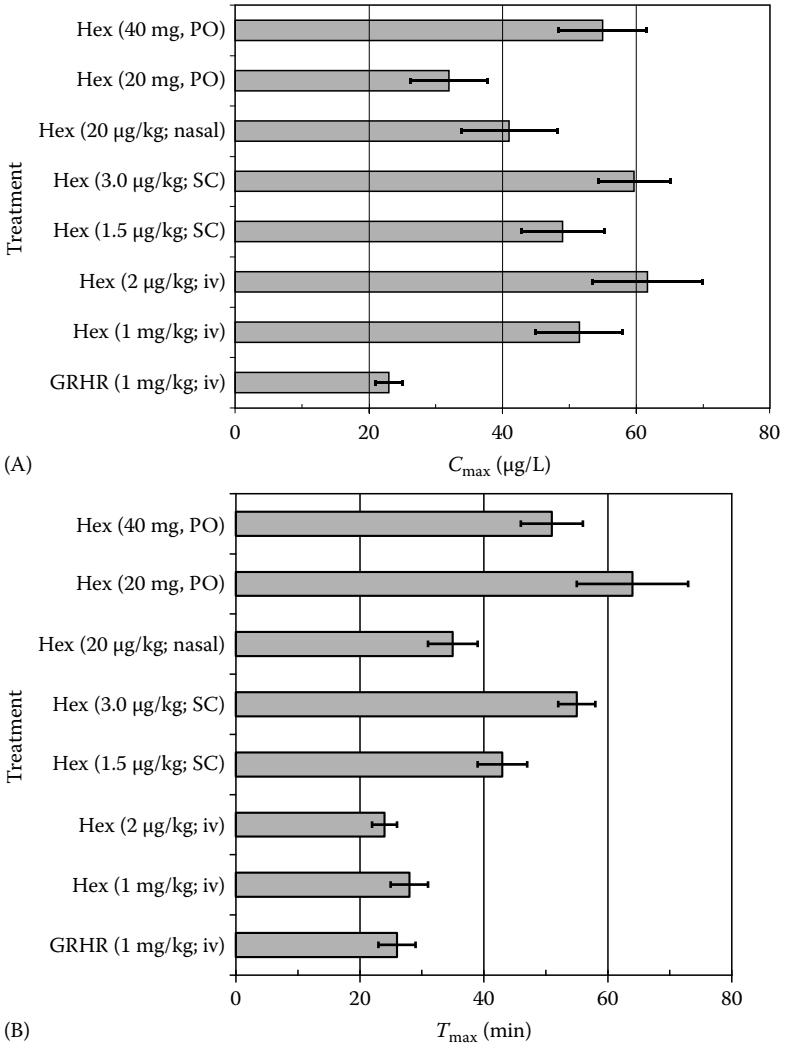

FIGURE 8.4 (A) Effect of different routes of hexarelin administration on growth hormone release-(A) serum maximum concentration (C_{max}). Notation: PO = per oral route, nasal = intranasal route, IV = intravenous route. Data is average of 6 men and 6 women. (B) Effect of different routes of administration of hexarelin on growth hormone release: Time to reach maximum concentration (T_{max}). Notation: PO = per oral route, nasal = intranasal route. Data is average of 6 men and 6 women.

(*continued*)

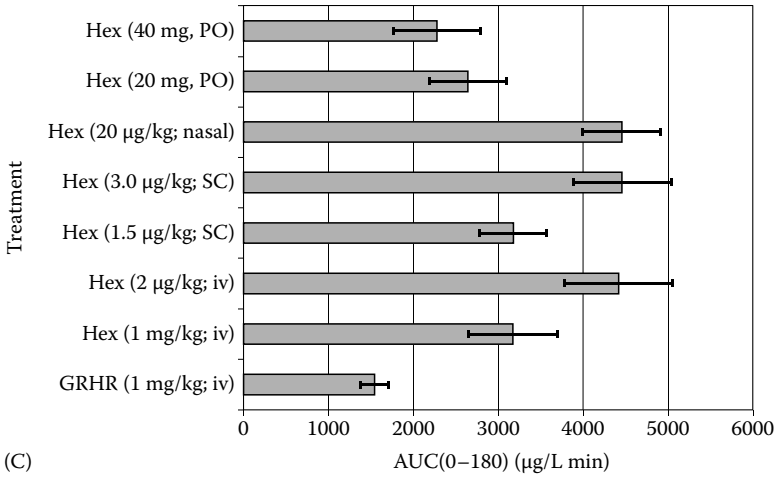

FIGURE 8.4 (continued) (C) Effect of administration routes on hexarelin growth hormone release–area under the curve (AUC; 0–180 min, (µg/L/h). Notation PO = per oral route, nasal = intranasal route, IV = intravenous route. Data is average of 6 men and 6 women. (From Ghigo et al., *J. Clin. Endocrinol. Metab.*, 78, 693, 1994.)

words, feeding reduces the growth hormone response to hexarelin and GHRH by 42% and 70%, respectively. Apparently, hexarelin therapy may be more resistant to the inhibitory effects of feeding compared to growth hormone release hormone.

Arvat et al.[155] demonstrated that IV injection of hexarelin (2 µg/kg, IV) to six healthy adults led to a greater growth hormone response compared to the response obtained with GHRH (2 µg/kg, IV). The combination of hexarelin + GHRH produced a greater growth hormone response compared to the arithmetic sum of the responses obtained with each agent alone (Figure 8.5). Pretreating participants with rhGH (2 U, IV) before growth hormone release hormone and/or hexarelin led to a decreased growth hormone response. Interestingly, rhGH feedback inhibition was greater for GHRH compared to hexarelin (87% vs. 32% inhibition). IGF-1 also exerts negative feedback inhibition of hexarelin activity. In a study reported by Ghigo et al. involving 8 women (age = 28.3 ± 1.2 years; BMI = 20.1 ± 0.5 kg/m^2), pretreatment with recombinant human IGF-1 (20 µg/kg, SC) inhibited the growth hormone response to hexarelin (2 µg/kg, IV) and growth hormone release hormone (2 µg/kg, IV) by 58% and 42% respectively.[156] Twice daily subcutaneous hexarelin therapy (1.5 µg/kg, SC) for 16 weeks led to a progressive fall in the growth hormone release following single injection monitored as AUC (µg/L/h).[157,158] Interestingly, discontinuing hexarelin therapy between 16 and 20 weeks restored the initial sensitivity to hexarelin.

8.4.3 Ghrelin

Synthetic GHRP described in Section 8.4.2 binds to the growth hormone secretagogue receptors (GHS-R), which, having no known natural ligands prior to

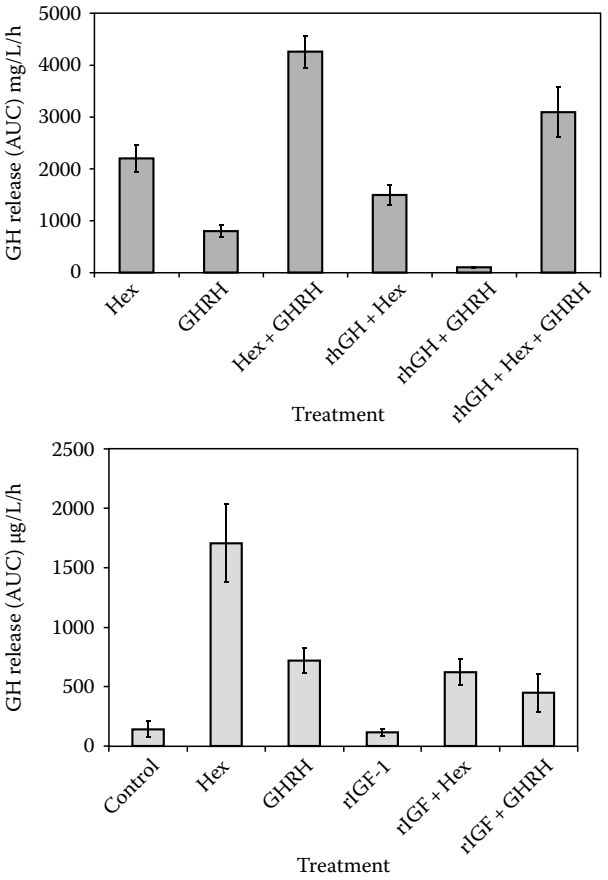

FIGURE 8.5 Feedback inhibition of growth hormone response to hexarelin. Data shows effect of pretreatment with rhGH (top), or recombinant insulin like growth factor1 (rhlGF1) (bottom). (From Arvat, E. et al., *J. Clin. Endocrinol. Metab.*, 79, 1440, 1994; Ghigo, E. et al., *J. Clin. Endocrinol. Metab.*, 84, 285, 1999.)

the 1990s, were considered to be under "orphan" status. The natural ligand for GHS-R is known to be ghrelin. Recent research suggests that GHS-R occurs in diverse tissue aside from the CNS. Ghrelin activation of the GHS-R located in the pituitary gland account for its GHS function. By comparison, GHS-R located in the arcuate nucleus is linked with the control of food intake. Host inflammatory cells express GHS-R, suggesting that ghrelin may be important for the innate immune response; these and other roles of ghrelin related to inhibition of myocyte apoptosis and vasodilatation are discussed elsewhere in the book.* The anabolic effects of ghrelin have led to suggestions for its use in treating cachexia (Section 8.6).

* For more information on ghrelin see Section 9.4 and Section 6.3.6.

8.5 LEUCINE, BCAA, AND RELATED PEPTIDES

8.5.1 Essential Amino Acids as Anabolic Agents

Supplementation using essential amino acids can improve muscle mass in the absence of detailed information concerning amino acid imbalances produced by ill health (Section 3.4.3). Volpi et al. found that an essential amino acid supplement had an anabolic effect on muscle protein synthesis as obtained using a mixture of 18 g essential amino acids and 22 g of nonessential amino acids.[159] Combining essential amino acids and carbohydrates promoted muscle protein synthesis following acute exercise (in healthy young adults) but there was no effect on protein degradation.[160] The anabolic response toward dietary essential amino acids has been demonstrated following bed rest[161] or age-related muscle wasting.[162,163] Supplementation using cysteine was beneficial during sepsis.[164] The branched-chain amino acids (BCAA) have a high muscle sparing action (Section 8.5.2). Moreover, of all the BCAA, leucine alone accounts for a large proportion of the anabolic effects of BCAA.[165] Leucine stimulates muscle protein synthesis and inhibits protein breakdown by stimulating the Akt/PKC/mTOR (Sections 3.5.2 and 3.5.6). Trial evidence for the benefits of leucine and BCAA supplementation is reviewed below.

8.5.2 The Branched-Chain Amino Acids

Recent developments in the use of branched-chain amino acids (BCAA) for supplementation were reviewed in the proceedings from the "Symposium on Branched-Chain Amino Acids" held in Versailles, France. The conference summary indicates that a major area of research up to 2005 concerned the role of BCAA in the regulation of muscle protein balance and the feasibility of treating cachexia and other wasting conditions using BCAA supplementation. Expert opinion suggested that BCAA supplementation is not justified for healthy adults based on estimates for daily BCAA requirements or possible changes in the rates of BCAA oxidation during exercise.[166] In contrast, there is a strong case for supplementation for various groups of patients to correct for decreases of plasma BCAA levels during illness. There is also a net release of BCAA from muscle and elevated utilization for oxidation or gluconeogenesis in response to stress.[167–169,*] Nutritional support using BCAA for burns, sepsis, or trauma patients has been appraised by Cynober and de Bandt.[170] According to these experts, the number of randomized placebo controlled trials of BCAA supplementation are still limited and the results are cryptic, though showing some indication of overall benefit. Past studies were also criticized on the basis of their limited power, poor study design, etc. (see Section 2.4). It is further questioned whether the BCAA-concept should be dropped all together in favor of leucine, which is the major active component within BCAA mixtures.[†] Nevertheless, it is important to

* An increase in BCAA requirements during illness provides a rationale for using this agent as medical food (see Chapter 2). BCAA supplementation is also described in Sections 3.5.2. and 3.5.6 and also in Section 4.5.3.

† The mixture of BCAA have a different bioactivity profile compared to leucine alone related to their effect on food intake. Leucine can produce a reduction in food intake whereas a mixture of all three BCAA appear to promote food intake (cf.Section 9.5.5.)

report that interest in BCAA supplementation has continued since 2006, especially in relation to liver disease, cancer, and surgical patients. Another group that appears to benefit from BCAA appear to be renal dialysis patients (Section 8.7.6). For convenience, later discussions of BCAA will be confined to those studies where leucine is not referred to as the bioactive agent. Leucine itself is discussed elsewhere.

8.5.3 Leucine

The effects of leucine on muscle protein balance were first tested in healthy young adults with the focus on athletic performance. A placebo-controlled RCT reported by Merro et al. in 1997 showed that dosing 10 track and field sportsmen with leucine (50 mg/kg/day; 10 weeks) helped to maintain blood levels of this amino acid in contrast to other amino acids that decreased by about 20%.[171] Blood leucine levels also fell by 20% for the placebo group ($n = 10$). Clearly, leucine supplementation could help maintain normal serum levels of this essential amino acid. Pitkanenm et al. later confirmed that leucine supplementation (50–200 mg/kg/day) helped to maintain serum levels during weeks of strenuous training, but that there was no significant effect on running performance.[172] Human trials by Melisa Crowe et al. from the Australian Institute of Sports found that 6 week supplementation with leucine (45 mg/kg/day) produced a significant increase in plasma levels of leucine as well as increasing upper body strength compared to controls. This study involving 10 women and 3 men found that leucine supplementation led to significant improvements in rowing performance.[173] A recent clinical trial from the University of Maastricht showed that consuming a carbohydrate (CHO) drink, or carbohydrate-protein drink, or carbohydrate-protein-leucine drink led to progressively higher rates of muscle protein synthesis (0.06%, 0.082%, and 0.095% per h, respectively).[174] A second Maastricht RCT comparing leucine effects in elderly and young subjects found no difference in the presence of carbohydrate and using free-living subjects allowed to undertake normal quota of daily exercise.[175] Apparently, healthy elderly and young subjects respond equally to the effects of leucine supplementation under circumstances resembling normal living. The finding from the Maastricht group also suggests that leucine supplementation does not affect strength and performance of healthy aging adults.[176] To summarize, research in healthy humans suggests leucine supplementation can produce a rise in plasma levels or maintain plasma levels. Supplementation results in an increase in muscle synthesis that may translate to improved performance for specific types of muscle groups. Study outcomes appear to be affected by gender, type of sports under consideration, and dietary components. More research is needed to determine whether leucine supplementation would be beneficial for the different populations of patients.

8.5.4 Whey Protein and Peptides

As noted for leucine, initial human trials of WP supplementation involved healthy, young, and highly active subjects. Indications are that WP supplementation can improve muscle mass and/or strength in young adult athletes but the results are far from unanimous.[177–179] Burke et al.[180] showed that WP supplementation (with creatine) led to significant improvements in lean body mass (compared to maltodextrin control), but

the increase in muscle mass was not translated into strength increases. Tipton et al.[181] reported that protein balance, measured in terms of phenylalanine exchange across the legs, was 58 or 66 mg/h following WP or casein compared to the placebo (–5 mg/h), but peak blood leucine levels were approximately threefold higher after supplementation with WP. Tipton et al. showed that the muscle protein response to WP was similar, whether supplement was given before or after exercise.[182] Coburn et al.[183] found that WP and leucine supplementation produced significant increases in leg muscle strength over and above those achieved with exercise alone. Post-training changes in muscle fiber cross-sectional area were increased equally in the supplemented and placebo control group. Apparently, WP had no effect on muscle size over and above improvements achieved with exercise alone. It suggested that WP and leucine supplementation produce a so-called ergogenic effect referring to improvements in muscle power/strength generation but no benefits in muscle growth. This study showed no changes in body composition during the 8 week trial. Tang et al. found that consumption of WP carbohydrate (10 g WP and 20 g fructose) beverage induced greater increases in muscle protein synthesis after resistance exercise compared to exercise-placebo group consuming a carbohydrate drink.[184] Kerksick et al.[185] showed via a 10 week double-blind RCT that a supplement containing WP (40 g/day) and casein (8 g/day) produced an increase in lean body mass for 36 trained male athletes (age = 30 +8 years; n = 10 per treatment group) following 10 weeks of resistance training. By comparison, WP supplementation with BCAA (5 g/day) or whey and glutamine (5 g/day) produced no changes in fat free mass compared to a carbohydrate placebo. It was concluded that WP-casein supplementation is highly effective in improving lean body mass and strength in resistance training but that WP per se produced no benefits beyond those seen with exercise alone. Cribb et al.[186] found that supplementation of young male athletes (24–25 years; Body mass 74–84 kg) with whey protein, creatine, or a combination of WP and creatine (1.5 g/kg/day) produced an increase in lean body mass, and higher contractile muscle content and fiber cross-sectional area over and above the effects of exercise only.

The anabolic effect of WP is only partly related to the leucine content.[187,188] The quantitative amount of leucine present in WP (~14%) is thought to be adequate to stimulate muscle protein synthesis. Consequently, extra intakes of leucine in addition to WP supplements leads to no further benefits. Amino acid balance measurements by Tipton et al. showed that whey supplementation produces similar levels of increase in leg protein synthesis achieved using WP and leucine supplements.[189] The results agree with reports from Koopman et al. that showed that addition of leucine to WP is excessive to requirements.[175] The consumption of animal milk was found to be associated with an increased rate of growth and increased serum levels of IGF-1 levels in 2.5 year old Finnish children.[190]

8.6 IN VIVO APPLICATIONS AND CLINICAL TRIALS

8.6.1 Short Bowel Syndrome

Short bowel syndrome (SBS) arises when a fraction of the colon and small intestines are surgically removed as treatment for patients suffering from various gastrointestinal diseases. SBS is characterized by limited nutrient absorption, diarrhea, and

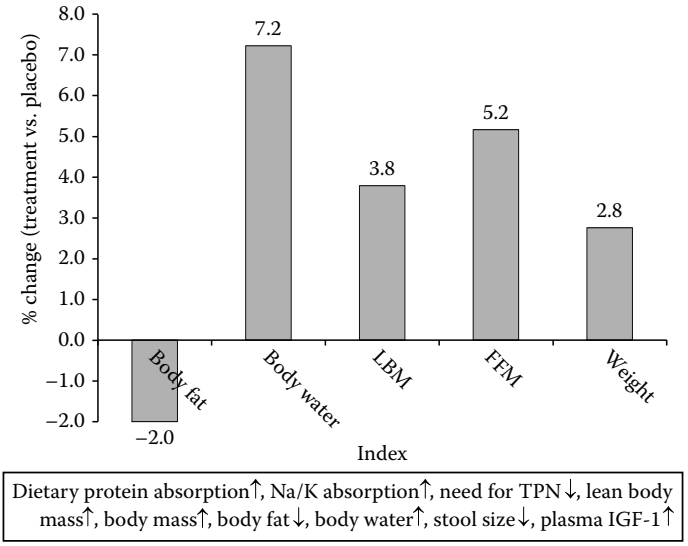

FIGURE 8.6 Effect of 8 week growth hormone therapy on body composition in ten patients suffering from SBS. Y-axis shows % change for treatment group compared with placebo. Panel shows summary of other changes. (From Ellegard, L. et al., *Ann. Surg.*, 225, 88, 1997.)

undernutrition. SBS patients undergo lifelong total parenteral nutrition (TPN), though some may be switched to enteral nutrition (EN) following partial recovery. Byrne and Wilmore proposed growth hormone therapy for SBS in 1992. In a 3 week trial using 47 patients (25 males, 22 females; age = 46; average colon length 50 cm), the growth hormone treatment regime led to improved protein and potassium absorption, decrease in stool size, and significant increases in body mass.[191,*] The study, which was not placebo controlled, was well received. The benefits of growth hormone therapy for SBS patients were later confirmed by a randomized, double-blind, placebo-controlled trial from Sweden using sixfold lower dose of growth hormone (0.024 mg/kg/day) for 8 weeks.[192] The merits of growth hormone therapy for treatment of SBS patients has been described by others[193–195] (Figure 8.6). A recent randomized, double-blind, placebo-controlled trial from Byrne et al. demonstrated that a regime led to a significant decline in the dependence of SBS patients on TPN[196].

It may be that more research is needed before the widespread adoption of growth hormone therapy for patients of SBS. Scolapio et al. reported that treatment of SBS patients for 21 days with growth hormone and a high carbohydrate diet, glutamine-supplemented diet, could produce small positive increases in body mass. However, the improvements in body mass were attributed to water retention rather than improvements in macronutrient (protein and fat) absorption.[249–251] Szkudlarek et al. found that growth hormone treatment had only modest effects on SBS patients, with

* The treatment consisted of subcutaneous applications of high doses of GH (0.14 mg/kg/day), combined with a high carbohydrate–low fat diet (60%:20%:20% energy as carbohydrates: fat: protein, respectively) and an oral/IV glutamine (0.6 g/kg/day) supplement.

any positive effect rapidly coming to an end following the end of the therapy.[252] In summary, the benefits of growth hormone therapy for SBS patients remains controversial. It has been suggested that the treatment therapy may be unsuitable for a subsection of SBS patients, which are (a) severely malnourished, (b) patients with micronutrient (e.g., Zn^{2+}, Mn) deficiency, and (c) SBS patients with modest or severe inflammatory response (e.g., IBD, Crohn's disease, etc.). For the latest commentary on the pros and cons of growth hormone therapy, see[253,254].

8.6.2 HIV Patients

HIV/AIDS is associated with muscle wasting and loss of body mass combined with the preservation of body fat.[255] Muscle wasting and attendant loss of muscle strength leads to increased risk of mortality for AIDS patients.[256] A loss of >10% of body mass is associated with twofold increased risk of mortality. Though highly active antiretroviral therapy (HAART) reduces the incidence of wasting, loss of body weight remains an independent risk factor for HIV-associated mortality. HIV/AIDS viral infection weakens the human immune system thereby increasing susceptibility to opportunistic infections. The likelihood of HIV-associated wasting is increased by factors including ethnicity (high risk for non-whites), history of drug use, CD4+ T lymphocyte count <200 cells/mL, high viral load, presence of anorexia, diarrhea, and feeling of nausea. Other common symptoms associated with HIV infection such as increased resting energy expenditure, decreased testosterone levels, and increased myostatin expression promote loss of muscle mass. Growth hormone resistance is common in HIV sufferers indicated by elevated plasma growth hormone coupled with an expectedly low IGF-1 value (81 vs. 179 µg/L for controls). Insulin concentrations tend to be lower than normal (29 vs. 237 pmol/L).

Schambelan et al.[257] found that treatment of HIV patients (n = 88) with growth hormone (0.1 mg/kg/day; average 6 mg/day) for 12 weeks led to increases in body weight (1.7 vs. 0.1 kg for placebo), increased lean body mass (3 vs. 0.1 kg), and decreased body fat (1.7 kg). Furthermore, the treadmill work output increased for the growth hormone group compared to HIV patients receiving placebo (99 vs. 22 kg/min). However, quality of life scores were not changed after growth hormone treatment, nor was the rate of disease progression measured in terms of CD4+ or CH8+ cell count or viral load. Mulligan et al. reported that improvements in lean body mass and overall weight in growth hormone-treated HIV patients occurred without a net increase in energy intake, indicating that hormone treatment alters the pattern of metabolism in favor of muscle and body weight accretion while increasing the rate of fat oxidation. These effects appear to be behind reduced visceral fat and other symptoms of lipodystrophy following growth hormone treatment of HIV/AIDS patients.[258,259] In summary, subcutaneous application of growth hormone appears to have protein-sparing action due to nitrogen retention and increased muscle protein synthesis. These results have been confirmed by numerous studies[260–265,*] (Table 8.6).

Lower dosage of growth hormone may be considered for HIV treatment in view of safety concerns (see below). Lo et al.[262] found that treatment with 3 mg growth

* Owing to space restrictions, only studies reported over the past 10 years are cited below.

TABLE 8.6
Trials for Growth Hormone Use as a Nutritional Adjuvant

Antiaging[197,198]
Arthritis[199]
Bone density[200–204]
Burns injury (and wound healing)[205,206]
Cancer cachexia[207]
Cardiac failure[208,209]
Chronic hepatitis[210]
Chronic renal failure and dialysis[211–215]
Critically ill patients[a,216–219]
Cystic fibrosis[220–223]
Elderly and aging[224–228]
GH deficiency and replacement[229–231]
Hemodialysis patients[232–235]
Hepatectomy[a,236]
HIV[b]
Inflammatory bowel disease[a,237]
Metabolic syndrome[238,239]
Multiple trauma patients[a,240]
Muscle strength, response to exercise[226]
Obesity[241–244]
Osteoporosis (see bone density)
Parenteral nutrition review[b]
Sepsis (see text)
Short bowel syndrome[b]
Surgical patients[a,127,245–247]
Undernutrition, anorexia[248]
Wound healing[206,207]

[a] GH for parenteral nutrition, otherwise administered by intravenous or subcutaneous injection.
[b] See text for further references.

hormone/day produced notable changes: increased IGF-1 concentration fourfold, while reducing the characteristic buffalo hump side effect of HIV therapy with protease inhibitors. Growth hormone therapy also reduces the levels of visceral adipose tissue, total body fat, and glucose intolerance. Lean body mass increased by an average of 5 kg over 6 months of growth hormone therapy. Though an open-label trial, the study suggests that low growth hormone doses could be considered for HIV treatment with the view of reducing side effects.[238] Growth hormone therapy was found to have no adverse effects on sleep patterns.[266] The present consensus view is that growth hormone therapy may be useful for HIV patients allowing for improved maintenance of body mass.[267]

8.6.3 Chronic Renal Failure

Chronic renal failure (CRF) is associated with protein under-nutrition attributed to anorexia, losses of amino acids during dialysis, and increased catabolic response to dialysis. Muscle wasting is associated with increased mortality in CRF patients.[268–271] A number of human trials and RCT, using rhGH to treat renal disease patients receiving maintenance hemodialysis or peritoneal dialysis, have been reported. Probably the first study of rhGH therapy on kidney dialysis patients was reported in 1991 by Ziegler et al.[276] The small pilot study included 5 elderly (~68 years old) patients (body weight ~68 kg) showed that hormone treatment produced no significant change in dietary protein intake though the rate of urea generation decreased. The biologically utilized nutrition (BUN) and protein catabolic rate also decreased, suggesting an overall decline in catabolism following growth hormone treatment.

The decrease in protein wasting in CRF patients following rhGH treatment has been confirmed by later studies (Table 8.6). Kopple et al.[233] reported that treatment of six hemodialysis patients with rhGH increased their circulating levels of IGF-1 2.25 fold and nitrogen balance 2-fold but serum nitrogen levels decreased (49.9 vs. 66.9 mg/dL). There were no changes in body weight. Interestingly, 2/6 patients showed a lack of response to rhGH therapy (see below). Pupim et al. reported that rhGH treatment improves the whole body protein balance for CRF patients by 22% (0.39 ± 0.04 vs. 0.50 ± 0.07 mg/kg FFM/min) coupled with a large decrease in the rate of essential amino acid loss during dialysis (18 ± 23 vs. 71 ± 20 mmol/L; $P < 0.05$).[234] Interestingly, Garibotto et al. recently identified a subgroup of uremic patients with inflammation that were insensitive to the anabolic action of GH.[24] Finally, the use of rhGH therapy for young prepubescent patients with/without CRF is worthy of mention. Young CRF patients treated with rhGH showed similar improvements as observed in adults as well as stimulation of linear growth rate. The general consensus appears to be that rhGH therapy ameliorates wasting associated with CRF, though the underlying mechanism has yet to be fully elucidated.

Plasma ghrelin levels are increased in CHF patients with cachexia (Section 8.4.3); in vivo trials by Nagaya et al. showed that additional IV administration of ghrelin (2 μg/kg twice daily; 3 weeks) resulted in positive outcomes for CHF patients ($n = 10$, age = 75 year old; 5 males and 5 females). Compared to placebo group, the ghrelin group showed increased lean body mass, increased food intake, and improved ventricular function compared to controls but body weight was not altered.[272] Ghrelin treatments for other muscle wasting conditions are described below.

8.6.4 Critical Illness and Sepsis

Exogenous growth hormone has a muscle-sparing effect and also promotes a positive nitrogen balance[273] even though critically ill patients exhibit growth hormone resistance.[274] An illustrative RCT is that produced by Voerman et al.[275] Growth hormone treatment for sepsis was proposed based on the immune-enhancing effect of this hormone.[276] Two clinical trials reported over the past 15 years suggest improvements in nitrogen balance following growth hormone treatment. However, the effects were

temporary and gains were lost when growth hormone therapy was discontinued. Growth hormone resistance is a feature of sepsis.[277,278] Growth hormone therapy is not recommended for critically ill patients.[219,279,280]

Several RCT reported in the 1990s suggest that GHRP-2 (in combination with thyroid stimulation hormone) could correct the GH derangements observed during some forms of critical illness. When 14 critically patients were treated with GHRP-2 for a period of five days, there were increases in growth hormone release and TSH release, and a decrease in proteolysis measured as a decline in the ratio of urea/creatinine.[281,282] Clearly, the effect of GHS on critically ill patients requires more study.

Recent research shows that insulin therapy to establish a strict control of blood glucose may improve the survival of critically ill patients. The findings are backed by several meta-analyses covering 38 related studies. Intensive insulin therapy has been found to decrease mortality of critically ill patients by about 7%. For surgical intensive care patients, the decline in mortality could be as high as 50%. Benefits of insulin therapy were also noted for surgical patients, diabetics, and patients with myocardial infarction.[283] Suggested explanations for the benefits of insulin therapy for IC patients include the following: (a) Prevention of hyperglycemia. It may be supposed that high concentrations of blood glucose may be harmful to cells. (b) Protection of mitochondrial function. (c) Improvements in serum lipids. (d) Reversal of muscle protein catabolism.[284–286]

8.6.5 Cancer Cachexia and Muscle Wasting

Insulin therapy has been considered as treatment for cancer cachexia that is linked with insulin resistance.[287] Morley et al. reported that insulin treatment (2 U/100 g) increased food intake in Fisher rats bearing W256 carcinoma.[288] Short-term treatment prevented the loss of body weight. Insulin administration also increased body fat in healthy rats and increased fat, water, and body protein in tumor-bearing rats. Tomas et al. reported that insulin treatment increased the rate of muscle protein synthesis by 34%–40%, in rats inoculated with breast cancer cells, while depressing the rate of protein breakdown by about 10%. Interestingly, insulin treatment led to a decrease in breast tumor size.[289] There are safety concerns in respect of using insulin as treatment for cancer weight loss. One study found that insulin treatment reduced the survival rate of tumor-bearing mice, though not by increasing tumor weight.[290] A recent study by Pfiffar showed that combination treatment with insulin, naproxen, and clenbuterol led to no improvements in outcomes for tumor-bearing rats compared with treatments lacking insulin—probably because of low concentrations used.[291]

Only a limited number of human trials examining the use of insulin for treating cancer cachexia have been published, perhaps related to the safety concerns discussed above. Lundholm et al.[292] found that insulin treatment (0.11 U/kg/day) for 150 days produced no changes in food intake, though carbohydrate intake increased. Body weight did not increase with insulin therapy but trunk and leg fat content increased. No biochemical or clinical indices were altered adversely with insulin treatment. Contrary to results in rodents, insulin treatment appears to increase the survival rates.[292] The mechanism by which insulin treatment improves weight in cancer patients remains uncertain, but presumably this is linked with the generalized

anabolic response. It has also been suggested that insulin administration may improve cachexia state by (a) stimulation of lymphocyte and macrophage metabolism, (b) a direct effect on tumor cell metabolism leading to decreased size, and (c) redirection of metabolites to host cells, for example, skeletal muscle and adipose tissue, decreasing the availability of metabolites to the tumor.[293]

BCAA supplementation for cancer therapy has been examined since the mid-1980s. Tayek et al. showed that TPN supplemented with BCAA (50% total protein intake) improved whole body protein synthesis and leucine balance in malnourished cancer patients.[294] Though nitrogen balance improved by 100%, the results were not statistically significant. Hunter et al. found that BCAA supplementation of cancer patients by TPN produced marked increases in protein breakdown and protein synthesis.[295] The rate of tyrosine oxidation was significantly reduced in the BCAA treatment group suggesting that supplementation may be beneficial for cancer patients. McNurlan et al. found that IV administration of TPN and BCAA increased host protein synthesis as well as tumor growth. However, BCAA suppressed tumor growth significantly compared to the normal TPN diet without BCAA. Recent investigations by Cangiona et al. showed that BCAA supplementation produced a significant rise in blood serum levels and increased food intake by 100%. The effects were explained in terms of the ability of neutral aliphatic amino acids to inhibit serotonin formation.[296] The potential benefits of BCAA use for nutritional support in cancer patients has been reviewed by Chaudry et al.[297] A recent RCT by Sun et al. showed that TPN using a BCAA enriched formula (30% vs. 24% dietary protein equivalent) produced significantly greater improvements in the CRP levels, higher alkaline phosphatase, and improved nitrogen balance in gastric cancer patients.[298]

There is currently ongoing work to evaluate ghrelin therapy for patients suffering from cardiac cachexia.[299,300] The ability of ghrelin to correct weight loss was attributed to several mechanisms, for example, the ability to stimulate growth hormone release, anti-inflammatory activity (Chapter 6), and appetite-stimulating effect (Chapter 9). Ghrelin and other GHS can also stimulate proliferation of myocytes.[301,302]

8.6.6 Liver Disease

There is evidence that BCAA nutritional therapy may be beneficial for liver diseases. Chin et al. found that a BCAA-supplemented formula led to improved weight and height gain in children (1.25 years old) awaiting liver transplantation compared to a control group fed a normal semi-elemental formula. The cross over RCT ($n = 19$ subjects, average age = 1.25 years) showed that BCAA therapy improved upper arm skin fold thickness, serum potassium levels, as well as serum albumin levels. A second, more extensive RCT involving 150 liver cancer patients who had surgery to remove their tumors showed that treatment with a proprietary formula (Aminoleban™; 100 g BCAA/day; ~36 months) led to increased body weight, higher red blood cell count, and higher serum albumin levels compared to controls fed with an isonitrogenous and isocaloric diet.[303] Meng et al. also found that a 12 week supplementation with Aminoleban for liver cancer patients that had undergone surgery resulted in

significant increases in blood hemoglobin and serum albumin levels while serum bilirubin decreased.[304] The treatment group also showed a significantly shorter length of hospital stay though overall mortality was unaffected. A large double blind RCT by Marchesini et al. found that 1 year supplementation of advanced liver cirrhosis patients with BCAA resulted in significant improvements in several key study outcomes (risk of death or deterioration; odds ratio 0.43; $P = 0.039$) measured against a lactalbumin diet but not a maltodextrin control. BCAA therapy led to reduced length of hospitalization, increased food intake, and general improvement in overall quality of life.[305] Broadly similar results are reported by Muto et al. who also found that BCAA supplementation improves quality of life indices and survival rates in patients.[306] Recent investigations by Fukushima et al. indicate that the timing of BCAA supplementation may have important consequences for liver cirrhosis patients. For reasons yet to be determined, nocturnal treatment with BCAA produced significantly greater nitrogen balance and serum albumin levels in patients compared to daytime feeding. Indeed, patients that had previously been unresponsive to daytime BCAA therapy were more sensitive to nocturnal treatment.[307] In summary, it can be seen from preceding studies mainly authored in Japan that BCAA treatment of liver cirrhosis patients is worthy of further trials. Recent investigations suggest that patients undergoing liver chemoembolization may also benefit from BCAA supplementation with possible improvements of nutritional status and general quality of life. However, there are no indications of improvements in overall mortality.[308,309] BCAA supplementation appears to be preferable to protein supplementation for liver cirrhosis patients who have a tendency to develop neurological symptoms linked with hepatic encephalopathy.[310] For a current appraisal of BCAA supplementation in the treatment of liver diseases refer to the following reviews.[311–313]

8.6.7 Adverse Effects and Safety Concerns

A number of adverse effects are reported for growth hormone therapy, mainly covering increased fluid retention (peripheral edema), joint pain (noninflammatory arthralgia, 36.4% incidence), muscular pain and myalgia (30% incidence), and glucose intolerance.[314,315] It has been suggested that growth hormone therapy may increase the rate of HIV virus replication or increase the risk of some forms of cancer.* Takala et al.[316] reported in 1999 that high doses of growth hormone increased the risk of mortality for critically ill patients. When a group of 247 critically ill Finnish patients (suffering from cardiac surgery, abdominal surgery, multiple trauma, or acute respiratory failure) were administered growth hormone (0.10 ± 0.02 mg/kg/day) for a maximum of 21 days, the treatment group showed increased length of hospital stay combined with an approximately twofold (40% vs. 18%) increased risk of mortality compared to untreated controls. These findings were confirmed by another study from the Finnish group.[314,317] Currently, the basis for growth hormone–induced increase in mortality has not been ascertained. Investigators have considered using IGF-1 as an alternative to growth hormone owing to the low side

* Only a limited number of studies have appeared on the use of GH for addressing cancer cachexia owing to concerns that this hormone may stimulate tumor growth.

effects associated with the former.[318] It has been also been suggested that low doses of growth hormone could be used for therapy in combination with glutamine[220,319] or alanyl-glutamine[320] for treatment of critically ill patients. Another possibility is to use GHS for treatment of intensive care patients rather than directly applying growth hormone.

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9 Bioactive Peptides for Alleviating Illness Anorexia

9.1 ILLNESS ANOREXIA

Inadequate food intake contributes to weight loss associated with illnesses.[1,2] Anorexia is observed in sufferers of HIV/AIDS, end-stage renal disease, chronic pulmonary disease, cardiac disease, and inflammatory bowel diseases. Chronic conditions such as aging, depression, pain, anxiety, and nausea also depress food intake.[3–5] There may be overt physical causes of anorexia, such as vomiting, obstruction of the gastrointestinal system, delayed digestion, malabsorption, gastric stasis and delayed emptying, and/or atrophic changes of the mucosa. Another important factor is stress, which is thought to activate the hypothalamus and pituitary glands to produce appetite-suppressing neuropeptides.

Bioactive peptides involved in the regulation of appetite are discussed in this chapter. Models for the regulation of food intake are briefly outlined in Section 9.1. Leptin and the melanocortin peptides, which are well-known anorectic agents, are discussed in Sections 9.2 and 9.3. The role of ghrelin in promoting food intake is covered in Section 9.4 followed by agouti-related peptides, serotonin antagonists, and miscellaneous orexigenic protein supplements in Section 9.5. In vivo studies and clinical trials examining the effect of bioactive peptides on appetite and food intake are discussed in Section 9.6. Ensuring adequate food intake is important for aiding recovery from illness and also for optimizing human performance in competitive situations. The material in this chapter is relevant for the control of obesity, but the focus of this discussion is on strategies for improving food intake and ensuring increases in lean body mass.

9.1.1 Models for the Regulation of Food Intake

Factors influencing food intake in healthy subjects were recently reviewed in a monograph by Harris and Mattes.[6] Historically, the regulation of food intake was described using the "glucostatic" or "lipostatic" model by analogy with mechanical thermostats. High levels of blood sugar and/or body fat stores were thought to provide feedback information for controlling food intake. According to the principles of regulatory physiology, homeostatic systems require sensors for monitoring a controlled variable (e.g., body weight or blood glucose) and a comparator to determine the degree of deviation of this variable from an idealized set point. When the observed value deviates significantly from the set point, a controller/actuator

device exerts corrective action to bring the measured variable within set limits. In principle, levels of metabolites (glucose, melonyl-COA, amino acids) and hormones can provide information on our energy balance. Brain cells located within the hypothalamus are thought to function as controllers for energy homeostasis. Feedback models for homeostasis have been widely applied to many physiological phenomena including thermoregulation and control of thirst.[6,*] In the case of food intake, the physical identities of many of the components of the control system have been difficult to establish.[7]

The regulation of food intake is somehow altered during illness such that there is a general decline in caloric intake. Infection-derived liposaccharide (LPS) are thought to activate frontline cells to produce inflammatory cytokines (Section 9.1.5) that ultimately reduce food intake. Figure 9.1 presents a schematic diagram indicating how LPS induces anorexia. The consequences of

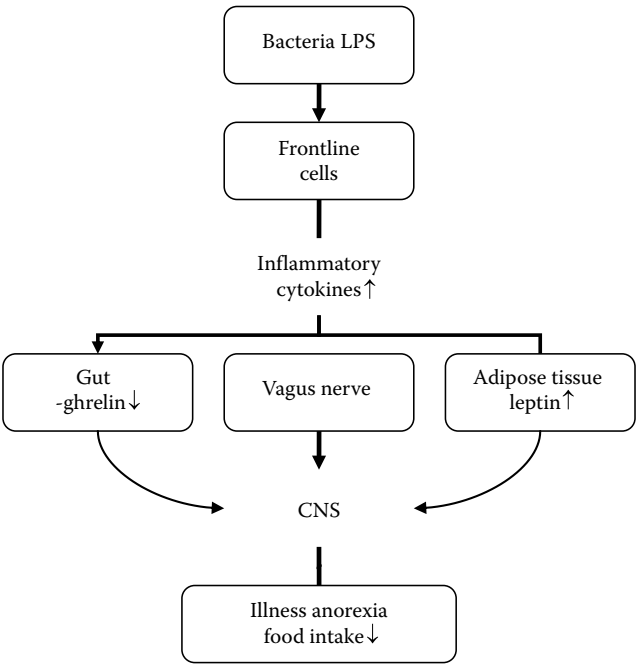


FIGURE 9.1 Schematic model showing how bacterial lipopolysaccharide (LPS) may include anorexia. Proinflammatory cytokines produced by LPS-activated frontline cells moderate hormone production from adipose tissue (leptin) or the gut (ghrelin), which then affect CNS centers for the control of appetite. Cf. elements of inflammatory signaling described in Chapters 6 and 7. (From Johnson, R.W., *Domest. Anim. Endocrinol.*, 15, 309, 1998; Johnson, R.W., *Vet. Immunol. Immunopathol.*, 87, 443, 2002.)

* The interested reader should refer to Ref. [6] for an authoritative account of the different models for appetite control. In this discussion we focus more narrowly on the role of bioactive peptides in the regulation of appetite.

illness anorexia include low nutritional status, fatigue, apathy, and depression[8,9]; see Section 1.3.2.

Food intake is dependent on the frequency of eating, duration of each meal, and meal size. These attributes are thought to be controlled by satiety and hunger centers identified, based on the effects of specific brain lesions on eating behavior.[10–12] Results from so-called knife-cut studies showed that an area of the brain stem called the nucleus tractus solitarius (NTS) receives signals traveling via the vagus nerves from the gut. Further experiments indicated that neurons within the hypothalamus, in particular the arcuate nucleus (ARC), paraventricular nucleus (PVN), and lateral hypothalamus (LH), were involved in the moderation of food intake. There is now increasing tendency to downplay the notion of discrete hunger or satiety centers in the brain. On the contrary, it is thought that interactions between different groups of neurons can increase or decrease food intake. Bundles of neurons from the ARC and pro-opiomelanocortin (POMC) regions (see below) are particularly important for moderating food intake.[13–16] It has emerged that diverse peptide hormones from the periphery of the body (e.g., adipose tissue and the gut) also affect brain centers for appetite.

9.1.2 Bioactive Peptides and Energy Homeostasis

A large number of bioactive peptides that reduce food intake is produced within the CNS (Tables 9.1 and 9.2), stomach, and pancreas. By contrast, cells within the ARC produce agouti-related protein (AgRP) and neuropeptide Y (NPY) that stimulate food intake (Section 9.5). The orexigenic peptides from the NPY/AgRP nuclei act to counterbalance the food inhibitory (anorectic) peptides produced from neurons from the POMC and cocaine-amphetamine stimulated transcript (CART) centers that signal the termination of food intake. Axonal interconnections occur between the NPY/AgRP and POMC/CART as well as between these sites and PVH and LHA. Furthermore, the ARC is located below the third ventricle near where there is increased permeability to blood-borne hormones and peptides. The anatomical basis for the gut–brain axis has been reviewed recently.[17–19] Leptin and ghrelin (discovered in 1994 and 1998, respectively) are key elements for controlling food intake.[20,21]

TABLE 9.1
Anorexigenic Peptides from the CNS

α-Melanocortin stimulation hormone (α-MSH)
Calcitonin-gene-related hormone
Cocaine and amphetamine-regulated transcript (CART)
Corticotrophin-releasing hormone (CRH)
Neuromedin U
Neurotensin
Oxytoxin
Serotonin
Somatostatin?
Thyrotropin-releasing hormone (TRH)

9.1.3 Anorectic Bioactive Peptides

Leptin and the gut peptides cross the blood–brain barrier (BBB) and so produce anorexigenic effects in the CNS. Alternatively, some peripheral peptides may stimulate vagus nerve endings thereby passing information directly to the brain from the GI tract. Orexigenic peptides that stimulate food intake are discussed in Section 9.5.

TABLE 9.2
Anorexigenic Peptides Produced in the Peripheral Regions of the Body

Gut Peptides and Hormones	Pancreatic Peptides	Adipokines
Amylin	CCK	Adiponectin
Bombesin	Insulin	Leptin
Gastrins		Resistin
GIP		
GLP		
GLP-1		
Motilin		
Obestatin		
Peptide YY		
Secretin		
VIP		

Abbreviations: CCK; cholecystokinin; GIP, gastric inhibitory polypeptide; GLP, glucagon-like peptides (GLP); GLP-1, glucagon-like peptide-1 (GLP-1); VIP, vasoactive intestinal peptide (see Table 9.10 for a list of orexigenic peptides).

9.1.4 Serotonin

High levels of brain serotonin or 5-hydroxytryptamine (5-HT) is thought to increase satiety and reduce food intake.[22,23] Classic investigations by Fernstrom and Wurtman showed that tryptophan is the precursor for 5-HT synthesis.[24,25] In addition, it was shown that high-protein diets do not increase brain tryptophan or 5-HT levels because of the low levels of tryptophan found in most proteins.[26,*] On the other hand, large neutral amino acids (LNAA) and the branched-chain amino acids (BCAA) act as competitive inhibitors for tryptophan transport at the BBB thereby lowering brain 5-HT levels (Section 9.5.4). Carbohydrates that increase insulin release stimulate *tissue* uptake of LNAA, thereby promoting tryptophan uptake at the BBB indirectly.[†]

Serotonin agonists that enhance 5-HT release or 5-HT receptor binding (5-HT agonists) or inhibit 5-HT reuptake at nerve endings (selective serotonin reuptake inhibitors; SSRIs) produce satiety, decreased food intake, antidepressant effects, and reduced antisocial behavior. 5-HT agonists decrease feeding frequency as well as meal size. By contrast, anti-serotogenic agents that reduce the concentration of brain serotonin enhance food intake. In rodents and primates, low brain 5-HT levels increase food intake and help sustain body mass. Human studies suggest that low 5-HT is also linked with aggressive behavior, depression, increased risk of suicide, and a tendency to undertake punished behavior[27,28] (Table 9.3).

* The following sites provide useful general introduction to diverse physiological actions of tryptophan and serotonin: http://en.wikipedia.org/wiki/Tryptophan and http://en.wikipedia.org/wiki/Serotonin.

† Note that protein is thought to be more satiating compared to complex carbohydrates or lipids.

TABLE 9.3
Physiological and Behavior Characteristics Affected by High Serotonin[a]

Behavior function	Aggression (–)
	Appetite (–)
	Circadian rhythm entrainment
	Insomniac (–)
	Learning and cognitive performance (+)
	Pain sensitivity (–)
	Sensory-motor reactivity
	Sexual behavior (–)
	Sleep disorder (–)
Psychiatric disorders	Depression (–)
	Anxiety disorders (–)
	Schizophrenia
	Anorexia (–)
Personality disorders	Aggression (–)
	Gambling(–), obsessive control (–)
	Attention-deficit disorder (–)

Sources: Adapted from Hosoda, H. et al., *Mol. Interv*. 2, 494, 2002; Arora, S. and Anubhuti, *Neuropeptides*, 40, 375, 2006.

[a] Behaviors either decrease (–) or increase (+) with high brain serotonin.

9.1.5 Cytokines and Food Intake

Proinflammatory cytokines (Section 9.3) depress food intake.[29–33] The underlying pathways involve elevated expression of leptin by adipose tissue as well as increased expression of leptin receptors within the CNS.[34–37] Cachexia is linked with increasing cytokine production probably as result of inflammation and malignancy. As supporting evidence for this model, past studies showed that injection of proinflammatory cytokines produce symptoms of cachexia.[33,38–40,*]

9.2 LEPTIN AND FOOD INTAKE

9.2.1 Characteristics of Leptin Receptor Signaling

Leptin is a 16 kDa hormone produced by adipose tissue.[41,42] Mutations in the gene coding for leptin (Ob-gene) are associated with obesity whereas mutations affecting the leptin receptor (Ob-R) gene lead to symptoms of Type 2 diabetes. The leptin membrane receptor (Ob-R) is a Type-1 cytokine receptor; the structure of leptin shows some similarity with cytokine family of proteins. So far six different isoforms of Ob-R are known (Ob-R_a, Ob-R_b, Ob-R_c, Ob-R_d, Ob-R_e, and Ob-R_f) that have the same

* Also see Figure 9.1 and associated text.

extracellular structure. However, only one (Ob-R_b) of the six isoforms is believed to possess the full structure required to sustain leptin signaling. Ob-R_e is lacking a transmembrane peptide sequence and is therefore a soluble protein.[43] Ligand binding leads to dimerization of Ob-R_b followed by activation of Janus tyrosine kinase 2 (JAK2) and the signal transducer and activator of transcription protein 3 (STAT3) or the so-called JAK2/STAT3 pathway.[44] Phosphorylated STAT-3 enters the nucleus where it stimulates the expression of genes that ultimately decrease food intake and increase energy expenditure. Signaling via Ob-R_b is regulated by two feedback systems: (a) Suppressor of cytokine-3 (SOC-3) protein and (b) protein tyrosine phosphatase 1B (PT1B). SOC-3 is produced in response to leptin signaling and switches of leptin signaling. PT1B3 catalyzes the dephosphorylation of the leptin receptor thereby curtailing further signaling.

9.2.2 Leptin Regulation of Food Intake

The ob/ob mice strain first described in the 1950s has a double recessive mutation in the gene coding for leptin, resulting in symptoms of obesity, including excessive food intake and raised blood insulin and glucose levels. Leptin deficiency is associated with other changes in the physiology of ob/ob mice, including the tendency for reduced physical activity, reduced body temperature, and depressed immune function. Parabiosis experiments showed that ob/ob mice lacked a serum hormone produced by normal mice.[45] Molecular biology studies reported in 1994 identified the ob/ob gene product as the hormone leptin. Ob/ob mice mutations lead to the formation of the inactivated hormone.* The role of leptin in anorexia or decreased appetite during illness is considered below.[46,47]

Plasma leptin concentrations are thought to have a major impact on food intake. Under normal circumstance, the concentration of leptin in the blood increase with increasing body fat. By contrast, decreases in fat mass and leptin concentration (due to fasting) increase appetite and food intake. Leptin functions as a negative feedback signal by which increasing body fat tends to limit food intake. The effect of leptin on food intake is mediated by neurons within the ARC, LH, and PVN, which express Ob-R.[48–52] In summary, leptin produced from adipose tissue is effectively transported to the CNS where it stimulates POMC/CART neurons to release appetite-suppressing melanocortin peptides (Section 9.3). By contrast, leptin binding to NPY/AgRP neurons decreases the synthesis of orexigenic peptides.

It is worth mentioning in passing that leptin is also implicated in reproductive growth. Accordingly, increased fat mass and serum leptin levels may partly explain the increased rate of maturation and earlier puberty in overweight humans. Extremely thin individuals with low fat mass and low circulating levels of leptin show delayed puberty. Leptin levels are decreased in anorexia nervosa contrary to the situation discussed for illness anorexia.[53,54]

* Five common murine models are used for obesity research designated obese (ob), diabetes (db), fat (fat), tubby (tub), and obese yellow (A^y).

9.2.3 Leptin Resistance during Obesity

The feedback control of food intake described in Section 9.2.2 appears to malfunction in obese subjects. Food intake remains elevated despite high plasma levels of leptin in obese subjects who may be considered leptin resistant.[55,*] CNS leptin resistance is observed in two genetic models for diabetes (db/db mice) and obesity (Zucker fat/fat rat) that carry a mutation in the Ob-R gene. It is thought that defects in Ob-R protein lead to an inability to switch off feeding impulse despite high fat mass and elevated serum leptin levels. CNS leptin resistance may also arise due to defects in the Ob-R signaling downstream of the receptor. For instance, chronically high leptin concentrations may lead to increased expression of SOC-3 which then deactivates leptin signaling. Polymorphism in the genes coding for leptin or the leptin receptor may decrease the efficiency of human response to leptin—though current research does not support this conclusion.[56] In summary, obese subjects exhibit high levels of leptin in their circulation (reflecting the higher body fat) and increased CNS resistance to leptin associated with defective Ob-R[57–59] (Table 9.4).

The second type of leptin resistance (peripheral leptin resistance) is ascribed to defective transport of this hormone across the BBB. Consequently, injection of the hormone directly into the CNS leads to depressed food intake but injection of leptin outside the CNS has no effect. The physical structure of BBB consists of a layer of endothelial cells. Transfer of leptin and other hormones across the BBB occurs via paracellular transfer across tight junctions or transcellular transport. Factors that

TABLE 9.4
Types of Leptin Resistance*

Diet-induced leptin resistance
High fat diet (p, CNS)
N-3 PUFA (p)
Genetically based leptin resistance
Diabetic (db/db) mice (CNS)
Zucker fatty (fa/fa) rat (CNS)
New Zealand obese mice (p)
Osborne–Mendel rat (p)
Chronic conditions
Aging (CNS, p)

Abbreviations: p, peripheral leptin resistance; CNS, central leptin resistance.

* ob/ob mice with defective leptin formation are not included as examples of leptin resistance.

* Two forms of leptin resistance may be distinguished: peripheral and central (CNS) leptin resistance.

moderate tight-junction permeability could be important though the major factors leading to impaired transport of leptin are poorly understood. According to recent observations, leptin transport is partly mediated by prior binding to a soluble form of the leptin receptor (Ob-R_b and Ob-R_e) levels of which fall with increasing BMI. Reductions in the plasma concentrations of soluble Ob-R protein lead to increased leptin resistance.[60–65]

9.2.4 Leptin Role in Illness Anorexia

Plasma concentrations of leptin increase under the influence of proinflammatory cytokines independently of fat mass. Grunfeld et al.[40] found that endotoxin increases the expression of leptin mRNA in hamsters leading to a decrease of food intake and weight loss.* Sarraf et al.,[34] found that IV administration of proinflammatory cytokines (TNF-α, IL-1, leukemia inhibitory factor [LIF]) raised serum leptin levels and leptin mRNA expression in adipose tissue in mice. Blood leptin concentration did not rise when cytokines not associated with an inflammatory response (IL-10, IL-4, IL-2, and neurotrophic factor) were administered. Luheshi et al.[66] reported that healthy rats show reduced food intake and a rise in core body temperature after injections with leptin intra-cerebroventricularly (ICV) or peripherally. Interestingly, leptin treatment increased levels of proinflammatory cytokines within the CNS. IL-1 receptor antagonist reduced the anorexic effect of leptin by 60%–80%. The fat/fat strain of rat that possess a defective Ob-R did not show the anorexic response to leptin administration. Other inflammatory conditions associated with increasing leptin include peritonitis,[35,36,67] chronic renal failure,[68] and endotoxemia.[69] Proinflammatory cytokines stimulate leptin gene expression and leptin receptor expression in the CNS. In addition to the effect on leptin secretion, bacterial LPS also produces illness-related behavior such as, reduced lick (grooming) frequency and wheel activity in rodents.[5,70]

Leptin antibodies have been shown to ameliorate illness anorexia. Thus, Harden et al.[71] showed that rodents injected with LPS showed a rise in body temperature, decreased food intake, and increased lethargy monitored as a decline in the voluntary running distances on their exercise wheel. LPS-induced sickness behavior could be eliminated using antibodies specific for leptin or IL-6. By comparison, anti-TNF-α and anti IL-1β had no impact on the LPS-induced sickness behavior. Somech et al.[72] found correlations between levels of CRP and leptin in pediatric patients with infection. Apparently, a rise in serum leptin levels may be a part of the acute phase response. High leptin concentration may also be important in age-related anorexia and anorexia associated with depression. Overall, there is strong support for the association between leptin concentrations and illness anorexia. LPS may also have direct effects on cells of the CNS without the intermediary role of leptin.[73]

* This research on the effect of bacterial endotoxin was completed prior to the discovery of toll-like receptors in adipose tissue (Chapter 3). Studies involving the incubation of lipopolysaccharide with cultured adipocytes could provide another test for results obtained with mice. The links between obesity and inflammatory status are discussed in Chapter 3.

9.3 MELANOCORTIN PEPTIDES

9.3.1 Melanocyte-Stimulating Hormone

The melanocortins are brain peptides linked with the regulation of skin tanning, pigmentation, and hair coloration. Melanocyte-stimulating hormones (MSH) and related peptides can also affect a range of other physiological responses, including, reproduction, cardiovascular health, and immune function. More importantly for the present discussion, MSH is implicated in the control of food/energy intake and symptoms of illness anorexia.[74–79]* Four closely related MSHs (α-, β-, δ-, and γ-MSH) and ACTH are produced from a single large POMC precursor protein by limited proteolysis catalyzed by prohormone-converting enzyme-1 and -2.[80] Cleavage of POMC is thought to occur at specific (LysLys, LysLysArgArg) residues leading to MSH derivatives and ACTH (Figure 9.2). With the exception of γ-MSH, the other MSHs contain a conserved tetra-peptide sequence [HisPheArgTrp] thought to be involved in receptor binding and bioactivity.[81]

9.3.2 Melanocortin Networks to Leptin and Serotonin Signaling

Central POMC/CART neurons express the Ob-R and it is thought that leptin bindin stimulates the synthesis and release of α-MSH.[82] Stimulation of Ob-R within the NPY/AgRP nucleus inhibits orexigenic peptide formation. Leptin signaling may also stimulate serotonin activity mediated by 5-HT receptors located on POMC-producing neurons, which extend to the PVH and LHA regions of the hypothalamus.[83,84] In conclusion, it can be inferred that proinflammatory cytokine formation, in response to injury, infection, and stress, can activate the leptin–MSH axis thereby reducing food intake and ultimately producing a state of illness anorexia. Therapeutic agents that potentiate melanocortin signaling, by-passing the effects of leptin resistance, could find applications in the treatment of obesity.[85] By contrast, antagonists for melanocortin receptor may be considered candidates for the treatment of anorexia (Section 9.5).

9.3.3 Melanocortin Receptors

Five different melanocortin receptors (MCR1–5) have been cloned and localized in different tissues and/or the CNS. Stimulation of MCR1–5 by MSH in different tissues regulates skin pigmentation, steroid genesis, reproductive function, anti-inflammatory activity, and food intake (Table 9.5). Skin, hair, and fur pigmentation is controlled by MC1R associated with pigment cells (melanocytes) of the skin and hair. Stimulation of MC1R by α-MSH and ACTH enhances the formation of dark coloration. MC2R associated with corticosteroids production and MC3R is associated with sexual dysfunction. Corticosteroids have an anti-inflammatory action. MCR4 and (and possibly MCR3) found in the brain is concerned with food intake.[86].

* The melanocortins system is a common pathway for the control of food intake and melanization response to illness. MSH-related peptides have anti-inflammatory action as discussed in Chapter 6.

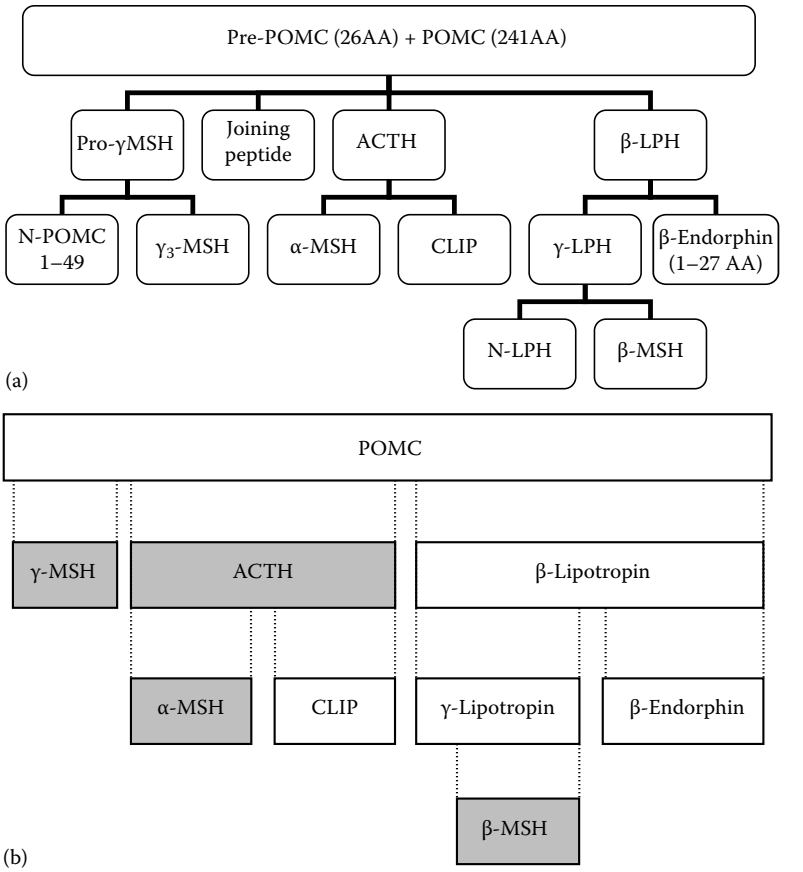


FIGURE 9.2 Pro-opiomelanocortin (POMC) peptide undergoes processing to form s bioactive peptides, melanocortin-stimulating hormone (MSH), lipotropin (LPH), corticotrophin-like intermediate lobe peptide (CLIP), and adrenocorticotrophin release hormone (ACTH). (Adapted from (a) Irani, B.G. et al., *Curr. Pharm. Des.*, 10, 3443, 2004 and (b) Wikipedia http://upload.wikimedia.org/wikipedia/commons/7/77/POMC.png (accessed May 2009)).

MCR1–5 are G-protein linked seven-helix transmembrane receptors whose stimulation leads to stimulation of adenylate cyclase activity, rising intracellular c-AMP levels, activation of protein kinase A (PKA), and increased gene expression. Recent investigations by Vongs et al.[87] indicate also that MRC signal the p42/p44 MAPK pathway conveyed through ionositol triphosphate though the full implication of this pathway in the control of food intake is uncertain.

So-called loss of function mutations affecting genes for POMC, MSH, or MCR4 result in excessive food intake in mice. POMC gene knockout mice exhibit increased food intake (hyperphagia), decreased production of adrenaline, decreased synthesis of corticosteroid hormones, and yellow coats vs. darker

TABLE 9.5
Melanocortins Receptor Function

Receptor	Ligand Affinity	Localization	Possible Function
MC1R	α-MSH	Melanocytes, immune cells, inflammatory cells, keratinocytes, endothelial cells, glial cells	Melanotropic, skin, fur, hair coloration, immune function, anti-inflammatory, antipyretic
MC2R	ACTH, α-MSH	Adrenal tissue	Corticosteroid formation
MC3R	α-MSH, γ-MSH	CNS, macrophages	Salt-sensitive hypertension, pro-erectile function
MC4R	α-MSH, β-MSH	CNS, brain: hypothalamus, olfactory cortex, brainstem, and spinal cord	Decreased food intake, anti-obesity, induces anorexia
MC5R	α-MSH, β-MSH	CNS, skin, muscle, spleen, and many organs	Uncertain
MC1R, MC3R, MC4R, and MC5R	ACTH	Adrenal glands	Improves blood pressure, pulse, and survival rate in stroke, arouse mating (erection, ejaculation, sexual posturing, and genital licking)

Sources: Summarized from Catania, A. et al., *Pharmacol. Rev*., 56, 1, 2004; Starowicz, K. and Przewlocka, B., *Life Sci*., 73, 823, 2003.

brown coats. The mice lacking POMC also showed early-onset obesity, increased adiposity, increased body length, normal lean body mass, increased serum insulin, raised leptin levels, reduced pO_2, and low sensitivity to leptin. Administration of melanocortins led to weight loss in the knockout mice and increased fur pigmentation. Knockout mice studies also showed that MC4R (and MCR3) are important for the control of food intake. Mice with a disrupted MCR4 gene showed increased obesity and increased food intake and raised blood insulin and glucose levels. Data from MCR4 knockout mice are similar to results achieved with receptor antagonist. MC4R is believed to affect only certain aspects of feeding behavior, for instance meal size and meal choice, whereas feed frequency is under the control of other satiety centers.[90–94]

Human polymorphisms for the POMC gene are described by Krude et al.[95,96] Two human children (boy and a girl) thought to have POMC gene defects showed orange-red hair, early-onset obesity, increased tendency for overeating, and lower than expected levels of adrenal gland hormones (epinephrine and corticosteroid hormones). Mutations in the POMC gene were indicated by PCR analysis to affect dysfunction in one or more *ligands* for melanocortins receptors. Natural mutations that affect MCR4 gene appear to contribute to genetic component of obesity in humans affecting between 1% and 6% of morbidly obese adults.[97,98]

9.3.4 Peptide Agonists and Antagonists for Melanocortin Receptors

Hruby et al. from the University of Arizona developed a series of α-MSH analogues in the 1980s that function as MCR super-agonists.[99–101] The MSH analogues were initially developed as patented agents for inducing sunless tanning, and as agents that correct erectile dysfunction.[102–104] The first analogues were linear synthetic peptides with limited stability in the circulation. These were later cyclicized by isosteric replacement of residues 4 and 10 with two cysteine residues followed by oxidation to form the intramolecular disulfide. Cyclization led to a more potent agent compared to the linear α-MSH when assayed by the ability to (a) stimulate melanosome dispersion in frog or lizard skin or (b) induce c-AMP production in cultured melanoma cells.

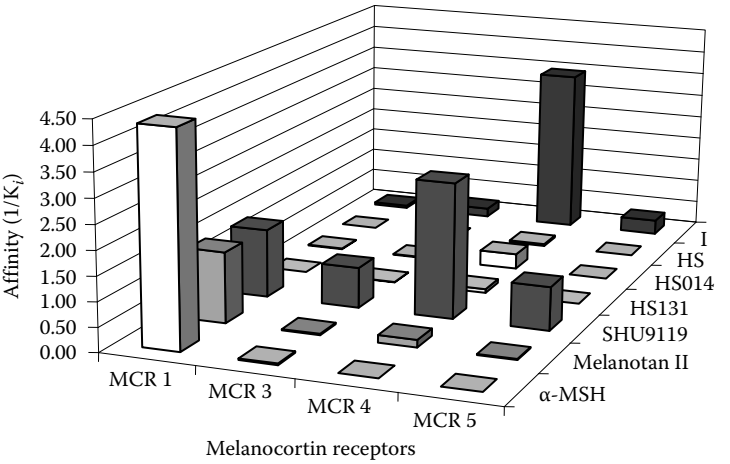
Melanotan I (MTI) and melanotan II (MTII) are arguably the best known of the α-MSA analogues. The α-MSH analogues may increase skin pigmentation and also decrease food intake.[97–102] The ring structure MTII is formed from an intramolecular amide link joining lysine and aspartate residues in the molecule. MTII functions as an *agonist* at MCR more potently than the α-MSH.[105] It has been shown that MTI and MTII enhance skin pigmentation without UV exposure leading to proposals for their use as bioactive sunscreen. Human clinical trials are underway to examine the ability of MTII to correct some forms of erectile dysfunction probably linked with bioactivity expressed at MCR5. Investigations summarized by Fan et al. (Table 9.6) showed that MTII inhibits food intake in several models of obesity due to its agonist effects at MCR4.[106] More intriguing still, some derivatives of MTII were found to behave as melanocortin inhibitors (antagonist) and therefore stimulate food intake (Figure 9.3; see Section 9.6).

MCR reverse agonists (e.g., AGP, AgRP (83–131), and SHU9119) have been proposed as treatment for illness anorexia.[107] The oldest melanocortin antagonist designated SHU9119 was chemically synthesized from MTII by replacing D-phenylalanine residue-7 with D-2′ napthylamine residue. The more bulky side-chain transformed MTII from a receptor agonists to a receptor antagonist SHU9119[108] (Figure 9.4).

TABLE 9.6
Effects of Melanotan II (MTII) on Food Intake

- Inhibits food intake in mice 1 h after ICV administration
- Anorexic effect lasts 4 h. Normal feeding resumed by 8 h
- IC_{50} (2 h) estimated at 0.6 nmol
- No adverse effects or indirect behavioral changes related to reduced food intake
- Slight reduction in water consumption noted at < 1 h
- Reduces obesity in three experimental models: (a) C57BL/6J-Lep^{Ob} mice, (b) C57BL-A^{y} mice, and (c) NPY injected C57BL mice
- Inhibits orexigenic effect of neuropeptide Y
- Direct metabolic effect, 58% decrease in serum insulin

Source: Summarized from Fan, W. et al., *Nature*, 385(6612), 165, 1997.
Abbreviation: ICV, intracerebroventricular.



	MCR1	MCR3	MCR4
α-MSH	4.35	0.03	0.00
Melanotan II	1.45	0.03	0.15
SHU9119	1.41	0.83	2.78
HS131	0.00	0.00	0.08
HS014	0.01	0.02	0.32
HS964	0.00	0.00	0.04

FIGURE 9.3 Summary data showing the structure and affinity of some well-known melanocortin receptor ligands. Binding affinity was determined from c-AMP production in CHO cells transfected with MCR genes (see Appendix 9.A.1 for amino acid sequence). (Drawn using data from Schioth, H.B. et al., *Biochem. Biophys. Res. Commun*., 301, 399, 2003.)

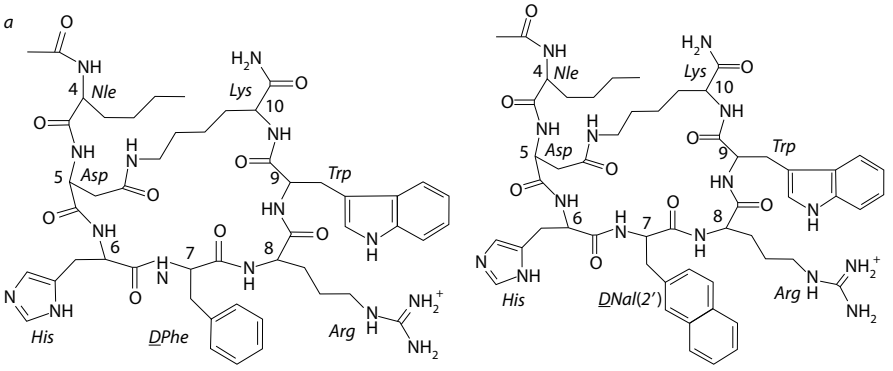


FIGURE 9.4 Structures of melanotan II and SHU9119. Each peptide has seven amino acids. Numbers shown in the diagram refer to residue positions using MSH as reference. Notice that the D-phenylalanine 7 is replaced with D-2′ napthyl-alanine in SHU9119. (From Fan, W. et al., *Nature*, 385(6612), 165, 1997.)

Another MCR active compound called HS131 was described that functions as an antagonist for all MCR though with 20-fold higher selectivity for MCR4 compared to MRC3.[109]

As discussed earlier, the core sequence required for α-MSH activity is the tetrapeptide sequence HisPheArgTrp. However, tests using Ac-His-Phe-Arg-Trp-NH2 showed that this sequence was 15,000–30,000 less potent compared to 13-AA residues for MSH.[110] Structure activity relations for MCR ligands were explored using a series of chemical alterations applied to α-MSH (Table 9.7). The studies helped to identify aspects of MCR ligands important for receptor activity, improved peptide stability, and potency. Replacement of histidine and phenylalanine residues with alanine produced a K_i value for peptide-MC4R binding of 20.3 and 1592 nM, respectively, compared to 1.32 nM for the control. Current data suggest that the core peptide residues are all essential for bioactivity and replacement of any of these residues has a tendency to decrease potency of the resulting ligand though exceptional response were observed. Alanine scanning studies by Holder et al. confirmed that Arg 8 is important for binding of HisArgPhePhe to MRC1–5.[111] Substitution of Arg 8 resulted in reductions in agonist activity expressed at MRC. Detailed analysis of results suggested that Arg was not crucial for ligand binding but was involved in orientation of HisArgPhePhe at the receptor binding site. Modification of Trp 9 residue led to ligands with 1000-fold higher reactivity at MRC1 and MRC5 compared to MRC4 and MCR3, which are involved in the regulation of food intake.

The pharmacological properties of MSH antagonist need further refinement to increase their stability, bioavailability, and receptor specificity. The last consideration is important since unwanted side effects could arise if all five MCR are activated indiscriminately. Strategies have appeared in literature for optimizing the function of ASP peptide analogues though much work remains to be done before wholly satisfactory drugs based on this template emerge. Cyclization of the 10 AA

TABLE 9.7
Structure–Activity Relations for Melanocortin Ligands

Method	Rational/Comment
1. Truncation studies—large peptide is systematically shortened	Identifies His-Arg-Phe-Trp- is core residues for activity
2. Alanine scanning—residues are systematically replaced by Ala	Substitution of His (6), etc., leads to loss of receptor activity
3. Modification of pharmacore—key residues modified by stereo chemical inversion by introduction of D-amino acid (D-AA) isomer, halogenation	D-AA increased potency of MRC ligands, various results, and increase in potency in some cases
4. N-terminal modification	Increases stability and potency
5. Cyclization	Increases conformational rigidity and increases potency and protease resistance

* Summarized from Refs.[111–113]

peptide resulting in increase of the backbone rigidity enhances receptor binding affinity. Another benefit of ring formation is the increased stability of the resulting peptide analogues to proteolytic digestion. Linear MSH analogues have a short lifetime in the circulation due to rapid clearance by enzymatic digestion. By contrast, ring analogues have reduced the susceptibility to enzymatic digestion and may therefore increase the lifetime in the circulation.[112,113]

9.4 GHRELIN

9.4.1 Characteristics of Ghrelin

Ghrelin is the natural ligand for the growth hormone secretagogue receptor (GHS-R). The discovery of ghrelin followed assays of stomach tissue extracts using Chinese hamster ovary (CHO) cells expressing GHS-R gene cloned some years previously.[114] Rat stomach tissue extracts contained a peptide that stimulates GHS-R transfected CHO cells resulting in rising intracellular Ca^{2+} levels. The endogenous ligand was later purified and named ghrelin (from "ghre" in the Proto-Indo-European language meaning "grow," and "relin" meaning to "release"). Active ghrelin is a 28-amino acid (~3 kDa) peptide formed by proteolysis of larger (12 kDa) precursor protein (Figure 9.5). The mature peptide carries an *n*-octanoyl substituent at Ser 3 that is required for bioactivity and the de-esterified ghrelin appears to be inactive. A second form of ghrelin possessing 27 amino acids (des-Gln-ghrelin) has a missing terminal glutamine derivative and is also inactive. Ghrelin not only stimulates GH release but is also found to stimulate food intake[20,115,116] and to act as an anti-inflammatory agent[117–119]; see Chapter 6.

9.4.2 The Ghrelin Receptor

Two different types of GHS receptors (GHS-R 1a and GHS-R 1b) have been identified for ghrelin but only GHS-R1a appears to be biologically active. The distribution of GHS-R1a appears to be widespread (e.g., CNS, thyroid, spleen, pancreas, myocardium, and adrenal glands) suggesting the hormone has diverse functions in addition to the regulation of food intake. The presence of GHSR-1a detected in human T cells, B cells, and neutrophils may explain the anti-inflammatory role of ghrelin and other GHS.[120–122,*] Since the synthetic GHRP (Chapter 9) also bind to GHS-R1a these compounds are now considered ghrelin agonists. The characteristics of the ghrelin receptor (GHS-R) are summarized in Table 9.8.

9.4.3 Factors Affecting Ghrelin Release

The naturally occurring growth hormone secretagogue ghrelin has been found to be important in the regulation of food intake and long-term weight control. There are good prospects that exogenous ghrelin may be useful in the treatment of illness

* Tissue-specific expression of hormone receptors allows one hormone to produce pleiotropic effects in different targets providing a simple explanation for the ability of ghrelin to produce different effects in different tissue.

Entry name	GHRL_HUMAN
Primary accession number	Q9UBU3
Protein name	Appetite-regulating hormone [Precursor]
Synonyms	Growth hormone secretagogue, Growth hormone-releasing peptide, Motilin-related peptide, M46 protein
Contains	Ghrelin-27, Ghrelin-28 (Ghrelin) Obestatin
Gene name	GHRL

Sequence information

```
        10         20         30         40         50         60
MPSPGTVCSL LLLGMLWLDL AMA [GSSFLSP EHQRVQQRKE SKKPPAKLQP R] ALAGWLRPE

        70         80         90        100        110
DGGQAEGAED ELEVRFNAPF DVGIKLSGVQ YQQHSQALGK FLQDILWEEA KEAPADK
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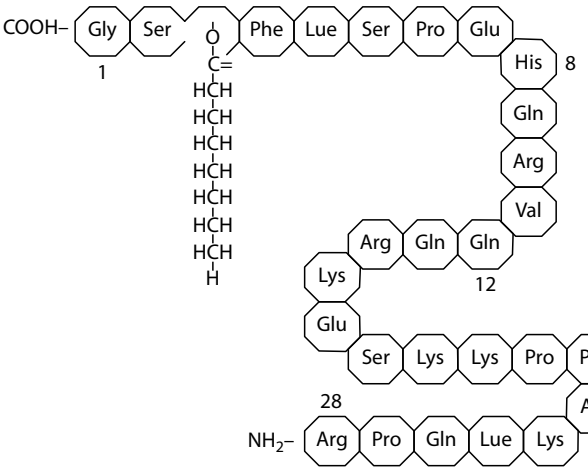

FIGURE 9.5 Structural characteristics of human gherelin-28 peptide. Serine 3 of ghrelin chain is acylated with C8 fatty acid residue.

TABLE 9.8
Characteristics of Growth Hormone Secretagogue Receptor (GHS-R)

Endogenous agonists	Ghrelin (IC50 = 8.1 nM/EC50 = 1.5 nM)
	des-Gln14-ghrelin (IC50 = 7.4 nM/EC50 = 1.5 nM)
Selective agonists	GHRP-2 (IC50 = 0.4 nM)
	MK-0677 (IC50 = 0.8 nM)
	GHRP-6 (IC50 = 1.5 nM)
Antagonists	[D-Lys3]-GHRP-6, EP-80317
Functions	Binding of ghrelin and GHRP results in growth hormone release, gastric acid secretion, gastric motility, increase appetite, vasodilatation, anti-inflammatory

Source: Adapted from Davenport, A.P. et al., *Pharmacol. Rev*., 57, 541, 2005.
Abbreviation: GHRP, growth hormone release peptides are discussed in this chapter.

anorexia (Section 9.6). This section consider some of the background science to the ghrelin. To indicate the role in energy homeostasis, blood levels of this ghrelin decline following eating and rise following weight loss. Periods of weight loss result in compensatory increases in ghrelin secretion, followed by increased food intake leading to weight gain. The ghrelin feedback system may ensure weight regain following weight-loss diets.[124,125] Feedback regulation of body weight may be impaired during cachexia.

Plasma ghrelin concentration is affected by a variety of factors including age, gender (women > men), food intake, body weight, and variations in hormone levels. In general, circulating ghrelin concentration is thought to be indicative of nutritional status being inversely related to BMI. Plasma ghrelin concentration decreases in response to high energy intake, weight gain, and obesity (Figure 9.6). High concentrations of ghrelin have been measured in human subjects experiencing weight loss, anorexia, cancer, and other catabolic conditions.[20,116,118,126] The association between high ghrelin concentrations and weight loss is consistent with the role of this hormone in the stimulation of food intake. However, the interpretation of serum ghrelin concentrations is fraught with some difficulty for several reasons: First, most investigators determine the total (acylated and non-acylated) ghrelin, whereas bioactivity is linked to the acylated form. Second, single-time point measures of plasma ghrelin may have doubtful value because the concentrations of this hormone appear to undergo diurnal changes (see below). Third, ghrelin levels reflect compensatory responses to wasting rather than being positively correlated to weight loss. Though high plasma levels of endogenous ghrelin occur under conditions of food deprivation and long-term weight loss, there is no indication the gut hormone promotes weight loss. Likewise, periods of inflammatory cachexia can lead to elevated levels of plasma ghrelin without the increases of food intake expected in healthy subjects

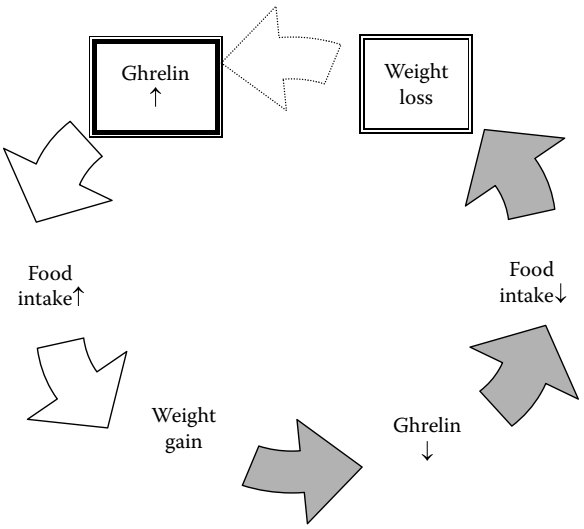

FIGURE 9.6 Feedback control of body weight involving ghrelin and food intake. Weight loss increases ghrelin secretion, food intake, and weight gain.

(Figure 9.6). Finally, only a limited number of studies measured ghrelin along with counter-regulatory hormones such as leptin.

9.4.3.1 Ghrelin Circadian and Ultradian Rhythms

Plasma ghrelin levels undergo diurnal changes in phase with changing levels of leptin. For human subjects (9 women, 1 man; age = 29–64 years; BMI = 22–30 kg/m^2), the ghrelin circadian rhythm led to maximum and minimum concentrations of hormone at 0100 and 0900. Detailed cyclical changes (frequency, amplitude) differed between subjects. Correlation analysis revealed that single-ghrelin measurements recorded at 6:00 and 9:30 h (before and after breakfast) were highly correlated with the 24 h integrated (area-under the curve) value for ghrelin (r = 0.873 and 0954, respectively). In other words, a single plasma ghrelin measurement could be used as a surrogate index to monitor true changes in plasma ghrelin. Diurnal changes in plasma ghrelin were similar for lean and obese subjects though the average plasma concentration is shifted upward following weight loss. Ghrelin levels increased prior to meals and decreased after breakfast and lunch indicating that this hormone may be important for meal initiation.[127,*]

Tolle et al. found diurnal changes in plasma ghrelin were partially related to GH pulsality in the rat. However, cluster analysis of ghrelin and GH profiles showed that there was no significant correlation between GH and ghrelin peak secretion but that the inter-peak intervals (frequency) of pulsations were correlated. Ghrelin waves peaked just prior to feeding and decreased by 26% following feeding. High plasma ghrelin levels "led to" increased food intake but only in the dark/feeding period.[128] The relation between ghrelin secretion peaks and feeding has been confirmed in animal studies involving sheep[129] and rat[130] but not always in man. Muller et al. found that plasma concentrations for 10 healthy subjects (10 males; age = 20–28; BMI = 21.8 ± 1/8 kg/m^2) remained constant as a function of time in the fed state, but that a diurnal pattern of secretion was initiated by fasting. Ghrelin levels did not peak prior to meals. Blood ghrelin levels were correlated to GH secretion but were not related to insulin, glucose, or free fatty acid.[131] More recently, Kalra et al. reported that pulsatility patterns of ghrelin increased during fasting with higher pulse number, amplitude, and cumulative hormone secretion. Derangements in ghrelin, leptin, and NPY pulsality under the influences of chronic increases in food intake was suggested a possible basis for obesity.[132]

Yildiz et al. suggested the dynamics of ghrelin secretion were altered in obese (BMI = 35 ± 1.3 kg/m^2) compared to nonobese subjects (BMI = 23 ± 0.3 kg/m^2).[133] The study looking at plasma ghrelin (adiponentin and leptin) changes employed >2000 serum samples over 24 h. There was no significant difference in the 24 h ghrelin values for obese versus lean subjects (409 ± 39 vs. 437 ± 9 pg/mL). However, the pulsatility frequency of ghrelin was significantly higher in lean subjects (21.8 ± 2 pulse/24 h) compared to obese subjects (16 ± 1.3 pulse/24 h). Plasma ghrelin concentration increased during sleep for lean subjects but not for obese subjects. In summary, hormonal signals contain information related to amplitude, frequency, and phase (related to the observed responses). Also, it is evident from the literature that the pulsality of ghrelin and the significance of such phenomenon cannot be considered

* This highly influential paper has received over 800 citations at the time of writing.

in isolation from changes in other appetite-related hormones, notably leptin which also undergoes pulsality. The pulsality of other hormones (e.g., growth hormone, luteinizing hormone, cortisol, etc.) may be relevant for energy homeostasis.[134,135]

9.4.3.2 Food Intake

Meal times coincide with peaks in ghrelin secretion that fell immediately after eating. Such observations were interpreted in terms of the role of ghrelin in meal initiation.[136] However, investigations using *grhl* –/– mice lacking a functioning ghrelin gene found no significant differences in appetite, food intake, or body weight compared to wild-type (*grhl* +/+ mice). Deletion of the ghrelin gene also had no impact on the expression of a range of CNS peptides associated with the regulation of food intake: NPY AgRP, MCH, and POMC peptide. The *grhl* –/– mice had increased tendency to utilize fat rather than carbohydrate as fuel. Apparently, one role of endogenous ghrelin is to promote fat utilization as fuel.[137] The implication of these results is that reported fall in ghrelin concentration in obese subject would promote fat utilization where a rise in ghrelin levels would be sparing of body fat.

9.4.3.3 Macronutrient Composition

Protein, lipid, and carbohydrate intake affect ghrelin secretion differently possibly accounting for their differential satiating effects. Macronutrients suppressed ghrelin secretion with the following order of effectiveness: protein > lipid > carbohydrate. Plasma concentrations of the bioactive (acylated) form of ghrelin declined for 16 healthy subjects (age = 34 ± 14; BMI = 24.1 ± 1 kg/m^2) following the administration of test beverages containing 80% energy contribution from protein, lipid, and glucose.[138] After 120 min of meal consumption, the concentration of serum ghrelin rebounded to levels ~10% higher than control values for high-carbohydrate meal but not with the high-protein or high-lipid meal. High circulating serum lipids decrease ghrelin levels.[139] Carbohydrate meals appear least likely to depress subsequent food intake owing to the more rapid recovery of ghrelin concentrations to pre-feeding levels. Other investigators reported more equivocal relations between dietary macronutrient composition and serum ghrelin levels. Moran et al. found no association between ghrelin levels and dietary macronutrients. By contrast, a 16-week calorie restricted diet produced weight loss (9.2 ± 0.7 kg) and increased fasting ghrelin levels (157.5 pg/mL vs. X) compared to control.[140]

9.4.3.4 Hormones and Ghrelin Secretion

Several hormones indicative of nutritional status affect ghrelin secretion. However, current data is mainly associative and no concrete mechanisms have been established. Insulin inhibits ghrelin secretion.[141–143] Insulin infusion produced a drop in circulating ghrelin (207 ± 12 vs. 169 ± 10 fmol/mL) and hypoglycemia while GH concentration increased from 4.1 to 28.2 µg/L. Serum ghrelin remained depressed during the subsequent increase in glucose concentration from 53 ± 2 to 163 ± 6 mg/dL. Insulin depressive effect on serum ghrelin may be linked with its stimulation of GH secretion, but alternative explanations exist (see below). Administration of glucagon-like peptide-1 (GLP-1; a gut peptide and insulin incretin) increased plasma insulin and decreased plasma ghrelin.[144]

The effect of GH therapy on ghrelin secretion has not received much attention.* Current data suggests that GH treatment suppress ghrelin secretion. A placebo controlled trial involving nine adults suffering from GH deficiency showed that recombinant GH increased blood glucose levels (10%) and insulin (48%). Ghrelin and leptin decreased by 26% and 24%, respectively, following GH therapy. The data is consistent with the view that GH/IGF axis has an inhibitory effect on ghrelin secretion.[145] A later study also involving GH-deficient adults and a slightly different experimental design suggested that GH depresses ghrelin levels by increasing serum free fatty acids following the GH activation of lipoprotein lipases.[146]

The sex hormones (testosterone and estrogen) produce different effects on plasma ghrelin depending on the age of test subjects. Estrogen therapy increased ghrelin secretion in adult females combined with decreased serum lipids (LDL, VLDL, cholesterol, and total triglycerides). However, estrogen had no effect in ghrelin (and leptin) concentrations in peri-pubertal girls (8–12 years old).[147–149] Testosterone treatment is reported to increase ghrelin levels in adult males.[150] However, studies in peri-pubertal males (8–12 years) found that ghrelin levels decreased following treatment with testosterone.

9.4.3.5 Ghrelin and Obesity

Ghrelin levels were lower or no different for obese compared to nonobese subjects.[161,†] Therefore, high food intake in obese subjects is not ascribable to the presence of high serum ghrelin. Tschop et al. found that obese subjects had 27% lower plasma ghrelin concentration compared to nonobese subjects. This influential study also showed that Parma Indians (a population with increased frequency of obesity) had decreased ghrelin levels compared to Caucasians of similar BMI and age. There were no gender differences in ghrelin concentrations but BMI was negatively correlated with serum ghrelin levels. In addition, plasma leptin and insulin levels were markedly elevated for obese subjects (Figure 9.7). It was theorized that the normal functions of leptin and ghrelin were deranged in obesity leading to compensatory changes (increase and decrease) in the secretion of both hormones. Morpurgo et al. reported that ghrelin levels were depressed in 10 severely obese subjects adults (3 males, 7 females; age = 35 ± 9.3 years old; BMI = 45 ± 10 kg/m^2) compared with aged-matched, none-obese controls (~100 vs. 350 pmol/L). Feeding resulted in a marked decline in the ghrelin levels in nonobese subjects but levels remained unchanged in obese subjects. Short-term (3 weeks) weight loss program had no effect on ghrelin levels.

9.4.3.6 Fasting Weight Loss

The effect of fasting on plasma ghrelin appears to be variable. Doucet and coinvestigators[162] found that a 4 day energy restriction diet produced no significant rise in plasma ghrelin for 15 healthy males (Age = 24.4 ± 4.4 years; BMI = 28.3 kg/m^2) despite

* The stimulation of GH secretion by ghrelin and other GH secretagogues is well known (Chapter 8, Section 8.2.4).

† A notable exception is found with obesity arising from the Prader–Willi Syndrome where patients have extremely elevated serum levels of ghrelin. It is also worth noting that the role of ghrelin in this rather complex genetic disorder is not well understood.

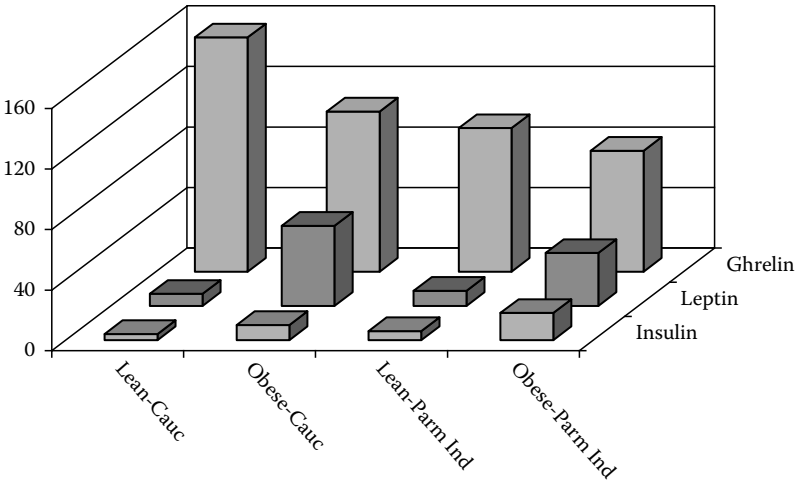

FIGURE 9.7 A comparison of plasma ghrelin levels in obese and nonobese Caucasians (Cauc) and Parma Indians (Parm Ind). (From Tschop, M. et al., *Nature*, 407, 908, 2000.)

significantly higher appetite scores, declining body weight (89.2 ± 15.5 vs. 87.9 ± 15.3 kg; $P < 0.01$), decreased waist circumference (95.4 ± 12.5 vs. 96.2 ± 13 cm vs.; $P < 0.05$), and decreased fat free mass (65.6 ± 6.2 vs. 67.2 ± 7.1 kg for controls; $P < 0.05$). By comparison, serum leptin concentrations fell by 27%–36% following a 4 day energy restriction. Blom et al.[163] reported that plasma ghrelin levels were not different for fasted and non-fasted lean young men (age = 23 ± 3 years; BMI = 27.3 kg/m^2). Preprandial ghrelin concentrations were not related to energy intake when subjects were granted free access to food. These studies suggest that short-term energy intake in lean health subjects may be affected by factors other than serum ghrelin levels.

Prolonged weight loss increases plasma ghrelin levels. Leidy et al. found that the 3 months exercise and diet program designed to produce a 45% deficit in energy intake led to increased 24 h ghrelin values along with weight loss and increased feelings of hunger[164] (Table 9.9). Plasma ghrelin were inversely related to weight. Garcia found that ghrelin levels increased significantly with weight loss following a 6 month exercise and diet program. However, ghrelin levels returned to baseline values after a further six months at the new reduced weight indicating that this hormone may not have a role in the long-term control of food intake.[165] This conclusion is in direct disagreement with those reached after a 12 month weight loss trial reported by Foster-Schubert et al.[154]

9.4.3.7 Aging and Ghrelin Secretion

Plasma ghrelin concentration were lower (158 ± 9 vs. 245 pg/mL) for elderly subjects (age = 79 years; BMI = 25 kg/m^2) compared to young subjects (age = 33.4 years, BMI = 21.2 kg/m^2).[166] Age differences in plasma ghrelin were also reported by Kozakowski et al.[167] Serum ghrelin varies inversely with increasing age between the ages 17–73 years. Women had higher concentrations of ghrelin compared to men. Sturm et al. found that older underweight women had higher ghrelin levels compared to normal weight controls despite lower appetite scores in the former

TABLE 9.9
Factors Linked with Plasma Ghrelin Changes in Humans

Increases Ghrelin	Decrease Ghrelin
Cachexia[a]	Aging[a]
Estrogen therapy	Colorectal cancer and *Helicobacter pylori* infection
Fructose[151]	Ethanol[156]
Multiple sclerosis[152]	Food intake[a]
Sleep curtailment[153]	GH administration
Undernourishment	Glucagon[157]
Undernutrition	Glucagon-like peptide[a]
Caloric restriction	Insulin[a]
Exercise[154,155]	Lipopolysaccharide treatment
Sauna treatment	Low carbohydrate/high-protein foods
Sepsis	Obesity[a]
	Weight gain[158,159]
	Breast feeding[160]
	Free fatty acids

[a] See text for discussions.

group. Clearly, serum concentrations of ghrelin may not always be a good indicator of appetite especially under (inflammatory) conditions where other anorexic factors may be operating.[168]

9.4.3.8 Cachexia

Plasma ghrelin levels are increased in certain wasting conditions and not others. Patients with a combination of chronic heart failure and cachexia had higher ghrelin levels (237 ± 17 fmol/mL) compared to patients with CHF alone (181 ± 10 fmol/mL) or healthy controls (140 ± 14 fmol/mL).[169] Cachexic COPD or lung cancer sufferers showed elevated ghrelin levels.* However, noncachectic patients of COPD showed no changes in ghrelin compared to controls though leptin levels were elevated.[170] The former group also had increased levels of GH, TNF-α, and epinephrine consistent with their catabolic state.[171] Ghrelin levels increased with anorexia nervosa[159] and diet-induced weight loss.[172,173]

The effects of cancer on ghrelin levels seem contradictory. Huang et al. found that ghrelin levels were not elevated in gastric or colorectal cancer patients whether cachexic or not.[174] The results are confirmed recently by D'Onghia et al. who found circulating ghrelin levels were not significantly different for patients of colorectal cancer.[175] In contrast to such results, increased ghrelin levels have been reported for patients suffering from lung cancer[170] and breast cancer.[176] Ghrelin response to cancer may differ according to tumor type and as well as other factors, perhaps the degree of cancer progression and general nutritional status. Plasma ghrelin levels display a biphasic response following exposure to bacterial endotoxin. Injection of

* Chronic obstructive pulmonary disease.

LPS (dose = 2 ng/kg BW) produced a surge in ghrelin within ~120 min (100 ± 30 vs. 7.2 ± 26 pg/mL) with corresponding increases in IL-6, GH, and ACTH. Ghrelin concentrations then declined from 25 h.[177]

9.4.4 Exogenous Ghrelin and Food Intake

There was little or no evidence to suggest that GH secretagogues could stimulate food intake prior to 2000.[178,179] Okada et al.[180] found that a synthetic peptide (KP-102) stimulates food intake based on prior reports that GH release factor had orexigenic properties.[181,*] When 12 obese subjects were injected daily with another GHS designated MC-677 for 8 weeks, there was a significant rise in GH secretion, coupled with increased muscle mass and basal metabolic rate. The treatment group showed increases in body weight consistent with increased food intake. The orexigenic effect of KP-102 was tentative because the food frequency tables applied in the study did not provide sufficiently precise information related to food intake. Ironically, it was the discovery that ghrelin is the natural ligand for GHS-R and accidental discovery of the former peptides effect on food intake that stimulated interest in GH release peptides as appetite stimulants. Research related to ghrelin and food intake is reviewed in this section.

Evidence showing that exogenous ghrelin stimulates food intake in animal models appeared from three different laboratories during 2000.[182–185] Wren et al. showed that peripheral injection of ghrelin (3–30 nmol) produced a dose-dependent increase in food intake in mice. The effects were not diminished by repeated injections of ghrelin. Mice treated with ghrelin by IV injection increased their 24 h food intake by 140%. The efficacy of ghrelin was comparable to NPY and superior to equivalent concentrations of the GHRR-6. The effects on food intake were independent of ghrelin effects on GH secretion which occurred over a timescale of 15–30 min. Ghrelin administration also increased plasma ACTH and decreased thyroid-stimulating hormone secretion.[184,†] Tschop et al. found that daily subcutaneous injection of ghrelin (2.4 μmol/kg) in mice increased weight gain without significantly changing food intake though there was a tendency for overeating. More significant increases in food intake and weight gain occurred following ICV compared to levels administered subcutaneously.[185,‡] Tang-Christinesen, Tchopp, and others reported that ghrelin effect on feeding behavior showed similarity to the effects of AgRP and NPY. Ghrelin treatment not only increased food intake in rats but it also inhibited spontaneous physical activity by 20%. The reductions in physical activity was greater than expected from increased periods of eating exhibited by ghrelin-treated animals. AgRP and ghrelin were similar in their effects on feeding and physical activity whereas NPY stimulated feeding only with no significant effect on physical activity.[186]

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* In hindsight, there was no reason to suspect that KP-102 would stimulate food intake based on data from GHRF because this hormone does not use the same receptor (GHS-R) employed by GH secretagogues such as GH release peptides (GHRP) or ghrelin. See discussion of GH secretagogues in Chapter 8.

† The study was first to confirm that administration of ghrelin at levels normally observed in starved animals could stimulate long-term food intake.

‡ ICV injection required 1000-fold lower concentration of ghrelin (~1–12 nmol/kg) compared to subcutaneous injection.

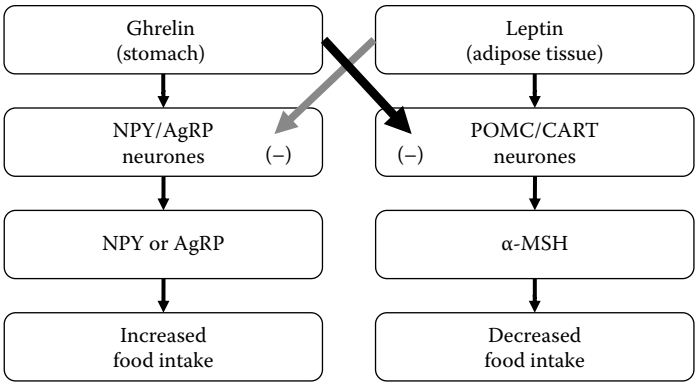

FIGURE 9.8 Modulation of food intake by ghrelin and leptin mediated by arcuate nucleus. AgRP, agouti-related protein; CART, cocaine–amphetamine-regulated transcript; NPY, neuropeptides Y; POMC, is the pro-opiomelanocortin. (Adapted from Meier, U. and Gressner, A.M., Clinical Chemistry, 50, 1511, 2004; Wu, J.T. and Kral, J.G., *Annals of Surgery*, 239, 464, 2004; Hosoda, H., Kokima, M., and Kangawa, K., *Journal of Pharmacological Sciences*, 100, 398, 2006.

9.4.5 Mode of Ghrelin Orexigenic Action

Investigation using rodents indicate that ghrelin and leptin function as counter-regulatory hormones in the regulation of food. The current model for ghrelin action supposes that (a) leptin and ghrelin receptors are located in the hypothalamic centers for regulation of food intake, (b) Stimulation of leptin receptor increase synthesis of anorexic peptides most notably α-MSH while inhibiting the production of NPY and AgRP (Figure 9.8). Exogenously ghrelin has opposing effects on leptin by stimulating the production of orexigenic peptides in the CNS, most notably AgRP and NPY.[187,188]

Ghrelin stimulation of food intake in healthy humans was first demonstrated in 2001.[189] Intravenous infusion of ghrelin (5.0 pmol/kg/min and 270 min) into 9 healthy subjects (5 males, 4 females; age = 25 ± 1.1; BMI = 23 ± 07 kg/m^2) increased food intake by 28 ± 3.9%. The randomized crossover study showed that ghrelin enhanced GH secretion in a dose-dependent manner but that the time-course of GH release was shorter than the effect on food intake. Hunger scores determined by a visual analogue score were significantly increased by ghrelin infusion while gut emptying rate and respiratory quotient were not altered in humans contrary to results from rodents. Interestingly, ghrelin treatment does not appear to moderate food intake in sufferers of anorexia nervosa.[190]

9.5 OTHER BIOACTIVE PEPTIDES FOR MODERATING APPETITE

Fasting leads to increased release of orexigenic hormones (ghrelin and orexin), which are thought to stimulate the vagus nerves running into the nucleus tractus solitarius (NTS). Serum ghrelin is also able to breach the blood–brain barrier (BBB) in the region of the brain stem (area postrema), which is accessible to blood-borne peptides. Receptors for ghrelin and other orexigenic peptides occur

within the ARC nucleus.[191] Gut peptides, whether inhibitory or stimulatory of food intake, are thought to signal via the ARC-NPY and also the POMC/CART nuclei, which therefore integrate signals from peripheral regions (gut, adipose tissue, and pancreas).[192] Further examples of bioactive peptides for moderating appetite are discussed in this section. Orexigenic peptides that improve food intake include the AgRP and synthetic MCR antagonists.[193] In contrast, many food proteins have been cited as inhibiting food intake.

9.5.1 Agouti-Signaling Protein and AgRP

Agouti-signaling protein (ASP) and AgRP are the natural antagonists or reverse agonists for MCR. ASP, which comprises 131 amino acids, regulates skin pigmentation by inhibiting MSH binding to MCR1. Over expression of ASP results in uniform yellow coat color, obesity, and metabolic defects similar to Type 2 diabetes.* Inhibition of α-MSH binding to MRC1 within hair follicles and skin leads to depigmentation or a switch from eumelanin (brown/black) to phaeo-melanin (yellow/red color) formation. Mutations in the somatic ASP gene, leading to increased expression of this protein throughout the body, is the basis for viable yellow (A^{VY}) or lethal yellow (A^{Y}) mice. The A^{VY} strain shows yellow coloration, increased food intake, maturity-onset obesity, increased serum glucose, increase serum lipids, as well as increased body length compared to wild-type mice. AgRP closely related to ASP is expressed mainly in the hypothalamus where it inhibits MSH binding to MCR4. Elevated production of AgRP is associated with obesity (Table 9.10). Administration of exogenous AgRP

TABLE 9.10
Orexigenic Peptides That Stimulate Food Intake

CNS-Derived Peptides	Peripheral Peptide
Agouti-signaling protein (ASP)[a]	Ghrelin
Agouti-related protein (AgRP)[b]	Agouti-signaling protein (ASP)
β-Endorphin	
Endo-cannabinoids	
Galanin	
Hypocretins	
Melanin concentrating hormone (MCH)	
Neuropeptides Y (NPY)[b]	
Norepinephrine	
Orexins A and B	
Substance P	

[a] ASP and AgRP are expressed in the periphery and CNS, respectively.
[b] See text for citations.

* ASP is found in a diverse range of tissue including adipose, gonads, and heart tissue. Over-expression of ASP in agouti mice results in uniform yellow coat color, obesity, and metabolic defects similar to Type II diabetes in humans. Mesh term introduced: 2008 (1997).

produces response similar to those observed naturally in the A^{VY} strain of mice: obesity, insulin resistance, and increases in food intake.* In humans, polymorphisms in the AgRP appears to be linked with inherited tendency toward leanness. Two SNP have been the focus of study. Amino acid substitution leading to the replacement of threonine residue with (Ala67Thr) may be associated with increased body weight in some populations. Another SNP linked with increased obesity was found in the promoter region of the AgRP involving C/T substitution (–38 C/T). Some 29%–35% of subjects carrying the T allele appear to have lower BMI and to show decreased risk of T2D.[194] The role of AgRP in the control of pigmentation and food intake have been reviewed.[106,195–197]

ASP and AgRP are both of interest as nutritional adjuvant for the treatment of anorexia. In small animal studies, prior administration of AgRP blocked LPS-induced anorexia and illness-related behavior. ASP and AgRP contain 130 amino acid residues and share peptide sequences homology with α-MSH. The stimulation of food intake by AgRP is attributed to a core tetra-peptide [His-Phe-Arg-Trp] sequence that also occurs at the C-terminal of α-MSH (Section 9.4.3.2). Though AgRP and ASP presumably compete with α-MSH for melanocortin receptor binding, it is of interest to determine whether the former peptides can inhibit and/or produce an "MSH-like" physiological response in the absence of the primary ligand.[198,†] This notion has been addressed in passing by Adan and Kass (2003) in terms of the behavior of AgRP and ASP as inverse agonists for MRC4. It has been suggested that MRC4 exhibits a level of constitutive activity in the absence of α-MSH leading a tonic decrease in food intake and that binding of AgRP may lead to increased food intake in the absence of MSH.[199,200]

9.5.2 Neuropeptide Y

Neuropeptide Y is a 36 AA peptide possessing tyrosine residues at both the N- and C-terminal endings (hence Y). NPY is produced by neurons in the ARC nucleus. These neurons appear to express leptin receptors. Normal production of NPY stimulates food intake. High levels of leptin is thought to reduce food intake, partly as a result of the inhibition of NPY-producing neurons while those that produce α-MSH are stimulated. So far five receptors have been identified for NPY with NPY5R most clearly linked with the stimulation of food intake.

9.5.3 Serotonin Antagonists

Current research suggests that BCAA increase food intake.[201] The appetite promoting action of BCAA is exerted through the serotonin pathway.[22,23,‡] Fernstrom and

* The nonviable lethal A^{Y} dies soon after birth?

† Ligands may function as antagonist or agonist depending on whether binding improves or inhibits the action of a primary ligand, respectively. According to the current literature, AgRP and related ligands bind to MC4R with lower affinity compared to α-MSH. In principle it should be very difficult to test whether AgRP produces the anorexic effect similarly to α-MSH when the former is administered "alone."

‡ Branched-chain amino acids were formerly called large neutral amino acids (LNAA) in the older literature. In this section we revert to the LNAA in order to retain accuracy of the discussions.

Wuetman showed that the serum concentrations of linear nonpolar amino acids (LNAA) including, tyrosine, leucine, isoleucine, methionine, phenylalanine, and valine increase following a protein meal whereas brain 5-HT and tryptophan levels decreased. When rats were fed synthetic diets containing different amino acids, the concentration of brain 5-HT was found to depend on the ratio of tryptophan: LNAA in the diet. A high-carbohydrate diet (and insulin) was found to enhance the uptake of LNAA by the peripheral tissue leading to a rise in the ratio of tryptophan: LNAA and increasing 5-HT production in the brain.[24,25] The BCAA, which are competitive inhibitors for tryptophan, uptake reduce brain serotonin levels and enhance food intake. During uremic anorexia, a disorder kidney function leads to a low serum concentration of LNAA that increases the uptake of tryptophan by the CNS. The resulting rise in brain serotonin levels may account for uremic anorexia.[202]

9.5.4 Dietary Proteins and Satiety

The possibility that protein supplements could decrease food intake in underweight patients cannot be wholly discounted. Cota et al.[203] found that intracerebroventricular (ICV) injection of leucine reduces food intake and body weight in rats. The anorectic effect of leucine was prevented by the mTOR inhibitor, rapamycin. Morrison et al.[204] showed that ICV injection of leucine depressed food intake and reduced AgRP mRNA expression. It was shown that leucine stimulates mTOR signaling in CNS linked with depressed levels of NPY/AgRP peptide and increasing levels of POMC/CART-derived peptides (Section 9.3). Dietary leucine also stimulates leptin production by adipocytes, which might be relevant in terms of changes in food intake.[205,*] Ropelle et al.[206] found that high-protein weight-loss diets may reduce food intake mediated by leucine activation of CNS-mTOR.[†] Animals fed a high-protein diet (or a leucine-enriched diet) showed reduced food intake and reduced weight gain compared to controls fed a standard chow. Supplementation resulted in reduced fat mass and reduced fat cell size (and possible increases in fat cell number). The anorectic effect of the high-protein diet was not due to increasing aversion to feed. Leucine and protein supplementation increased the phosphorylation p70S6k and the eukaryotic initiation factor 4E (eIF4E) thought to be medicated by mTOR. In addition to dietary studies, ICV injection of leucine (3 μL; 2–4 mM) reduced the 24 h food intake compared to controls injected with vehicle. Biochemical analysis showed that ICV injection of leucine produced a pattern of changes in AMPK, mTOR, and eIF4E similar to those observed with high-protein or leucine dietary supplementation. The possible involvement of mTOR in the regulation of energy balance by leucine has been reviewed.[207–209]

Atkins and other high-protein programs are thought to induce weight loss by various mechanisms: (a) reduction of appetite—protein is thought to be more satiating than fat or carbohydrate; (b) increased the thermic effect of feeding; (c) reduced energy density of meals compared to carbohydrates; and (d) decreased insulin

* Leptin levels can moderate food intake, subject to the phenomenon of leptin resistance.

† An undefined protein or 20 g leucine was added to standard rat chow containing 20% casein. Animals were male 8-week-old Wistar rats or 10-week-old ob/ob mice.

response compared to a carbohydrate meal that avoids postprandial hunger pangs. According to experts in weight-loss diets, the ideal Atkins diet should maintain muscle mass.[210–213] The anorectic effect of leucine (previous section) is one of the latest explanations for the ability of high-protein diets to produce weight loss.

There is currently little information available on the anabolic versus anorectic effect of bioactive peptides and protein supplements. Lang et al.[214] reported that three different proteins (casein, gelatin, soy) had no effect on subjective measures of satiety of nine healthy adults given a test meals comprising 25% energy intake as protein. Though protein choice affected the kinetics of amino acid absorption and insulin profile, these factors apparently did not translate into changes in food intake when subjects were given a mixed meal. Hall et al.[215] reported that whey protein is more satiating than casein. Prefeeding healthy subjects with a liquid meal containing 48 g whey protein produced significantly higher increase in satiety-related hormones (CCK, GLP-1, GIP) compared to casein. The higher satiating effect of whey protein was ascribed to the faster amino acid absorption kinetics. Anderson et al.[216] reported that preloading healthy subjects early in the morning (8:30–9:30 h) with a 400 mL liquid meal containing 45–50 g whey or soy protein reduced food intake 60 min later. By contrast, an egg-protein supplement increased food intake while soy protein also increased food intake if given at 11:00 h. The satiating effect of dietary proteins was short term, dependent on the protein choice and the time of feeding. Bowen et al.[217] found that the satiating effect of dietary protein was independent of protein type and BMI (20–39.9 kg/m^2) of test subjects. Pre-feeding lean subjects ($n = 25$) or obese subjects ($n = 47$) with 400 mL drinks supplemented with 50 g of soy, whey, or gluten protein reduced their subsequent food intake by 10%. Profiles of appetite-related hormones (GLP-1, CKK) were found to be elevated for all proteins preloads compared with a glucose control. Ghrelin levels were depressed for a longer period following protein preload compared to glucose. The changes in plasma levels of appetite generally reached a maximum after 60 min and returned to baseline in 3 h.

Veldhorst et al.[218] found that supplementation of custard breakfast with 25% of whey protein, soybean protein, or casein depressed food intake at lunch. At 10% protein supplementation, whey protein had a more satiating effect compared to soy protein or casein. Whey protein administration increased serum GLP-1 levels compared to other proteins. Veldhorst et al.[219] also found that a breakfast meal containing 25% casein was rated as more satiating by subjects compared to a breakfast meal supplemented with 10% casein in agreement with the higher plasma amino acid profile achieved with the former. However, a 25% casein supplement did not produce a significantly higher reduction in food intake compared to the 10% supplement. Recent investigations using the custard-breakfast model showed that supplementation with alpha-lactalbumin or gelatin (with added tryptophan) led to higher levels of satiety compared with casein, whey protein, or soy protein.[220] To summarize, so-called preloading studies suggest that feeding dietary proteins may impact negatively on subsequent food intake. The relative satiating effects of different proteins appear to be related to several attributes: the content of leucine, the ability to stimulate gut peptide release, the inhibiting serotonin formation, the degree of insulin response, the physical attributes including energy density, and rheological

properties. Perhaps more crucially, we found no concerted studies of the effect of protein supplementation on food intake in sick and convalescent patients. Different proteins are more satiating if they produce a lower insulin response compared to carbohydrate. Refer to the following reviews for additional information on protein foods and satiety.[221–224]

9.6 IN VIVO STUDIES AND CONTROLLED TRIALS

9.6.1 Cancer Anorexia

Animal studies as well as human clinical trial data suggest that ghrelin may be useful for the treatment of illness anorexia associated with cancer. Hanada et al.[225] showed that ghrelin administration (3 nmol/100 μL per mice, 2× per day for 6 days) led to increases in food intake and body weight in normal nude mice and nude mice inoculated with human SEKI tumor cells 14 days prior to hormone treatment. Ghrelin also increased fat mass in normal nude mice and increased serum leptin levels in the tumor-implanted mice, though a direct increase in white adipose tissue (WAP) was not observable by eye. Ghrelin also increased food intake in cachexic nude mice implanted with tumor. DeBoer et al.[226] compared ghrelin effects in a rat model for cachexia with synthetic GHS-R agonists (BIM28131) with improved pharmacokinetic properties. Ghrelin administration decreased wasting due to the loss of lean body mass. The study also revealed that ghrelin SC injection increased the expression of mRNA for orexigenic peptides (AgRP and NPY) while expression of POMC mRNA fell (Table 9.11).

Several Phase 1 human trials involving ghrelin have been reported. Vestergaard et al.[227] found that continuous IV administration of ghrelin (5 pmol/kg/min; 180 min infusion) in healthy humans resulted in biphasic elimination kinetics with a half-life of 24 (+2) min and terminal half-life of 146 (36) min. The mean residence time for ghrelin was positively correlated with HDL levels and BMI. IV ghrelin decreased insulin sensitivity and increased plasma glucose levels and free fatty acid levels. There were transient increases in GH as well as the anti-inflammatory hormones cortisol and ACTH. A recent extensive RCT by Strasser et al. into the safety of ghrelin showed there was little adverse effects in patients suffering from cancer cachexia—according a number of measured indices: bowel activity, abdominal pain, dry mouth, dizziness, chest pain, nausea, or constipation.[228] Akamizu et al. found little or no

TABLE 9.11
Effect of Ghrelin and Synthetic GHS-R Receptor Agonist on Food Intake and Body Mass of Cachectic Rat

Treatment	Food Intake (g)	Δ Fat Mass (%)	Δ Lean Body Mass (%)
Saline	41.4 ± 3.3	−50.8	−12.6 ± 6
Ghrelin	66 ± 3	−51.6 ± 3.4	−1.0 ± 1.9
BIM28131	72.5 ± 3	−31 ± 4.3	−2.7 ± 1.2

Source: Data from Tschop, M. et al., *Nature*, 407, 908, 2000.

adverse effects on ghrelin administration with indications of increased bowel movement, increased bowel activity, hunger, sleepiness, and warm feeling.[229,230]

Two recent Phase II human clinical trials of the effect of ghrelin on cancer anorexic and cachexia have been published. Neary, Wren, and coinvestigators showed that infusion of ghrelin (5.0 pmol/kg/min for 270 min) increased food intake in nine cancer patients by about 31% ± 7% ($P = 0.005$), while meal appreciation improved by 28% ± 8%. The patients for this clinical trial consisted of six, two, and one patient suffering breast cancer, colon cancer, and malignant melanoma.[231] Levin et al. found in a study of healthy humans ($n = 8$, 5 men) and GH-deficient subjects ($n = 6$, 3 men) that ghrelin infusion (10 pmol/kg/min and 180 min) increased gastric emptying rate (1.26% vs. 0.83% per min for saline control). The half-time for gastric emptying decreased from 75.6 ± 4.9 to 49.4 ± 3.9 min following ghrelin infusion. Analysis of gut hormone expression showed that ghrelin treatment increased the secretion of CCK and GLP-1, which may be related to the effects of gastric emptying. There was no effect on PPY or motilin secretion.[232] Subcutaneous administration of ghrelin (3.6 nmol/kg) on 12 healthy subjects (8 men, 8 women) stimulated food intake during buffet breakfast (5076 ± 691 vs. 4230 ± 607) and the palatability score of the participants.[233] Potential applications of ghrelin in the treatment of cachexia have been reviewed.[234–238]

9.6.2 Pulmonary Obstructive Disease

Ghrelin has undergone trials as an adjuvant for treating COPD. A trial by Nagaya et al.[239] showed that a single IV administration of ghrelin (2 μg/kg) increased plasma GH levels in patients 21–25 fold in COPD patients ($n = 7$ subjects). Three week treatment (2 μg/kg, twice daily; 3 weeks) with ghrelin also improved food intake, increased lean body mass, increased body weight, and improved lung function. According to such findings, the ability of ghrelin to correct weight loss is partly due to its anabolic effects arising from the stimulation of GH release. Clearly, other attributes of ghrelin such as the stimulation of food intake and its anti-inflammatory activity may also be important (Chapter 6).

9.6.3 Chronic Kidney Failure and Dialysis Patients

Wynne et al.[240] from Imperial College, Hammersmith, and Charring Cross hospitals, London (United Kingdom) demonstrated that ghrelin can improve food intake in renal dialysis patients. The randomized, double-blind, placebo controlled, crossover study involved mildly malnourished renal dialysis patients ($n = 9$; age = 39–55 years) injected subcutaneously with a single dose of ghrelin (3.6 nmol/kg) or saline. The experimental group showed a twofold increase in food intake (690 ± 60 vs. 440 ± 80 kcal for controls). Ghrelin treatment also produced a 10-fold increase in plasma ghrelin resulting in an attendant increase GH. Ghrelin treatment was followed by significant decrease in blood pressure. As discussed previously, the effects of ghrelin in food intake is believed to be independent of the changes GH. The results are supported by subsequent studies reported by Deboer et al. using animal models for

renal failure.[241] These studies showed that ghrelin treatment not only improved food intake and lean muscle mass, but also inhibited muscle protein breakdown linked with falling levels of proinflammatory cytokines. Apparently, the benefits of ghrelin treatment could be ascribed to the anti-inflammatory activity (Chapter 6) in renal dialysis patients.

9.6.4 HIV Infection Anorexia

Despite the emphasis on protein synthesis and degradation in other parts of this text, there is evidence that weight loss in HIV patients is more determined by general energy balance.[242] McCallan et al. showed that periods of marked weight loss in HIV patients were associated with decreased total energy expenditure owing physical inactivity. Weight loss was strongly associated with a deficit in energy intake arising from "voluntary" reductions in energy intake. Diarrhea, persistent infections, and decreased food intake are major factors that affect HIV-related weight loss.[243–245]

9.6.5 Melanocortin Antagonists for Anorexia Treatment

Though MCR4 is implicated in the development of cachexia,[74–76,246,247] there are currently no well-publicized human RCT for melanocortin antagonists for promoting food intake.[77–79] Several reasons may be suggested for the lack of human studies on MCR antagonists: (a) the present range of compounds lack receptor specificity, (b) peptide MCR antagonists tend to show low stability in the plasma, (c) the AgRP do not appear to cross the BBB and are typically injected via the ICV route, and (d) possibility of side effects, such as tanning, immune effects, etc. However, there may be reason for optimism in this field because MCR agonists for decreasing food intake are currently under active study. Anti-obesity compounds, which show limited bioavailability due to poor transport across the BBB, were successfully administered by intranasal administration.[248,249] The intranasal route is therefore worth considering for CNS acting peptides intended to treat anorexia.

Animal studies by Giraudo et al. showed that injection with 10 or 50 pmol of the nonspecific MCR antagonist SHU9119 increased 4 h food intake in rats by 25% and 52%, respectively. The effect of SHU9119 reached a maximum after 2 h but lasted for 24 h. SHU9119 could be administered by IV injection though at higher concentrations than the levels applied directly to the brain.[250] Kask et al.[251] screened seven cyclical MSH analogues using cells engineered to express MCR1–5 proteins. Binding studies were conducted using ^{125}I-labeled peptides and also by measuring intracellular c-AMP. Of the seven peptides tested, HS024 was the most active melanocortin receptor antagonist with some specificity for MCR4. Administration of HS024 produced a dose-dependent increase in food intake when injected in the central ventricle of free-feeding rats. Estimates showed that H20S4 had similar potency to SHU9119 though the former could be expected to be more selective.

Interestingly, tests showed that high concentrations of HS024 and SHU9119 were toxic to rats, producing disturbances in locomotion termed "barrel rolling," and death. A further analogue of SH9119 designated HS131 has been shown to

increase food intake in rodents. Intracranial injection (0.1 μg) or intravenous injection (1 mg/kg BW) of HS131 increased food intake in within the first 2 h of administration but did not affect 24 h food consumption though long-term studies suggests an ability to increase fat mass. The short-term effects of HS131 suggest that this agent is quickly broken down within the body and that further research is needed to improve the pharmacokinetic characteristics.[109] Recent investigations by Sutton et al. show that SHU9119 and related analogues PG932 increased food intake when administered peripherally. Intraperitoneal injection of mice with 0.4–4 mg/kg (BW) produced a dose-dependent increase in food intake within 1–4 h. Daily injections of these peptides produced a cumulative increase in food intake lasting over 8 days. Interestingly, gene knockout mice lacking a functioning MCR4 did not respond to SHU9119 and PG932.* both peptides were able to reduce the anorexic response and illness behavior (lethargy, reduced grooming) induced by LPS demonstration but only for a short time.[252]

Joppa et al.[253] reported that CNS infusion of 0.3 nmol of the AgRP (83–131) reduced the cachectic effects of induced irradiation (300 RAD, ~3 min) or inoculation with murine colon-26 cells. Treatment with AgRP (83–131) increased food intake and reduced losses of fat and muscle mass compared to untreated controls. Body composition data showed that percentage fat decreased by 20%, 40%, and 5% for controls, tumor-bearing mice, and tumor-bearing mice treated with AgRP, respectively. The control group and AgRP-treated mice both retained higher muscle mass (7%) compared to 2% for tumor-bearing mice. Clearly, MRC4 blockade using AgRP could be a potentially useful strategy for treatment of cancer anorexia–cachexia. Cheung et al. found that ICV administration of AgRP reduced uremic cachexia in a mouse model resulting in increased body weight independently of food and protein intake. The effects appeared to be partly mediated by changes in myostatin and IGF-1 gene expression that were decreased and increased by AgRP administration, respectively. AgRP administration also decreased levels of SOCS2 protein levels of which were elevated in uremic cachexic animals.[254]

9.7 SUMMARY AND CONCLUSIONS

The control of food intake involves neuroendocrine mechanisms interacting with signals from the peripheral tissues, mainly the gut and adipose tissue. Current evidence suggest that the gut–hypothalamus axis offers many potential targets for the development of anorexia. A number of mutations affecting the leptin and melanocortin genes or their receptors are linked with increased food intake and obesity. Current strategies to combat anorexia have been described using a wide range of orexigenic agents to increase food intake including the growth hormone secretagogue ghrelin, melanocortin receptor antagonist, and branched-chain amino acids. Clinical trials conducted so far show some promising data suggesting that bioactive peptides may be useful for promoting appetite.

* SHU9119 is considered a nonselective antagonist of the melanocortin receptors.

APPENDIX 9.A.1
Amino Acid Sequences of Some Melanocortin Receptor Ligands[a]

AA Sequence	3	4	5	6	7	8	9	10	11	12	13
α-MSH	Ser	Met	Glu	***His***	***Phe***	***Arg***	***Trp***	Gly	Lys	Pro	Val
Melanotan II	—	nLe	Asp	His	D-Phe	Arg	Trp	Lys			
SHU9119[a]		nLe	Asp	His	D-Nal	Arg	Trp	Lys			
HS131			Cys	Gly	D-Nal	**Arg**	**Trp**	Cys			
HS014		Cys	Glu	His	D-Nal	**Arg**	**Trp**	Gly	Cys		
HS024[a]	Cys	Nle	Arg	His	D-Nal	**Arg**	**Try**	Gly	Cys		

[a] Ligands are those listed in Table 9.4.

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Food Science and Technology

Critically Appraises the Efficacy of Bioactive Peptides Using Evidence from Human Clinical Trials & Animal Models

Chronic illnesses, injury, or infections produce a decline in muscle mass—leading to delayed recovery, more post-treatment complications, longer hospital stays, and higher mortality rates. Therefore, ensuring adequate lean body mass is of major concern in health care. Presenting data from human studies and trials, along with recent research findings, **Bioactive Peptides: Applications for Improving Nutrition and Health** summarizes the applications, and the benefits of bioactive peptides used to mitigate major metabolic derangements that arise from chronic illnesses and result in unwanted weight loss.

Reviews the Latest Theories Explaining Muscle Loss and Accretion during Illness & Infection

In chapters one through five, the book presents the background science on the relationship between illness and muscle weight loss, highlighting bioactive peptides' ability to enhance the body's antioxidant status, antisepsis capacity, immune function, anti-inflammatory capacity, growth potential, and appetite. Chapters six through nine deal with the use of bioactive peptides to modify aspects of the host response to illness, including inflammation, antimicrobial activity, anabolic dysfunction, and anorexia.

This state-of-the-art reference also includes case studies on aging, AIDS, COPD, diabetes, inflammatory bowel disease, kidney failure, and tuberculosis. It is a valuable resource for dieticians, doctors, nutritionists, and manufacturers of medical foods, tube feeds, supplements, and nutraceuticals.

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