Nutritional interventions to promote post-exercise muscle protein synthesis

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Nutritional interventions to promote post-exercise muscle protein synthesis

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1

Introduction

Skeletal muscle is one of the major organs involved in metabolism. It accounts for ~50% of total body mass and is therefore one of the major contributors to whole-body metabolic rate. In addition, skeletal muscle plays a central role in the regulation of whole-body glucose metabolism. By virtue of its mass, skeletal muscle is the primary tissue responsible for insulin-dependent glucose clearance, accounting for up to 80% of whole-body glucose uptake following meal ingestion.
Aging and diseases like cancer and chronic obstructive pulmonary disease (COPD) are associated with a significant loss of muscle mass. For example, a 20-40% reduction in fat free mass has been reported in COPD patients. Muscle wasting in aging and these catabolic diseases eventually leads to muscle weakness and major disabilities in day-to-day life. The latter is accompanied by a reduction in physical performance, the loss of functional capacity and an increased likelihood of developing chronic metabolic diseases like obesity, type 2 diabetes and osteoporosis.
Various interventions have been proposed to reduce or reverse the losses of muscle mass and function. It has been suggested that physical activity (i.e. resistance exercise) and nutritional interventions (i.e. protein and amino acids) could represent effective strategies to reduce or counteract the loss of muscle tissue that occurs with aging and disease. In this thesis we focus on the beneficial effects of resistance exercise and the potential of dietary supplementation with carbohydrate, protein and a specific amino acid to enhance muscle protein anabolism.

Resistance exercise

Resistance exercise or strength training involves a small number of muscle contractions against a heavy load (63). This kind of exercise has been demonstrated to result in an increase in muscle size and strength (34, 93, 113). Although the metabolic and morphological adaptations resulting from resistance and endurance exercise are quite different, both training methods have been shown to augment whole-body insulin sensitivity (16, 23, 29-31, 35, 55, 73, 82, 92). While endurance training can effectively increase mitochondrial density and oxidative capacity (51, 52), resistance training should not be considered as a primary means of training for increasing VO_2max. However, this type of exercise can effectively increase muscular strength, fat free mass and augment physical fitness. The latter is partly due to an increased amount of contractile protein within the muscle (113). This is particularly important in the elderly where the loss of muscle mass and strength are prominent deficits. As such, the American College of Sports Medicine currently recommends strength or resistance training as an important component of an overall fitness program (1).

Resistance exercise and muscle protein synthesis

Resistance exercise can provoke profound increases in muscle mass, or hypertrophy. However, it generally takes weeks or months before these changes become

apparent (89). This prolonged time course for hypertrophy is a reflection of the slow turnover rate of muscle proteins. The turnover rate of contractile protein in the fasted state in skeletal muscle, for example, is as low as 1% per day (7, 77, 105). Hypertrophy can only occur when protein synthesis is exceeding protein breakdown for an extended period. In conclusion, it is the balance between muscle protein synthesis and breakdown rates that determines net protein balance and the rate of net protein gain (89).
Resistance exercise can effectively stimulate muscle protein synthesis (21, 68, 69, 84). It has been shown that after a single session of resistance exercise, muscle protein synthesis is rapidly stimulated within 2 to 4h (84). Moreover, the stimulation of protein synthesis has been reported to last for at least 24h (21, 68), up to 48h (84). The increase in muscle protein synthesis or fractional synthetic rate (FSR), can be largely attributed to an increase in myofibrillar protein synthesis rates. Proteins, likely to be upregulated following resistance exercise are most likely myosin heavy chain (MHC) and actin, since those proteins are most abundant in skeletal muscle. This is supported by observations showing that synthesis rates of MHC are increased after a single resistance exercise session (11, 112, 119). Interestingly, muscle protein breakdown is also stimulated following resistance exercise; although, to a lesser extent than protein synthesis. The result is an increased net muscle-protein balance that persist up to 48h (84). On the other hand, net muscle protein balance is negative in the absence of nutrient intake, indicating that the muscle remains in a catabolic state. Therefore, muscle hypertrophy requires the interaction of exercise and nutrition, which will be further discussed in chapter 1.4.
In addition to the acute effects of resistance exercise on muscle protein synthesis rates, prolonged resistance exercise training results in an elevated resting mixed muscle protein FSR (83, 85). This training induced elevation in muscle protein turnover indicates that the processes of protein synthesis and degradation are both increased. The latter may indicate that a more rapid protein remodeling is taking place (83).

Muscle protein synthesis

The primary determinant of skeletal muscle size attained during resistance training is the external load that is applied. Several animal models have shown that increased loading results in an increased muscle mass and protein content per muscle (6, 10, 90). Increased loading enhances muscle protein synthesis and breakdown and this training induced rise in muscle protein turnover leads to a more rapid protein remodeling (83).
Factors that lead to muscle hypertrophy are: muscle cell stretch (56, 70), increases in intracellular calcium (56), as well as exercise-induced increases in circulating and locally produced growth-factors such as insulin-like growth factor-1 (IGF-1; 42) and muscle growth factor (MGF; 47, 49). An acute bout of resistance exercise has been shown to activate transcription factors like Myo-D and myogenin (115), and to increase the rate of mRNA formation encoding for myosin heavy chain (66,

115). However, interestingly, changes in the rate of protein synthesis occur before changes in mRNA accumulation (111). Therefore, activation of protein synthesis must be controlled by a post-transcriptional mechanism (64). The post-transcriptional regulation of protein synthesis involves mRNA translation initiation, elongation, termination and post-translational modification. The initiation of mRNA translation is thought to be most important for the overall control of muscle protein synthesis (14).

Translation initiation

The initiation of mRNA translation is a complex multi-step process requiring more than a dozen eukaryotic initiation factors (eIFs) (59, 81). At least two steps in the initiation pathway are subject to regulation *in vivo*: 1) binding of initiator methionyl-tRNA (met-tRNA) to the 40S ribosomal subunit, and 2) binding of mRNA to the 43S pre-initiation complex (2). In the first step, met-tRNA binds to the 40S ribosomal subunit as a ternary complex with eIF2 and GTP. A second initiation factor, eIF2B, mediates guanine nucleotide exchange on eIF2 (figure 1.1). Inhibition of eIF2B activity results in a decrease in the amount of eIF2·GTP available to form the ternary complex, thereby limiting translation initiation. The activity of eIF2B is reciprocally regulated in part by phosphorylation of the α-subunit of eIF2, as it converts eIF2 from a substrate to a competitive inhibitor of eIF2B (60).
Another potential rate-controlling step in the process of translation initiation involves the recognition and unwinding of the mRNA to allow binding to the 40S ribosome (2). This step requires a group of proteins referred to as eIF4F (figure 1.1), which is a multi-subunit complex (consisting of both eIF4E and eIF4G). The eIF4F complex collectively serves to recognize, unfold and guide the mRNA to the 43S pre-initiation complex (81). Modulation of eIF4E availability for binding eIF4G and forming the active eIF4F complex can alter translation initiation. The availability of eIF4E for eIF4F complex formation appears to be regulated in part by 4E-BP1, which compete with eIF4G for binding eIF4E (46), and is able to sequester eIF4E into an inactive complex. The binding of 4E-BP1 to eIF4E is regulated by phosphorylation of 4E-BP1, with increased phosphorylation of the protein causing a decrease in the affinity of 4E-BP1 for eIF4E.

Intracellular signaling pathways that regulate translation initiation

It has been shown that the phosphatidylinositol-3 kinase (PI-3 kinase)/mammalian target of rapamycin (mTOR) signal transduction pathway plays a major role in the regulation of translation initiation. This signaling pathway includes several protein kinases, including PI-3 kinase, protein kinase B (PKB, or Akt), mTOR and p70/p85 ribosomal protein S6 protein kinase (S6K1). Akt, downstream of PI-3 kinase, phosphorylates and inactivates another protein kinase, glycogen synthase kinase (GSK-3). Consequently GSK-3 phosphorylates eIF2B, resulting in inhibition of its guanine nucleotide exchange activity, thereby increasing protein

synthesis rates (figure 1.1). In addition to GSK-3, Akt also phosphorylates mTOR on Ser^{2448}, which subsequently phosphorylates and activates 4E-BP1 and S6K1. This allows the eIF4F complex formation and the activation of ribosomal protein S6, resulting in enhanced mRNA translation (figure 1.1).

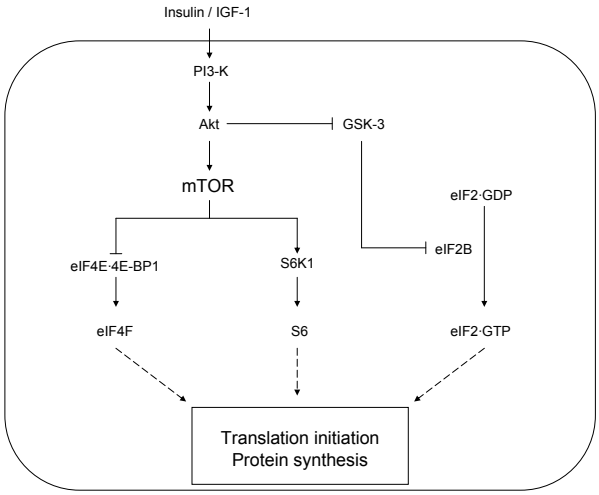

Figure 1.1 Intracellular signaling and protein signaling. Details of the individual steps are discussed in the text. PI3-K, phosphatidylinositol-3 kinase; Akt, protein kinase B; mTOR, mammalian target of rapamycin protein kinase; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eIF4E binding protein 1; S6K1; p70/p85 ribosomal protein S6 protein kinase; eIF4F, eukaryotic initiation factor 4F; S6, ribosomal protein S6; GSK-3, glycogen synthase kinase 3; eIF2B, eukaryotic initiation factor 2B; eIF2·GDP, inactive eukaryotic initiation factor 2; eIF2·GTP, activated eukaryotic initiation factor 2.

Chronic overloading of the plantaris muscle in rodents has been shown to increase phosphorylation of Akt/PKB and proteins downstream of mTOR, such as 4E-BP1 and S6K1 (10). In addition, increased phosphorylation of Ser^{2448} on mTOR after overload has been reported in rats (90). Moreover, in other animal studies phosphorylation of both 4E-BP1 and S6K1 has been shown to be enhanced in response to muscle loading (6, 10, 75). However few studies have examined the impact of exercise on translation initiation factors in humans *in vivo*. A recent study in humans investigated the effects of a single session of resistance exercise on S6K1 activation (57). Although phosphorylation of S6K1 was increased following exercise, S6K1 activity was unaltered, as evidenced by unchanged S6 phosphorylation. These data indicate that a single session of resistance exercise does not fully activate S6K1. Interestingly, Karlsson et al. (15) showed that post-exercise ingestion of branched chain amino acids has a striking effect on S6K1 phosphorylation, which was evident by site-specific phosphorylation at Thr^{389}. These data indicate that intake of protein/amino acids is needed to fully activate skeletal muscle translation initiation.

Measuring protein synthesis

Our knowledge of the regulation of protein metabolism at the whole-body, muscle and organ levels in various physiological conditions is based on direct and indirect measurements of protein synthesis and breakdown, and exchange rates of amino acids and amino acid tracers between tissues (109). In the studies that are described in this thesis we used amino acid tracers to study the effect of several exercise and nutritional interventions on whole-body and muscle protein turnover.

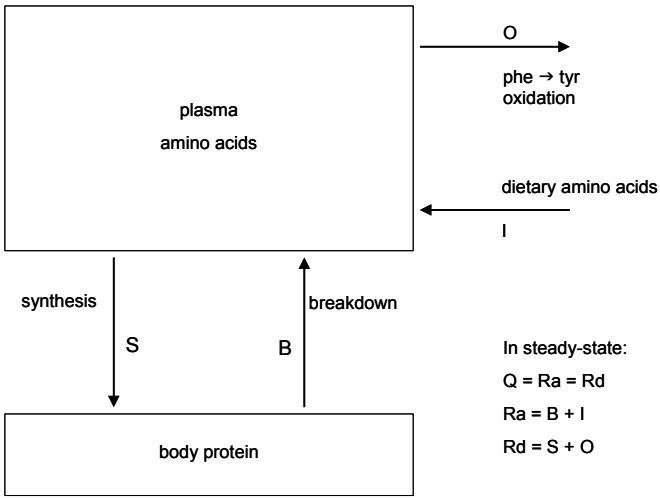

Figure 1.2 General model of protein metabolism used in the whole-body methods. Q, whole-body amino acid turnover or flux; Ra, rate of appearance in the plasma free amino acid pool; Rd, rate of disappearance from the plasma free amino acid pool; phe, phenylalanine; tyr, tyrosine. Adapted from Wagenmakers (109).

Measuring whole-body protein turnover

Stable isotope tracers are widely used in biomedicine to study metabolic pathways *in vivo*, because they are functionally identical to the compound of interest (tracee), but distinct in some physical characteristics that enable their precise detection (116). Calculation of substrate kinetics traditionally measures the rate of appearance (Ra) which in steady state conditions equals the rate of disappearance (Rd) of the substrate (=flux). In a single pool model it is assumed that the infusion of tracer, sampling, and Ra of substrate occurs from a single, homogenous, instantly mixing pool (109). In metabolic studies, the blood compartment is usually viewed as a single pool, which implicates that blood sampling is allowed for the calculation of whole-body flux (109, 116). In this model the rate of disappearance of amino acids from the blood compartment equals the rate of oxidation + protein synthesis, whereas the rate of appearance of amino acids equals the rate of protein breakdown + the rate of appearance of meal protein from the gut (figure 1.2) (109).

The use of the (whole-body) tracer balance methodology to investigate the effect of post-exercise nutrition on protein metabolism has some important limitations, as it does not allow the direct measurement of muscle protein synthesis rates. As such, it does not provide information on the contribution of individual tissues to protein metabolism, their response to nutritional and/or exercise intervention and it does not enable the discrimination between active and inactive muscle.

Measuring muscle protein synthesis

To circumvent the limitation of whole-body tracer methodology, methods were introduced to measure tissue or protein-specific synthesis rates; the amino acid tracer incorporation method. In most of these studies the amino acid tracer is provided by a continuous intravenous infusion until a steady state is obtained in the precursor pool for protein synthesis. Repeated muscle samples are then taken at steady-state. The protein is precipitated from the biopsy samples, hydrolyzed and the amino acids, after derivatization are analyzed for tracer enrichment using gas chromatography-mass spectrometry (GC-MS) or gas chromatography-isotope ratio mass spectrometry (GC-IRMS) methodology. The rise in tracer enrichment in the protein-bound amino acid fraction, over a given sampling time, is divided by the steady-state tracer enrichment in the precursor pool to give the fractional synthetic rate (FSR) of the protein, which is the percentage of the existing pool that has been synthesized over that time period (109). Although there has been a lot of discussion in the literature on which amino acid pool represents the best reflection of the precursor pool for protein synthesis during continuous-infusion studies, the intracellular amino acyl-tRNA is generally regarded to be the true protein synthesis precursor pool (109). However, due to the large muscle sample size that is required to make an estimate of the enrichment in the amino acyl-tRNA pool and the inherent analytical complexity of such measurements, the plasma and free muscle amino acid enrichments are generally used as surrogates to provide an estimate of the lower boundary (based on plasma precursor enrichments) and the higher boundary (based on intracellular muscle precursor enrichments) for the true fractional synthetic rate of mixed muscle proteins.

Nutrition and muscle protein synthesis

Physical activity is the most powerful stimulus to promote net muscle protein anabolism. After a single bout of resistance exercise, muscle protein synthesis and degradation rates are elevated. As a result, net muscle protein balance will actually remain negative in the absence of food intake. Skeletal muscle hypertrophy can only take place when food is ingested during the early phases of post-exercise recovery (32). Consequently, the combined ingestion of carbohydrate and protein/amino acids during recovery from exercise forms an effective strategy to stimulate muscle protein synthesis, inhibit protein degradation and, as such, to obtain a positive muscle protein balance by optimizing circulating insulin and amino acid concentrations.

Carbohydrate

The ingestion of glucose during the post-exercise recovery phase has been shown to significantly increase circulating plasma insulin levels (62, 95, 102, 103). The latter has been shown to result in a decrease in urinary 3-methylhistidine and urea excretion, suggesting that protein degradation is reduced (95). However, muscle protein synthesis rates are not affected following carbohydrate ingestion (17, 95). Although carbohydrate ingestion improves net leg amino acid balance compared with water or placebo intake, the net balance remains negative (17). The effects of carbohydrate supplementation on protein metabolism can be in part attributed to the increase in circulating insulin. The elevation of plasma insulin levels results in an increase in net muscle protein anabolism *in vivo* in humans (42, 45, 53). However, insulin should not be regarded as a primary regulator of muscle protein synthesis rates as insulin levels exert only a modest effect on muscle protein synthesis in the absence of elevated amino acid concentrations (8). These observations are further supported by measurements in rats, showing that increased circulating insulin concentrations alone (induced by glucose supplementation) do not stimulate translation initiation post-exercise in rats (44). In addition, a nutritionally complete meal was shown to be more effective than feeding carbohydrate alone for post-exercise stimulation of protein synthesis (44). It is therefore suggested that most probably the protein in the diet is necessary for full stimulation of translation initiation and thus protein synthesis in skeletal muscle during exercise recovery.

Protein and amino acids

Amino acids have more metabolic roles than to merely serve as the building blocks of proteins. In addition, amino acids have been shown to induce the secretion of insulin, glucagon, growth hormone, and IGF-1 (37). The infusion of free amino acids *in vivo* in humans was shown to result in a significant increase in plasma insulin levels (38-41). More recently, reports from our laboratory show that co-ingestion of a protein hydrolysate and free leucine and phenylalanine with carbohydrate effectively increases the plasma insulin response in type 2 diabetes patients (101), healthy controls (104) and endurance trained athletes (102, 103). Amino acids, and leucine in particular, can act as potent stimuli for the secretion of insulin from the pancreatic β-cell. The various mechanisms by which amino acids promote and/or enhance insulin secretion are diverse and have not yet been fully elucidated (79). Similar to glucose mediated insulin secretion (28), intracellular catabolism of the metabolizable amino acids will increase substrate availability for the tricarboxylic acid (TCA) cycle. Increased TCA cycle activity and oxidative phosphorylation will result in an increased ATP/ADP ratio, which will lead to the closing of ATP-sensitive K^+ channels. The latter will lead to the depolarization of the plasma membrane, thereby opening up voltage activated Ca^{2+}-channels, resulting in Ca^{2+}-activated insulin exocytosis. Leucine-induced insulin secretion is mediated by its oxidative decarboxylation as well as by its ability to allosterically activate glutamate dehydrogenase (79, 96, 117). Both the generation of acetyl-CoA

and α-ketoglutarate are needed for leucine to fully stimulate mitochondrial activity in the pancreatic β-cell. The metabolically linked secondary signals that subsequently lead to insulin exocytosis remain to be established, and also seem responsible for the leucine-induced activation of the mTOR signaling pathway (100, 117).

There is a substantial amount of evidence that supplementation with protein/amino acids can effectively stimulate muscle protein synthesis rates. Biolo et al. (9) demonstrated that hyperaminoacidemia, resulting from intravenous infusion of amino acids, increases post-exercise muscle protein synthesis rates and prevent the exercise-induced increase in protein degradation. Moreover, amino acid infusion in the fasted state, without preceding (resistance) exercise, rapidly increased muscle protein synthesis (105). Since intravenous infusion of free amino acids is not a practical method for amino acid delivery, oral ingestion of protein and/or amino acids has been studied intensively. The oral ingestion of a large amount (30-40 g) of amino acids after exercise effectively stimulates muscle protein synthesis (99). Moreover, ingestion of a small amount of essential amino acids (EAAs) with and without carbohydrates has also been shown to increase post-exercise protein synthesis and improve net protein balance (18, 72, 87). Thus, post-exercise amino acid ingestion represents an effective method to maximize the anabolic effect of exercise. However, ingestion of only a small amount (6 g) of EAA 1h and 2h post-exercise resulted in a positive net protein balance for only two hours, after which net protein balance over the muscle became negative (18). This indicates that amino acid and/or energy delivery was not sufficient to maintain an anabolic state (enhanced protein synthesis).

The precise mechanisms responsible for this amino acid-induced anabolic response have not yet been established. Amino acids play an important role in the control of mRNA translation and thus protein synthesis in skeletal muscle (58). Increased amino acid availability, particularly the branched-chain amino acid leucine, has been shown to activate several key-factors (mTOR, S6K1) of the mTOR signal transduction pathway, thereby increasing the rate of mRNA translation (61).

Leucine

The role of leucine as a regulator of protein metabolism has been studied extensively (43). In the 1970s, a number of laboratories performed *in vitro* investigations of the factors that control protein turnover in tissues (19, 65, 74). These studies showed that high concentrations of all amino acids stimulated protein synthesis and inhibited protein degradation. In particular, it was shown in isolated diaphragm muscle, that leucine alone could effectively stimulate protein synthesis (19). This group of studies, from several laboratories, initiated a continuing series of investigations into the role of leucine in the control of tissue protein mass, its mechanism of action, and its possible value for enhancing muscle protein deposition in healthy subjects or moderating muscle protein loss in catabolic states in patients.

In general, most animal studies support the findings by Buse and Reid (19) and report that leucine inhibits muscle protein breakdown and stimulates muscle protein synthesis. For example, Anthony et al. (3, 4) have reported a direct, stimulating effect of leucine ingestion on muscle protein synthesis in rodents. In line with those data, the same group showed that leucine supplementation enhances protein synthesis in skeletal muscle from diabetic rats through insulin-independent mechanisms (5). These studies have increased the understanding of the mechanisms of nutritional regulation of protein synthesis at the molecular level and indicate that leucine ingestion could stimulate muscle anabolism in several ways, as it can further increase plasma insulin concentrations and also directly stimulate protein synthesis. Leucine has the ability to function as a nutritional signaling molecule that modulates muscle protein synthesis and/or breakdown following food intake. The stimulatory effect of leucine on protein synthesis occurs at the level of translation initiation and involves signaling through mTOR (61, 86), which is thought to serve as a convergence point for leucine- and insulin-mediated effects on translation initiation (61, 86). In addition, leucine has been shown to have the potential to affect muscle protein metabolism by decreasing the rate of protein degradation (78), most likely via an increase in circulating insulin, and phosphorylation of key proteins involved in the regulation of protein synthesis (57, 67, 86).

Though most *in vitro* and *in vivo* animal studies report that leucine administration can inhibit protein breakdown and stimulate protein synthesis, most *in vivo* human studies report that leucine and/or BCAA administration reduces muscle protein breakdown, without stimulating muscle protein synthesis (71). The apparent discrepancy between observations in humans and animal studies remains to be elucidated. Maximal rates of protein synthesis rates during post-exercise recovery probably require both leucine and insulin signaling and the anabolic signal of resistance exercise. However, under normal *in vivo* conditions in humans it is impossible to discriminate between the effects of leucine and insulin and resistance exercise (IGF-1 and MGF) on muscle protein synthesis.

In conclusion, due to anabolic properties of insulin and amino acids (leucine in particular), it has been suggested that a dietary supplement containing free leucine, protein and carbohydrate could be an effective tool to further increase post-exercise muscle protein synthesis and/or to inhibit protein degradation.

Aging

The progressive aging of our population imposes a major threat to welfare in the Western society. Population demographics in the Netherlands show that by 2025 more than 20% of the entire Dutch population will be aged 65 years or older (91). This aging of our society is responsible for a major increase in population morbidity, which will put an increasing demand on our health care system. The increased morbidity in the elderly is associated with the progressive decline in skeletal muscle mass and function. This process of age-related loss of muscle mass and function, or sarcopenia, has substantial health consequences, as it is associated

with decreased basal metabolic rate, increased body fatness, loss of bone mass and reduced strength and functional capacity. Sarcopenia is facilitated by a combination of factors, which include a more sedentary lifestyle and sub-optimal diet. The age-related changes in skeletal muscle mass are attributed to a disruption in the regulation of muscle protein turnover, which results in a chronic imbalance between muscle protein synthesis and degradation.

Aging and muscle function

It has been reported that both muscle cross-sectional area and fiber numbers decreased from as early as the fourth decade of life. Short et al. (97, 98) measured the cross-sectional area of muscle in the mid-thigh using computed tomography imaging in healthy human subjects (18 to 88 y) and observed an age-related decrease in muscle area starting from the fourth decade of life. In addition, a parallel decrease in muscle strength (knee extension) occurred with the decrease in muscle mass. Accordingly, data from our laboratory shows a similar an age-related decrease in muscle strength (figure 1.3, L. Verdijk, unpublished observations). Maximal oxygen uptake is another physical performance related factor which has been shown to decline with aging (97). As a result, the muscles of older individuals are thought to fatigue faster than those of young adults.

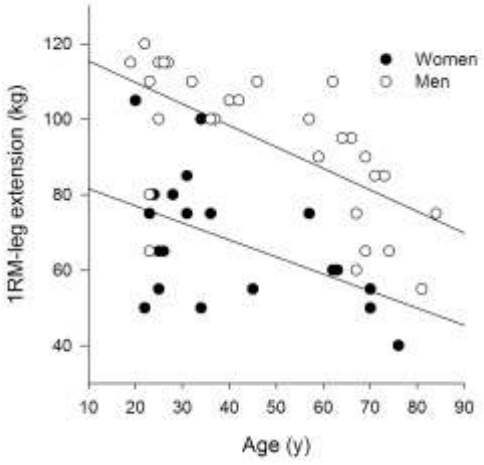

Figure 1.3 Age-related decrease in knee extensor strength as observed in our laboratory. Knee extensor strength was determined on a leg-extentions machine. 1RM, one-repetition maximum or maximal strength.

The observed decrease in muscle mass and muscle strength with aging, in combination with a reduced endurance capacity, is linked to reduced levels of physical activity (76), which results in a decrease in total energy expenditure in older persons. The latter is likely to attribute to an increased prevalence of obesity, especially abdominal fat accumulation. Such alterations in body composition can

cause insulin resistance and contribute to the development of type 2 diabetes, hyperlipidemia, and hypertension. The combined effect of these metabolic abnormalities is increased cardiovascular and other morbidities (76).

Aging and muscle protein turnover

The age-related changes in skeletal muscle mass are attributed to a disturbance in the regulation of muscle protein turnover, which results in a chronic imbalance between muscle protein synthesis and degradation. The concept of aging is strongly associated with a decrease in protein synthesis, including changes in genome integrity and gene expression (54), and in translation and post-translational modifications of proteins (88). Recently, it has been shown that muscles of elderly humans have significantly lower amounts of total mTOR, S6K1 and eIF2B, all key-factors in the regulation of translation initiation (24). Many studies have reported either similar (80, 106-108), or reduced (7, 50, 94, 112, 113, 118, 119) skeletal muscle protein synthesis rates in the elderly compared with young adults. More specifically, the synthesis rates of myofibrillar proteins (112), but not of sarcoplasmic proteins (7) have been shown to be reduced in the elderly compared with young adults. The synthesis of myosin heavy chain was dramatically reduced (7), without any change in its rate of transcription (110). In addition to the reduced MHC synthesis, aging is also characterized by a reduced rate of mitochondrial protein synthesis, which may contribute to the depressed aerobic capacity and muscle performance (94). Restoring the balance between protein synthesis and breakdown should form the main therapeutic target in intervention programs designed to improve muscle mass and function in the elderly.

Interventions to improve muscle protein synthesis in the elderly

There is substantial evidence that the muscle protein synthesis rate is responsive to exercise (89). In studies performed in young and aged persons, resistance exercise (50) and endurance exercise (97) stimulate the synthesis rate of mixed muscle proteins. Resistance exercise training mainly affects muscle strength and muscle mass and can increase specific muscle proteins, such as MHC proteins, in the elderly (50). In contrast, endurance exercise improves many metabolic functions, including insulin-induced glucose disposal and mitochondrial function, which is likely to be mediated by changes in specific proteins/enzymes (15). The specific adaptations that occur depend on the frequency, intensity and the duration of the exercise regimen, as it has been shown that only modest increases in strength are achieved by elderly subjects when exercise intensity is low (33).
In addition to exercise, it has been hypothesized that the ingestion of sufficient amounts of protein or specific mixtures of amino acids can effectively increase the anabolic response in older individuals. In accordance, infusion of amino acids has been shown to increase muscle protein synthesis in the elderly (105). However, intravenous infusion of amino acids is not a physiologic route for amino acid delivery, as it circumvents both intestinal absorption and first-pass splanchnic

processing. Interestingly, it has been shown that protein digestion rates can differentially affect the anabolic response in young and older adults (25). Slowly absorped protein (casein) stimulates protein anabolism in the young (12), whereas faster absorped proteins (whey protein) favor anabolism in the elderly (25). In addition, a percentage of the ingested amino acids will not be made available for muscle protein synthesis due to utilization in the splanchnic tissues. The latter has been suggested to increase with age (13, 107). Nevertheless, Volpi et al. (107) demonstrated that despite a greater first-pass extraction by the gut and liver of orally administered amino acids, the amino acid-induced muscle protein anabolism in older subjects is not different from that of younger individuals.

In contrast to these finding, there are indications that the anabolic response to mixed meal, consisting of carbohydrate and protein (and fat), is substantially blunted in the elderly. For example, most interventions using nutritional supplements to attenuate muscle loss in older individuals were unsuccessful in humans (20, 36, 114). However, Esmarck et al. (32) showed that in the elderly the timing of protein ingestion following resistance exercise is crucial, as immediately post-exercise supplement ingestion resulted in significantly greater gains than when the supplement was ingested after several hours of recovery. Volpi et al. (106) reported a blunted anabolic response in older individuals to the combined ingestion of glucose and essential amino acids. The latter was confirmed using the hyperinsulinemic, hyperaminoacidemic clamp technique showing an impaired activation of S6K1 as a potential mechanism for the attenuated protein synthetic response in the elderly (48).

Interestingly, recent studies in rodent have suggested a possible role for oral leucine supplementation to restore normal postprandial anabolism in aged animals (26, 27). When supplemented with leucine, muscle protein synthesis in old rats was stimulated and similar to that observed in mature rats. Therefore, it was concluded that meal supplementation with leucine was sufficient to restore postprandial stimulation of muscle protein synthesis (27) and inhibition of proteasome-dependent proteolysis (22) in old rats. Whether leucine supplementation can effectively increase muscle protein synthesis and net muscle protein balance and quality under normal physiological conditions in humans remains to be established.

Outline of this thesis

This thesis describes a series of studies that investigate the effects of exercise and nutrition on muscle protein synthesis, substrate utilization and insulin sensitivity. In **chapter 2**, we first describe a study about the acute effects of a single session of resistance exercise on whole-body insulin sensitivity. **Chapter 3** describes a study in which we focused on the use of intramyocellular substrate sources during a single session of resistance exercise. Based on the observed changes in muscle lipid and glycogen content we discuss possible interactions with the observed improvement in insulin sensitivity following such an exercise bout. In **chapter 4**, we describe the use of different amino acid tracers to determine whole-body protein metabolism and discuss which amino acid should be preferentially used as

a tracer in metabolic research. In addition, we investigated the effects of the ingestion of different nutritional supplements on whole-body protein metabolism. **Chapters 5** and **6** describe the effects of amino acid and/or protein ingestion on muscle protein synthesis. In **chapter 5**, we investigated the effects of the ingestion of carbohydrate, protein and leucine on skeletal muscle protein synthesis rates after resistance exercise in young healthy males. **Chapter 6** describes a study in which we compared muscle protein synthesis following activities of daily living between young and elderly subjects while ingesting carbohydrates. In addition we investigated the effects of the combined ingestion of protein and leucine with carbohydrate on muscle protein synthesis rates in the young and the elderly. In **chapter 7**, we provide information on the effect of a single session of resistance exercise on the phosphorylation status of several proteins that are involved in the regulation of muscle protein synthesis and investigate whether changes in phosphorylation are muscle fiber-type specific. Finally, **chapter 8** discusses the results of the above described studies and provides an overview of the main conclusions. Based on these conclusions the practical implications of the performed research are discussed and subsequent aims for future research are provided.

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2

A single session of resistance exercise enhances insulin sensitivity for at least 24hours in healthy men

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Abstract

The aim of the present study was to determine whether a single session of resistance exercise improves whole-body insulin sensitivity in healthy men for up to 24h. Twelve male subjects (23±1 y) were studied over a period of 4 days during which they consumed a standardized diet, providing 0.16±0.01 $MJ \cdot kg^{-1} \cdot day^{-1}$ containing 15±0.1 Energy% (En%) protein, 29±0.1 En% fat and 55±0.3 En% carbohydrate. Insulin sensitivity was determined 24h before and 24h after a single resistance exercise session (8 sets of 10 repetitions at 75% of 1-repetition maximum for 2 leg exercise tasks) using an intravenous insulin tolerance test. Insulin sensitivity index was calculated by the decline in arterialized blood glucose concentration following intravenous administration of a single bolus of human insulin (0.075 $IU \cdot kg^{-1}$ fat free mass). Basal glucose and insulin concentrations were not changed 24h after resistance exercise. However, a substantial 13±5% improvement in whole-body insulin sensitivity was observed 24h after resistance exercise ($P<0.05$). This study shows that even a single session of resistance exercise improves whole-body insulin sensitivity for up to 24h in healthy men, which is consistent with earlier observations following endurance exercise tasks.

Introduction

Skeletal muscle tissue is responsible for most of the insulin stimulated glucose uptake in humans. The capacity for insulin-mediated glucose uptake is directly related to total muscle mass and inversely associated with fat mass (58). In type 2 diabetes patients, insulin-stimulated glucose uptake is substantially impaired in liver and skeletal muscle tissue, leading to the development of a hyperinsulinemic and/or hyperglycemic state (9). The development of insulin resistance and/or type 2 diabetes is strongly associated with the presence of obesity and physical inactivity (18, 31, 44). Weight loss (23, 30, 45), increased physical activity (27), as well as pharmacological interventions (33, 34, 49) have been shown to form effective strategies to improve insulin sensitivity.

Studies investigating the role of physical activity as a means to improve insulin sensitivity generally apply endurance exercise as a model. Endurance exercise allows the use of a relatively large amount of muscle for a prolonged period of time, and, as such, forms a safe and effective means to elevate energy expenditure and promote weight loss (16). Prolonged endurance exercise training has been shown to improve insulin sensitivity in young (11), elderly (36) and/or insulin-resistant subjects (4, 10, 12, 32, 46, 48). However, even a single bout of moderate-to-high-intensity endurance exercise has been shown to acutely improve insulin sensitivity and/or glucose tolerance (14, 15, 26, 42, 46). This effect has been reported to persist for a period ranging from 2h (42), 4-6h (56), 12-16h (14, 15, 26) to up to 48h post-exercise (42, 46).

In contrast to endurance exercise, limited information is available on the potential of resistance exercise to affect insulin sensitivity and/or glucose tolerance (51, 52). Some studies have demonstrated that 6-12 wk of progressive resistance training improves glucose tolerance (8, 19, 43). These changes are generally attributed to the concomitant gain in skeletal muscle mass. Unfortunately, the few studies that investigated the more acute effects of resistance exercise have provided contradictory findings. Whereas some have reported an improved glucoregulatory response 12-24h after a single bout of resistance exercise (19, 20), others have failed to observe any change in insulin sensitivity (6). The apparent discrepancy in the literature is likely due to the methods used to determine insulin sensitivity and/or glucose tolerance. Most studies have applied an oral glucose tolerance test (OGTT) as a surrogate measure of insulin sensitivity. However, the OGTT has been reported to have a questionable reproducibility (37). In contrast to the OGTT, the insulin tolerance test (ITT) directly measures insulin sensitivity (28) and has been validated using the euglycaemic hyperinsulinemic clamp (1). Therefore, in the present study we applied the ITT to investigate the short-term effects of a single resistance exercise session on whole-body insulin sensitivity.

Methods

Subjects

Twelve healthy male volunteers with no history of participating in any regular exercise program were recruited for the present study. Subject characteristics are shown in table 2.1. All subjects were informed on the nature and possible risks of the experimental procedures before their written informed consent was obtained. The latter after approval by the Medical Ethical Committee of the Academic Hospital Maastricht, The Netherlands.

Table 2.1 Subjects' characteristics

	Mean ± SEM
Age (yrs)	23.0 ± 1.0
Weight (kg)	74.3 ± 2.8
Height (m)	1.79 ± 0.02
BMI ($kg \cdot m^{-2}$)	23.1 ± 0.7
% bodyfat (%)	17.1 ± 2.2
Fat free mass (kg)	61.2 ± 1.8
Fat mass (kg)	13.1 ± 1.9
HbA_{1C} (%)	5.23 ± 0.08
1RM leg press (kg)	198 ± 8
1RM leg press ($kg \cdot BW^{-1}$)	2.67 ± 0.08
1RM leg extension (kg)	108 ± 4
1RM leg extension ($kg \cdot BW^{-1}$)	1.46 ± 0.05

Values are expressed as means±SEM.

Pretesting

All subjects reported to the laboratory in the morning after an overnight fast for measurement of body composition, assessed using the hydrostatic weighing method. Residual lung volume was measured by the helium-dilution technique using a spirometer (Volugraph 2000, Mijnhart, Bunnik, The Netherlands). Body weight was measured with a digital balance with an accuracy of 0.001 kg (E1200, August Sauter GmbH, Albstadt, Germany). Body fat percentage was calculated using Siri's equation (50). Fat Free mass (FFM) was calculated by subtracting fat mass from total body mass.

Thereafter, subjects participated in an exercise trial to become familiarized with the exercise protocol and the equipment. Proper lifting technique was demonstrated and practiced for each of the 2 lower-limb exercises (leg press and leg extension) and for the 3 upper-body exercises (chest press, shoulder press and lat-pulldown). Thereafter maximum strength was estimated using the multiple repetitions testing procedure (41). In another session, at least 1 wk before the first experimental trial, subjects' 1 repetition maximum (1RM) was determined (38). After warming up, the load was set at 90-95% of the estimated 1RM, and increased after each successful lift until failure. A 5 min resting period between subsequent attempts was allowed.

A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance.

Standardization diet and activity prior to testing

All subjects received a standardization diet for 4 days, i.e. the day prior to the Insulin Tolerance Test (ITT) (on day 1), the ITT (on day 2), the resistance exercise session (on day 3), and the second ITT (on day 4), which were performed at exactly 8.30 am in the morning after an overnight fast. Subjects were provided with a pre-weighed amount of food products, beverages, and instant meals and were allowed to drink water ad libitum. All main meals (breakfast, lunch, and dinner) and between-meal snacks were instructed to be taken at predetermined time intervals during each day. Subjects were asked to record their food intake during the entire testing period. Energy intake averaged 0.16±0.01 MJ·kg bodyweight^{-1}·day^{-1} containing 15±0.1 Energy% (En%) of protein, 29±0.1 En% of fat and 55±0.3 En% of carbohydrate. Energy intake did not differ significantly between days, even though subjects were free to increase or decrease portion size of all meals and snacks. All volunteers were instructed to refrain from any sort of heavy physical exercise during the entire period except for the resistance exercise session.

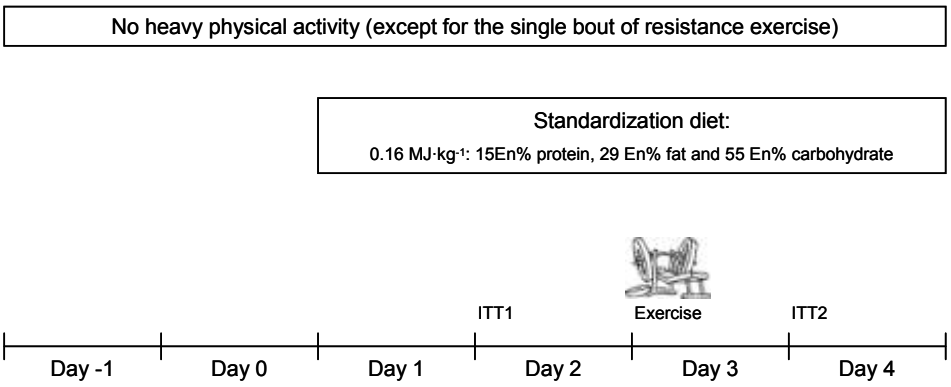

Figure 2.1 Overview of the study design. All subjects received a standardization diet during the entire study period; prior to the day preceding the first Insulin Tolerance Test (ITT) (day 1), the ITT (day 2), the resistance exercise session (day 3), and the second ITT (day 4), which were performed at exactly 8.30 am in the morning after an overnight fast. All volunteers were instructed to refrain from any sort of heavy physical exercise during the entire period (6 days) except for the resistance exercise session.

Experimental trials

An overview of the study protocol is provided in figure 2.1. One week after the subjects completed their 1RM-test, insulin sensitivity was measured using a short Insulin Tolerance Test (ITT). 24h after the ITT, subjects exercised for ~1 h using

the resistance exercise machines. The resistance exercise was followed 24h later with a second ITT.

ITT

The day before and after the resistance exercise session, subjects arrived at the laboratory by car or public transportation (to avoid disturbances in insulin sensitivity due to physical activity: i.e. cycling, stair walking) at 8.00 am after an overnight fast. A Teflon catheter was inserted into an antecubital vein for insulin infusion and a second Teflon catheter was inserted retrogradely in a dorsal vein of the contralateral hand, which was placed in a hot-box (60°C), for arterialised blood sampling. After 30 min of incline bed rest, a basal blood sample was collected (t=-5 min). Another blood sample (t=0 min) was obtained before administration of a single intravenous dose of human insulin (Actrapid®, Novo Nordisk A/S, Bagsværd, Denmark) of 0.075 $IU \cdot kg^{-1}$ fat free mass. Thereafter, blood samples were collected every 2 min until t=16 min. Thereafter, the test was terminated and subjects consumed a standardised breakfast.
Blood samples were taken for blood glucose measurement (see analyses section). In addition, blood glucose concentrations were directly monitored using a blood glucose monitor (Precision Xtra, MediSense, Amersfoort, The Netherlands) at t=0, 6, 12, 16 min. If blood glucose <2.5 $mmol \cdot L^{-1}$ at t=16 min a 10 ml bolus of 20% dextrose (Baxter B.V., Utrecht, The Netherlands) was injected, to prevent severe hypoglycaemia. Blood glucose was checked again at 22 and 30 min after insulin injection. No subjects reported symptoms of hypoglycaemia during the ITT, and/or during the 20-30 min after insulin administration.

Resistance exercise

The day after the first ITT subjects arrived at the laboratory by car or public transportation at 8.00 am, in an overnight fasted state. Subjects performed a general warm-up of 5 min using a Stairmaster, followed by 3 sets of 10 repetitions on 3 resistance exercise machines targeting the upper-body (chest-press, shoulder-press and lat-pulldown, Jimsa Benelux BV, Rotterdam, The Netherlands). The latter were included to provide a whole-body warm-up and to reduce the risk of injury. Thereafter, the resistance exercise session targeted the legs, with 8 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, The Netherlands) and 8 sets of 10 repetitions on the leg extension machine (Technogym). Both exercises were performed at 75% of the subjects' individual 1RM with 2 min rest intervals between sets and in total required ~40 min to complete. All subjects were verbally encouraged during the test to complete the entire protocol. At the end of the exercise session, subjects consumed a standardized breakfast. Energy expenditure during the exercise session was not measured. Based on indirect calorimetry measurements during similar resistance exercise protocols, others have shown energy expenditure rates ranging between 14 and 27 $kJ \cdot min^{-1}$ (2, 5, 47).

Analysis

Blood samples (4 ml) were collected in tubes containing a glycolytic inhibitor (sodium fluoride) and anticoagulant (potassium oxalate) and placed on ice. After centrifugation at 1000·g and 4ºC for 5 min aliquots of plasma were frozen immediately in liquid nitrogen and stored at –80ºC until analyses. Plasma glucose (Uni Kit III, 07367204, Roche, Basel, Switzerland) concentrations were analyzed with the COBAS FARA semi-automatic analyzer (Roche). Plasma insulin was measured by radioimmunoassay (HI-11K, Linco Research Inc., St. Charles, MO, USA). To determine basal fasting blood HbA_{1C} content a 3 ml blood sample was collected in EDTA containing tubes and analyzed by high-performance liquid chromatography (Bio-Rad Diamat, Munich, Germany).

Calculation of insulin sensitivity

The decline in blood glucose between 4 and 16 min during the ITT was used to determine insulin sensitivity (ISindex) (4). Linear regression was used to calculate the slope of the decline in log transformed blood glucose concentration against time during the first 4-16 min (1, 4, 28). The slope was multiplied by –100 to derive the rate constant (K_{ITT}) which is equivalent to the percentage decline in blood glucose per min (28).
Insulin sensitivity was also estimated by the homeostasis model assessment or HOMA-Insulin Resistance (IR) index which is calculated by dividing the product of fasting plasma glucose ($mmol \cdot L^{-1}$) and insulin concentrations ($mU \cdot L^{-1}$) by 22.5 (40).

Statistics

All data are expressed as means ± SEM. IS_{index} (K_{ITT}) calculated from data obtained during ITT1 and ITT2 were compared using a two-tailed, paired t-test. In addition, simple regression analysis was performed to calculate correlations between basal insulin, glucose, IS_{index} and HOMA index. Statistical significance was set at $P<0.05$.

Results

Resistance exercise

Mean 1 repetition maximum (1RM) measured during the pre-testing was 198±8 kg on the horizontal leg press and 108±4 kg on the leg extension. Therefore, average weights used during the resistance exercise were 148±6 and 81±3 kg for the leg press and leg extension, respectively. All subjects completed 8 sets with 10 repetitions on the leg press. However, during the 6th set, 2 subjects could not finish all 10 repetitions, after which weight was reduced to 65% of the individual 1RM.

Ten subjects completed 8 sets of 10 repetitions on the leg extension. Two subjects were not able to finish the last 2 sets due to dizziness.

Plasma analyses

Table 2.2 Blood parameters

	ITT 1	ITT 2
Basal glucose (mmol·L^{-1})	5.44 ± 0.12	5.45 ± 0.10
Glucose at t=16 (mmol·L^{-1})	3.00 ± 0.18	2.73 ± 0.19 *
KITT (%·min^{-1})	5.07 ± 0.37	5.77 ± 0.53 *
Basal insulin	5.35 ± 0.43	5.51 ± 0.35
HOMA-(IR) index	1.29 ± 0.11	1.33 ± 0.08

Values are expressed as means±SEM. * Significantly different from ITT1, P<0.05.

All subjects showed normal fasting plasma glucose (5.42 ± 0.13 mmol·l^{-1}) and insulin concentrations (5.35 ± 0.43 mU·L^{-1}) before the start of the first ITT. Basal plasma glucose concentrations were similar before the ITT1 and ITT2 trial (5.42 ± 0.13 vs. 5.45 ± 0.10 mmol·L^{-1}, respectively). Basal plasma insulin concentrations did not differ between the ITT1 and ITT2 (5.35 ± 0.43 and 5.51 ±0.35 mU·L^{-1}, respectively). The HOMA-(IR) index before ITT1 and ITT2 averaged 1.29 ± 0.11 and 1.33 ± 0.08, respectively (NS). An overview of the plasma data is presented in table 2.2.

Intravenous insulin tolerance test

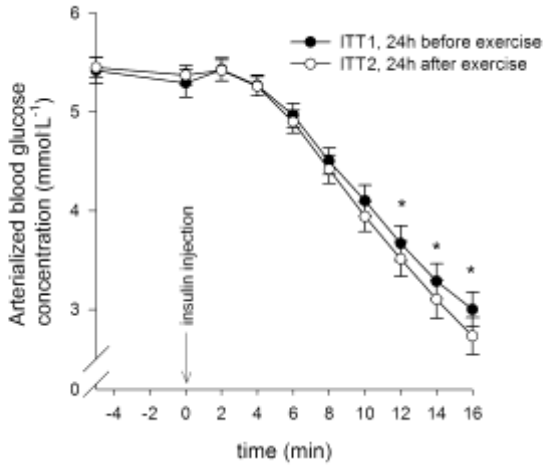

Figure 2.2 Plasma glucose concentrations following injection of a bolus of insulin (0.075 IU·kg^{-1} fat free mass) 24h before and 24h after a single bout of resistance exercise. Values are expressed as means±SEM. * Significantly different from basal blood glucose concentrations during ITT1 and ITT2 (P<0.01)

During the first ITT, the administration of insulin resulted in a decline in plasma glucose concentration from 5.27±0.11 mmol·L^{-1} at t=4 min to 3.00±0.18 mmol·L^{-1} at t=16 min. During the ITT 24h after resistance exercise (ITT2), plasma glucose concentrations were reduced from 5.26 ± 0.10 mmol·L^{-1} at t=4 min to 2.73 ± 0.19 mmol·L^{-1} at t=16 min. Plasma glucose levels at t= 16 min were significantly lower in ITT2 compared to ITT1 ($P<0.05$). The decline in blood glucose concentration is presented in figure 2.2.
Calculated rate constants for the glucose disappearance (KITT, %·min^{-1}) between 4-16 min following insulin injection during a short insulin tolerance test (ITT) before, and 24h after resistance exercise are shown in figure 2.3A. KITT values were significantly increased with 13.4 ± 4.8% 24h post-exercise compared to pre-exercise values (5.1±0.4 vs. 5.8±0.5 %·min^{-1}, $P<0.05$). The individual KITT values of blood glucose after insulin administration during the ITT before and 24h after resistance exercise are shown in figure 2.3B.

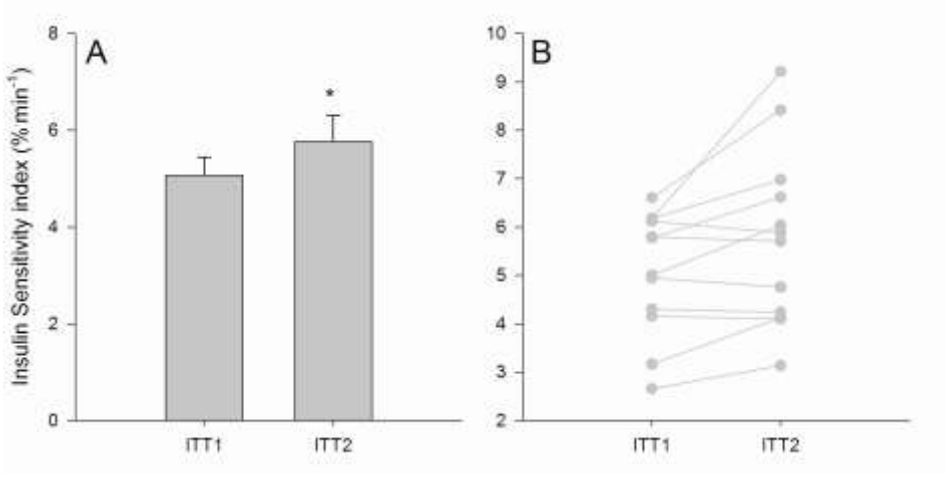

Figure 2.3 Insulin Sensitivity index presented as the mean rate constants for the disappearance (K_{ITT}) of blood glucose following insulin injection during a short insulin tolerance test (ITT) before, and 24h after resistance exercise (A). Values are expressed as means±SEM. * significantly different from values observed during ITT1 ($P<0.05$). Individual results showing KITT values of blood glucose after insulin administration during the ITT before and 24h after resistance exercise (B).

Discussion

It has been well established that a single bout of endurance exercise improves whole-body insulin sensitivity for up to 24h. In the present study, we extend on those findings by showing that a single session of resistance exercise can also effectively increase whole-body insulin sensitivity for up to 24h in healthy males.
Endurance exercise training has been reported to improve whole-body insulin sensitivity in young (11), elderly (36) and insulin-resistant subjects (4, 10, 12, 32, 46, 48). The latter is attributed to the concomitant induction of weight loss (16) and the upregulation of skeletal muscle GLUT-4 expression (7). Besides the more

prolonged adaptive response to endurance training, it has been firmly established that even an acute bout of endurance exercise elevates whole-body insulin sensitivity for a prolonged period, ranging from 2 (42), 4-6 (56), 12-16 (14, 15, 26) to up to 48h following cessation of exercise (42, 46). The latter is generally attributed to attenuated muscle GLUT-4 translocation (53) as well as increased GLUT-4 expression in muscle tissue (39). Factors thought to play a major regulatory role in this process include muscle lipid content (54) in relation with physical inactivity, AMPK activation (21, 25, 55), muscle glycogen content and subsequent activation of glycogen synthase activity (3, 13, 22, 46, 56, 57), which enhance insulin signalling downstream of the receptor (24, 27).

In addition to prolonged endurance training, resistance exercise interventions have also been reported to improve glucose tolerance and/or whole-body insulin sensitivity (8, 17, 19, 29, 35, 43). The latter is generally attributed to a concomitant gain in skeletal muscle tissue, which improves whole-body glucose disposal capacity (8, 19, 43). Besides this increase in lean muscle tissue, resistance exercise also improves functional capacity, thereby supporting a more active, healthy lifestyle. However, in the development of most exercise intervention programs, the focus generally lies on the implementation of endurance exercise because of its acute stimulating effect on whole-body insulin sensitivity. Consequently, endurance exercise sessions are generally implemented at the expense of resistance exercise when designing such intervention programs. In the present study, we speculated that a single resistance exercise session could also acutely improve whole-body insulin sensitivity. The latter would imply that there is no need to restrict the inclusion of resistance exercise when designing effective lifestyle intervention programs.

So far, the few studies that investigated the acute effects of resistance exercise on insulin sensitivity have provided contradictory findings (6, 19, 20). In the present study, we show that a single resistance exercise session (mainly consisting of 16 sets of leg-exercise using 75% of the individual 1RM) improves whole-body insulin sensitivity by as much as 13.4±4.8% when measured 24h after exercise. However, a large inter-subject variation was observed. Whereas 5 subjects did not show a measurable increase in plasma glucose clearance 24h after resistance exercise, the 7 other subjects showed improvements in insulin sensitivity ranging between 13 and 49%. The average increase in whole-body insulin sensitivity following resistance exercise seems to be of a similar magnitude as the ~20% improvement reported following an acute (~60 min) bout of endurance exercise (42, 46). Previous studies, investigating the acute effects of resistance exercise on insulin sensitivity, have applied either oral (19, 20) or intravenous (6) glucose tolerance tests (OGTT and IVGTT, respectively) to estimate insulin sensitivity. These studies, which use the glucose and/or insulin response following glucose administration as a representative of insulin action, have provided discrepant findings. Fluckey et al. (1994) reported no change in integrated glucose concentrations following glucose ingestion 18 h after resistance exercise, but observed a lower insulin response (20). Fenicchia et al. (2004) reported a lower glucose response 12-24h after resistance exercise in female type 2 diabetes patients, without observing any difference in

insulin levels. In that study as well as in a study by Chapman et al. (2002), no changes in glucose or insulin responses were observed 15h after a single bout of resistance exercise in the healthy, non-obese, normoglycaemic controls (6, 19). Clearly, in the literature the acute effects of resistance exercise on whole-body insulin sensitivity have always remained equivocal.

The discrepancy in these earlier findings could likely be attributed to the use of glucose tolerance tests, which do not measure insulin sensitivity directly. In accordance, the OGTT has been reported to have a questionable reproducibility as a measure of whole-body insulin sensitivity (37). Therefore, in the present study, we applied an intravenous insulin tolerance test (ITT) 24h before and 24h after performing a resistance exercise session. We observed a greater arterial blood glucose clearance rate following an intravenous dose of insulin 24h after resistance exercise compared with pre-exercise values (figure 2.3). During the ITT, insulin sensitivity index was calculated by the observed decline in arterial blood glucose following insulin administration. In contrast to the OGTT, the ITT provides a reproducible method that more directly assesses insulin sensitivity (28), and has been validated using the euglycaemic hyperinsulinemic clamp (1). Other factors that might explain the discrepant findings in the literature include the intensity and/or duration of the resistance exercise session, the standardization of dietary intake and daily physical activity as well as the population studied. The resistance exercise protocol used both by Fluckey et al. (1994) and Chapman et al. (2002) may not have been of sufficient intensity to invoke a large significant improvement in whole-body insulin sensitivity as observed in the present study. Furthermore, many of the earlier studies did not standardize food intake and/or physical activity prior to the OGTTs (19, 20). In the present study, both food intake and physical activity were strictly standardized to reduce any confounding effects of diet on insulin sensitivity.

In the present study we show that resistance exercise acutely stimulates insulin sensitivity. Similar to the reported effects of a single bout of endurance exercise, this is likely attributed to attenuated muscle GLUT-4 translocation (53) and/or elevated GLUT-4 expression (39). Though information on the metabolic demand imposed upon by resistance exercise is scarce, we speculate that resistance exercise activates AMPK and substantially reduces muscle glycogen and/or IMTG content. More research is warranted to elucidate the exact mechanisms responsible for the observed increase in insulin sensitivity following exercise. In conclusion, an acute bout of intense resistance exercise substantially improves whole-body insulin sensitivity for up to 24h after cessation of exercise. Therefore, the present data indicate that both endurance as well as resistance type exercise tasks stimulate insulin sensitivity. As such, there should be no restriction in combining the benefits of both types of exercise in future lifestyle intervention programs.

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3

Intramyocellular lipid and glycogen content are reduced following resistance exercise in untrained healthy males

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Abstract

Resistance exercise has recently been shown to improve whole-body insulin sensitivity in healthy males. Whether this is accompanied by an exercise-induced decline in skeletal muscle glycogen and/or lipid content remains to be established. In the present study, we determined fiber-type specific changes in skeletal muscle substrate content following a single resistance exercise session. After an overnight fast, eight untrained healthy lean males participated in a ~45 min resistance exercise session. Muscle biopsies were collected before, following cessation of exercise, and after 30 and 120 min of post-exercise recovery. Subjects remained fasted throughout the test. Conventional light and (immuno)fluorescence microscopy were applied to assess fiber-type specific changes in intramyocellular triacylglycerol (IMTG) and glycogen content. A significant 27±7% net decline in IMTG content was observed in the type I muscle fibers ($P<0.05$), with no net changes in the type IIa and IIx fibers. Muscle glycogen content decreased with 23±6, 40±7 and 44±7% in the type I, IIa and IIx muscle fibers, respectively ($P<0.05$). Fiber-type specific changes in intramyocellular lipid and/or glycogen content correlated well with muscle fiber-type oxidative capacity. During post-exercise recovery, type I muscle fiber lipid content returned to pre-exercise levels within 120 min. No changes in muscle glycogen content were observed during recovery. We conclude that intramyocellular lipid and glycogen stores are readily used during resistance exercise and this is likely associated with the reported increase in whole-body insulin sensitivity following resistance exercise.

Introduction

Fat and carbohydrate are the principal substrates that fuel ATP resynthesis in skeletal muscle tissue. Endogenous carbohydrates are mainly stored as muscle and liver glycogen and represent less than 5% of total energy storage. The vast majority of our energy reserves is stored as fat, mainly deposited as triacylglycerol (TG) in subcutaneous and deep visceral adipose tissue. Smaller quantities of TG are present as lipid droplets inside the muscle fibers, intramyocellular triacylglycerol (IMTG) (19).

There has been much controversy on the actual contribution of the IMTG pool as a substrate source during exercise (47). The apparent discrepancy in the literature likely stems from the methodological difficulties that have been associated with the biochemical triacylglycerol extraction method that has been used to determine IMTG content in muscle biopsies collected before and after exercise (47). In accordance, more recent studies using stable isotope methodology, ^{1}H-magnetic resonance spectroscopy, electron and/or immunofluorescence microscopy all support the contention that the IMTG pool can function as an important substrate source during submaximal exercise (45). Besides the intramyocellular lipid deposits, muscle glycogen stores represent another important substrate source. In accordance, substantial net reductions in both type I and II muscle fiber glycogen content have been reported following prolonged endurance exercise tasks (42). These intramyocellular lipid and glycogen stores play a major regulatory role in skeletal muscle metabolism. Muscle glycogen use and subsequent post-exercise glycogen synthesis rates have been shown to be functionally coupled to the exercise-induced increase in skeletal muscle insulin sensitivity in humans and rodents (2, 6, 14, 35, 48, 49). Furthermore, interventions known to improve insulin sensitivity, like weight loss in humans (15, 20), dietary lipid withdrawal (33), the use of thiazolidinediones in rodents (34) as well as an acute bout of endurance exercise in rodents and humans (33, 45) have all been associated with a reduction in skeletal muscle lipid content. Because of its capacity to reduce both intramyocellular lipid and glycogen content, endurance exercise is generally considered an effective interventional strategy to improve skeletal muscle insulin sensitivity.

However, resistance exercise should also be regarded as an essential component of effective intervention programs designed to improve health. Resistance training increases skeletal muscle mass (22) and, as such, can augment whole-body glucose disposal capacity (5, 11, 31). In addition, resistance training also improves muscle strength, power and functional capacity, which facilitates the adaptation towards a more active lifestyle (10, 28). Though there is some discrepancy in the existing literature, we (24) as well as others (11, 12) have recently shown that even a single resistance exercise session can improve whole-body insulin sensitivity for up to 24h after cessation of exercise. In line with the acute stimulating effects of endurance exercise on post-exercise insulin sensitivity (2), it has been speculated that the acute effects of resistance exercise on whole-body insulin sensitivity are also attributed to the reduction of the intramyocellular glycogen and/or lipid stores

(24). However, studies on the metabolic demands of resistance exercise are scarce, which is likely due to the methodological difficulties associated with the non-steady state conditions of this type of exercise. Therefore, in the present study, we determined fiber-type specific changes in intramyocellular glycogen and lipid content during and immediately after a single resistance exercise session.

Methods

Subjects

Eight healthy untrained male volunteers with no history of participating in any regular exercise program were recruited for the present study. Subjects' characteristics are shown in table 1. All subjects were informed on the nature and possible risks of the experimental procedures before their written informed consent was obtained, the latter after approval by the Medical Ethical Committee of the Academic Hospital Maastricht.

Table 3.1 subjects' characteristics

	Mean ± SEM
Age (yrs)	22.5 ± 0.9
Weight (kg)	74.9 ± 2.8
Height (m)	1.80 ± 0.01
BMI (kg.m-2)	23.3 ± 0.7
% bodyfat (%)	17.8 ± 2.4
Fat free mass (kg)	61.3 ± 2.1
Fat mass (kg)	13.6 ± 2.1
HbA_{1C} (%)	5.3 ± 0.1
1RM leg press (kg)	198 ± 7
1RM leg extension (kg)	105 ± 3

Values are expressed as means±SEM.

Pretesting

Body composition was assessed using the hydrostatic weighing method in the morning following an overnight fast. Residual lung volume was measured by the helium-dilution technique using a spirometer (Volugraph 2000, Mijnhart, Bunnik, The Netherlands). Body mass was measured with a digital balance with an accuracy of 0.001 kg (E1200, August Sauter GmbH, Albstadt, Germany). Body fat percentage was calculated using Siri's equation (39). Fat Free mass (FFM) was calculated by subtracting fat mass from total body mass.
To familiarize subjects with the resistance exercise protocol and the equipment, a familiarization trial was performed. Proper lifting technique was demonstrated and practiced for each of the 2 lower-limb exercises (leg-press and leg-extension) and for the 3 upper-body exercises (chest-press, shoulder-press and lat-pulldown). Thereafter, maximum strength was estimated using the multiple repetitions testing procedure (29).

In an additional exercise session, at least 1 wk before the first experimental trial, the subjects' 1 repetition maximum (1RM) was determined (26). After warming up, the load was set at 90-95% of the estimated 1RM, and increased after each successful lift until failure. A 5 min resting period between subsequent attempts was allowed. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance.

Standardised diet and activity prior to testing

All subjects received a strict standardized diet for 2 days prior to the resistance exercise test trial, which was performed in the morning after an overnight fast. Subjects were provided with a pre-weighed amount of food products, beverages, and instant meals and were allowed to drink water ad libitum. Subjects were instructed to take all main meals (breakfast, lunch, and dinner) and between-meal snacks at predetermined time intervals during each day. The standardized diet provided 0.15 $MJ \cdot kg^{-1} \cdot day^{-1}$ containing 15 Energy% (En%) of protein, 30 En% of fat and 55 En% of carbohydrate. All volunteers were instructed to refrain from any sort of heavy physical exercise during the 2 days prior to the test-trial.

Experimental trial

The day of the test, subjects arrived at the laboratory by car or public transportation at 8.00 am, following an overnight fast. A Teflon catheter was inserted into an antecubital vein for blood sampling. After 30 min of supine rest, a basal blood sample was collected and a muscle biopsy was taken from the m. vastus lateralis. Thereafter, subjects performed a 5 min low-intensity warm-up on a Stairmaster, followed by 3 sets of 10 repetitions on 3 resistance exercise machine targeting upper-body muscle groups (chest-press, shoulder-press and lat-pulldown, Jimsa Benelux BV, Rotterdam, The Netherlands). The latter were included to provide a whole-body warm-up to reduce the risk of injury. Thereafter, subjects performed 8 sets of 10 repetitions on the horizontal leg-press machine (Technogym BV, Rotterdam, The Netherlands) and 8 sets of 10 repetitions on the leg-extension machine (Technogym). Both exercises were performed at 75% of the individual 1RM with 2 min rest intervals between sets and required ~45 min to complete. All subjects were verbally encouraged during the exercise session to complete the entire protocol. Immediately after cessation of exercise, a second muscle biopsy sample was taken, after which subjects rested supine for 2h. After 30 min and 2h of post-exercise recovery, additional muscle biopsies were taken. Blood samples were collected before, after 30 min of exercise, immediately after cessation of exercise and following 30, 60, 90 and 120 min of post-exercise recovery. Subjects remained fasted throughout the test.

Muscle biopsy samples were collected from both legs. The first two biopsies were taken from the same incision in one leg; the last two were taken from the same incision in the contralateral leg. When biopsy samples were taken from the same incision, the first sample was taken from a different region (distal of the incision,

with the needle pointing inwards) than the second (proximal with the needle pointing outwards). Muscle biopsies were obtained from the middle region of the m. vastus lateralis (15 cm above the patella) and approximately 3 cm below entry through the fascia using the percutaneous needle biopsy technique (1). Muscle samples were dissected carefully, freed from any visible non-muscle material, embedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and rapidly frozen in liquid nitrogen-cooled isopentane.

Plasma sample analysis

Blood samples (6 ml) were collected in EDTA-containing tubes and centrifuged at 1000 g at 4°C for 10 min. Aliquots of plasma were frozen immediately in liquid nitrogen and stored at -80°C. Plasma glucose (Uni Kit III, Roche, Basel, Switzerland), lactate (Gutmann & Wahlefeld, 1974) and FFA (Wako NEFA-C test kit, Wako Chemicals, Neuss, Germany) concentrations were analysed with a COBAS FARA semi-automatic analyser (Roche).

Muscle sample analysis

Multiple serial sections (5 μm) from biopsy samples collected before, immediately after, 30 and 120 min after exercise were thaw-mounted together on uncoated, pre-cleaned glass slides for each subject. To permit the determination of muscle fiber IMTG content stained by oil red O together with immunolabelled cellular constituents we used the protocol as previously described (25, 44-46). The proportion of type I, IIa, and IIx muscle fibers was determined by ATPase staining (27). To assess intramyocellular glycogen content we used the modified Periodic Acid Schiff reagent stain (PAS) as recently described (37), allowing direct, fiber-type-specific determination of muscle glycogen content. Muscle fiber-type specific oxidative capacity was estimated by determining succinate dehydrogenase activity (SDH) in the muscle cross-sections using histochemical analyses (16). Histological techniques, like oil red O, PAS and SDH staining combined with either immunofluorescence or conventional light microscopy are semi-quantitative. As a consequence, the present study does not provide absolute measures of skeletal muscle lipid and/or glycogen concentrations. After 24h, glass slides were examined using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan color CCD camera, with a Bayer color filter. Epifluorescence signal was recorded using a Texas red excitation filter (540-580 nm) for oil red O, a fluorescein isothiocyanate (FITC) excitation filter (465-495 nm) for muscle fiber-type, and a 4',6-diamidino-2-phenylindole (DAPI) UV excitation filter (340-380 nm) for laminin.
Digitally captured images (240x magnification) with a minimum of six fields-of-view per muscle cross-section (12±1 fibers per field-of-view), were processed and analyzed using Lucia 4.8 software (Nikon, Düsseldorf, Germany). The signal derived from the antibody against laminin was used to select single muscle fibers and with the antibody against human myosin heavy chain I, we were able to

differentiate between type I and II muscle fibers. To differentiate between type IIa and IIx fibers we used matching serial cross-section stained using routine ATPase staining. The oil red O epifluorescence signal was recorded for each muscle fiber, resulting in a total of 70±5 muscle fibers analyzed for each muscle cross-section (35±2 type I, 25±4 type IIa and 10±1 type IIx muscle fibers). An intensity threshold representing minimal intensity values corresponding to lipid droplets was set manually and uniformly used for all images. Total area measured and the area as well as the number of objects emitting oil red O epifluorescence signal were recorded. Fiber-type-specific IMTG content was expressed as the percentage of the measured area that was stained with oil red O. In a previous study, two different muscle samples from two different subjects were analysed; showing a coefficient of variance of 10.5 and 38.6% for the type I and II muscle fibers (45). Mixed muscle fiber lipid content, as determined by oil red O staining, has been shown to correlate significantly with measures of intramyocellular lipid content as assessed by ^{1}H-magnetic resonance spectroscopy (46). Average lipid droplet size was calculated by dividing the total area lipid stained by total number of lipid droplets. Lipid droplet density was calculated by dividing the total number of lipid droplets by the total area measured.

PAS and SDH stained sections were captured in full color using bright field light microscopy. Digitally captured images (120x magnification) with a minimum of four fields-of-view per muscle cross-section (37±2 fibers per field-of-view), were processed and analyzed using the Lucia 4.8 software package. The PAS and SDH signals were recorded for each muscle fiber, resulting in a total of 142±8 muscle fibers analyzed for each muscle cross-section (64±4 type I, 57±5 type IIa and 21±2 typ IIx muscle fibers). The bright-field images of the PAS and SDH stains were converted post hoc to 8-bit grayscale values. The mean optical density of the PAS or SDH-raised signal per individual fiber was determined by averaging the optical density measured in every pixel in the cell, corrected for the mean optical density of the background stain measured in a field-of-view containing no muscle fibers. Mixed muscle glycogen content, as determined by PAS staining, correlates significantly with muscle glycogen content as measured using biochemical analyses (37).

Statistics

All data are expressed as means±SEM. Analyses of variance (ANOVA) for repeated measures were applied to determine differences in muscle lipid and glycogen content over time in type I, IIa and IIx fibers with time and muscle fiber-type as factors. In case of a significant F-ratio, a Scheffé post-hoc test was applied to locate the differences. Statistical significance was set at $P<0.05$. Simple regression analysis was performed on mean muscle fiber-type oxidative capacity and glycogen/lipid content.

Results

Resistance exercise

Mean 1 repetition maximum (1RM) measured during the pre-test was 198±7 kg on the horizontal leg press and 105±3 kg on the leg extension. Therefore, average weight lifted during the resistance exercise was set at 148±5 and 79±3 kg for the leg press and leg extension respectively. All subjects completed 8 sets with 10 repetitions on the leg press. One subject was unable to finish 10 repetitions during the 6th set, after which resistance was reduced to 65% of the individual 1RM. All subjects completed 8 sets of 10 repetitions on the leg extension machine.

Plasma analyses

Plasma glucose, lactate and FFA concentrations are presented in figure 3.1. Plasma glucose concentrations increased during exercise and peaked immediately post-exercise ($P<0.05$), after which they returned to baseline values. Plasma lactate concentrations markedly increased during exercise ($P<0.0001$), and decreased during recovery. Plasma FFA concentrations tended to decrease during exercise ($P=0.08$), and markedly increased during recovery ($P<0.001$).

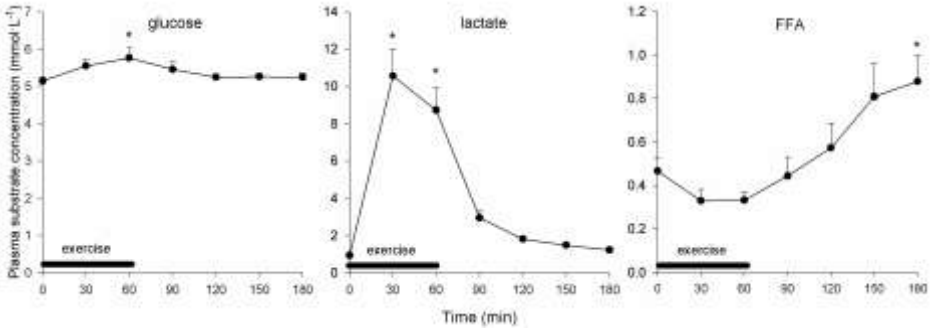

Figure 3.1 Plasma glucose, lactate and free fatty acid concentrations at rest, during resistance exercise and subsequent recovery. Data provided are means±SEM. * Significantly different from pre-exercise values ($P<0.05$)

Muscle glycogen content

Representative images of muscle cross-sections obtained before (A) and immediately after (B) exercise following PAS staining for analyses of intramyocellular glycogen content are shown in figure 3.2. Muscle fiber glycogen content was similar at rest in all fiber-types. Mixed muscle glycogen content declined by 33±7% following the exercise session ($P<0.0001$). Glycogen content had declined by 23±6% in the type I fibers ($P<0.01$), 40±7% in the type IIa fibers ($P<0.001$), and 44±7% in the type IIx fibers ($P<0.001$) compared to pre-exercise values (figure 3.3). The observed decrease over time in glycogen content was

significantly greater in the type IIx fibers compared to the type I muscle fibers ($P<0.05$). During post-exercise recovery muscle glycogen content remained below pre-exercise levels, with no significant changes over time in the type I, IIa and IIx muscle fibers.

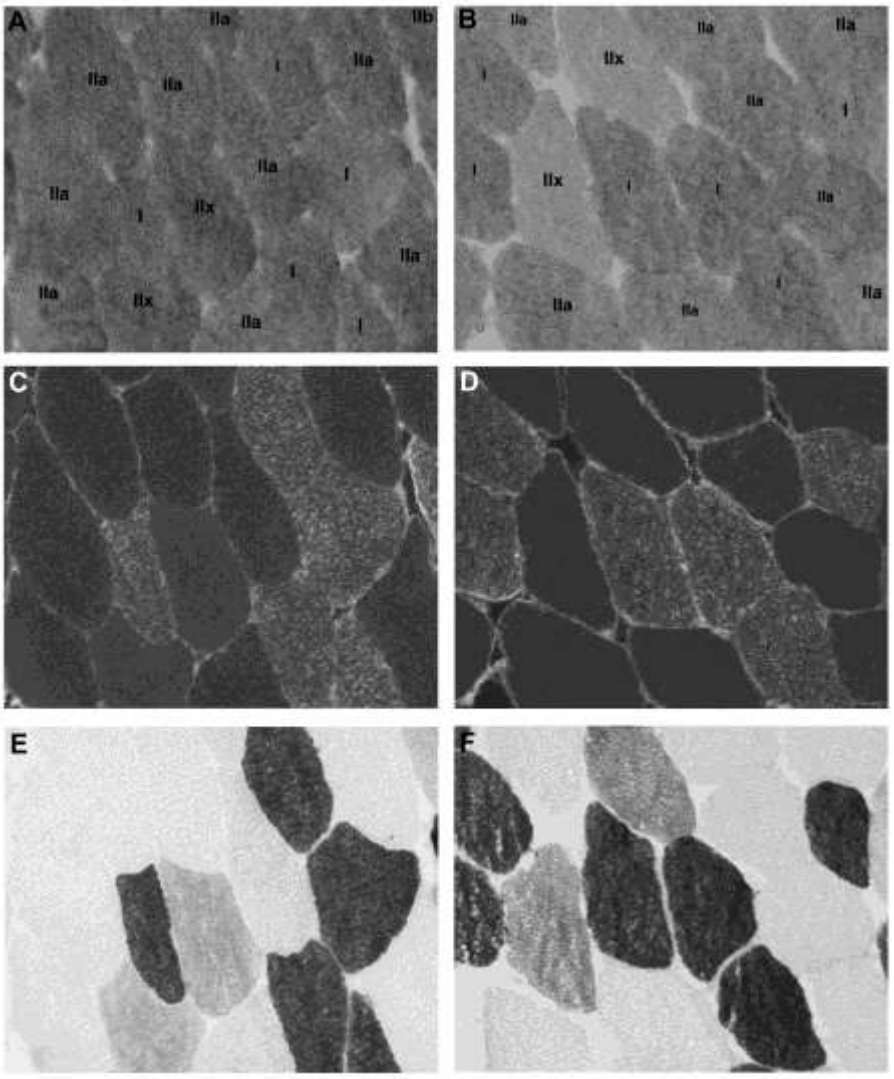

Figure 3.2 Images (120x magnification) of representative cross-sections of vastus lateralis muscle obtained before (left) and immediately after exercise (right) with sections stained for glycogen content (A/B), and laminin and myosin heavy chains I (C/D). The muscle fibers are labeled as type I, type IIa, and type IIx. Differentiation between type IIa and IIx fibers was made based on ATPase staining (E/F).

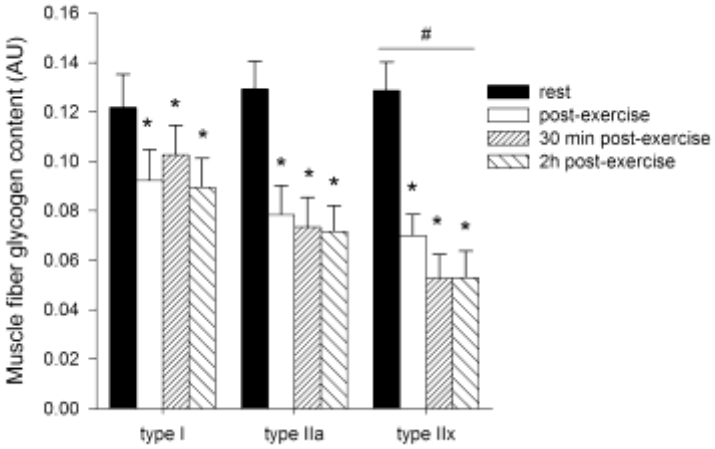

Figure 3.3 The effect of resistance exercise on muscle glycogen content. Fiber-type specific glycogen content (expressed in arbitrary units) before and immediately after exercise, and following 30 and 120 min of post-exercise recovery as determined by brightfield microscopy on PAS-stained muscle cross-sections. Data provided are means±SEM. * Significantly lower than pre-exercise values (P<0.05); # significantly different over time compared to type I muscle fibers (P<0.05).

IMTG content

Representative images of muscle cross-sections obtained before (A) and immediately after (B) exercise stained for intramyocellular lipid content are shown in figure 3.4. Muscle tissue analyses for IMTG content applying fluorescence microscopy on oil red O stained muscle cross-sections showed that at rest IMTG content in type I fibers was higher compared with type II fibers (P<0.05). Mixed muscle IMTG content tended to decline with 25±10% following the exercise session (P=0.087). In addition, a substantial 27±7% decline in lipid content in the type I fibers (P<0.05) was found, with IMTG content in the type I muscle fibers being significantly lower both immediately post-exercise as well as following 30 min of post-exercise recovery compared to pre-exercise values (figure 3.5). After 2h of post-exercise recovery, IMTG content had returned to values similar to pre-exercise values. The observed decrease in IMTG content was accounted for by a significant decrease in lipid droplet size (from 0.69±0.08 to 0.53±0.07 and 0.53±0.07 μm^2, P<0.01) without any changes in lipid droplet density (from 0.049±0.003 to 0.045±0.004 and 0.041±0.003 droplets·μm^{-2}) as determined before, immediately after and after 30 min of post-exercise recovery exercise, respectively. Net changes in IMTG content over time in the type I fibers were significantly different compared to the type IIa and IIx fibers (P<0.05). No significant changes over time were observed in intramyocellular lipid content in the type IIa or IIx muscle fibers.

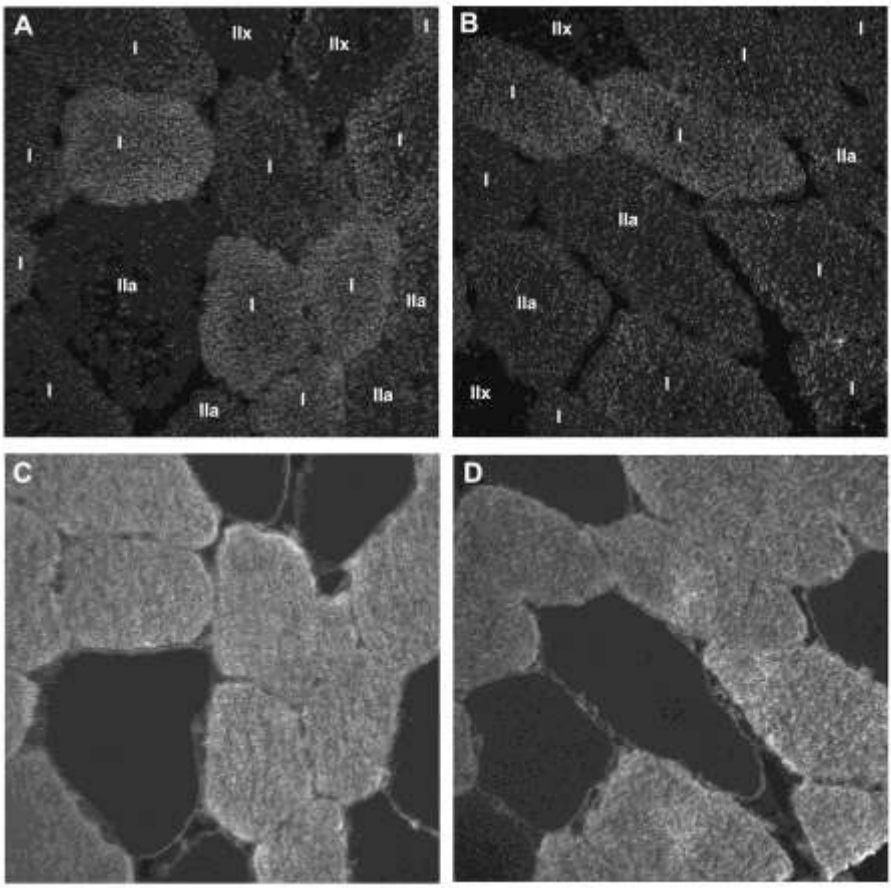

Figure 3.4. Images (240x magnification) of representative cross-sections of vastus lateralis muscle obtained before (left) and immediately after exercise (right) with sections stained for lipid content (A/B), and laminin and myosin heavy chains (C/D). The muscle fibers are labeled as type I, type IIa, and type IIx. Differentiation between type IIa and IIx fibers was made, based on standard ATPase staining.

Muscle fiber oxidative capacity

A histochemical analysis for succinate dehydrogenase (SDH) activity in muscle cross-sections was performed as a measure of muscle fiber oxidative capacity and results are shown in table 3.2. SDH activity was significantly greater in the type I versus the type II fibers ($P<0.05$). Type IIa muscle fiber SDH activity was substantially greater when compared to the IIx fibers ($P<0.05$). Positive correlations were observed between muscle fiber-type oxidative capacity and baseline muscle fiber lipid content ($r=0.573$, $P<0.01$), and muscle fiber-type oxidative capacity and the net reduction in muscle fiber lipid content observed following exercise ($r=0.482$, $P<0.05$). No significant correlation was observed between muscle fiber-type specific oxidative capacity and resting muscle fiber glycogen content. A significant negative correlation was observed between muscle

fiber-type oxidative capacity and the net reduction in muscle fiber glycogen content observed during exercise (r=-0.556, P<0.05).

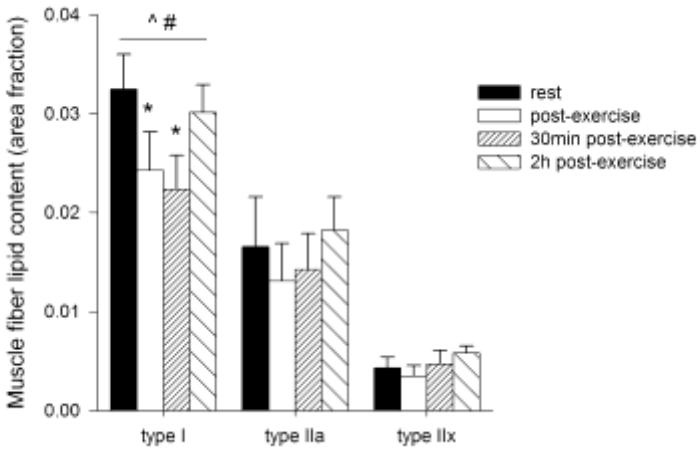

Figure 3.5 The effect of resistance exercise on muscle lipid content. Mean fiber-type-specific intramyocellular lipid content (expressed as percentage of area lipid stained) before exercise, immediately after exercise and following 30 and 120 min of post-exercise recovery as determined by fluorescence microscopy on oil red O stained muscle cross-sections Data provided are means±SEM. *: significantly lower than pre-exercise values (P<0.05); #: significantly different over time compared to type IIa fibers (P<0.05); ^: significantly different over time compared to type IIx fibers (P<0.05).

Table 3.2 Muscle SDH activity, glycogen and lipid content at rest

	Type I	**Type IIa**	**Type IIx**
Fiber-type distribution (%)	46 ± 4	39 ± 3	15 ± 2
SDH activity (AU)	73 ± 6	45 ± 5 *	27 ± 5 *#
IMTGcontent (area fraction)	0.032 ± 0.004	0.017 ± 0.005 *	0.004 ± 0.001 *^
Glycogen content (AU)	0.122 ± 0.038	0.129 ± 0.032	0.129 ± 0.032
Lipid content / SDH activity	0.47 ± 0.07	0.42 ± 0.13	0.19 ± 0.05
Glycogen content / SDH activity	1.85 ± 0.41	3.41 ± 0.85	6.74 ± 1.87 *#

Values are expressed as means ± SEM. * Significantly different from type I fibers (P<0.05), # significantly different from type IIa fibers (P<0.05), ^ different from type IIa fibers (P=0.07). AU Arbitrary Units.

Discussion

In the present study, we show that resistance exercise can substantially reduce skeletal muscle glycogen and lipid content. A single resistance exercise session reduces both type I and II muscle fiber glycogen content, with most pronounced changes located in the type IIx fibers. Furthermore, resistance exercise also reduces intramyocellular lipid content, which is restricted to the type I muscle fibers. Fiber-type specific substrate utilisation during resistance exercise seems to be tightly coupled to muscle fiber-type oxidative capacity.

The exercise-induced reduction in skeletal muscle glycogen and/or lipid content has been associated with an increase in insulin sensitivity in humans (2, 35, 48, 49). Numerous studies have reported intramuscular substrate availability to play a key regulatory role in skeletal muscle insulin signaling (18, 50). For example, skeletal muscle glucose uptake and glycogen synthase activity strongly depend on muscle glycogen availability (32, 50). The mechanism for this phenomenon is related to increased GLUT4 translocation under reduced muscle glycogen availability, and is likely coupled to an increased activation of AMP-activated protein kinase (AMPK) (50). Furthermore, a decrease in muscle lipid content following exercise has also been linked to greater insulin sensitivity in rodents (33). Although a variety of mechanisms have been suggested, recent studies seem to indicate that the relationship between IMTG content and skeletal muscle insulin sensitivity is not functional (44).

Aging, disability and functional incapacity often result in the inability/inefficacy to perform moderate-intensity endurance exercise. Therefore, resistance exercise training is now more frequently advocated as a necessary component of effective exercise intervention programs to augment muscle mass (22), strength, power and functional capacity (10, 28). However, only few studies have investigated the acute effects of resistance exercise on insulin sensitivity (4, 11, 12). Recently, we showed that a single resistance exercise session improves whole-body insulin sensitivity by ~13% for up to 24h after exercise (24). The latter seems to be of a similar magnitude as the ~20% increase in whole-body insulin sensitivity that has been reported after ~60 min of endurance exercise (7, 8, 30, 35). It could be speculated that the increase in insulin sensitivity following resistance exercise is also associated with a net reduction in skeletal muscle glycogen and/or lipid content. However, literature on the metabolic demands of resistance exercise is scarce. Early reports suggest that energy needs during resistance exercise can be provided with only minor activation of glycogenolysis or lipolysis (23).

In the present study, we investigated fiber-type specific changes in skeletal muscle glycogen and lipid content following resistance exercise and subsequent recovery by using semi-quantitative histological techniques. The latter provides an effective strategy to assess fiber-type specific changes in intramyocellular lipid and/or glycogen content, without the methodological limitations associated with biochemical extraction analyses. In accordance, we recently reported a good correlation between IMTG content as determined by ^{1}H-magnetic resonance spectroscopy (MRS) and fluorescence microscopy (45). In addition, strong correlations between data on (mixed-muscle) glycogen content obtained by the use of both biochemical and histochemical methods have recently been reported (37). In the present study, mixed muscle glycogen content declined by 33±7% following the resistance exercise session. The latter seems to be in line with previous studies applying biochemical analysis on mixed muscle tissue (9, 41). We extent their findings by showing that these changes in muscle glycogen are fiber-type specific, and seem to be tightly coupled to (non-)oxidative capacity (table 3.2, figure 3.3). Linear regression revealed a negative correlation between the net reduction in muscle fiber-type glycogen content and fiber-type oxidative capacity (r=-0.556,

P<0.05). As such, net changes in muscle fiber glycogen content were more pronounced in type IIx fibers compared to type I muscle fibers, which can be attributed to the greater recruitment of these fibers during high-intensity resistance exercise (13). During post-exercise recovery no significant differences in muscle glycogen content were observed over time in the type I, IIa or IIx muscle fibers. Furthermore, we observed a significant 27±7% reduction in type I muscle fiber lipid content (P<0.05). No changes in IMTG content were observed in the type IIa and IIx fibers (figure 3.5). The net reduction in IMTG content was significantly different compared to the type II fibers (P<0.05). On average, type I muscle fiber lipid content was 2.5±0.3 and 8.8±1.4 times greater when compared to the type IIa and IIx fibers, respectively. Due to the many methodological difficulties with the biochemical triacylglycerol extraction analyses on mixed muscle biopsy samples (21), we applied (immuno)histochemical IMTG analyses on oil red O stained muscle cross section to enable fiber-type specific determination of intramyocellular lipid content. The latter might be the reasons why Essen-Gustavsson and Tesch (9) failed to detect a significant reduction in mixed muscle lipid content following resistance exercise. In accordance, we observed a non-significant 25±10% net decline in calculated mixed muscle lipid content (P=0.087).

The reported net changes in muscle fiber-type specific lipid content following exercise were positively correlated with muscle fiber-type oxidative capacity (table 3.2). Interestingly, within 120 min of post-exercise recovery, muscle fiber lipid content had returned to pre-exercise values. This could be attributed to the substantial increase in circulating plasma FFA concentrations during post-exercise recovery. Which is similar to reports on IMTG accumulation under conditions of elevated plasma FFA availability, like prolonged fasting (38, 40), prolonged endurance exercise in regard to inactive muscle tissue (38) and/or 48h of post-exercise recovery on a relatively high-fat diet (46). The absence of a prolonged net reduction in skeletal muscle lipid content implies that the reported stimulating effects of resistance exercise on whole-body insulin sensitivity is unlikely to be attributed to a reduction in intramuscular lipid content. The latter is in line with the fact that the proposed relationship between IMTG deposition and insulin sensitivity does not appear functional (44). It has been suggested that the ratio between IMTG content and oxidative capacity likely represents a more appropriate parameter to assess the risk of developing insulin resistance and/or type 2 diabetes (43). This is especially important when evaluating the benefits of exercise intervention programs, as intervention studies have actually shown IMTG contents to remain stable or even increase despite substantial improvements in muscle oxidative capacity and/or whole-body insulin sensitivity (3, 17, 36).

In conclusion, a single resistance exercise session substantially reduces intramyocellular lipid and glycogen content. These changes are fiber-type specific and are closely related to fiber-type oxidative capacity. The present data suggest that earlier reports on the acute improvements in whole-body insulin sensitivity following resistance exercise could, at least partly, be attributed to the net reduction in skeletal muscle glycogen content.

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Changes in protein metabolism during and following prolonged endurance exercise: effect of nutritional supplements and discrepancies between tracers

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Abstract

The aims of this study were to compare different tracer methods to assess whole-body protein turnover during 6h of prolonged endurance exercise when carbohydrate was ingested throughout and to investigate whether the addition of protein can improve protein balance. Eight endurance trained athletes were studied on 2 different occasions at rest (4h), during 6h of exercise at 50% VO_2max (in sequential order: 2.5h of cycling, 1h running and 2.5h of cycling) and during subsequent recovery (4h). Subjects ingested either carbohydrate (CHO trial; 0.7 g CHO·kg^{-1}·h^{-1}) or carbohydrate/protein beverages (CHO+PRO trial; 0.7 g CHO·kg^{-1}·h^{-1}and 0.25 g PRO·kg^{-1}·h^{-1}) at 30 min intervals during the entire study. Whole-body protein metabolism was determined by infusion of L-[1-^{13}C]leucine, L-[$^{2}H_5$]phenylalanine and [$^{15}N_2$]urea tracers with sampling of blood and expired breath. Leucine oxidation increased from rest to exercise (CHO: 27±2.5 vs. 74±8.8; CHO+PRO: 85±9.5 vs. 200±16.3 mg protein·kg^{-1}·h^{-1}; $P<0.05$), whereas phenylalanine oxidation and urea production did not increase with exercise. Whole-body protein balance during exercise with carbohydrate ingestion was negative (-74±8.8; -17±1.1; -72±5.7 mg protein·kg^{-1}·h^{-1}, when using L-[1-^{13}C]leucine, L-[$^{2}H_5$]phenylalanine and [$^{15}N_2$]urea as tracer). Addition of protein to the carbohydrate drinks resulted in a positive or less negative protein balance (-32±16.3; 165±4.6; 151±13.4 mg protein·kg^{-1}·h^{-1}, when using L-[1-^{13}C]leucine, L-[$^{2}H_5$]phenylalanine and [$^{15}N_2$]urea as tracer). We conclude that even during 6h of exhaustive exercise in trained athletes using carbohydrate supplements; net protein oxidation does not increase, compared with the resting state and/or post-exercise recovery. Furthermore, the combined ingestion of both protein and carbohydrate improves net protein balance at rest as well as during exercise and post-exercise recovery.

Introduction

In the nineteenth century, skeletal muscle protein was thought to be the main fuel used to generate energy for muscle contractions (33). However, controlled nitrogen balance studies invalidated the proposed hypothesis as they showed no substantial increase in nitrogen loss during and/or following prolonged exercise (9, 14). Since then various methods have been applied to investigate the effects of exercise on protein metabolism. This has resulted in many discrepant findings in the literature, which are due in part to differences in the methodology employed and in part to the conditions under which different exercise interventions have been performed.

Since the introduction of stable isotope tracers in metabolic research, various methods have been applied to study whole-body protein metabolism. Most studies have used plasma L-[1-^{13}C]leucine kinetics as a model for whole-body protein metabolism. Whereas several studies have reported increases in whole-body protein degradation during exercise (23, 25, 39, 40), others have failed to observe such changes (6, 26). Wolfe et al. (39) measured the rate of appearance of $^{13}CO_2$ in the expired breath from infused ^{13}C-labeled leucine and reported a 3-fold increase in leucine oxidation rates during exercise. The latter was shown to occur in the absence of a change in total leucine flux, which implies that the observed increase in protein breakdown was accompanied by a reduction in the rate of protein synthesis. Another tracer method that has been developed to determine whole-body protein metabolism, without the necessity of analyzing breath gasses, is the use of L-[$^{2}H_5$]phenylalanine according to the model of Clarke and Bier (10) and Thompson et al (27). However, that method has not yet been applied to investigate the effect of prolonged endurance exercise. Studies applying [^{15}N]urea to determine the rate of urea production as a measure of the amount of amino acids that are liberated by net protein degradation (degradation – synthesis) and oxidized (with conversion of the amino group to urea) during exercise, have not confirmed the catabolic nature of prolonged endurance exercise (7, 39).

The reports using urea as a tracer, indicate that protein synthesis and degradation rates do not change during endurance type activities. However, nitrogen balance data from field studies suggest that prolonged endurance exercise, leading to glycogen depletion, is accompanied by a substantial increase in net protein breakdown (13). In accordance, activation of the branched-chain α-keto acid dehydrogenase complex, the enzyme responsible for leucine oxidation in the muscle, has been shown to increase during exercise when glycogen stores are low (34, 35). During competition many endurance athletes (e.g. cyclists, triathletes and ultra-marathon runners) exercise for more than 5h at relatively high workloads and deplete their glycogen stores to a large extent. However, whole-body protein turnover measurements using multiple amino acid tracers have not yet been performed during such prolonged exhaustive exercise activity.

Endurance trained athletes try to optimize carbohydrate availability through carbohydrate ingestion before, during and after exercise. However, most studies

measuring protein turnover during exercise have been performed following an overnight fast and with nutritional interventions generally applied during the post-exercise recovery phase. Little information is available about the effects of carbohydrate or carbohydrate and protein supplementation on protein metabolism during exercise. The available literature indicates that combined ingestion of carbohydrate and protein in the post-exercise recovery phase improves net protein balance (12, 22), at least after resistance exercise (2, 3, 24, 28, 29). Ingestion of such a substrate mixture during exercise could possibly also affect protein balance during and after prolonged endurance exercise.
In the present study, we determined whole-body protein synthesis and degradation rates at rest, during prolonged exhaustive exercise and during subsequent recovery. A multiple tracer approach, applying L-[1-^{13}C]leucine, L-[$^{2}H_5$]phenylalanine and [$^{15}N_2$]urea infusions was chosen to investigate whether there are differences in outcome depending on the applied tracer model. These studies were performed in a laboratory setting that resembles real-life endurance exercise competition in elite triathletes, combining 6h of exercise with regular carbohydrate supplementation. In addition, we investigated whether the addition of protein to carbohydrate ingestion can improve net protein balance during exercise and subsequent recovery compared with the ingestion of only carbohydrate.

Methods

Subjects

Eight well-trained male subjects (age: 31±3 y; height: 1.84±0.03 m; weight: 72.4±2.3 kg; body mass index (BMI): 21.4±0.3 kg·m^{-2};% bodyfat: 9.6±0.8%, fat free mass: 65.7±1.3 kg; maximal workload capacity (Wmax): 383±13 W; maximal oxygen uptake capacity (VO_2max): 4.9±0.2 L·min^{-1}) participated in this study. Subjects trained around 15h a week (3h swimming, 7h cycling and 5h running) and had a training history > 5y. Subjects were informed about the nature and risks of the procedures before their written informed consent was obtained. This study was approved by the local medical ethical committee of the Academic Hospital Maastricht.

Pre-testing

VO_2max and Wmax were measured on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) during an incremental exhaustive exercise test (17) one week before the first trial to determine the 45% Wmax workload (179±8.6 W; ~50% VO_2max), which was applied in these studies. Maximum running velocity was determined on a motor driven treadmill with a 1% incline (to represent outdoor running) using an incremental exhaustive exercise test to determine the 45% maximal running velocity (Vmax) (11.0±0.3 km·h^{-1}; ~50% VO_2max). After a 5 min warm-up at 10 km·h^{-1}, speed was increased every 3

minutes by 2 km·h^{-1}, and from a heart rate of 85% of maximal heart rate with 1 km·h^{-1} every 3 minutes until the subject was unable to continue.

Diet and activity prior to testing

All subjects were instructed not to consume any products with a high natural abundance of ^{13}C (carbohydrates derived from C4 plants: corn, sugar cane) one week before the first trial and during the entire test period. This has been shown to minimize possible shifts in background enrichment due to changes in endogenous substrate utilization (36). All subjects were also instructed to refrain from heavy physical exercise and to maintain normal dietary habits for 3 days prior to each trial. In addition, we asked subjects to record their food intake for 48h before the start of the first experimental trial and to consume exactly the same food 48h before the start of the second test.

Studies

All subjects were studied on two different occasions. Each test day consisted of three parts during which whole-body protein turnover and whole-body urea production rate, were measured at rest, during prolonged exercise and subsequent recovery. Via a randomized cross-over design, subjects received beverages containing either carbohydrate (CHO) or carbohydrate with protein hydrolysate (CHO+PRO) during each trial. An outline of the study design is presented in figure 4.1.

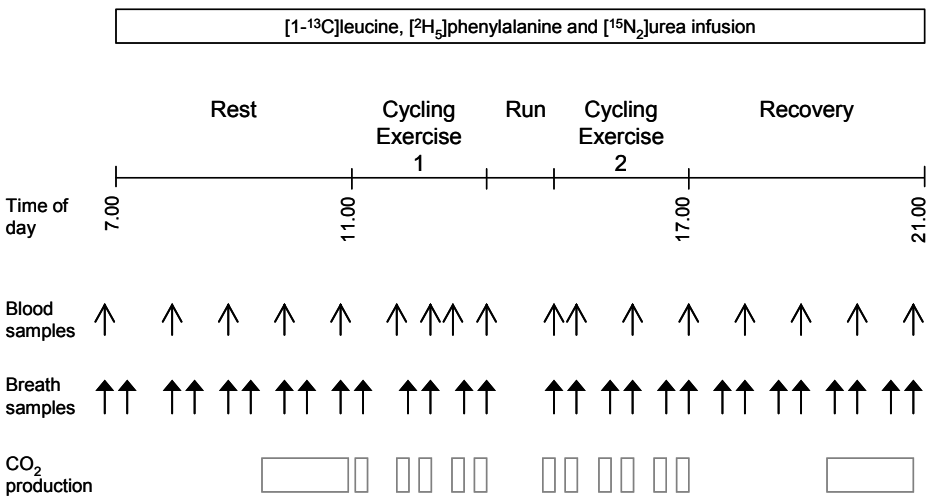

Figure 4.1 Schematic outline of the study protocol. Cycling and running exercises were performed at 50% VO_2max. Every 30 min throughout the entire test, subjects received a beverage volume of 4 ml·kg^{-1} to ensure a given dose of 0.7 g·kg^{-1}·h^{-1} carbohydrates (maltodextrin) in the control trial (CHO) and 0.7 g·kg^{-1}·h^{-1} carbohydrate and 0.25 g·kg^{-1}·h^{-1} protein hydrolysate in the CHO+PRO trial.

Protocol

The subjects reported at the laboratory at 7.00 am after an overnight fast. A Teflon catheter (Baxter BV, Utrecht, The Netherlands) was inserted into an antecubital vein of one arm for blood sampling; another catheter was inserted in the contralateral arm for isotope infusion. A resting blood sample was drawn and expired breath samples were collected into vacutainer tubes (Becton Dickinson, Meylan, France) to measure baseline enrichments. Thereafter, a single intravenous dose of $NaH^{13}CO_3$ (5.9 μmol·kg^{-1}) was administered to prime the bicarbonate pool, which was followed by a L-[1-^{13}C]leucine (7.6 μmol·kg^{-1}), L-[$^2H_5$]phenylalanine (3.0 μmol·kg^{-1}), [$^{15}N_2$]urea (88 μmol·kg^{-1}) and L-[$^2H_4$]tyrosine (0.46 μmol·kg^{-1}) prime. Thereafter (t=0), continuous infusion of L-[1-^{13}C]leucine (7.6 μmol·kg^{-1}·h^{-1}), L-[$^2H_5$]phenylalanine (3 μmol·kg^{-1}·h^{-1}), [$^{15}N_2$]urea (88 μmol·kg^{-1}·h^{-1}) was started via a calibrated IVAC 560 pump (San Diego, CA, USA) and continued for 14h. After the start of the tracer infusion, subjects rested for 4h (resting period). Subsequently, subjects exercised for 6h at a moderate intensity (45% Wmax), starting with 2.5h of cycling, followed by 1h of treadmill running, and another 2.5h of cycling exercise. Subjects were not allowed to rest between the different exercise bouts apart from the time necessary to change from ergometer to treadmill and back. After cessation of exercise, subjects rested for 4h (recovery). During the pre-exercise resting period and during the post-exercise recovery phase breath and blood samples were obtained every 30 and 60 minutes, respectively. During cycling exercise periods blood and breath samples were collected every 30 minutes and VO_2 and VCO_2 were measured (Oxycon-β, Mijnhardt, Bunnik, The Netherlands) at 30 min intervals for 5 min.

Beverages

Directly after the start of the stable isotope infusion and every 30 min throughout the entire trial (before, during and after exercise), subjects received a beverage volume of 4 ml·kg^{-1} to ensure a given dose of 0.7 g·kg^{-1}·h^{-1} carbohydrates (maltodextrin) in the control trial (CHO) and 0.7 g·kg^{-1}·h^{-1} carbohydrate and 0.25 g·kg^{-1}·h^{-1} protein hydrolysate in the CHO+PRO trial. The drinks were prepared by Quest International (Naarden, The Netherlands). L-[$^2H_5$]phenylalanine and L-[1-^{13}C]leucine enrichment in the beverages was measured using GC-MS and did not differ from the L-[$^2H_5$]phenylalanine and L-[1-^{13}C]leucine enrichment in the plasma background samples. Therefore, beverage ingestion did not alter plasma enrichment and corrections were not required. The beverages contained per liter: 80 g maltodextrin, 25 g glucose syrup, 0.3 g sodium-benzoaat, 2.25 g citrus compound, 1 g citric acid and 0.2 g ascorbic acid with or without the addition of 30 g rice hydrolysate (Hyprol Dev 8115, Quest International). Amino acid profile of the rice hydrolysate is listed in table 4.1.

Table 4.1 Amino acid composition of the protein hydrolysate

Amino acids	Rice hydrolysate
L-Arginine (Arg)	5.6
L-Histidine (His)	1.5
L-Asparagnine (Asn)	4.1
L-Glutamine (Gln)	7.7
L-Lysine (Lys)	2.8
L-Glycine (Gly)	2.7
L-Alanine (Ala)	3.5
L-Tryptophan (Trp)	0.6
L-Serine (Ser)	3.4
L-Proline (Pro)	2.4
L-Valine (Val)	3.9
L-Threonine (Thr)	2.4
L-Cysteine (Cys)	0.6
L-Isoleucine (Ile)	2.8
L-Leucine (Leu)	5.2
L-Aspartate (Asp)	2.4
L-Glutamate (Glu)	4.5
L-Methionine (Met)	1.4
L-Phenylalanine (Phe)	3.6
L-Tyrosine (Tyr)	3.3

Values expressed in g·100g dry product^{-1}.

Plasma analyses

Blood samples were collected in EDTA containing tubes and centrifuged at 1000 g and 4°C for 5 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C. Plasma [1-^{13}C]alpha-Ketoisocaproate (KIC) enrichment was measured using a quinoxalinoltrimethylsilyl derivate (31), whereas L-[$^{2}H_5$]phenylalanine and L-[$^{2}H_4$]tyrosine enrichments were measured using their t-butyldimethylsilyl derivates on a gas chromatography mass spectrometer (GC-MS) (Finnigan: Incos XL, Bremen, Germany) (27). Plasma [$^{15}N_2$]urea enrichment was measured using the N,N'-bistrimethylsilyl derivate of urea on a GC-MS (39). Breath $^{13}CO_2$ enrichment in the expired air was measured by GC continuous flow-IRMS (Finnigan, MAT-252).

Calculations

The rate of protein turnover was calculated as described previously (20). Briefly, leucine turnover (flux, Q) was measured from the dilution of L-[1-13C]leucine infusion in plasma alpha-KIC at isotopic steady state:

$$Q = i \cdot \left(\frac{Ei}{Ep} - 1 \right)$$

where i is the [1-^{13}C]leucine infusion rate (μmol·kg body weight^{-1}·h^{-1}), Ei is the enrichment of the [1-^{13}C]Leucine infused (expressed in atom percent excess; APE)

and Ep is the [1-^{13}C]alpha-KIC enrichment in the plasma at steady state (APE). The background enrichment of expired $^{13}CO_2$ was measured before the start of tracer infusions. This background enrichment was subtracted from the isotopic plateau value for the calculation of leucine oxidation. Leucine oxidation rates were also corrected for bicarbonate retention. In endurance trained subjects, employed bicarbonate retention factors were 83.1% for rest and 98.9% for exercise (11). The rate of leucine oxidation is calculated using,

$$O = {}^{13}CO_2 \cdot \left[\left(\frac{1}{Ep} \right) - \left(\frac{1}{Ei} \right) \right] 100$$

where $F^{13}CO_2$ is the rate of $^{13}CO_2$ production and release in the expired breath (μmol $^{13}CO_2$·kg^{-1}·h^{-1}). At isotopic steady state, protein flux (Q) equals the sum of protein synthesis (S) and oxidation (O) as well as the sum of the rate of appearance of meal protein from the gut (I) and protein breakdown (B), whole-body protein synthesis rate was calculated as flux minus oxidation.

$$Q = S + O = B + I$$

$$S = Q - O$$

The leucine parameters mentioned above were converted to corresponding estimates of whole-body protein turnover by multiplying the leucine values by the constant 1/(590 μmol leucine·g^{-1}protein) to give values of g protein·kg^{-1}·h^{-1} (20). The 590 μmol leucine·g protein^{-1} factor corresponds to a protein leucine content of 7.8% and was derived from averaged values for leucine content of protein of human and other mammal muscles (20).

In addition, we used the phenylalanine balance model as described by Thompson et al (27). In this model, phenylalanine flux (Qp) can be calculated by isotope dilution using equation 1, where i is the L-[$^2H_5$]phenylalanine infusion rate (μmol·kg^{-1}·h^{-1}), Ei is the enrichment of the L-[$^2H_5$]phenylalanine infused (expressed in APE) and Ep is the L-[$^2H_5$]phenylalanine in the plasma at steady state (APE). At isotopic steady state, whole-body phenylalanine oxidation can be determined from the conversion (hydroxylation) of L-[$^2H_5$]phenylalanine to L-[$^2H_4$]tyrosine. The rate of hydroxylation (Qpt) was calculated (27) using the formula

$$Q_{pt} = \frac{P_t \cdot Q_p^2}{P_p \cdot \left(\frac{E_p}{E_t} - 1 \right) i_p - Q_p}$$

where P_t/P_p (=0.73) is the molar ratio of the fluxes of tyrosine and phenylalanine arising from protein catabolism (27), Qp is the phenylalanine flux, Et is the L-[$^2H_4$]tyrosine enrichment, and ip is the L-[$^2H_5$]phenylalanine infusion rate (μmol·kg^{-1}·h^{-1}). Protein synthesis and degradation using this approach was calculated using

$$Q = S + Q_{pt} = B + I$$

Rates of protein synthesis and degradation (g protein·kg bodyweight^{-1}·h^{-1}) are calculated using the phenylalanine content of protein of 280 μmol·g^{-1}protein (27).

The 280 μmol phenylalanine·g protein^{-1} factor was derived from protein composition measurements in animals (21).
The net protein balance (table 4.2) was calculated from the difference between whole-body protein synthesis (S) and degradation (B) determined using the leucine and phenylalanine model as described above.
The rate of urea production was calculated using equations described previously (38). In short, at isotopic steady state during the constant infusion of [$^{15}N_2$]urea, two enrichments (expressed in tracer tracee ratio; TTR) were measured, A and B

$$A = \frac{\text{singly labeled urea}}{\text{unlabeled urea}} \qquad B = \frac{\text{doubly labeled urea}}{\text{unlabeled urea}}$$

The rate of urea production was calculated using

$$Q = \frac{2F}{A + 2B}$$

where F is the [$^{15}N_2$]urea infusion rate (μmol·kg^{-1}·h^{-1}).
The net balance of the amount (g·kg^{-1}·h^{-1}) of protein that was broken down in the CHO trial and converted to urea was calculated by converting urea production rates to mg N produced per kg per h and multiplying with 6.25 (1 g of protein contains 160 mg of nitrogen). The positive protein net balance (protein synthesis – protein degradation) reported in table 4.2 was calculated in this case by again converting the observed urea production rate to protein oxidation rates and then taking the difference between protein ingestion and protein oxidation (both in g protein·kg^{-1}·h^{-1}).

Statistics

All data are expressed as mean±SEM. To compare tracer kinetics, protein synthesis and degradation rates between the different interventions over time, a two way repeated measures ANOVA was used with both subject and treatment as factors. A Scheffé's post-hoc test was applied in case of a significant F-ratio to locate specific differences. Student's t-tests for paired observations were used to compare differences in protein synthesis, breakdown, oxidation, net balance and urea production between the CHO and CHO+PRO trial. Significance was set at the 0.05 level of confidence.

Results

Tracer kinetics

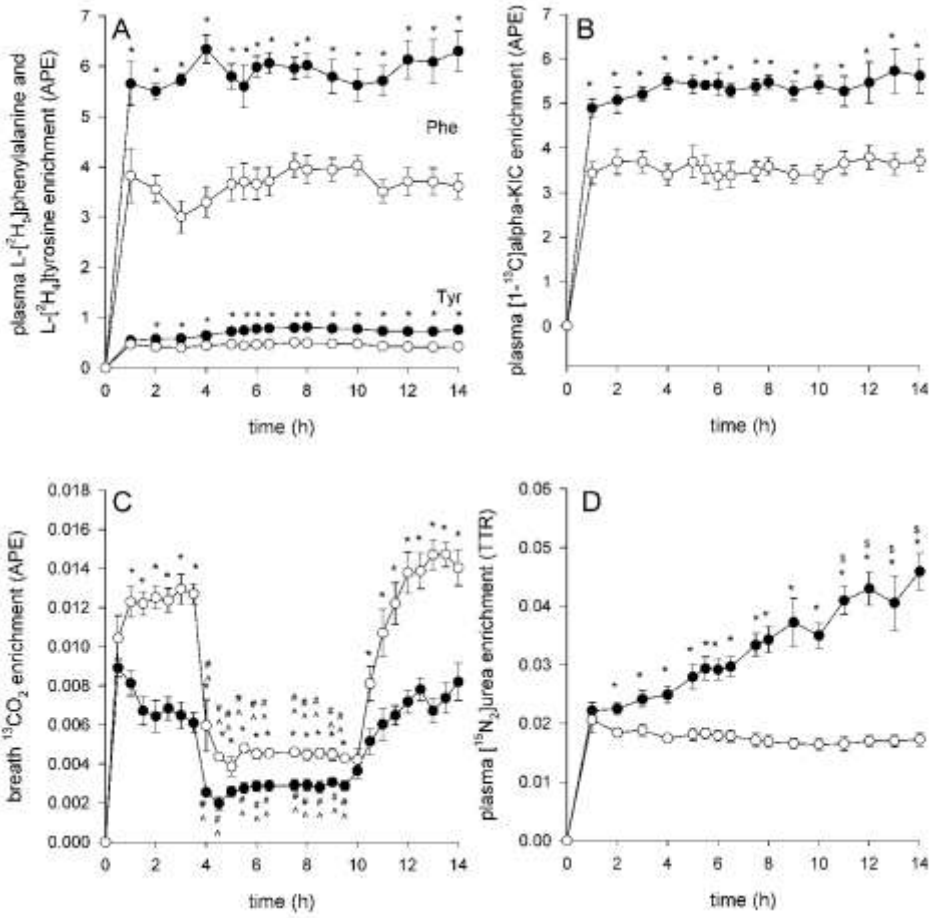

Figure 4.2 Tracer data. A, plasma L-[$^{2}H_5$]phenylalanine (Phe) and L-[$^{2}H_4$]tyrosine (Tyr) enrichment; B, plasma [^{13}C]alphaKIC; C, breath $^{13}CO_2$ enrichment; D, plasma [$^{15}N_2$]urea enrichment. APE, atom percent excess. Data are means±SEM. * Significantly different between studies (P<0.05); ^ significantly different from rest (P<0.05); # significantly different from recovery (P<0.05)

Plasma L-[$^{2}H_5$]phenylalanine and L-[$^{2}H_4$]tyrosine enrichment, plasma [1-^{13}C]alpha-KIC enrichment, breath $^{13}CO_2$ enrichment and plasma [$^{15}N_2$]urea enrichment are shown in figure 4.2A, B, C and D, respectively. Plasma L-[$^{2}H_5$]phenylalanine, L-[$^{2}H_4$]tyrosine, [1-^{13}C]alpha-KIC reached a steady state after 2h of infusion. Breath $^{13}CO_2$ enrichment was significantly lower during exercise (P<0.05) compared with

both resting conditions and post-exercise recovery. Plasma [$^{15}N_2$]urea enrichment slightly increased over time during the CHO trial, with significantly higher plasma [$^{15}N_2$]urea enrichment during recovery compared with pre-exercise resting values ($P<0.05$). In contrast, during the CHO+PRO trial plasma [$^{15}N_2$]urea enrichment remained constant throughout the entire trial. During the CHO+PRO trial plasma L-[$^2H_5$]phenylalanine, L-[$^2H_4$]tyrosine, [1-^{13}C]alpha-KIC and [$^{15}N_2$]urea enrichments were significantly lowered compared with the CHO trial ($P<0.05$). Breath $^{13}CO_2$ enrichment was elevated during the CHO+PRO trial compared with the CHO trial ($P<0.05$).

Phenylalanine model

Protein flux was similar at rest, during exercise 1 and 2 and during post-exercise recovery (176±6, 170±6, 172±7 and 162±15 mg protein·kg^{-1}·h^{-1}respectively) in the CHO trial and (303±26, 294±14, 264±14 and 293±21 mg protein·kg^{-1}·h^{-1} respectively) in the CHO+PRO trial. However, in the CHO+PRO trial, protein flux was ~70% increased at rest, during exercise and in the post-exercise recovery phase, when compared with the CHO trial ($P<0.01$).

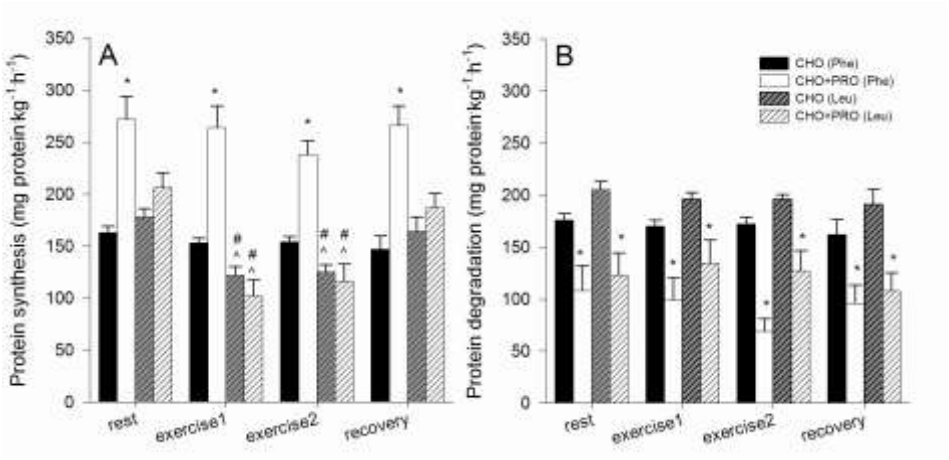

Figure 4.3 Whole-body protein synthesis (A) and degradation (B) calculated on basis L-[1-^{13}C]leucine and L-[$^2H_5$]phenylalanine at rest, during exercise 1 and 2 and during recovery. Data are means±SEM; ^ significantly different from rest ($P<0.01$); # significantly different from recovery ($P<0.01$); * significantly different between studies ($P<0.05$).

Whole-body protein synthesis (mg protein·kg^{-1}·h^{-1}) during the CHO and CHO+PRO trials is shown in figure 4.3A. No differences were found in protein synthesis rates during exercise compared to rest and recovery phase. However, under all conditions protein synthesis rates were higher in the CHO+PRO trial, when compared with the CHO trial ($P<0.01$).

Protein degradation rates (mg protein·kg^{-1}·h^{-1}) did not change during exercise (figure 4.3B). A marked decrease in protein breakdown was observed throughout the CHO+PRO trial, when compared with the CHO trial ($P<0.01$).
Whole-body protein oxidation (mg protein·kg^{-1}·h^{-1}) is shown in figure 4.4. Whole-body protein oxidation was similar at rest, during exercise and during post-exercise recovery (13±1, 17±1, 19±2 and 15±2 mg protein·kg^{-1}·h^{-1} respectively) in the CHO trial and (31±4, 30±3, 25±2 and 27±3 mg protein·kg^{-1}·h^{-1} respectively) in the CHO+PRO trial. During the CHO+PRO trial protein oxidation rates were higher at rest, during exercise and recovery when compared with the CHO trial ($P<0.05$).
During exercise, whole-body protein balance (mg protein·kg^{-1}·h^{-1}) was not different from resting conditions and post-exercise recovery (figure 4.5). In the CHO+PRO trial, protein balance was positive whereas protein balance was negative in the CHO trial.

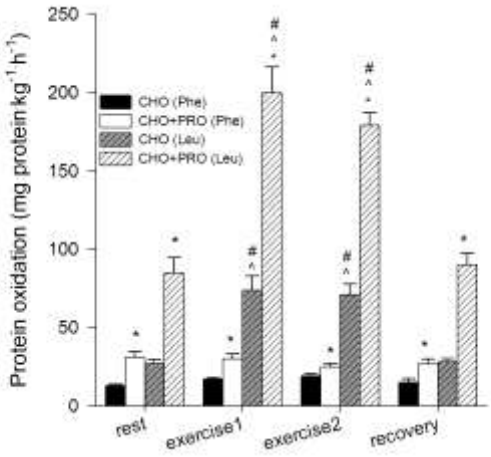

Figure 4.4 Whole-body protein oxidation calculated on basis L-[1-^{13}C]leucine and L-[$^{2}H_5$]phenylalanine at rest, during exercise 1 and 2 and during recovery. Data are means±SEM; ^ significantly different from rest ($P<0.01$); # significantly different from recovery ($P<0.01$); * significantly different between studies ($P<0.05$).

Leucine model

Protein flux (mg protein·kg^{-1}·h^{-1}) was not different during exercise compared with fluxes at rest and during post-exercise recovery. Protein flux was ~50% higher at rest, during exercise and in the post-exercise recovery phase ($P<0.01$) in the CHO+PRO trial, when compared with the CHO trial.
Whole-body protein synthesis (mg protein·kg^{-1}·h^{-1}) in the CHO and CHO+PRO studies is shown in figure 4.3A. Protein synthesis rates were decreased during exercise compared with rates at rest and during post-exercise recovery ($P<0.01$). Whole-body protein synthesis tended to be increased at rest ($P=0.09$), but was similar during exercise and post-exercise recovery in the CHO+PRO trial, when compared with the CHO trial.

Protein degradation rates (mg protein·kg^{-1}·h^{-1}) did not change during exercise (figure 4.3B). A marked decrease in protein breakdown was observed throughout the CHO+PRO trial, when compared with the CHO trial ($P<0.01$).
Whole-body protein oxidation (mg protein·kg^{-1}·h^{-1}) is shown in figure 4.4. Protein oxidation rates were 2-3 fold higher during exercise than at rest and during post-exercise recovery ($P<0.01$) in the CHO and CHO+PRO trial. In the CHO+PRO trial, protein oxidation was increased at rest, during exercise and recovery when compared with the CHO trial ($P<0.05$).
During the CHO trial whole-body protein balance (mg protein·kg^{-1}·h^{-1}) was negative at rest, during exercise and during subsequent recovery. Protein balance was more negative during exercise compared with the rest and recovery period ($P<0.01$) (figure 4.5). During the CHO+PRO trial, protein balance was less negative at rest, during exercise and during post-exercise recovery, when compared with the CHO trial ($P<0.01$), however protein balance during exercise remained negative.

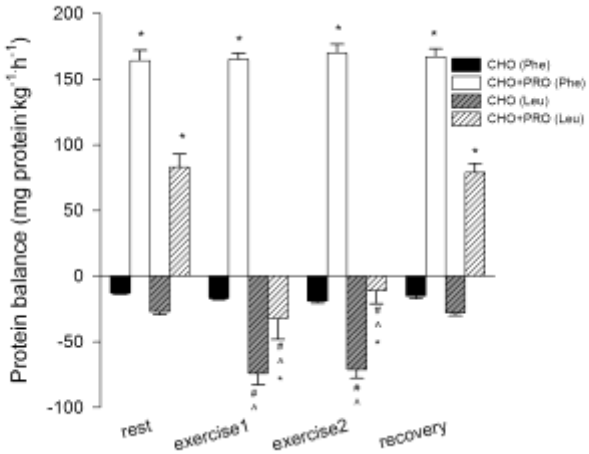

Figure 4.5 Net whole-body protein balance calculated on basis L-[1-^{13}C]leucine and L-[$^{2}H_5$]phenylalanine at rest, during exercise 1 and 2 and during recovery. Data are means±SEM; ^ significantly different from rest ($P<0.01$); # significantly different from recovery ($P<0.01$); * significantly different between studies ($P<0.05$).

Phenylalanine model vs. leucine model

The relative increase in protein flux in the CHO+PRO trial compared with the CHO trial was similar for both applied tracers (L-[$^{2}H_5$]phenylalanine, +70±14%; L-[1-^{13}C]leucine, +48±12%).
Protein synthesis was 69±14% increased in the CHO+PRO trial compared with the CHO trial when phenylalanine was used as a tracer. The leucine model showed no differences in protein synthesis rates between trials.

The relative decrease in protein degradation in the CHO+PRO trial compared with the CHO trial was similar for both applied amino acid tracers (L-[$^2H_5$]phenylalanine, -55±11%; L-[1-^{13}C]leucine, -62±8%).
Protein oxidation was increased with both tracer models during the CHO+PRO trial when compared with the CHO trial. The relative increase in protein oxidation in the CHO+PRO trial compared with the CHO trial was higher when measured with L-[1-^{13}C]leucine than with L-[$^2H_5$]phenylalanine (190±30% vs. 82±22%, $P<0.05$).
Protein balance was improved with both tracer models during the CHO+PRO trial when compared with the CHO trial ($P<0.05$). The relative increase in protein balance in the CHO+PRO trial compared with the CHO trial was higher when measured with L-[$^2H_5$]phenylalanine than with L-[1-^{13}C]leucine ($P<0.01$).

Urea model and comparison with other tracers

Urea kinetics were calculated using a [$^{15}N_2$]urea tracer and results are illustrated in figure 4.6. During the CHO trial urea production decreased during exercise and recovery compared with resting values, whereas during the CHO+PRO trial urea production remained constant. Urea production was higher during the CHO+PRO trial compared with the CHO trial ($P<0.05$).

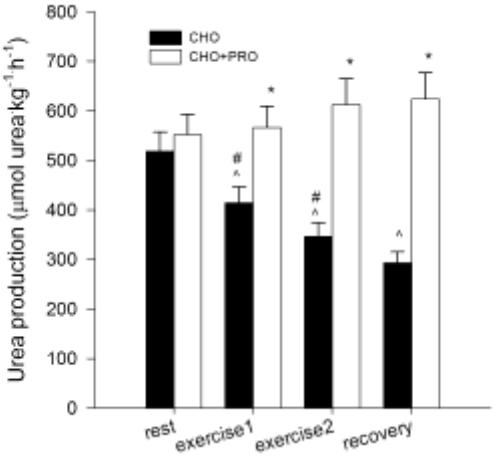

Figure 4.6 Urea production calculated on basis [$^{15}N_2$]urea data at rest, during exercise 1 and 2 and during recovery. Data are means±SEM; ^ significantly different from rest ($P<0.01$); # significantly different from recovery ($P<0.01$); * significantly different between studies ($P<0.05$).

Net protein balance calculated from urea production rates was less negative during exercise and recovery compared with resting values in the CHO trial ($P<0.05$). No changes in net protein balance were observed in the CHO+PRO trial. Calculated net protein balance was positive in the CHO+PRO trial, whereas during the CHO trial net protein balance was negative.

In the CHO trial, whole-body protein oxidation did not change in time with L-[$^{2}H_5$]phenylalanine applied as a tracer, increased during exercise with L-[1-^{13}C]leucine used as a tracer to return to resting values in the recovery period, and gradually decreased in time with [$^{15}N_2$]urea as tracer. In the CHO+PRO trial, whole-body protein oxidation did not change in time with the L-[$^{2}H_5$] phenylalanine and [$^{15}N_2$]urea as tracer and increased during exercise with L-[1-^{13}C] leucine as tracer to return to resting values in the recovery period.
Protein balance was improved with all tracer models applied in this trial during the CHO+PRO trial when compared with the CHO trial. The phenylalanine model and the urea model both indicate that the net protein balance is positive in the CHO + PRO trial at rest, during exercise and during recovery.

Discussion

In the present study we show that whole-body estimates of protein metabolism during exercise are highly dependent on the specific tracermethod that is applied. Using L-[1-^{13}C]leucine as a tracer to study the effects of exercise on protein metabolism shows that protein oxidation is increased 2-3 fold during exercise. However, when using L-[$^{2}H_5$]phenylalanine as a tracer, protein oxidation, synthesis and degradation rates were not affected by exercise. In addition, urea production, measured using [$^{15}N_2$]urea, decreased in time during the CHO trial. Thus, two of the three methods indicate that prolonged exhaustive exercise does not lead to increased protein oxidation and/or a more negative protein balance. Although the application of different tracer methods produce different outcomes upon the effect of exercise on protein metabolism, each method shows that protein balance is negative when only carbohydrates are ingested. The addition of protein to carbohydrate ingestion improves whole-body protein balance, when measured using both the phenylalanine and the leucine model used in the present study.
Urea production in subjects not ingesting any protein is generally considered a reflection of the amount of amino acids that are released by net protein degradation (degradation - synthesis) and subsequently oxidized with conversion of the amino group to urea. Studies on the effect of exercise on urea production have consistently found no significant change from the basal rate (7, 39, 40), leading to the conclusion that the rate of net protein catabolism (degradation – synthesis) and amino acid oxidation was not changed. However, data obtained using another approach to determine whole-body protein turnover, involving the infusion of L-[1-^{13}C]leucine and the determination of leucine turnover and $^{13}CO_2$ appearance in the expired air, indicated that leucine oxidation was markedly increased during exercise (4, 5, 39, 40). Because of this discrepancy between methods, we applied both tracer methods in the same study. In addition, we also applied a third tracer, L-[$^{2}H_5$]phenylalanine, which has been successfully applied at rest (10, 27), but not during exercise conditions. In contrast to most studies we used an experimental setup, which included exercise and the continuous ingestion of carbohydrate (CHO) or carbohydrate and protein supplements (CHO+PRO) as athletes always ingest carbohydrates during exercise (training or competition) of this duration and

intensity. With leucine as tracer, protein oxidation was 2 to 3-fold higher during exercise than in the resting or recovery period (figure 4.4). The widely used L-[1-^{13}C]leucine model for the calculation of whole-body protein synthesis and breakdown therefore suggests that exercise inhibits protein synthesis (figure 4.3A). However, with phenylalanine used as tracer, protein oxidation, synthesis and degradation were similar at rest, during exercise and recovery (figure 4.4, 4.3A and B, respectively). Moreover, urea production and the amount of protein oxidation calculated from it also did not increase during exercise (figure 4.6). Thus, both the phenylalanine model and urea data show that prolonged moderate intensity exercise does not lead to an imbalance between protein synthesis and degradation and subsequent oxidation of the liberated amino acids. This is in line with tracer incorporation studies (8, 30), which reported no change in muscle protein synthesis both during moderate endurance exercise and recovery. However, results from both the phenylalanine model and the leucine model in the present study show that whole-body protein balance is negative at rest, during exercise and during post-exercise recovery when only carbohydrates are ingested (figure 4.5).

A several fold increase in leucine oxidation without changes in urea production during exercise has been previously observed (39, 40). Moreover, other studies also showed no effect of exercise on plasma urea concentration (26), production (7) or urinary excretion (6, 18, 23). An explanation for the discrepancy between the leucine and urea data is that leucine metabolism during exercise is not representative of the fate of the other amino acids that are present in proteins. The stochastic leucine model, therefore, cannot be used to investigate whole-body protein metabolism during exercise. A difference in time response of leucine and urea kinetics theoretically also leads to an apparent absence of an increase in urea production during high rates of leucine oxidation. The urea pool is large and turns over with a half-life of 8-10h (16, 19, 37). This implies that any increase in urea production due to an accelerated protein oxidation rate during exercise should have become apparent either during or following the 6h exercise period. However, in the CHO trial we observed a gradual decrease instead of an increase in urea production over time. Such a decrease in urea production in time has previously been reported in studies at rest after an overnight fast (37). The latter can be attributed to the delayed response of the urea production following changes in protein intake (16, 37).

In the present study, we also investigated phenylalanine metabolism. We determined the rate of conversion (hydroxylation) of L-[$^{2}H_5$]phenylalanine to L-[$^{2}H_4$]tyrosine at rest, during exercise and during recovery. This is the first step of phenylalanine oxidation and we found that its rate did not change during exercise and/or recovery in comparison to the resting state. This is in line with findings reported by Wolfe et al. (40) using a lysine tracer. In the latter study the increase in lysine oxidation during exercise was small compared with the increase in leucine oxidation. In addition, a discrepancy existed between the [^{15}N]- and [^{13}C]lysine flux data and the [^{13}C]- and [^{15}N]leucine flux data, which is similar to the discrepancy between phenylalanine and leucine observed in the present study.

One of the likely causes of the discrepancy between the lysine and/or phenylalanine tracer and the leucine tracer methods is the fact that during exercise, uptake (1, 32) and oxidation (32, 34, 35) of branched chain amino acids (leucine, isoleucine and valine) are increased several fold in contracting muscle. As such, the leucine tracer does not seem to be representative of the fate of the other essential amino acids during exercise. The latter means that the stochastic leucine model of whole-body protein metabolism (20) becomes invalid during exercise and cannot be used to estimate qualitative or quantitative changes in whole-body protein synthesis and/or degradation.

Most studies measuring protein turnover during exercise were performed in the morning after an overnight fast, which does not represent every day practice in the competitive athletes. Athletes optimize carbohydrate availability before and during exercise to enhance endurance performance capacity. During subsequent recovery they ingest mixed meals or carbohydrate/protein recovery drinks. The present study demonstrates that net protein degradation does not increase during moderate intensity exercise when carbohydrate is ingested. This implies that net protein breakdown and oxidation is minimal during athletic events such as (ultra)marathons and prolonged cycling races when carbohydrate is ingested. Although protein degradation and synthesis are unaltered by exercise when CHO ($0.7\ g \cdot kg^{-1} \cdot h^{-1}$) are ingested, whole-body protein balance measured with L-[$^2H_5$]phenylalanine was slightly negative during exercise and during recovery in the present study. However, the imbalance between protein synthesis and degradation and the amount of protein oxidized was not larger than in the resting state (figure 4.5).

It has previously been shown that infusion or ingestion of amino acids alone or in combination with carbohydrate increases protein synthesis and reduces protein degradation both at a whole-body level and in skeletal muscle tissue under resting conditions (12, 15, 22) and after resistance exercise (2, 3, 24, 28, 29), resulting in a positive protein balance. We aimed to investigate whether the addition of protein to a carbohydrate supplement can improve whole-body protein balance during and following prolonged endurance exercise leading to a (more) positive protein balance, as previously observed in the resting state. Combined ingestion of protein and carbohydrate resulted in an increased whole-body protein synthesis rate at rest, during exercise and recovery when compared with carbohydrate supplementation only. Although protein oxidation was increased ~2 fold at rest, during exercise and recovery with the protein supplement, whole-body protein degradation decreased by ~60%. Thus, the present study shows that the combined ingestion of protein and carbohydrate improves net protein balance at rest, during prolonged moderate intensity exercise and subsequent recovery in elite endurance athletes. With phenylalanine and urea as tracer a positive net protein balance was achieved, whereas ingestion of only carbohydrate resulted in a negative protein balance.

The L-[$^2H_5$]phenylalanine tracer method indicate that protein flux and oxidation rates are increased as expected when protein is orally ingested. In addition, co-ingestion of protein with carbohydrate results in a decreased rate of protein degradation and improved protein balance. The [$^{15}N_2$]urea tracer provides a similar

outcome upon the effect of exercise on net protein balance as the L-[$^{2}H_5$]phenylalanine tracer. Urea production slightly increases when protein is ingested (figure 4.6). This is due to the fact that more protein is ingested than is used for protein synthesis. The excess is directly oxidized or used for gluconeogenesis and/or lipogenesis with the aminogroup being disposed as urea. The balance between protein synthesis and degradation calculated from the difference between urea production observed and the urea equivalent of the ingested amount of protein (table 4.2) indicates that there is net positive protein balance, which is quantitatively similar to the estimates made from the figures generated by the phenylalanine tracer both at rest, during exercise and during recovery. This implies that the information generated by the phenylalanine tracer and urea tracer on the effect of protein ingestion is similar in all time periods studied, while the leucine data generate a different message during exercise (positive balance at rest and during recovery and negative balance during exercise).
In summary, this study shows that the L-[$^{2}H_5$]phenylalanine, L-[1-^{13}C]leucine and [$^{15}N_2$]urea tracers models reveal the same qualitative effect of the combined ingestion of protein and carbohydrate on whole-body protein balance at rest and during recovery. However, the L-[1-^{13}C]leucine model for measurements of whole-body protein turnover overestimates protein oxidation rates and underestimate protein synthesis rates during exercise. We conclude that prolonged exhaustive moderate intensity exercise does not result in increased protein degradation and/or a decrease in protein synthesis compared with resting situations in endurance-trained athletes. Whereas whole-body protein balance remains negative when only carbohydrates are ingested, co-ingestion with protein improves protein balance by increasing synthesis and decreasing breakdown, resulting in a positive net protein balance both at rest, during prolonged moderate intensity exercise and during recovery.

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5

The combined ingestion of protein and free leucine with carbohydrate increases post-exercise muscle protein synthesis *in vivo* in male subjects

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Abstract

The present study was designed to determine post-exercise muscle protein synthesis and whole-body protein balance following the combined ingestion of carbohydrate with or without protein and/or free leucine. Eight male subjects were randomly assigned to 3 trials in which they consumed drinks containing either carbohydrate (CHO), carbohydrate and protein (CHO+PRO), or carbohydrate, protein and free leucine (CHO+PRO+leu) following 45 min of resistance exercise. A primed, continuous infusion of L-[ring-$^{13}C_6$]phenylalanine was applied, with blood samples and muscle biopsies collected to assess fractional synthetic rate (FSR) in the m. vastus lateralis as well as whole-body protein turnover during 6h of post-exercise recovery. Plasma insulin response was higher in the CHO+PRO+leu compared to the CHO and CHO+PRO trials (+240±19% and +77±11%, respectively, $P<0.05$). Whole-body protein breakdown rates were lower, and whole-body protein synthesis rates were higher in the CHO+PRO and CHO+PRO+leu trials compared to the CHO trial ($P<0.05$). Addition of leucine in the CHO+PRO+leu trial resulted in a lower protein oxidation rate compared to the CHO+PRO trial. Protein balance was negative during recovery in the CHO trial, but positive in the CHO+PRO and CHO+PRO+leu trials. In the CHO+PRO+leu trial, whole-body net protein balance was significantly greater compared to values observed in the CHO+PRO and CHO trials ($P<0.05$). Mixed muscle fractional synthetic rate, measured over a 6h period of post-exercise recovery, was significantly greater in the CHO+PRO+leu trial compared to the CHO trial (0.095±0.006 %·h^{-1} vs. 0.061±0.008 %·h^{-1}, respectively; $P<0.05$), with intermediate values observed in the CHO+PRO trial (0.0820±0.0104 %·h^{-1}). We conclude that the co-ingestion of protein and leucine stimulates muscle protein synthesis and optimizes whole-body protein balance when compared to the intake of carbohydrate only.

Introduction

Resistance training can substantially augment skeletal muscle size and it has been shown that after resistance exercise, mixed muscle protein synthesis is stimulated for up to 48h (12, 35). Simultaneously, the rate of muscle protein degradation is also increased after exercise (7, 35). However, in the absence of food intake net muscle protein balance remains negative (7, 35, 36). Carbohydrate supplementation in the post-exercise recovery phase has been shown to result in a decrease in urinary 3-methylhistidine and urea excretion, suggesting that protein degradation is reduced (40). However, muscle protein synthesis does not seem to be affected following carbohydrate ingestion (10, 40). Although carbohydrate ingestion improves net leg amino acid balance compared with water or placebo intake, the net balance remains negative (10). It has been shown using different tracer methods/models (e.g. tracer incorporation measurements in muscle, leg amino acid exchange methods or whole-body measurements) that the combined infusion or ingestion of carbohydrate and protein/amino acids is needed to increase protein synthesis rate, to reduce protein degradation, and thus to elicit a positive net protein balance under resting conditions (13, 33) and during recovery from resistance exercise (8, 11, 39, 43, 44). Moreover, in a recent study (25), we have shown that the combined ingestion of protein and carbohydrate leads to a positive protein balance during ultra endurance exercise and subsequent recovery. The combined ingestion of carbohydrate and protein/amino acids in the post-exercise recovery phase can stimulate protein metabolism in several ways. Besides providing amino acids as precursors for protein synthesis, combined ingestion of carbohydrate and protein/amino acids can elicit a strong insulinotropic response (34, 38, 49).

Combined ingestion of carbohydrate with protein and/or free amino acids (34, 38, 47-49), as well as intravenous infusion of free amino acids (16, 17) has been shown to further elevate plasma insulin levels. It has been speculated that such elevated insulin concentrations can stimulate the uptake of selected amino acids (6) and muscle protein synthesis rate (6, 20). In addition, insulin has been reported to effectively inhibit proteolysis (9, 18, 19). Nonetheless, the exact mechanisms responsible for the stimulatory effects of carbohydrate and protein/amino acid ingestion on muscle protein synthesis during recovery from resistance exercise have not yet been established in detail. For example, in animal models, free leucine has been shown to stimulate protein synthesis by an insulin-independent mechanism (2, 3). Consequently, it has been speculated that a nutritional supplement containing carbohydrate, protein and free leucine could represent an effective tool to further increase post-exercise muscle protein synthesis and/or to inhibit protein degradation.

The main aim of the present study was to investigate the effect of carbohydrate (CHO), carbohydrate and protein (CHO+PRO) as well as carbohydrate, protein and leucine (CHO+PRO+leu) ingestion on the anabolic response to resistance exercise. By measuring the incorporation rate of labeled amino acids in the skeletal

muscle protein pool, mixed muscle protein synthesis rates were directly measured during 6h of recovery from strenuous resistance exercise. A primed constant infusion of L-[ring-$^{13}C_6$]phenylalanine was combined with plasma and muscle tissue sampling to simultaneously measure mixed muscle fractional protein synthetic rate and whole-body protein turnover.

Methods

Subjects

8 healthy, untrained male volunteers (age: 22.3±0.9 y; weight: 74.1±3.5 kg; height: 1.81±0.02 m; BMI: 22.5±0.9 kg·m^{-2}; % body fat: 16.0±3%), with no history of participating in any regular exercise program, were recruited for the present study. All subjects were informed on the nature and possible risks of the experimental procedures before their informed consent was obtained. The latter after approval by the Medical Ethical Committee of the Academic Hospital Maastricht, The Netherlands.

Pretesting

All subjects participated in an orientation trial to become familiarized with the exercise protocol and the equipment. Proper lifting technique was demonstrated and practiced for each of the 2 lower-limb exercises (leg press and leg extension). Thereafter maximum strength was estimated using the multiple repetitions testing procedure (29). In another session, at least 1 wk before the first experimental trial, subjects' 1 repetition maximum (1RM) was determined (26). After warming up, the load was set at 90-95% of the estimated 1RM, and increased after each successful lift until failure. A five-minute resting period between the subsequent attempts was allowed. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance. The average 1RM for the leg press was 185±9 kg and the mean 1RM for the leg extension was 106±4 kg.

Diet and activity prior to testing

All subjects consumed a standardized meal (66.1±2.7 kJ·kg^{-2}, consisting of 72 Energy% (En%) carbohydrate, 23 En% protein and 5 En% fat) the evening before the trials. All volunteers were instructed to refrain from any sort of heavy physical exercise and to keep their diet as constant as possible 3 days before the trials. In addition, subjects were asked to record their food intake for 48h before the start of the first experimental trial and to consume the same diet 48h before the start of the second and third test.

Experimental trials

Each subject participated in 3 trials, separated by at least 7 days, in which recovery drinks containing either carbohydrate (CHO), carbohydrate and protein (CHO+PRO) or carbohydrate, protein and leucine (CHO+PRO+leu) were tested in a randomized and double-blind fashion. Each trial lasted ~8 h. Repeated boluses of a given test-drink were ingested following the exercise protocol to ensure a continuous supply of ample glucose and amino acids to the muscle. Plasma and muscle samples were collected during a 6 h post-exercise period. These trials were designed to simultaneously assess whole-body amino acid kinetics and fractional synthetic rate (FSR) of mixed muscle protein by the incorporation of L-[ring-$^{13}C_6$]phenylalanine in the m. vastus lateralis.

Protocol

At 8.00 am, after an overnight fast, subjects reported at the laboratory and a Teflon catheter was inserted into an antecubital vein for stable isotope infusion. A second Teflon catheter was inserted in a heated dorsal hand vein of the contralateral arm, which was placed in a hot-box (60°C) for arterialized blood sampling. After collection of a resting blood sample, a single intravenous dose of L-[ring-$^{13}C_6$]phenylalanine (2 μmol·kg^{-1}) was administered to prime the phenylalanine pool. Thereafter, tracer infusion was started with subjects resting in a supine position. After 75 min of infusion, a second blood sample was collected and subjects started the resistance exercise protocol. The exercise session consisted of 8 sets of 8 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, The Netherlands) and 8 sets of 8 repetitions on the leg extension machine (Technogym). Both exercises were performed at 80% of the subjects' individual 1RM with 2 min rest intervals between the sets and in total required ~45 min to complete. All subjects were verbally encouraged during the test to complete the whole protocol. At the end of the resistance exercise protocol (t=0 min), subjects rested supine and a arterialized blood sample from the heated hand vein as well as a muscle biopsy from the m. vastus lateralis muscle were collected. Subjects then received an initial bolus (3 ml·kg^{-1}) of a given test drink. Repeated drinks (3 ml·kg^{-1}) were taken every 30 min until t=330 min post-exercise. Blood samples were subsequently taken from the heated hand vein at t= 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min with an additional muscle biopsy taken at 360 min post-exercise.

Muscle biopsies

Muscle biopsies were obtained from the middle region of the m. vastus lateralis (15 cm above the patella) and approximately 2 cm below entry through the fascia using the percutaneous needle biopsy technique (5). Muscle samples were immediately freed from blood, visible fat and connective tissue, and rapidly frozen in liquid nitrogen, and stored at –80ºC for measurement of the amino acid enrichment in the muscle free amino acid pool and in mixed muscle protein.

Beverages

Subjects received a beverage volume of 3 ml·kg^{-1} every 30 minutes to ensure a given dose of 0.3 g carbohydrate·kg^{-1} (50% as glucose and 50% as maltodextrin) and 0.2 g·kg^{-1}of a protein hydrolysate every h, with or without the addition of 0.1 g·kg^{-1}·h^{-1} leucine. The total amount of protein (0.2 g·kg^{-1}·h^{-1}) provided in both the CHO+PRO and CHO+PRO+leu trials by far exceeded the calculated amount of protein that was estimated to provide sufficient precursor substrate to sustain maximal protein synthesis rates for at least 6h (50). Repeated boluses were administered to enable a continuous ample supply of amino acids in the circulation, preventing perturbations in L-[ring- $^{13}C_6$]phenylalanine enrichments. The whey protein hydrolysate used in the present study contained 9.95% leucine and consequently the total amount of leucine administered in the CHO+PRO and CHO+PRO+leu trial was 0.02 vs. 0.12 g·kg^{-1}·h^{-1}, respectively.
The compositions of all test drinks are listed in table 5.1. Glucose and maltodextrin were obtained from AVEBE (Veendam, The Netherlands). Whey protein hydrolysate was prepared by Numico Research (Wageningen, The Netherlands). Leucine was purchased from BUFA (Uitgeest, The Netherlands). To make the taste comparable in all trials, beverages were uniformly flavored by adding 0.2 g sodium-saccharinate solution (25% w/w), 1.8 g citric acid solution (50% w/w) and 5 g of cream vanilla flavor (Numico Research) for each liter of beverage. Trials were performed in a randomized order, with test-drinks provided in a double-blind fashion.

Table 5.1 Composition of beverages

Test drink	CHO	CHO+PRO	CHO+PRO+leu
Whey protein	-	33.3	33.3
Leucine	-	-	16.6
Glucose	25	25	25
Maltodextrin	25	25	25
Sodium saccharinate	0.2	0.2	0.2
Citric acid	1.8	1.8	1.8
Cream vanilla	5	5	5
Water	up to 1.00 L		

Values expressed in g·L^{-1} beverage.

Tracer infusion

L-[ring-$^{13}C_6$]phenylalanine (99% enriched, Cambridge Isotopes, Andover, MA, USA) was dissolved in 0.9% saline before infusion. The phenylalanine pool was

primed with an infusion dose of 2 μmol·kg^{-1}. Thereafter, continuous intravenous infusion of the isotopes was performed using a calibrated IVAC 560 pump (San Diego, CA, USA) with an average infusion rate of 0.046±0.001 μmol·kg^{-1}·min^{-1}.

Analysis

Blood samples were collected in EDTA containing tubes and centrifuged at 1000 g and 4°C for 5 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C. Insulin was analyzed by radio immunoassay (Insulin RIA kit, LINCO Research Inc., St. Charles, MO, USA). Plasma (500 μL) for amino acid analysis was deproteonized on ice with 100 μL of 24% (w/v) 5-sulphosalicylic acid, mixed (vortexing) and the clear supernatant was collected after centrifugation. Plasma amino acid concentrations were analyzed on an automated dedicated amino acid analyzer (LC-A10, Shimadzu Benelux, Den Bosch, The Netherlands), using an automated precolumn derivatization procedure and a ternary solvent system (15). The exact phenylalanine concentration in the infusates (8.06±0.21 mmol·L^{-1}) was measured using the same method. Plasma phenylalanine and tyrosine were derivatized to their tert-butyldimethylsilyl (TBDMS) derivatives and their ^{13}C enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS, Finnigan Incos XL) using selected ion monitoring of masses 336 and 342 for unlabeled and labeled phenylalanine respectively and masses 466 and 472 for unlabeled and labeled tyrosine (51).
For measurement of L-[ring-$^{13}C_6$]phenylalanine enrichment in the free amino acid pool and mixed muscle protein, 55 mg of wet muscle was freeze-dried. Collagen, blood and other non-muscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (2-3 mg) was weighed and 2 ml of ice-cold 2% perchloric acid (PCA) was added. The tissue was then homogenized and centrifuged. The supernatant was collected and processed in the same manner as the plasma samples, such that intracellular free L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{13}C_6$]tyrosine enrichments could be measured using their t-butyldimethylsilyl derivatives on a GCMS. The protein pellet was washed with 3 additional 2 ml washes of 2% PCA, dried and the proteins were hydrolyzed in 6M HCl at 120°C for 15-18h. The hydrolyzed protein fraction was dried under a nitrogen stream while heated to 120°C, then dissolved in a 50% acetic acid solution and passed over a Dowex exchange resin (AG 50W-X8, 100-200 mesh hydrogen form, Biorad, Hercules, CA, USA) using 2M NH_4OH. Thereafter, the eluate was dried and the purified amino acid fraction was derivatized into the N-acetyl-methyl (NAM)-esters to determine the ^{13}C-enrichment of protein bound phenylalanine enrichment using GC-IRMS (Finnigan, MAT 252).

Calculations

Infusion of L-[ring-$^{13}C_6$]phenylalanine with muscle and arterialized blood sampling was used to simultaneously assess whole-body amino acid kinetics and FSR of mixed muscle protein. Whole-body rates of appearance (Ra) and disappearance

(Rd) of phenylalanine were calculated using the non-steady state Steele equations adapted for stable isotope methodology (41).

$$R_a = \frac{F - V[(C_2 + C_1)/2][(E_2 - E_1)/(t_2 - t_1)]}{(E_2 + E_1)/2}$$

$$R_d = R_a - V \cdot \left(\frac{C_2 - C_1}{t_2 - t_1}\right)$$

where F is the infusion rate (μmol·kg^{-1}·min^{-1}); V= distribution volume for phenylalanine (125 ml·kg^{-1}) (14); C1 and C2 are the phenylalanine concentrations (mmol·L^{-1}) in arterialized plasma at time 1 (t1) and 2 (t2), respectively and E1 and E2 are the plasma L-[ring- $^{13}C_6$]phenylalanine enrichments (expressed in tracer-tracee ratio; TTR). As whole-body Ra comprise phenylalanine rate of appearance arising from protein breakdown (B) and protein intake (I), whole-body protein breakdown can be calculated as follows.

$$B = R_a -$$

As whole-body Rd comprise the rate of phenylalanine disappearance from the free amino acid pool in the blood due to protein synthesis (S) and oxidation, whole-body protein synthesis can be calculated as Rd minus oxidation. The rate of phenylalanine oxidation was calculated using the phenylalanine balance model from Thompson et al. (42). Whole-body phenylalanine oxidation (Qpt) can be determined from the conversion (hydroxylation) of L-[ring- $^{13}C_6$]phenylalanine to L-[ring- $^{13}C_6$]tyrosine, without the necessity to measure $^{13}CO_2$ enrichment in breath gasses (42). The rate of hydroxylation can be calculated using the formula,

$$Q_{pt} = \frac{P_t}{P_p} \cdot \left(\frac{\frac{E_p}{E_t} - 1}{\quad}\right) \frac{Qp^2}{\quad}$$

where Pt/Pp (=0.73) is the molar ratio of fluxes of tyrosine and phenylalanine arising form protein catabolism (31); Qp equals Rd under the steady state conditions that we observed. Ep and Et are the L-[ring- $^{13}C_6$]phenylalanine and L-[ring- $^{13}C_6$]tyrosine enrichments (expressed in Atom Percent Excess; APE). Whole-body protein synthesis was calculated using

$$S = R_d -$$

Fractional rate of mixed muscle protein synthesis was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[ring-$^{13}C_6$]phenylalanine (TTR), by the enrichment of the precursor. Due to the large muscle sample size that is required to make an estimate of the enrichment in the aminoacyl-tRNA pool (generally regarded to be the true protein synthesis precursor pool) and the inherent analytical complexity of such measurements, we used plasma L-[ring-$^{13}C_6$]phenylalanine (TTR) and free muscle L-[ring-$^{13}C_6$]phenylalanine (TTR) enrichments as surrogates to provide an estimate of the lower boundary (based on plasma precursor enrichments) and the higher boundary

(based on intracellular muscle precursor enrichments) for the true fractional synthetic rate of mixed muscle proteins. Muscle FSR's were calculated as follows,

$$FSR = \frac{\Delta Ep \cdot 2}{E_{precursor} \cdot t} \cdot 100$$

where ΔEp is the delta increment of protein bound L-[ring- $^{13}C_6$]phenylalanine during incorporation periods. Eprecursor is (I) the average plasma L-[ring-$^{13}C_6$]phenylalanine enrichment during the time period for determination of amino acid incorporation; (II) the free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle biopsy taken at 6h post-exercise; (III) the free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle biopsy taken at 6h post-exercise corrected for the contribution of extra-cellular water, as previously described (50). t indicates the time interval (h) between biopsies and the factor 100 is needed to express the FSR in percent per hour (%·h^{-1}). The factor 2 arises because in the NAM-ester of L-[ring-$^{13}C_6$]phenylalanine molecule six of a total of twelve carbon atoms are labeled.

Statistics

All data are expressed as means±SEM. The plasma essential amino acid, insulin and glucose responses were calculated as area under the curve above pre-drink values. Statistical analysis of the data was performed using a one factor repeated measures analysis of variance (ANOVA) for non-time dependent variables. A two-factor repeated measures ANOVA with time and treatment as factors was used to compare differences between treatments over time. In case of significant difference between trials, a Tukey's post hoc test was applied to locate these differences. Statistical significance was set at $P<0.05$.

Results

Plasma insulin levels increased in all trials during the first 15-30 min after initial beverage ingestion. After this strong initial increase, insulin concentrations plateaued after 3-4h of recovery. Plasma insulin concentrations in the CHO+PRO and CHO+PRO+leu trials increased more compared to the CHO trial (figure 5.1A). The insulin response, expressed as area under the curve (above baseline values) during the entire 6h post-exercise period (figure 5.1B), was substantially greater in the CHO+PRO+leu trial compared to the CHO and CHO+PRO trials (12.6±1.2 vs. 3.7±0.4 and 7.4±0.9 U·6h·L^{-1}, respectively; $P<0.01$, which represent a +240±19 and +77±11% increase, respectively). The observed insulin response during the recovery period in CHO+PRO trial was increased with +98±17% compared to the CHO trial ($P<0.05$). Plasma glucose levels significantly increased following administration of the first beverage, after which they tended to decrease. The glucose response, expressed as area under the curve above baseline values, was significantly lower in the CHO+PRO+leu trial compared with the CHO trial

(96±62 vs. 320±65 mmol·6h·L^{-1}, P<0.05), with an intermediate value for the CHO+PRO trial (216±48 mmol·6h·L^{-1}).

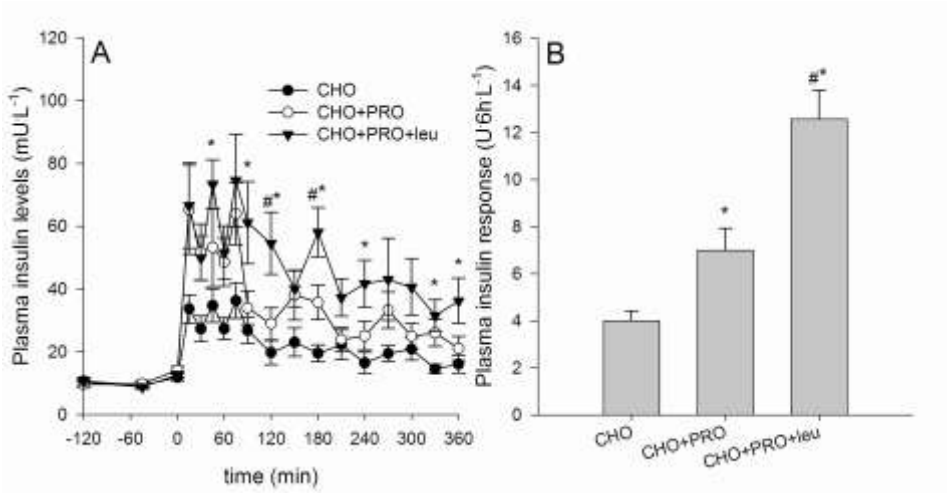

Figure 5.1 A, Plasma insulin concentration (expressed in mU·L^{-1}); B, plasma insulin response (expressed as area under the curve minus baseline values) during the different trials, while ingesting carbohydrate (0.3 g·kg^{-1}·h^{-1}; CHO), carbohydrate and protein (0.3 and 0.2 g·kg^{-1}·h^{-1}, respectively; CHO+PRO), or a carbohydrate and protein plus leucine mixture (0.3, 0.2 and 0.1 g·kg^{-1}·h^{-1}, respectively; CHO+PRO+leu). Values are means±SEM; * significantly different from CHO; # significantly different from CHO+PRO.

Plasma leucine, phenylalanine and tyrosine concentrations are reported in figure 5.2. Plasma essential amino acid (EAA) responses (area under the curve above baseline) are reported in figure 5.3. Resting plasma phenylalanine concentration averaged 53.6±1.4 µmol·L^{-1}. After ingestion of the first bolus plasma phenylalanine concentrations increased in the CHO+PRO and CHO+PRO+leu trials (P<0.05), whereas a slight non-significant decrease was observed in the CHO trial during the first 2h of post-exercise recovery (figure 5.2B).
The plasma phenylalanine response (figure 5.3) was negative in the CHO trial, whereas it was positive in the CHO+PRO and CHO+PRO+leu trials (-2.92±1.00 vs. 3.25±0.72 and 1.47±0.81 mmol·6h·L^{-1}, respectively; P<0.01). Plasma leucine concentrations (figure 5.2A) strongly increased after ingestion of the first bolus (P<0.01) in the CHO+PRO+leu and CHO+PRO trials whereas a slight decrease (P<0.05) was observed in the CHO trial during the first 2h of recovery. The plasma leucine response (figure 5.3) was negative in the CHO trial, positive in the CHO+PRO trial and significantly higher in the CHO+PRO+leu trial compared to both other trials (-9±5, 46±12 and 468±62 mmol·6h·L^{-1}, respectively; P<0.05). The observed plasma insulin response was strongly correlated with the observed plasma leucine concentration (r=0.77; P<0.001). Basal plasma tyrosine concentrations averaged 65.3±0.8 µmol·L^{-1} in all trials. Plasma tyrosine concentrations increased after ingestion of the first bolus in the CHO+PRO and CHO+PRO+leu trials

(P<0.05). In the CHO trial the concentration decreased (P<0.05) during the first 2h after exercise (figure 5.2C). Whereas plasma tyrosine responses were negative in the CHO trial, they were positive in the CHO+PRO and CHO+PRO+leu trials (-5.0±1.5 vs. 11.2±2.3 and 4.3±0.8 mmol·6h·L^{-1}, respectively; P<0.01).

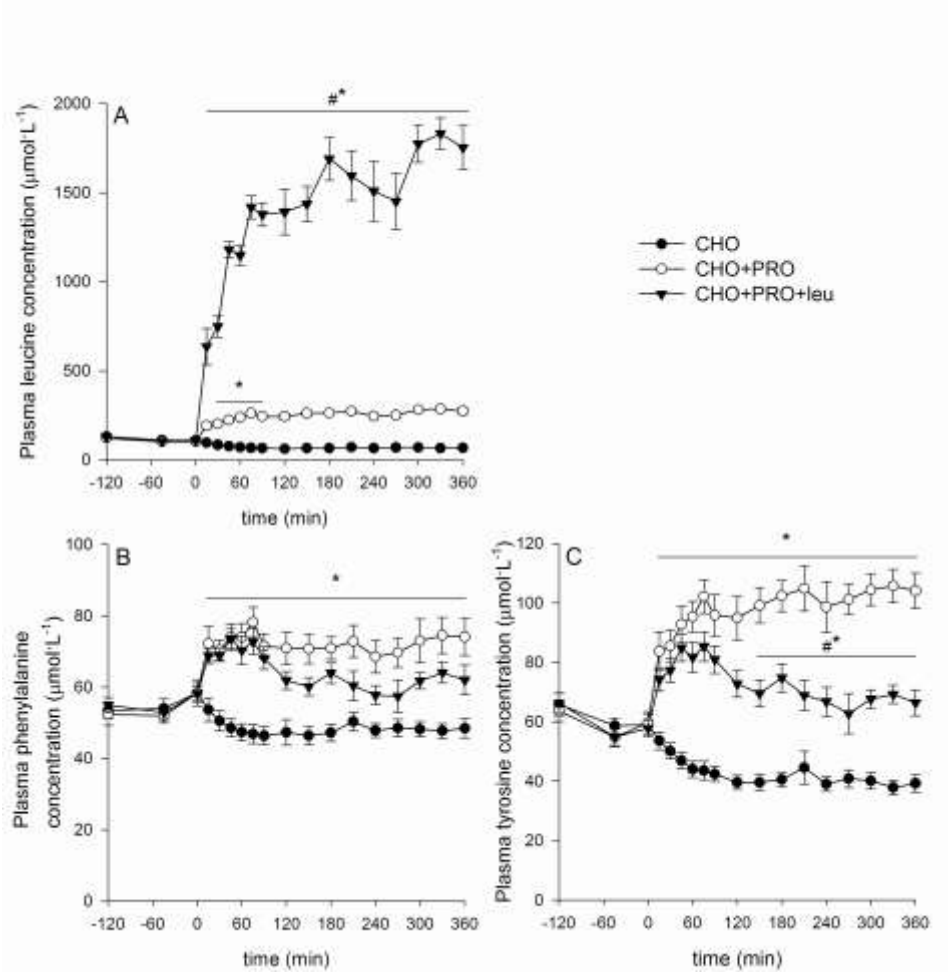

Figure 5.2 Plasma leucine (A); phenylalanine (B); plasma tyrosine (C) concentration (µmol·L^{-1}), during the CHO, CHO+PRO and CHO+PRO+leu trials. Values are means±SEM. * significantly different from CHO; # significantly different from CHO+PRO.

The time course of the plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{13}C_6$]tyrosine enrichments are shown in figure 5.4. Plasma L-[ring-$^{13}C_6$]phenylalanine enrichment increased after exercise in the CHO trial. In the CHO+PRO and CHO+PRO+leu trials, plasma L-[ring-$^{13}C_6$] phenylalanine enrichment slightly decreased to reach plateau values at t=60 min. Plasma L-[ring-$^{13}C_6$]phenylalanine enrichment was significantly greater in the CHO trial compared to the CHO+PRO and CHO+PRO+leu trials (P<0.05). Plasma L-[ring-$^{13}C_6$]tyrosine enrichment

increased after exercise in the CHO trial, but remained unchanged in the CHO+PRO and CHO+PRO+leu trials. Plasma L-[ring-$^{13}C_6$]tyrosine enrichment was significantly higher in the CHO compared to the CHO+PRO and CHO+PRO+leu trials ($P<0.05$). Mean plasma amino acid enrichment, plasma phenylalanine rate of appearance (Ra) and rate of disappearance (Rd), muscle free amino acid enrichment and the increment in muscle protein enrichment are presented in table 5.2.

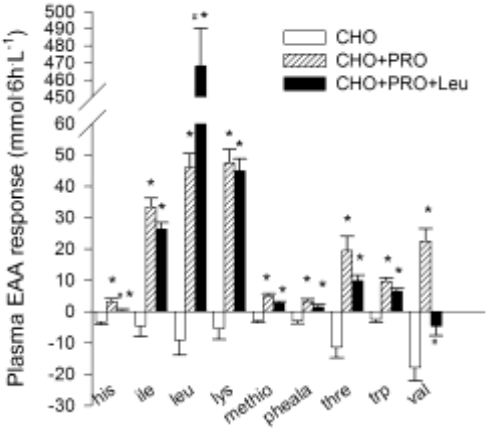

Figure 5.3 Plasma essential amino acid response (expressed as area under the curve minus baseline values) during the CHO, CHO+PRO and CHO+PRO+leu trials. Values are means±SEM; * significantly different from CHO; # significantly different from CHO+PRO.

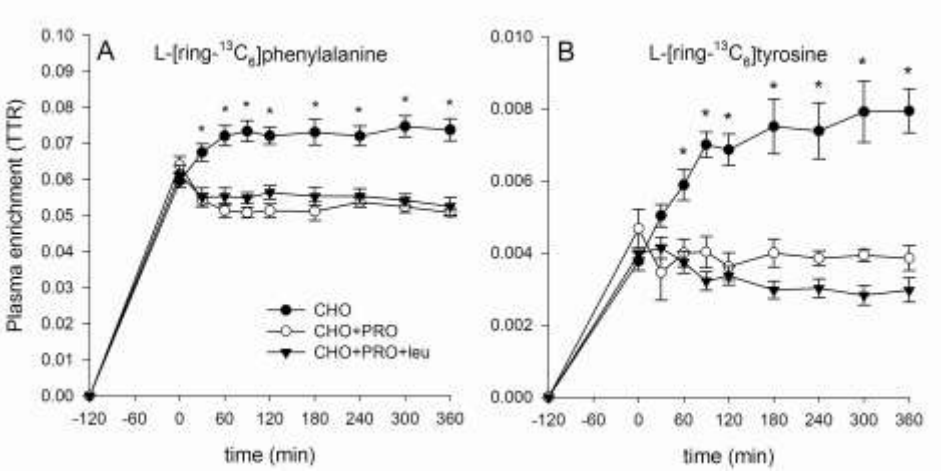

Figure 5.4 Plasma L-[ring-$^{13}C_6$]phenylalanine enrichment (A) and plasma L-[ring-$^{13}C_6$]tyrosine enrichment (B) during the CHO, CHO+PRO and CHO+PRO+leu trials. Values are means±SEM; * significantly different from CHO+PRO and CHO+PRO+leu.

Whole-body protein breakdown (figure 5.5), calculated over the 6h of post-exercise recovery, was lower in the CHO+PRO and CHO+PRO+leu trials compared to the CHO trial ($P<0.05$). Whole-body protein synthesis (figure 5.5), as calculated by Rd - Rox, was increased in the CHO+PRO and CHO+PRO+leu trial compared to the CHO trial ($P<0.05$). The rate of post-exercise whole-body phenylalanine oxidation (figure 5.5), as calculated from the conversion of phenylalanine to tyrosine, was decreased in the CHO+PRO+leu trial compared to the CHO+PRO and CHO trials ($P<0.05$). Whole-body protein balance (figure 5.5) was negative in the CHO trial, whereas protein balance was positive in the CHO+PRO and CHO+PRO+leu trials. In addition, whole-body protein balance was higher in CHO+PRO+leu than in CHO+PRO ($P<0.01$). Whole-body protein breakdown correlated with the plasma insulin and leucine responses ($r=-0.670$ and $r=-0.647$, respectively; $P<0.01$). Whole-body net protein balance was positively correlated with the plasma insulin and leucine response ($r=0.678$ and $r=0.626$, respectively; $P<0.01$).

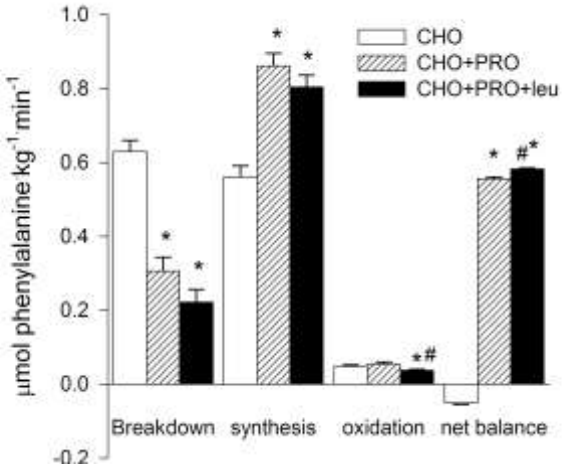

Figure 5.5 The rate of whole-body protein breakdown, synthesis, oxidation and net balance (expressed as µmol phenylalanine.kg.min-1) during the CHO, CHO+PRO and CHO+PRO+leu trials. Values are means+SEM; * significantly different from CHO; # significantly different from CHO+PRO.

Mixed muscle protein fractional synthesis rates (FSR) were calculated using the precursor-product equation outlined in the methods section. FSR, with the mean plasma L-[ring-$^{13}C_6$]phenylalanine enrichment as precursor (figure 5.6), was significantly higher in the CHO+PRO+leu trial compared to the CHO trial (0.095 ± 0.005 vs. 0.061 ± 0.009 %·h^{-1}, respectively; $P<0.05$), with intermediate values observed in the CHO+PRO trial (0.0820 ± 0.0104 %·h^{-1}). When the free intracellular L-[ring-$^{13}C_6$]phenylalanine enrichment was used as the precursor, FSR values were higher then when calculated with plasma L-[ring-$^{13}C_6$]phenylalanine enrichment as precursor, but revealed the same intervention effect ($P=0.052$). FSR

values averaged 0.089±0.012, 0.110±0.013, and 0.128±0.007 %·h^{-1} for the CHO, CHO+PRO and CHO+PRO+leu trial, respectively. When these values were corrected for the contribution of extracellular water to the measured free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment (50), FSR values averaged 0.097±0.013, 0.117±0.014, and 0.136±0.007 %·h^{-1} for the CHO, CHO+PRO and CHO+PRO+leu trial, respectively, with the same intervention effect (P=0.073). Significant correlations were observed between FSR and the amount of leucine ingested (r=0.471; P<0.05), phenylalanine Rd (r=0.531; P<0.01), whole-body protein synthesis (r=0.548; P<0.01), and whole-body protein balance (r=0.507; P<0.02).

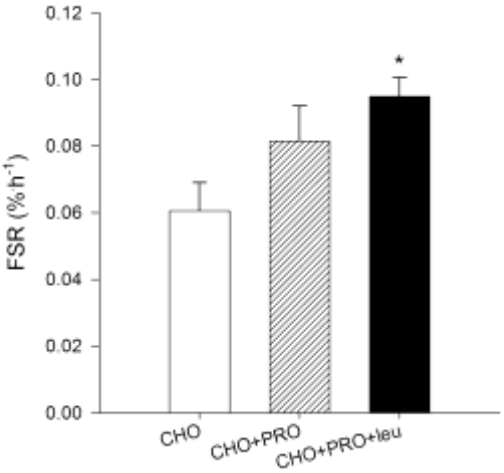

Figure 5.6 Fractional synthetic rate (FSR) of mixed muscle protein during the recovery phase from resistance exercise during the CHO, CHO+PRO and CHO+PRO+leu trials. Values are means±SEM; * significantly different from CHO.

Discussion

In the present study we simultaneously assessed whole-body protein turnover as well as the fractional synthetic rate (FSR) of mixed muscle protein by the incorporation of labeled phenylalanine in the m. vastus lateralis after resistance exercise. The co-ingestion of leucine and protein with carbohydrate significantly increased whole-body net protein balance compared to carbohydrate intake only and the combined ingestion of carbohydrate and protein. In addition, muscle protein synthesis rates were higher when protein and free leucine were co-ingested compared to the ingestion of carbohydrate only. Ingestion of carbohydrate and protein resulted in intermediate muscle protein synthesis rates, which were not significantly different from rates observed after co-ingestion of protein and leucine and carbohydrate ingestion only. Thus, the present study shows that the combined ingestion of protein and leucine with carbohydrate stimulates protein anabolism, measured using both tracer incorporation and whole-body tracer methodology.

It has been shown that both muscle protein synthesis (7, 35, 44) as well as protein breakdown rates (7, 35) are accelerated following resistance exercise. Resistance exercise exerts its effect either via the increased local production of IGF-1 in the muscle (21) and/or other contraction induced signaling events. Although muscle protein synthesis is stimulated during the post-exercise recovery phase (35), net muscle protein balance remains negative in the absence of food intake (7, 35, 36). It has been shown that the administration of amino acids with or without carbohydrate results in a rapid increase in muscle protein synthesis rate, whereas protein degradation rate is decreased (8, 11, 39, 44, 45). This results in an improved (positive) net muscle protein balance at rest and/or during post-exercise recovery.
The combined intake of free leucine and protein with carbohydrate has been shown to increase the plasma insulin response (47-49). Increased amounts of circulating insulin have been reported to increase net muscle protein balance during conditions of hyperaminoacidemia (18, 19, 22), which occurs primarily by inhibition of muscle protein breakdown, rather than by stimulating protein synthesis. Furthermore, leucine ingestion has been reported to stimulate muscle protein synthesis, independent of circulating plasma insulin levels, by increasing the phosphorylation (activation) of key proteins involved in the regulation of protein synthesis in rat (1, 4) and human skeletal muscle (23, 28). An insulinotropic mixture containing carbohydrate, protein and leucine could therefore likely represent an efficient nutritional intervention to optimize net muscle accretion during post-exercise recovery.
Using whole-body tracer balance methodology we observed that protein breakdown rates exceeded protein synthesis rates in the CHO trial, leading to a negative net protein balance (figure 5.5). The latter is in line with data from Borsheim et al. (2004) who showed that protein balance over the (leg) muscle remains negative after resistance exercise when only carbohydrate is ingested (10). However, measurements over the muscle do not necessarily account for all of the changes observed on a whole-body level. Whole-body protein breakdown rates were significantly suppressed in the CHO+PRO and CHO+PRO+leu trials compared to the CHO trial, whereas protein synthesis rates were increased (figure 5.5). Co-ingestion of protein or protein and leucine with carbohydrate resulted in a 50±2% and 62±2% reduction in protein degradation, respectively, when compared to carbohydrate ingestion only. In addition, protein synthesis was 54±5% and 45±5% increased in the CHO+PRO and CHO+PRO+leu trials, respectively, compared to the CHO trial. When whole-body protein synthesis rates were expressed as a percentage of the rate of disappearance (Rd) of phenylalanine, we found the highest values in the CHO+PRO+leu trial (95.6±0.1% compared to 92.0±0.4% and 94.2±0.4% in the CHO and CHO+PRO trials respectively; $P<0.05$), which could be attributed to a decreased rate of phenylalanine oxidation in the CHO+PRO+leu compared to the CHO+PRO trial, with intermediate values found in the CHO trial. Such a reduction in amino acid oxidation after administration of leucine has previously been established (32). Our findings are in accordance with earlier reports (27, 30, 39) showing that the combined ingestion of a single bolus of protein/amino acids and carbohydrate improves protein balance

in the post-resistance exercise period. In the present study we extend on these findings by investigating whether additional leucine could further promote protein anabolism. Therefore, in both the CHO+PRO and CHO+PRO+leu trials we provided an ample supply of protein (0.2 $g \cdot kg^{-1} \cdot h^{-1}$). This amount by far exceeds the amount of protein that was estimated to allow sustained maximal protein synthesis rates (50), and was selected to ensure that precursor substrate availability would not be limiting. Repeated boluses were administered, as opposed to a single bolus approach, to ensure a continuous supply of amino acids. Consequently, we show that the co-ingestion of leucine even further increases the net protein balance compared to carbohydrate and protein ingestion (figure 5.5).

Most studies have applied the (whole-body) tracer balance methodology to investigate the effect of post-exercise nutrition on protein metabolism. Unfortunately, whole-body tracer methods have some important limitations, as they do not allow the direct measurement of muscle protein synthesis rates. As such, they do not provide information on the contribution of individual tissues to protein metabolism, their response to nutritional and/or exercise intervention and do not enable the discrimination between active and inactive muscle. Therefore, we also measured the incorporation rate of labeled phenylalanine in skeletal muscle tissue after resistance exercise to determine FSR of mixed muscle protein in the active m. vastus lateralis. We used plasma L-[ring-$^{13}C_6$]phenylalanine and free muscle L-[ring-$^{13}C_6$]phenylalanine enrichments to provide an estimate of the lower boundary (based on plasma precursor enrichments) and the higher boundary (based on intracellular muscle precursor enrichments) for the true fractional synthetic rate of mixed muscle proteins. Both methods provided similar results. As we observed a higher variation in intracellular muscle precursor enrichment, we based the FSR values on plasma precursor enrichments. Mixed muscle FSR averaged 0.0605 ± 0.0085 $\%\cdot h^{-1}$ during 6h of post-exercise recovery while ingesting carbohydrate. Combined ingestion of leucine and protein with carbohydrate resulted in a significantly increased FSR compared to carbohydrate ingestion alone (figure 5.6). The combined ingestion of protein and carbohydrate without free leucine resulted in an intermediate value, and was not significantly different from the lower values in the CHO trial nor the higher values reported in the CHO+PRO+leu trials (figure 5.6). Therefore our data on the muscle level also show that the combined ingestion of leucine and protein with carbohydrate can effectively stimulate muscle protein synthesis.

The presented FSR data are in line with our observations using whole-body tracer methods, showing that co-ingestion of leucine and protein with carbohydrate accelerates protein synthesis and improves net protein balance when compared to carbohydrate ingestion only. Using whole-body tracer methodology, our data indicate that the combined ingestion of leucine and protein with carbohydrate can reduce protein breakdown. The latter seems to be in line with earlier reports by Nair et al (1992), who showed that leucine infusion significantly decreases protein breakdown as well as amino acid oxidation rate. In addition, data from the same study showed a decrease in the plasma concentrations of the other essential amino acids (EAA) during leucine infusion. In the present study we also observed a

reduced plasma EAA response in the CHO+PRO+leu trial compared to the CHO+PRO trial, although the same amount of EAA (with the exclusion of the supplemented leucine) was consumed. However, no differences were observed in phenylalanine Rd between the CHO+PRO+leu and CHO+PRO trials. This diminished plasma EAA response following leucine supplementation could be attributed to a reduced release from the muscle, and indicating a reduced protein breakdown and/or reduced protein oxidation rate (figure 5.5).

We recently reported that co-ingestion of leucine, phenylalanine and protein with carbohydrate results in an increased plasma insulin response in the post-absorptive resting state (46, 49) and during recovery from endurance exercise (47, 48). In the present study we demonstrate that combined ingestion of leucine and protein with carbohydrate (without phenylalanine) can substantially increase the insulin response by ~250% when compared to the ingestion of only carbohydrate. Circulating insulin concentrations play an important role in regulating protein metabolism. The mechanism by which insulin mediates muscle hypertrophy is still a subject of debate as some reports (9, 18, 19) indicate that insulin infusion reduces protein breakdown while others (6, 20) have reported an enhanced muscle protein synthesis rate when sufficient amino acids were made available. However, in the absence of an ample supply of amino acids, insulin does not seem to stimulate muscle protein synthesis (9). In the present study, we found plasma insulin responses to be negatively correlated with whole-body protein degradation ($r=-0.641$; $P<0.01$), whereas whole-body protein synthesis was positively correlated with plasma insulin response ($r=0.451$; $P<0.05$). Muscle protein synthesis rates (FSR) did not correlate with the plasma insulin response, whereas mixed muscle protein FSR did correlate with the amount of leucine that was ingested ($r=0.471$; $P<0.05$). Whether the observed differences can be attributed to the insulin or non-insulin dependent stimulatory effects of leucine remains to be established.

The combined ingestion of leucine and protein with carbohydrate in the recovery period from resistance exercise could stimulate muscle protein synthesis in several ways. It provides amino acids as precursors for muscle protein synthesis and the added leucine further increases plasma insulin concentrations and could also directly stimulate protein synthesis. Leucine has been shown to have the potential to affect muscle protein metabolism by decreasing the rate of protein degradation (32), most likely via increases in circulating insulin, and the phosphorylation of key proteins involved in the regulation of protein synthesis (23, 28, 37). The latter has been shown to occur even in the absence of an increase in circulating insulin concentrations. These studies indicate that leucine has the ability to function as a nutritional signaling molecule that modulates muscle protein synthesis and/or breakdown following food intake. The stimulatory effect of leucine on protein synthesis occurs at the level of translation initiation and involves signaling through mTOR (24, 37). This protein kinase referred to as mammalian target of rapamycin is thought to serve as a convergence point for leucine- and insulin-mediated effects on translation initiation (24, 37). Maximal rates of protein synthesis rates probably require both leucine and insulin signaling and the anabolic signal of resistance exercise. However, under normal *in vivo* conditions it is impossible to discriminate

between the effects of leucine and insulin and resistance exercise (IGF-1 and MGF) on muscle protein synthesis as all these signals are substantially elevated in the CHO+PRO+leu trial.

In conclusion, the combined ingestion of protein and leucine with carbohydrate improves whole-body protein balance during recovery from resistance exercise when compared to the ingestion of carbohydrate or carbohydrate with protein. The combined ingestion of both leucine and protein with carbohydrate augments post-exercise mixed muscle protein synthesis when compared to the ingestion of only carbohydrate. The present data indicate that the additional ingestion of free leucine in combination with protein and carbohydrate likely represents an effective strategy to increase muscle anabolism following resistance exercise.

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6

Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly men

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Abstract

The progressive loss of skeletal muscle mass with aging is attributed to a disruption in the regulation of skeletal muscle protein turnover. We investigated the response to the ingestion of carbohydrate with or without protein and free leucine following simulated activities of daily living (ADL) on whole-body protein balance and mixed muscle protein synthesis rates. Eight elderly (75±1 y) and eight young (20±1 y) lean men were randomly assigned to 2 cross-over experiments in which they consumed either carbohydrate (CHO) or carbohydrate, protein and free leucine (CHO+PRO+leu) after performing 30 min of standardized ADL activities. Primed, continuous infusions with L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine were applied, and blood and muscle samples were collected to assess whole-body protein turnover as well as protein fractional synthetic rate (FSR) in the vastus lateralis muscle over a 6h period. Whole-body phenylalanine and tyrosine flux were significantly higher in the young versus the elderly ($P<0.01$). Protein balance was negative in the CHO, but positive in the CHO+PRO+leu experiment in both groups. Mixed muscle protein synthesis rates (FSR) were significantly lower in the elderly compared with the young subjects ($P<0.05$). FSR values were significantly greater in the CHO+PRO+leu compared with the CHO experiment in both the young (0.082±0.005 %·h^{-1} vs. 0.060±0.005 %·h^{-1}, respectively; $P<0.01$), and elderly subjects (0.072±0.006 %·h^{-1} vs. 0.043±0.003 %·h^{-1}, respectively; $P<0.01$). Co-ingestion of protein and leucine with carbohydrate following ADL activities improves whole-body protein balance and increases muscle protein synthesis rates to the same extent in lean, young and elderly men.

Introduction

Aging is associated with a slow progressive loss of skeletal muscle mass, which is also called sarcopenia (1). Sarcopenia is generally accompanied by a reduction in strength, the loss of functional capacity and an increased risk of developing chronic metabolic diseases like obesity, type 2 diabetes and osteoporosis. Sarcopenia is facilitated by a combination of factors, which include a sedentary lifestyle and a less than optimal diet (2). The age-related changes in skeletal muscle mass are attributed to a disruption in the regulation of skeletal muscle protein turnover (3), which results in a chronic imbalance between muscle protein synthesis and breakdown rates. It has been reported that basal protein synthesis rates are either similar (4-7), or reduced (8-14) in the elderly compared with young adults. Furthermore, muscle protein breakdown rates tend to be greater in the elderly, resulting in a gradual loss of skeletal muscle mass (6).

Protein turnover in skeletal muscle tissue is highly responsive to nutrient intake in healthy, young individuals (15). In contrast, the anabolic effect of food intake on muscle protein synthesis seems to be substantially blunted in the elderly (5, 16-18). The latter has been proposed to represent a key-factor in the etiology of sarcopenia. In addition to food intake, physical activity can also effectively modulate protein metabolism, as it stimulates both protein synthesis and breakdown (19). However, in the absence of food intake net muscle protein balance will remain negative under these conditions (19, 20), leading to net muscle protein loss. Carbohydrate ingestion effectively reduces the activity-induced stimulation of muscle protein degradation, but does not affect protein synthesis (21, 22). As a consequence, the protein balance will remain negative (21, 22) unless protein and/or amino acids are co-administered (23-25). Interestingly, supplementation with leucine has been proposed as an effective strategy to reduce muscle protein breakdown and to further stimulate muscle protein synthesis (24, 26-29). The latter could be attributed to the potential of leucine to stimulate protein anabolism by activating the mRNA translational machinery through the mammalian target of rapamycin (mTOR) in an insulin-dependent and independent manner (30-32). As a result, it has been suggested that the co-ingestion of a mixture of protein and additional free leucine with carbohydrate, could represent an effective strategy to augment muscle protein synthesis and/or to inhibit protein degradation (24).

Under normal living conditions, ADL activities in the morning or afternoon are generally followed by food intake. As such, it is important to determine the combined effects of food intake and physical activity on skeletal muscle protein balance, when studying the proposed changes in skeletal muscle protein metabolism with aging. In the present study, we investigated the differential effects of carbohydrate and carbohydrate with protein and free leucine ingestion on skeletal muscle protein synthesis rates following simulated ADL type activity in young (~20 y) and elderly (~75 y) lean men. Infusions of L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine were combined with plasma and

skeletal muscle tissue sampling to simultaneously measure whole-body protein turnover as well as mixed muscle protein fractional synthetic rates *in vivo* in men.

Methods

Subjects

Eight healthy, elderly men (75±1 y) and eight weight-matched, young controls (20±1 y), with no history of participating in any regular exercise training program, were selected to participate in the present study. Subjects' characteristics are shown in table 6.1. All subjects were informed about the nature and risks of the experimental procedure before their written informed consent to participate was obtained. This study was approved by the local Medical Ethical Committee.

Table 6.1 Subjects' characteristics

	Young (n=8)	Elderly (n=8)
Age (yrs)	20 ± 1	75 ± 1.2 *
Weight (kg)	73.7 ± 3.2	75.5 ± 2.1
Height (m)	1.81 ± 0.03	1.72 ± 0.01 *
BMI ($kg \cdot m^{-2}$)	22.5 ± 1.1	25.7 ± 0.8 *
Leg volume (L)	9.43 ± 0.39	8.23 ± 0.32 *
HbA_{1C} (%)	5.03 ± 0.17	5.71 ± 0.11 *
Basal glucose ($mmol \cdot L^{-1}$)	5.26 ± 0.12	5.51 ± 0.10
Basal insulin ($mU \cdot L^{-1}$)	11.15 ± 0.75	10.15 ± 0.85
HOMA-(IR)	2.61 ± 0.19	2.48 ± 0.20
1RM leg press (kg)	203.8 ± 7.4	151.3 ± 7.6 *
1RM leg press ($kg \cdot BW^{-1}$)	2.80 ± 0.17	2.00 ± 0.06 *
1RM leg extension (kg)	107.5 ± 3.8	78.1 ± 4.3 *
1RM leg extension ($kg \cdot BW^{-1}$)	1.48 ± 0.09	1.03 ± 0.04 *

Values are expressed as means ± SEM. HOMA-IR, Homeostasis model assessment of insulin resistance (22); 1RM, one-repetition maximum. * Significantly different from young, $P<0.05$.

Pretesting

Before selection in the study, all volunteers were subjected to an oral glucose tolerance test (33, 34). Leg volume was determined as described previously (35), after which all subjects participated in an orientation trial to become familiarized with the physical activity protocol and the equipment. Proper lifting technique was demonstrated and practiced for each of the 2 lower-limb exercises (leg press and leg extension). Subsequently, maximal strength (one-repetition maximum, 1RM) was estimated using the multiple repetitions testing procedure (36).

Diet and activity prior to testing

All subjects consumed a standardized meal (64.1±2.0 $kJ \cdot kg^{-1}$ body weight, consisting of 65 Energy% (En%) carbohydrate, 15 En% protein and 20 En% fat) the evening before the trials. All volunteers were instructed to refrain from any

sort of heavy physical exercise and to keep their diet as constant as possible 3 d before the trials. In addition, subjects were asked to record their food intake for 48h before the start of the first experimental trial and to consume the same diet 48h before the start of the second trial.

Experimental trials

Each subject participated in 2 experiments, separated by 7 d, in which drinks containing carbohydrate (CHO) or carbohydrate, protein and leucine (CHO+PRO+leu) were administered in a randomized and double-blind fashion. Each experiment lasted ~8h. Repeated boluses of a given test-drink were ingested following the physical activity protocol to ensure a continuous supply of glucose and amino acids. Plasma and muscle samples were collected during a 6h period. These experiments were designed to simultaneously assess whole-body amino acid kinetics and FSR of mixed muscle protein by the incorporation of L-[ring-$^{13}C_6$]phenylalanine in the mixed protein of muscle biopsies collected from the vastus lateralis muscle.

Protocol

At 8.00 am, after an overnight fast, subjects arrived at the laboratory by car or public transportation and a Teflon catheter was inserted into an antecubital vein for stable isotope infusion. A second Teflon catheter was inserted in a heated dorsal hand vein of the contra-lateral arm, placed in a hot-box (60°C), for arterialized blood sampling. After basal blood sample collection, a single intravenous dose of L-[ring-$^{13}C_6$]phenylalanine (2 μmol·kg^{-1}) and L-[ring-$^{2}H_2$]tyrosine (0.775 μmol·kg^{-1}) was administered to prime the phenylalanine and tyrosine pool. Thereafter, tracer infusion (average infusion rate (IR) of 0.049±0.001 μmol·kg^{-1}·min^{-1} for L-[ring-$^{13}C_6$]phenylalanine and 0.019±0.001 μmol·kg^{-1}·min^{-1} for L-[ring-$^{2}H_2$]tyrosine) was started and subjects rested in a supine position for 1h, before engaging in the standardized physical activity protocol. The protocol was designed to simulate 30 min of moderate intensity physical activity (for example garden tasks as lawn mowing) as has been recommended by several public health authorities (37, 38). The energy consumption during such an activity pattern is estimated to be ~650 kJ·30 min^{-1} (39), and was simulated by combining low-intensity cycling and light resistance-type exercise. After 5 min of self-paced cycling, subjects performed 6 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, The Netherlands) and 6 sets of 10 repetitions on the leg extension machine (Technogym BV). The first 2 sets of both resistance exercises were performed at 40% of the subjects' 1RM. Sets 3-4, and 5-6 were performed at 55% and 75% of 1RM, respectively, with 2 min rest intervals between sets. As such, the young and elderly, lean men performed exercise at the same relative intensity. At the end of the exercise protocol (t=0 min), subjects rested supine and an arterialized blood sample and a muscle biopsy from the vastus lateralis muscle were collected.

Subjects then received an initial bolus (1.33 ml·kg^{-1}) of a given test drink. Repeated boluses (1.33 ml·kg^{-1}) were ingested every 30 min until t=330 min. Arterialized blood samples were collected at t= 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min with a second muscle biopsy taken at t=360 min from the contralateral limb.

Table 6.2 Beverage composition

Test drink	CHO	CHO+PRO+leu
Whey protein	-	60
Leucine	-	10
Glucose	92	92
Maltodextrin	92	92
Sodium saccharinate	0.2	0.2
Citric acid	1.8	1.8
Cream vanilla	5	5
Water	up to 1.00 L	

Values expressed in g·L^{-1} beverage.

Beverages

Subjects received a beverage volume of 1.33 ml·kg^{-1} every 30 min to ensure a given dose of 0.49 g carbohydrate·kg^{-1} (50% as glucose and 50% as maltodextrin) with or without the addition of 0.16 g·kg^{-1} of a whey protein hydrolysate and 0.03 g·kg^{-1} leucine every h. The total amount of protein provided in the CHO+PRO+leu experiment by far exceeds the calculated amount of protein needed to provide sufficient precursor substrate to sustain maximal protein synthesis rates for at least 6 h (40). Repeated boluses were administered to enable a continuous supply of amino acids in the circulation, preventing perturbations in L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments. The whey protein hydrolysate (68.8% protein) contained 10.3% leucine and consequently the total amount of leucine administered in the CHO and CHO+PRO+leu experiment was 0 vs. 0.041 g·kg^{-1}·h^{-1}, respectively.

The compositions of all test drinks are listed in table 6.2. Glucose and maltodextrin were obtained from AVEBE (Veendam, The Netherlands). Whey protein hydrolysate was prepared by DSM Food Specialties (Delft, The Netherlands). Leucine was purchased from BUFA (Uitgeest, The Netherlands). To make the taste comparable in all experiments, beverages were uniformly flavored by adding 0.2 g sodium-saccharinate solution (25% w/w), 1.8 g citric acid solution (50% w/w) and 5 g of cream vanilla flavor (Numico Research, Wageningen, The

Netherlands) for each liter of beverage. Experiments were performed in a randomized order, with test-drinks provided in a double-blind fashion.

Analysis

Blood samples were collected in EDTA containing tubes and centrifuged at 1000 g and 4°C for 5 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C. Plasma glucose (Uni Kit III, 07367204, Roche, Basel, Switzerland) concentrations were analyzed with the COBAS-FARA semi-automatic analyzer (Roche). Insulin was analyzed by radio immunoassay (Insulin RIA kit, LINCO Research Inc., St. Charles, MO, USA). Plasma (500 μL) for amino acid analyses was deproteinized on ice with 100 μL of 24% (w/v) 5-sulphosalicylic acid, mixed and the clear supernatant was collected after centrifugation. Plasma amino acid concentrations were analyzed on an automated dedicated amino acid analyzer (LC-A10, Shimadzu Benelux, Den Bosch, The Netherlands), using an automated precolumn derivatization procedure and a ternary solvent system (14). The exact phenylalanine and tyrosine concentrations in the infusates (4.442±0.005 and 1.762±0.003 $mmol \cdot L^{-1}$, respectively) were measured using the same method. Plasma phenylalanine and tyrosine were derivatized to their tert-butyldimethylsilyl (TBDMS) derivatives and their ^{13}C and/or ^{2}H enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS, Agilent 6890N GC/5973N MSD Little Falls, USA) using selected ion monitoring of masses 336 and 342 for unlabeled and labeled phenylalanine respectively and masses 466, 468 and 472 for unlabeled and labeled tyrosine (46).
For measurement of L-[ring-$^{13}C_6$]phenylalanine enrichment in the free amino acid pool and mixed muscle protein, 55 mg of wet muscle was freeze-dried. Collagen, blood and other non-muscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (2-3 mg) was weighed and 8 volumes (8x dry weight of isolated muscle fibers x wet/dry ratio) of ice-cold 2% perchloric acid (PCA) were added. The tissue was then homogenized and centrifuged. The supernatant was collected and processed in the same manner as the plasma samples, such that intracellular free L-[ring-$^{13}C_6$]phenylalanine, L-[ring-$^{2}H_2$]tyrosine and L-[ring-$^{13}C_6$]tyrosine enrichments could be measured using their t-butyldimethylsilyl derivatives on a GCMS. The free amino acid concentration in the supernatant was measured using an HPLC technique, after precolumn derivatization with o-phthaldialdehyde (35). The protein pellet was washed with 3 additional 1.5 ml washes of 2% PCA, dried and the proteins were hydrolyzed in 6M HCl at 120°C for 15-18h. The hydrolyzed protein fraction was dried under a nitrogen stream while heated to 120°C, then dissolved in a 50% acetic acid solution, and passed over a Dowex exchange resin (AG 50W-X8, 100-200 mesh hydrogen form, Biorad, Hercules, CA, USA) using 2M NH4OH. Thereafter, the eluate was dried and the purified amino acid fraction was derivatized into the N-acetyl-methyl (NAM)-esters to determine the ^{13}C-enrichment of protein bound phenylalanine enrichment using GC-IRMS (Finnigan, MAT 252).

Calculations

Infusion of L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine with muscle and arterialized blood sampling was used to simultaneously assess whole-body amino acid kinetics and fractional synthetic rate (FSR) of mixed muscle protein. Whole-body kinetics for phenylalanine and tyrosine were calculated using the equations described by Thompson et al. (34) and Short et al. (32). Briefly, phenylalanine and tyrosine turnover (flux, Q) was measured from the isotope dilution at isotopic steady state:

$$Q = i \cdot \left(\frac{Ei}{Ep} - 1 \right)$$

where i is the isotope infusion rate (μmol·kg body weight^{-1}·h^{-1}) and Ei and Ep correspond to the enrichments of infusate and plasma amino acids, respectively. At isotopic steady state, protein flux (Q) equals the sum of protein synthesis (S) and oxidation (O) as well as the sum of the rate of appearance of meal protein from the gut (I) and protein breakdown (B), whole-body protein synthesis rate was calculated as flux minus oxidation.

$$Q = S + O = B +$$

$$S = Q - O$$

At isotopic steady state, whole-body phenylalanine oxidation can be determined from the conversion (hydroxylation) of L-[ring-$^{13}C_6$]phenylalanine to L-[ring-$^{13}C_6$]tyrosine. The rate of hydroxylation (Qpt) was calculated (32) using the formula,

$$Q_{pt} = Q_t \cdot \frac{E_t}{E_p} \cdot \frac{Qp}{(- Q_p)}$$

where Qt and Qp are the flux rates for L-[ring-$^{2}H_2$]tyrosine and labeled phenylalanine, respectively. Et and Ep are the L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{13}C_6$]phenylalanine enrichments in plasma, respectively and ip is the infusion rate of the phenylalanine tracer.

Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[ring-$^{13}C_6$]phenylalanine, by the enrichment of the precursor. Plasma L-[ring-$^{13}C_6$]phenylalanine and free muscle L-[ring-$^{13}C_6$]phenylalanine enrichments were used to provide an estimate of the lower boundary (based on plasma precursor enrichments) and the higher boundary (based on intracellular muscle precursor enrichments) for the true fractional synthetic rate of mixed muscle proteins. Muscle FSR's were calculated as follows (20):

$$FSR = \frac{\Delta Ep \cdot 2}{E_{precursor} \cdot t} \cdot 100$$

Where ΔEp is the delta increment of protein bound L-[ring-$^{13}C_6$]phenylalanine during incorporation periods. $E_{precursor}$ is (I) the average plasma L-[ring-$^{13}C_6$]phenylalanine enrichment during the time period for determination of amino acid incorporation; (II) the free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle biopsy taken at 6h post-exercise; (III) the free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle biopsy taken at 6h post-exercise corrected for the contribution of extra-cellular water, as previously described (41). t indicates the time interval (h) between biopsies and the factor 100 is needed to express the FSR in percent per hour (%·h^{-1}). The factor 2 arises because in the NAM-ester of L-[ring-$^{13}C_6$]phenylalanine molecule 6 of a total of 12 carbon atoms are labeled.

Statistics

All data are expressed as means±SEM. The plasma essential amino acid, insulin and glucose responses were calculated as area under the curve above baseline values. A three-factor repeated measures ANOVA with age-group, time and treatment as factors was used to compare differences between treatments over time between groups. For non-time dependent variables, a two-factor ANOVA with age-group and treatment as factors was used to compare differences in treatment effects between groups. In case of significant difference between experiments, a Scheffé post-hoc test was applied to locate these differences. Statistical significance was set at $P<0.05$. All calculations were performed using StatView 5.0 (SAS Institute inc., Cary, NC, USA).

Results

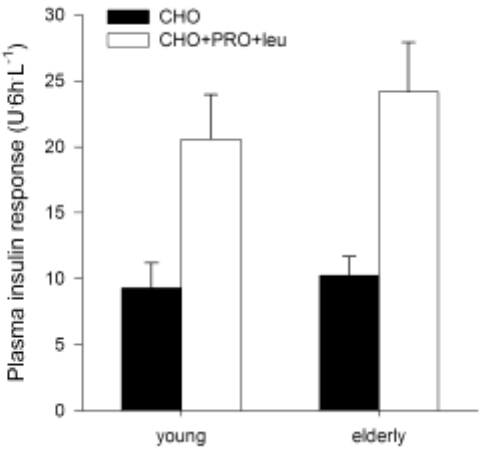

Figure 6.1 Plasma insulin response (expressed as area under the curve minus baseline values) in lean young (n=8) and elderly (n=8) men while ingesting carbohydrate (0.49 g·kg^{-1}·h^{-1}; CHO), or carbohydrate with protein and leucine (0.49, 0.16 and 0.1 g·kg^{-1}·h^{-1}, respectively; CHO+PRO+leu). Values are means±SEM. Data were analyzed with ANOVA (age-group x treatment): age-group effect, P=0.423; treatment effect, P<0.001; interaction of age-group and treatment, P=0.636.

Plasma analyses

The insulin response, expressed as area under the curve above baseline values (AUC) during the entire 6h post-exercise period is shown in figure 6.1. The glucose response (AUC), was significantly lower in the young subjects compared with the elderly (estimated marginal means: 587±42 vs. 869±39 mmol·6h·L^{-1}, respectively, $P<0.01$).

Plasma leucine, phenylalanine and tyrosine concentrations over time are reported in figure 6.2. Basal (t=-120 min) plasma leucine concentrations were significantly lower in the elderly compared with the young (99.0±3.8 vs. 119.7±2.7 μmol·L^{-1}, respectively, $P<0.05$). No differences were observed between the young and elderly in basal (t=-120 min) plasma phenylalanine and tyrosine concentrations. The plasma leucine response (AUC) was negative in the CHO and positive in the CHO+PRO+leu experiment (estimated marginal means: -13.9±2.9 vs. 137.2±2.9 mmol·6h·L^{-1}, respectively, $P<0.01$. The observed plasma insulin responses were positively correlated with the observed plasma leucine concentration ($r=0.675$, $P<0.001$). The plasma phenylalanine response (AUC) was negative in the CHO, whereas it was positive in the CHO+PRO+leu experiment (estimated marginal means: -3.24±0.34 vs. 1.87±0.34 mmol·6h·L^{-1}, respectively; $P<0.01$). Plasma tyrosine responses (AUC) were negative in the CHO and positive in the CHO+PRO+leu experiment in the young (-5.8±0.5 vs. 3.1±0.6 mmol·6h·L^{-1}, respectively; $P<0.01$) and elderly (-5.6±0.3 vs. 5.7±0.8 mmol·6h·L^{-1}, respectively; $P<0.01$). The plasma tyrosine response in the CHO+PRO+leu experiment was significantly greater in the elderly compared with the young ($P<0.05$). The plasma essential amino acid (EAA) response (area under the curve above baseline, with the exception of the supplemented leucine) was negative in the CHO and positive in the CHO+PRO+leu experiment in the young (-67.8±3.7 vs. 61.6±4.9 mmol·6h·L^{-1}, respectively, $P<0.01$) and in the elderly (-57.1±5.0 vs. 74.1±8.1 mmol·6h·L^{-1}, respectively, $P<0.01$), with no differences between the young and elderly.

The time course of the plasma L-[ring-$^{13}C_6$]phenylalanine, L-[ring-$^{2}H_2$]tyrosine and L-[ring-$^{13}C_6$]tyrosine enrichments are shown in figure 6.3. Plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments increased in the CHO experiment in both the young and elderly. In the CHO+PRO+leu experiment, plasma L-[ring-$^{13}C_6$] phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments did not change. Plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments were significantly greater in the CHO compared with the CHO+PRO+leu experiment ($P<0.05$). Plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments were significantly higher in the elderly in the CHO and CHO+PRO+leu experiments compared with the young subjects ($P<0.05$). Plasma L-[ring-$^{13}C_6$]tyrosine enrichment increased in the CHO, but remained unchanged in the CHO+PRO+leu experiment in the young and the elderly subjects. Plasma L-[ring-$^{13}C_6$]tyrosine enrichment was significantly higher in the CHO compared with the CHO+PRO+leu experiment ($P<0.05$).

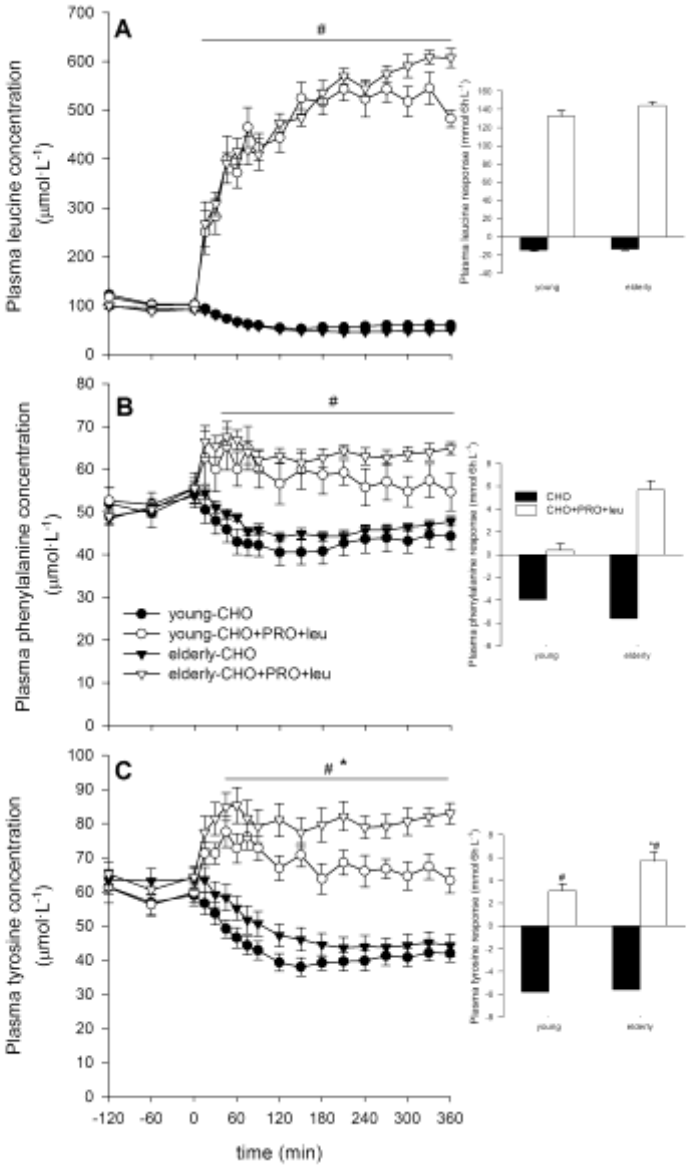

Figure 6.2 Plasma leucine (A), phenylalanine (B), and tyrosine (C) concentrations (µmol.L^{-1}) and responses, expressed as area under the curve (AUC) above baseline, during the CHO and CHO+PRO+leu experiments in lean young (n=8) and elderly (n=8) men. Values are means±SEM. AUC data were analyzed with a two-factor ANOVA (age-group x treatment). Plasma leucine response: age-group effect, P=0.10; treatment effect, P<0.001; interaction of age-group and treatment, P=0.186. Plasma phenylalanine response: age-group effect, P<0.05; treatment effect, P<0.001; interaction of age-group and treatment, P=0.596; Plasma tyrosine response: age-group effect, P<0.05; treatment effect, P<0.001; interaction of age-group and treatment, P<0.05; # significantly different from CHO experiment (Scheffé's test, P<0.05); * significantly different compared to the young within the CHO+PRO+leu experiment (Scheffé's test, P<0.05).

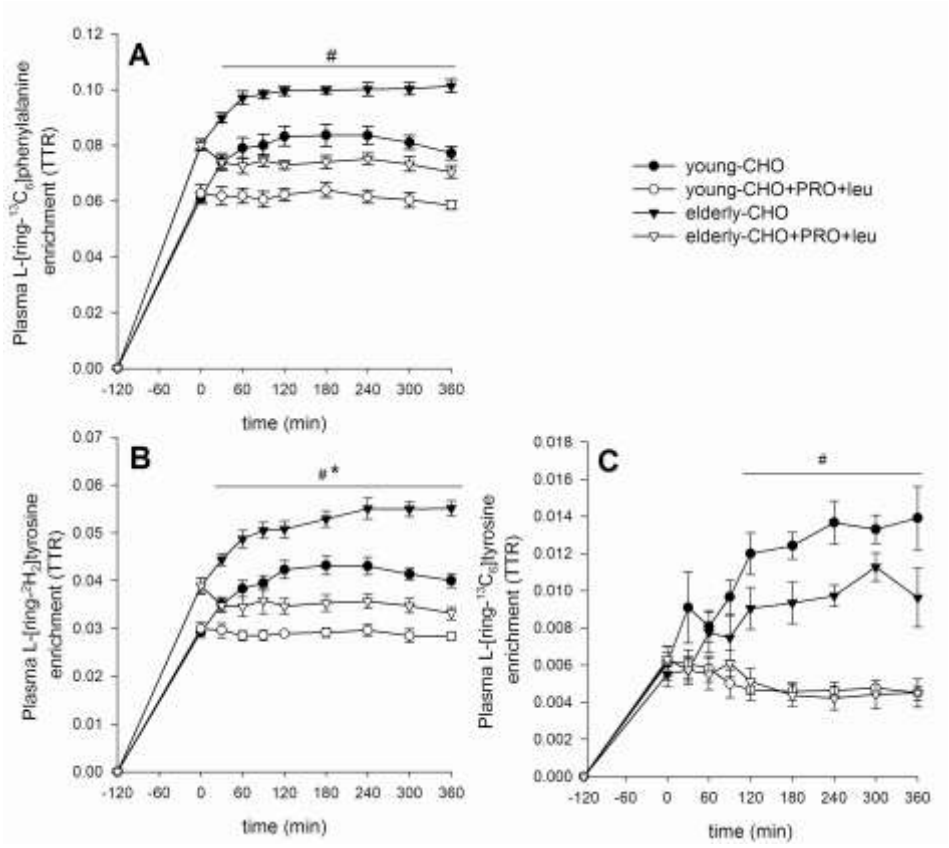

Figure 6.3 Plasma L-[ring-$^{13}C_6$]phenylalanine (A), L-[ring-$^{2}H_2$]tyrosine (B), and L-[ring-$^{13}C_6$]tyrosine enrichment (TTR) (C) during the CHO and CHO+PRO+leu experiments in lean young (n=8) and elderly (n=8) men. Values are means±SEM. Data were analyzed with ANOVA repeated measures (age-group x treatment x time). Plasma L-[ring-$^{13}C_6$]phenylalanine enrichment: age-group effect, P<0.001; treatment effect, P<0.001; time effect, P<0.001; interaction of age-group and treatment, P=0.191; interaction of age-group and time, P<0.001; interaction of treatment and time, P<0.001; interaction of age-group, treatment and time, P=0.339. Plasma L-[ring-$^{2}H_2$]tyrosine enrichment: age-group effect, P<0.001; treatment effect, P<0.001; time effect, P<0.001; interaction of age-group and treatment, P=0.081; interaction of age-group and time, P<0.001; interaction of treatment and time, P<0.001; interaction of age-group, treatment and time, P<0.001. Plasma L-[ring-$^{13}C_6$]tyrosine enrichment: age-group effect, P=0.053; treatment effect, P<0.001; time effect, P<0.001; interaction of age-group and treatment, P=0.053; interaction of age-group and time, P=0.415; interaction of treatment and time, P<0.001; interaction of age-group, treatment and time, P=0.478. * significant differences between young and elderly (Scheffé's test, P<0.05); # significantly different between treatments (Scheffé's test, P<0.05).

Muscle analysis

No differences in free leucine and tyrosine concentrations in the muscle biopsies taken at t=0 min were observed between the young and elderly. Muscle free phenylalanine concentrations were lower in the elderly compared with the young (77±7 vs. 160±32 μmol·L^{-1}, respectively, P<0.05). At t=360 min muscle leucine concentrations were significantly higher during the CHO+PRO+leu trial compared with the CHO trial in the young (322±35 vs. 83±11 μmol·L^{-1},

respectively, P<0.05) and elderly (232±38 vs. 103±10 μmol·L^{-1}, respectively, P<0.05). Muscle free tyrosine concentrations were significantly higher in the CHO+PRO+leu trial when compared with the CHO trial in the young only (44±4 vs. 57±4 μmol·L^{-1}, respectively, P<0.05). No differences were observed in muscle free phenylalanine concentrations between trials and between the young and elderly at t=360 min.

Table 6.3 Plasma and muscle tracer enrichments in young and elderly lean men

		Experiment		P values
		CHO	CHO+PRO+leu	Age/treatment/interaction
Plasma AA enrichment				
L-[ring-$^{13}C_6$]phenylalanine				<0.001 / <0.001 / 0.109
	Young	0.0817±0.0012	0.0614±0.0009	
	Elderly	0.1002±0.0003	0.0731±0.0008	
L-[ring-$^{2}H_2$]tyrosine				<0.001 / <0.001 / 0.214
	Young	0.0420±0.0006	0.0289±0.0002	
	Elderly	0.0524±0.0013	0.0347±0.0004	
L-[ring-$^{13}C_6$]tyrosine				<0.01 / <0.01 / <0.05
	Young	0.0131±0.0004	0.0046±0.0001 *	
	Elderly	0.0098±0.0004 #	0.0045±0.0002 *	
Muscle AA enrichment				
L-[ring-$^{13}C_6$]phenylalanine				<0.001 / <0.001 / 0.573
	Young	0.0556±0.0010	0.0429±0.0020	
	Elderly	0.0639±0.0036	0.0537±0.0013	
L-[ring-$^{2}H_2$]tyrosine				0.105 / <0.050 / <0.050
	Young	0.0226±0.0026	0.0218±0.0014	
	Elderly	0.0297±0.0016 #	0.0209±0.0010 *	
L-[ring-$^{13}C_6$]tyrosine				0.858 / 0.070 / <0.05
	Young	0.0214±0.0024	0.0153±0.0043	
	Elderly	0.0214±0.0020	0.0163±0.0021	
Δ enrichment muscle protein				
L-[ring-$^{13}C_6$]phenylalanine				0.398 / 0.149 / 0.442
	Young	0.0556±0.0010	0.0429±0.0020	
	Elderly	0.0639±0.0036	0.0537±0.0013	

Values are means±SEM. n=8 elderly and 8 young persons. CHO, carbohydrate; CHO+PRO+leu, carbohydrate, protein and leucine; enrichments are expressed as tracer/tracee ratio (TTR). Data were analyzed with ANOVA (age x treatment). Mean plasma amino acid (AA) enrichments during the last 4h of recovery. Muscle free amino acid enrichments in the 6h post-exercise biopsy. Increments in muscle protein enrichment from 0 to 6h following ADL. * Significantly different from CHO experiment (Scheffé's test, P < 0.05). # Significantly different from values observed in the young (Scheffé's test, P < 0.05).

Mean plasma amino acid enrichment during the last 4h of recovery, muscle free amino acid enrichment in the 6h post-exercise biopsy and the increment in muscle protein enrichment are presented in table 6.3. Free L-[ring-$^{13}C_6$]phenylalanine enrichments in the 6h post-exercise biopsy were significantly higher in the elderly compared with the young in both trials (P<0.05), while L-[ring-$^{2}H_2$]tyrosine enrichments were higher in the elderly in the CHO trial only (P<0.05). No

differences in muscle free L-[ring-$^{13}C_6$]tyrosine enrichments were observed between the young and elderly in the CHO and CHO+PRO+leu trials and between trials. Free L-[ring-$^{13}C_6$]phenylalanine enrichments in the 6h post-exercise biopsy were significantly higher in the CHO trial compared with the CHO+PRO+leu in both the young and elderly ($P<0.05$), while L-[ring-$^{2}H_2$]tyrosine enrichments were lower in the CHO+PRO+leu trial in the elderly only ($P<0.05$). No differences were observed in the increase in protein-bound L-[ring-$^{13}C_6$]phenylalanine enrichment during the CHO and CHO+PRO+leu trials between the young and elderly.

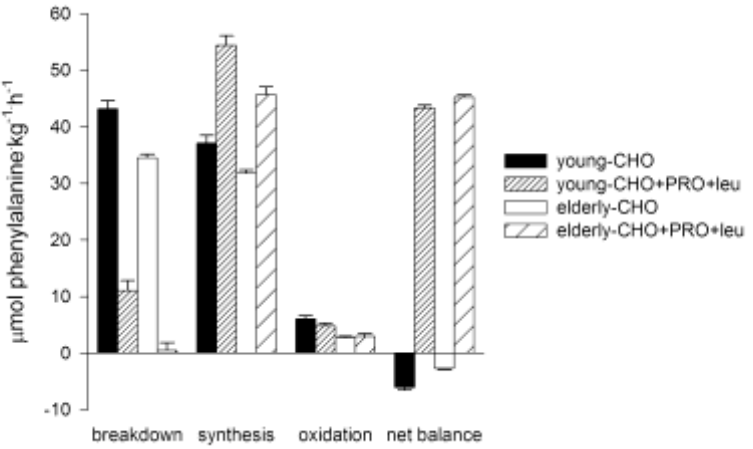

Figure 6.4 The rate of whole-body protein breakdown, synthesis, oxidation and net protein balance (expressed as µmol phenylalanine·kg·h^{-1}) during the CHO and CHO+PRO+leu experiments in lean young (n=8) and elderly (n=8) men. Values are means+SEM. Data were analyzed with ANOVA (age-group x treatment). Breakdown: age-group effect, $P<0.001$; treatment effect, $P<0.001$; interaction of age-group and treatment, $P=0.483$. Synthesis: age-group effect, $P<0.001$; treatment effect, $P<0.001$; interaction of age-group and treatment, $P=0.202$. Oxidation: age-group effect, $P<0.001$; treatment effect, $P=0.225$; interaction of age-group and treatment, $P=0.092$. Net balance: age-group effect, $P<0.001$; treatment effect, $P<0.001$; interaction of age-group and treatment, $P=0.092$.

Whole-body protein metabolism

Both phenylalanine and tyrosine fluxes were higher in the CHO+PRO+leu trial compared with the CHO trial in the young (59.2±1.8 vs. 43.2±1.5 µmol phenylalanine·kg^{-1}·h^{-1}, respectively, $P<0.01$) and elderly subjects (48.7±1.35 vs. 34.6±0.6 µmol phenylalanine·kg^{-1}·h^{-1}, respectively, $P<0.01$). Phenylalanine and tyrosine fluxes in the CHO trial were 20-25% higher in the young compared with the elderly subjects ($P<0.01$). In the CHO+PRO+leu trial, phenylalanine and tyrosine fluxes were 30-40% higher in the young compared with the elderly subjects ($P<0.01$). Whole-body protein breakdown (figure 6.4), calculated over the 6h of post-exercise recovery, was lower in the CHO+PRO+leu trial compared with the CHO trial ($P<0.05$) in the young and elderly. Whole-body protein synthesis (figure 6.4), as calculated by Qp-Qpt, was increased in the CHO+PRO+

leu trial compared with the CHO trial ($P<0.01$) in both groups. The rate of post-exercise whole-body phenylalanine oxidation (figure 6.4), as calculated from the conversion of phenylalanine to tyrosine, was decreased in the CHO+PRO+leu trial compared with the CHO trial ($P<0.05$) in the young but not in the elderly. Whole-body protein balance (figure 6.4) was negative in the CHO trial, whereas protein balance was positive in the CHO+PRO+leu trial in the young and elderly. Protein breakdown, synthesis and oxidation rates were higher in the young compared with the elderly ($P<0.05$). Protein synthesis efficiency (whole-body protein synthesis as a percentage from phenylalanine flux) was significantly higher in the elderly compared with the young subjects in the CHO trial ($P<0.01$), but not in the CHO+PRO+leu trial ($P=0.10$). Protein synthesis efficiency was increased in the CHO+PRO+leu trial compared with the CHO trial in the young (91.9±0.7 vs. 86.0±1.1%, respectively, $P<0.01$), but not in the elderly subjects.

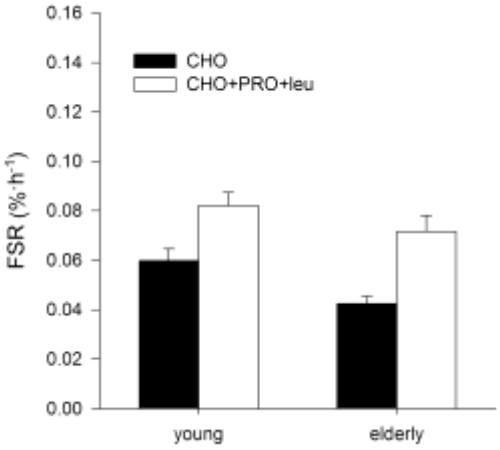

Figure 6.5 Fractional synthetic rate (FSR) of mixed muscle protein following the ingestion of carbohydrate (CHO) or carbohydrate with protein and leucine (CHO+PRO+leu) in lean young (n=8) and elderly (n=8) men using plasma phenylalanine enrichment as a precursor. Values are means±SEM. Data were analyzed with ANOVA (age-group x treatment). Age-group effect, $P < 0.05$; treatment effect, $P < 0.001$; interaction of age-group and treatment, $P = 0.583$.

Mixed muscle protein synthesis rates

Mixed muscle protein fractional synthesis rates (FSR), with the mean plasma L-[ring-$^{13}C_6$]phenylalanine enrichment as precursor (figure 6.5), was significantly higher in the CHO+PRO+leu trial compared with the CHO trial in the young (0.082±0.005 %·h^{-1} vs. 0.060±0.005 %·h^{-1}, respectively; $P<0.01$), and in the elderly (0.072±0.006 %·h^{-1}vs. 0.043±0.003 %·h^{-1}, respectively; $P<0.01$). The observed FSR values in the elderly subjects were significantly lower than those in the young ($P<0.05$). Net differences in the mixed muscle protein synthesis rates between trials were not different between young and elderly subjects (0.022±0.007 %·h^{-1} vs.

0.029±0.004 %·h^{-1}, respectively; NS). When the free intracellular L-[ring-$^{13}C_6$]phenylalanine enrichment was used as the precursor, FSR values were higher, but revealed the same intervention effect. FSR values in the young subjects averaged 0.088±0.008 and 0.124±0.011 %·h^{-1} for the CHO and CHO+PRO+leu trial, respectively (P<0.05). In the elderly, FSR values averaged 0.068±0.005 and 0.097±0.008 %·h^{-1} for the CHO and CHO+PRO+leu trial respectively (P<0.01). When these values were corrected for the contribution of extra-cellular water to the measured free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment (41), FSR values averaged 0.095±0.009 and 0.135±0.014 %·h^{-1} for the CHO and CHO+PRO+leu trial, respectively (P<0.05). In the elderly, FSR values averaged 0.076±0.007 and 0.102±0.008 %·h^{-1} for the CHO and CHO+PRO+leu trial respectively (P<0.01).

Discussion

In the present study, we assessed whole-body protein turnover and determined mixed muscle protein synthesis rates by measuring the incorporation of labeled phenylalanine in the vastus lateralis muscle following the ingestion of carbohydrate with or without protein and free leucine in young and elderly men. Co-ingestion of protein and leucine is shown to improve whole-body protein balance in both the young and elderly when compared with the ingestion of carbohydrate only. Direct measurement of mixed muscle protein synthesis rates reveals that protein synthesis rates are lower in the elderly compared with the young when ingesting only carbohydrate. However, co-ingestion of protein and leucine substantially increases muscle protein synthesis rates to the same extent in both young and elderly men.
It has been reported that basal muscle protein synthesis rates are similar (25, 38-40) or reduced in the elderly (6, 16, 30, 42, 43, 48, 49), while protein degradation rates are increased (40). In addition, there are indications that the stimulating effect of food intake on muscle protein synthesis is blunted in the elderly (38, 44, 45). Besides food intake, physical activity can also effectively modulate protein metabolism. Under normal living conditions, activities of daily living (ADL) are generally followed by food intake. Therefore, determining the combined effects of food intake and physical activity on skeletal muscle protein metabolism is of crucial importance when investigating the proposed changes in skeletal muscle protein metabolism with aging. In the present study, we investigated the anabolic response to carbohydrate (CHO) ingestion following simulated ADL type activities *in vivo* in young (~20 y) and elderly men (~75 y). Consequently, we show that muscle protein synthesis rates are significantly lower in the elderly (~75 y) compared with the young (~20 y) under such conditions. The observed mixed muscle FSR values following ADL activities were ~30% lower in the elderly compared with the young subjects (figure 6.5), which would represent a ~5% decline per decade. These observations are in line with previous reports showing muscle protein synthesis rates to be 20-30% lower in elderly humans (60-84 yrs) than in young adults in a basal, fasted state (16, 33, 44). Our findings on the muscle level are in line with our observations using the whole-body tracer methods showing whole-body phenylalanine and tyrosine turnover to be 20-25% lower in the elderly compared

with the young. Thus, on a whole-body level, protein synthesis, oxidation and breakdown are reduced in the elderly compared with the young subjects. In accordance to our earlier findings (20), we observed that whole-body net protein balance remains negative when only carbohydrate is ingested. To our knowledge we are the first to show that muscle protein synthesis is reduced in the elderly in practical daily living conditions in which ADL activities are followed by ingestion of carbohydrate only.

Administration of amino acids with or without carbohydrate results in a rapid increase in muscle protein synthesis in young adults, whereas protein degradation rates are being reduced (10, 11, 20, 24, 25, 29). The latter can be attributed to the role of amino acids as precursors for protein synthesis (47), the potential of amino acids to stimulate insulin secretion (36) and the proposed property of amino acids, and leucine in particular, to stimulate protein synthesis by activating the mRNA translational machinery through the mTOR-pathway (19). The latter has been reported for leucine administration *in vivo* in rodents (3-5). Therefore, a mixture containing carbohydrate, protein and additional free leucine could therefore likely represents an effective nutritional intervention to optimize net muscle accretion. Recently, we showed that the combined ingestion of protein and free leucine augments skeletal muscle protein synthesis rates in healthy young adults following intense resistance exercise when compared with the ingestion of carbohydrate only (20). In the present study, we aimed to investigate whether the proposed anabolic response to the combined ingestion of protein and leucine would also be present in the elderly when compared with the ingestion of carbohydrate only, and we questioned whether this response is of a similar or lower magnitude in the elderly.

In accordance to our earlier findings (20), we observed that whole-body protein breakdown rates were significantly suppressed following co-ingestion of leucine and protein with carbohydrate, whereas protein synthesis rates were increased (figure 6.4). Co-ingestion of protein and leucine with carbohydrate resulted in a 47±3% and 44±4% increase in whole-body protein synthesis in the young and elderly, respectively, when compared with the ingestion of carbohydrate only. As a result, whole-body protein net-balance became positive in the CHO+PRO+leu trial, which verifies that protein/amino acid ingestion is necessary for net muscle anabolism to occur. The co-ingestion of protein and leucine with carbohydrate increased FSR compared with carbohydrate ingestion only (figure 6.5). The net differences in the mixed muscle protein synthesis rates between the CHO and CHO+PRO+leu trials did not differ between the young and elderly subjects. As such, our data imply that the combined ingestion of protein and leucine with carbohydrate can stimulate muscle protein synthesis to the same extent in young and elderly men. This would be in line with more recent observations, showing a similar response of muscle protein turnover to an amino acid load in healthy elderly and younger adults (25, 37, 39). However, our FSR data seem to be in contrast with data presented by Volpi et al (38), who showed that the response of muscle protein synthesis to the combined ingestion of amino acids and glucose is impaired in the elderly. The latter could be related to the blunted insulin response in the elderly compared with the young controls that were reported by Volpi et al

(38). In contrast, we observed similar plasma insulin responses in the young and elderly volunteers following the co-ingestion of protein and leucine with carbohydrate. The observed differences might be attributed to the property of leucine to stimulate insulin secretion (36) and/or mRNA translation (3). In this study, we investigated the potential differences in the capacity to stimulate muscle protein synthesis in the young and elderly following the ingestion of a theoretically optimal anabolic nutritional intervention. The latter shows that these properties are as effective in the young as in the elderly.

In conclusion, co-ingestion of protein and leucine improves whole-body protein balance when compared with the ingestion of carbohydrate only in young and elderly men. Mixed muscle protein synthesis is reduced in the elderly under conditions where ADL activities are followed by food ingestion. However, co-ingestion of protein with leucine substantially increases mixed muscle protein synthesis rates to the same extent in the young and the elderly, thereby improving whole-body protein balance.

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7

The increase in S6K1 phosphorylation in human skeletal muscle following resistance exercise occurs mainly in type II muscle fibers

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Abstract

To investigate the *in vivo* effects of resistance exercise on translational control in human skeletal muscle, we determined the phosphorylation of AMP activated kinase (AMPK), eukaryotic initiation factor 4E-binding protein (4E-BP1), p70/p85-S6 protein kinase (S6K1) and ribosomal S6 protein (S6). Furthermore, we investigated whether changes in the phosphorylation of S6K1 are muscle fiber-type specific. Eight male subjects performed a single high-intensity resistance exercise session. Muscle biopsies were collected before, immediately after exercise, and following 30 and 120 min of post-exercise recovery. The phosphorylation status of AMPK, 4E-BP1, S6K1 and S6 was determined by western blotting with phospho-specific and pan antibodies. To determine fiber-type specific changes in the phosphorylation status of S6K1, immunofluorescence microscopy was applied. AMPK phosphorylation was increased ~3 fold immediately following resistance exercise, while 4E-BP1 phosphorylation was reduced to 27±6% of pre-exercise values. Phosphorylation of S6K1 at Thr^{421}/Ser^{424} was increased 2-2.5 fold during recovery, but did not induce a significant change in S6 phosphorylation. Phosphorylation of S6K1 was more pronounced in the type II vs. type I muscle fibers. Before exercise, phosphorylated S6K1 was predominantly located in the nuclei. After 2h of post-exercise recovery, phospho-S6K1 was primarily located in the cytosol of type II muscle fibers. We conclude that resistance exercise effectively increases the phosphorylation of S6K1 on Thr^{421}/Ser^{424}, which is not associated with a substantial increase in S6 phosphorylation in a fasted state.

Introduction

Skeletal muscle protein turnover has been shown to be stimulated after an acute bout of resistance exercise (8, 26). The process of mRNA translation initiation forms an important regulatory site in the overall control of muscle protein synthesis (5). Recent evidence suggests that the phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a key role in this process (18, 28). The activity of the signaling cascade controlling muscle protein synthesis is largely determined by the phosphorylation of the mammalian target of rapamycin (mTOR) and its subsequent activation of the p70/p85 S6 protein kinase (S6K1) and the eukaryotic initiation factor 4E-binding protein (4E-BP1) (18).

Both S6K1 and 4E-BP1 can modulate translation initiation, and control the binding of mRNA to the 40S ribosomal subunit (18). 4E-BP1 can bind to the initiation factor eIF4E, thereby acting as a translational repressor preventing the formation of the eIF4F scaffolding complex, which is necessary for efficient binding of the 40S ribosomal subunit to mRNA (1, 14). Activation of the mTOR pathway results in 4E-BP1 hyperphosphorylation and a subsequent dissociation of the 4E-BP1·eIF4E complex, allowing it to participate in the translation initiation process. Another mechanism regulating the binding of mRNA to the 40S ribosomal subunit involves the phosphorylation of ribosomal protein S6, which is controlled by the activity of S6K1 (1). S6K1 consist of two isoforms: a 70-kDa cytoplasmic isoform (p70-S6K) and an 85-kDa nuclear isoform (p85-S6K) (21). The activation of both S6K1 isoforms has been shown to phosphorylate S6 on the 40S subunit in close proximity of the eIFs and mRNA (21). Phosphorylation of S6 is thought to alter the interaction of the protein with these components, thereby promoting mRNA translation (7). Interestingly, in rodents the phosphorylation status of S6K1 following resistance exercise has been reported as an excellent marker for the long-term increase in skeletal muscle mass (2).

It has been speculated that the anabolic response to resistance exercise is muscle fiber-type specific. In accordance, it has been shown that human soleus as opposed to vastus lateralis muscle is less responsive to resistance exercise (32). In rats, the phosphorylation of protein kinase B (PKB or Akt), mTOR and S6K1 following resistance exercise has been reported to be more pronounced in muscle tissue containing a greater proportion type II muscle fibers (2, 25, 28), as opposed to those muscle groups containing more type I fibers (25). Moreover, by applying immuno-histochemistry in rat tibialis muscle, it was recently shown that mTOR phosphorylation is selectively increased in type IIa fibers for up to several h after resistance exercise (25). Data on the activation of the translation initiation machinery in human skeletal muscle remain scarce (9, 16). Information on the potential muscle fiber-type specific changes in the phosphorylation status of proteins involved in translation initiation response to exercise in humans is still lacking.

Both S6K1 and mTOR have been reported to be localized in the cytosol (36). However, recent studies have reported these proteins to be localized both in the

cytosol as well as in the nuclei of human embryonic kidneys (HEK) 293 cells (17). The latter implies that sub-cellular localization of mTOR and/or S6K1 could play a functional role. In HEK 293 cells, nuclear import of mTOR has been shown to enhance 4E-BP1 phosphorylation and S6K1 activation, whereas nuclear export of mTOR attenuated the phosphorylation/activation, suggesting that both activation of S6K1 and phosphorylation of 4E-BP1 require nuclear mTOR (17). Similar nuclear transport has also been reported for S6K1 (17). These observations suggest that the intracellular cytosolic and/or nuclear localization of these key regulatory proteins represent a level of control in the regulation of skeletal muscle protein synthesis. So far, the sub-cellular localization of S6K1 and the subsequent changes that occur in response to resistance exercise have not been studied in human muscle.

The purpose of the present study was to investigate the mode of activation of the mRNA translation initiation machinery downstream of mTOR in skeletal muscle tissue following a single bout of resistance exercise *in vivo* in humans. Besides measuring the phosphorylation state of S6K1, 4E-BP1 and S6, we aimed to investigate whether changes in S6K1 phosphorylation are specific for muscle fiber-type and/or sub-cellular localization.

Methods

Subjects

Eight healthy male volunteers with no history of participating in any regular exercise program were recruited to participate in the present study. Subjects' characteristics are shown in table 7.1. All subjects were informed on the nature and possible risks of the experimental procedures, before their written informed consent was obtained. This study was approved by the Medical Ethical Committee of the Academic Hospital Maastricht.

Table 7.1 Subjects' characteristics

	Mean ± SEM
Age (yrs)	22.5 ± 0.9
Weight (kg)	74.9 ± 2.8
Height (m)	1.80 ± 0.01
BMI ($kg \cdot m^{-2}$)	23.3 ± 0.7
% bodyfat (%)	17.8 ± 2.4
Fat free mass (kg)	61.3 ± 2.1
Fat mass (kg)	13.6 ± 2.1
HbA1C (%)	5.3 ± 0.1
1RM leg press (kg)	198 ± 7
1RM leg extension (kg)	105 ± 3

Values are expressed as means±SEM.

Pretesting

Body composition was assessed using the hydrostatic weighing method in the morning following an overnight fast. Residual lung volume was measured by the helium-dilution technique using a spirometer (Volugraph 2000, Mijnhart, Bunnik, The Netherlands). Body weight was measured with a digital balance with an accuracy of 0.001 kg (E1200, August Sauter GmbH, Albstadt, Germany). Body fat percentage was calculated using Siri's equation (30). Fat free mass (FFM) was calculated by subtracting fat mass from total body mass.
To familiarize subjects with the resistance exercise protocol and the equipment, a familiarization trial was performed. Proper lifting technique was demonstrated and practiced for each of the 2 lower-limb exercises (leg press and leg extension) and for the 3 upper-body exercises (chest press, shoulder press and lat-pulldown). Thereafter, maximum strength was estimated using the multiple repetitions testing procedure (22).
In an additional exercise session, at least 1 week before the experimental trial, the subjects' 1 repetition maximum (1RM) was determined (20). After warming up, the load was set at 90-95% of the estimated 1RM, and increased after each successful lift until failure. A 5 min resting period between subsequent attempts was allowed. A repetition was valid if the subject was able to complete the entire lift in a controlled manner without assistance.

Standardization diet and activity prior to testing

All subjects received a strict standardization diet for 2 days prior to the resistance exercise test trial, which was performed in the morning after an overnight fast. Subjects were provided with a pre-weighed amount of food products, beverages, and instant meals and were allowed to drink water ad libitum. Subjects were instructed to take all main meals (breakfast, lunch, and dinner) and between-meal snacks at predetermined time intervals during each day. The standardization diet provided 0.15 $MJ \cdot kg^{-1} \cdot day^{-1}$ containing 15 Energy% (En%) of protein, 30 En% of fat and 55 En% of carbohydrate. All volunteers were instructed to refrain from any sort of heavy physical exercise during the 2 days prior to the test-trial.

Experimental trial

The day of the test, subjects arrived at the laboratory by car or public transportation at 8.00 am, following an overnight fast. After 30 min of supine rest, a basal blood sample was collected and a muscle biopsy was taken from the vastus lateralis muscle. Thereafter, subjects performed a 5 min low-intensity warm-up on a cycle-ergometer, followed by 3 sets of 10 repetitions on 3 resistance exercise machines targeting upper-body muscle groups (chestpress, shoulderpress and lat-pulldown, Jimsa Benelux BV, Rotterdam, The Netherlands). The latter were included to provide a whole-body warm-up to reduce the risk of injury. Thereafter, subjects performed 8 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, The Netherlands) and 8 sets of 10 repetitions on the

leg extension machine (Technogym). Both exercises were performed at 75% of the individual 1RM with 2 min rest intervals between sets and required ~45 min to complete. All subjects were verbally encouraged during the exercise session to complete the entire protocol. Immediately after cessation of exercise, a second muscle biopsy sample was taken, after which the subjects rested supine for 2h. After 30 min and 2h of post-exercise recovery, additional muscle biopsies were taken.

Muscle biopsies

Muscle biopsy samples were collected from both legs. The first two biopsies were taken from the same incision in one leg; the last two were taken from the same incision in the contra-lateral leg. When biopsy samples were taken from the same incision, the first sample was taken from a different region (distal of the incision, with the needle pointing inwards) than the second (proximal with the needle pointing outwards). Muscle biopsies were obtained from the middle region of the m. vastus lateralis (15 cm above the patella) and approximately 3 cm below entry through the fascia using the percutaneous needle biopsy technique (3).
Muscle samples were freed from any visible non-muscle material and rapidly frozen in liquid nitrogen. Muscle samples (~40 mg) for Western blotting analyses were freeze-dried and collagen, blood and other non-muscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (6-10 mg) was weighed and 8 volumes (8x dry weight of isolated muscle fibers x wet/dry ratio) of ice-cold buffer (20 mmol·L^{-1} HEPES (pH 7.4), 100 mmol·L^{-1} KCl, 50 mmol·L^{-1} beta-glycerophosphate, mmol·L^{-1} NaF, 1 mmol·L^{-1} dithiothreitol, 0.5 mmol·L^{-1} Na_3VO_4, 0.2 mmol·L^{-1} EDTA, 0.1 mmol·L^{-1} PMSF and 1 mmol·L^{-1} benzamidine) was added (4). The tissue was then homogenized, after which homogenates were centrifuged for 5 min at 1000 g and at 4ºC. Thereafter, the supernatant was centrifuged at 10 000 g at 4ºC for 10 min, resolved in SDS-buffer and boiled for 5 minutes at 100ºC.
About 20 mg of each muscle sample were frozen in liquid nitrogen-cooled isopentane and embedded in Tissue-Tek for immunohistochemical analysis (Sakura Finetek, Zoeterwoude, The Netherlands).

Muscle sample analysis

Antibodies

Polyclonal primary phospho-specific antibodies (anti-phospho S6K1 (Thr^{421} /Ser^{424}), anti-phospho S6 (Ser$^{235/236}$), anti-phospho 4E-BP1 (Thr^{37}) and anti-phospho AMPK$\alpha_{1,2}$ (Thr^{172}) and anti-S6K1, anti-S6, anti-4E-BP1 and anti-AMPK$\alpha_{1,2}$ were purchased from Cell Signaling Technologies (Beverly, MA, USA). Anti-caveolin-3 was from BD biosciences (San Jose, CA USA) and the monoclonal antibody raised against adult human slow myosin heavy chain (or A4.951) was from Developmental Studies Hybridoma Bank, developed by Dr. Blau. Appropriate secondary conjugated antibodies (GARIgGAlexa555 and

GAMIgG1Alexa488) were purchased from Molecular Probes (Leiden, The Netherlands).

Western blotting

Equal amounts of protein (40 μg/lane) were run on either 10% (S6K1 and S6) or 12% (AMPK and 4E-BP1) SDS-polyacrylamide gels (200 V, mini protein 3 cell, Biorad, Hercules, CA) and proteins were transferred (2h 250 mA, Criterion blotter, Biorad, Hercules, CA) to 0.45 mm nitrocellulose membranes. After Ponceau S staining and de-staining, membranes were blocked in 5% Non Fat Dry Milk Powder (NFDM) (Biorad, Hercules, CA) in Tris buffered saline containing 0.1% Tween-20 (TBS-T) for 1h. Thereafter, a 1:1000 dilution of the primary (phospho) specific antibody (all from Cell Signaling Technology) in 5% NFDM/TBS-T was added and incubated overnight at 4ºC on a shaker. After washing the membranes 4 times 5 min in 15 ml 5% NFDM/TBS-T, the membranes were incubated with a 1:10 000 dilution of the horseradish peroxidase-conjugated secondary antibody (Pierce) in 5% NFDM/TBS-T. Thereafter, the membranes were washed in 25 ml of TBS-T for 5, 15, 5 and 5 min. Light sensitive film (CL-Xposure; Pierce) was used to detect immunoreactive bands using chemiluminescent substrate (SuperSignal CL; Pierce). Autoradiographic film was scanned densitometrically and quantification was performed using the program Quantity One version 4.2.1. (Biorad). Alpha actin was used to standardize for the amount of protein loaded. Phosphorylation of AMPK, 4E-BP1, S6K1 and S6 was expressed relative to the total amount of each protein. Data was analyzed as the % change in phosphorylation state from pre-exercise values for each subject.

Immunohistochemistry

Multiple serial sections (5 μm) from biopsy samples collected before, immediately after, 30 and 120 min after exercise were thaw-mounted together on uncoated, pre-cleaned glass slides for each subject, carefully aligned for cross-sectional analysis. Sections were fixed for 5 min in methanol, followed by 1 min in acetone. Slides were then incubated overnight at 4 °C with anti-caveolin-3 and anti-phospho S6K1 antibodies (1:200 and 1:50 in PBS, respectively). Slides were rinsed for 3x 5 min with PBS and then incubated for 45 min with appropriate secondary antibodies diluted together with 5 $\mu g \cdot ml^{-1}$ 4',6-Diamidino-2-phenylindole (DAPI; to visualize nuclei) in PBS. After several washes with PBS, stained sections were embedded in Mowiol® and covered with a coverslip. All muscle cross-sections were stained and prepared within a single batch using the same antibody-preparation to minimize variability in staining efficiency. Serial sections were stained for slow MHC with the monoclonal A4.951 antibody to determine the proportion and cross-sectional area of type I and II muscle fibers, which was measured as previously described (33).

After 24h, glass slides were examined using a Nikon E800 fluorescence microscope (Uvikon, Bunnik, The Netherlands) coupled to a Basler A113 C progressive scan color CCD camera, with a Bayer color filter. Epifluorescence signal was recorded using a Texas red excitation filter (540-580 nm) for S6K1, a

fluorescein isothiocyanate (FITC) excitation filter (465-495 nm) for caveolin-3, and a 4',6-diamidino-2-phenylindole (DAPI) UV excitation filter (340-380 nm) for the nuclei.
Digitally captured images (240x magnification) with five fields-of-view per muscle cross-section (12±1 fibers per field-of-view), were processed and analyzed using Lucia 4.81 software (Nikon, Düsseldorf, Germany). The phospho-S6K1 specific fluorescence signal was quantified for each muscle fiber, resulting in a total of 60±2 muscle fibers analyzed for each muscle cross-section (32±2 type I and 27±2 type II muscle fibers) and for each muscle nuclei, resulting in a total of 117±7 muscle fiber nuclei analyzed for each muscle cross-section (58±5 nuclei of type I and 59±5 nuclei of type II muscle fibers). Muscle nuclei were selected based on the DAPI staining. An intensity threshold representing minimal intensity values corresponding to nuclear phospho-S6K1 was set manually and uniformly used for all images. Phospho-S6K1 content of muscle nuclei was expressed as the percentage of the nuclear area that was stained. Intracellular phospho-S6K1 content was expressed as mean staining intensity.

Statistics

All data are expressed as means±SEM. Analyses of variance (ANOVA) for repeated measures were applied to determine differences in phosphorylation status in the protein of interest over time. In case of a significant F-ratio, a Scheffé post-hoc test was applied to locate the differences. AMPK phosphorylation status before and after exercise were compared using a paired t-test. Statistical significance was set at $P<0.05$.

Results

Resistance exercise

Mean 1 repetition maximum (1RM) measured during the pre-test was 198±7 kg on the horizontal leg press and 105±3 kg on the leg extension exercise. Therefore, average weight lifted during the resistance exercise was set at 148±5 and 79±3 kg for the leg press and leg extension respectively. All subjects completed 8 sets with 10 repetitions on the leg press. One subject was unable to finish 10 repetitions during the 6th set, after which resistance was reduced to 65% of the individual 1RM. All subjects completed 8 sets of 10 repetitions on the leg extension machine.

Western blotting results

Western blotting with phospho-specific antibodies showed that AMPK phosphorylation was increased 3-fold following resistance exercise ($P<0.05$). Thr^{172} phosphorylation of AMPK assessed with western blotting is shown in figure 7.1. Thr^{37} phosphorylation of 4E-BP1 assessed with western blotting is presented in figure 7.2. 4E-BP1 phosphorylation was reduced immediately after resistance

exercise (P<0.05), after which they returned to baseline values after 30 min of recovery. After 120 min of post-exercise recovery, 4E-BP1 phosphorylation was significantly reduced compared to resting values (P<0.05).

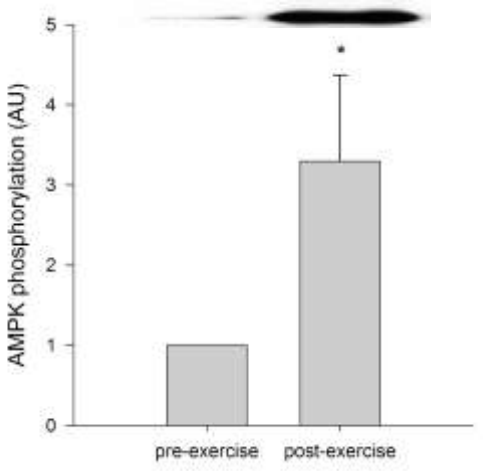

Figure 7.1 AMPK phosphorylation at Thr[172] in vastus lateralis muscle at rest, immediately post-exercise and after 30 and 120 min of post-exercise recovery. Representative immunoblots are shown (top). Values are means+SEM; * significantly different from resting values.

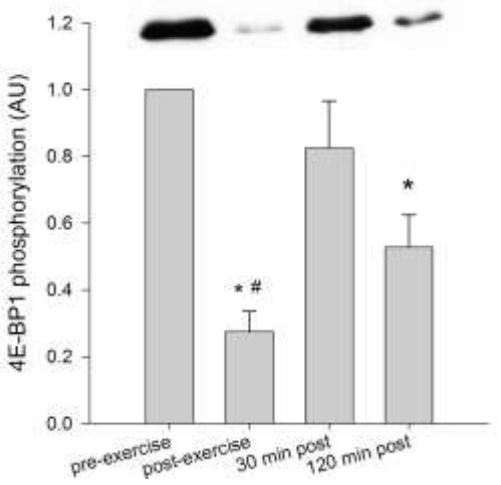

Figure 7.2 4E-BP1 phosphorylation at Thr[37] in vastus lateralis muscle at rest, immediately post-exercise and after 30 and 120 min of post-exercise recovery. Representative immunoblots are shown (top). Values are means+SEM; * significantly different from resting values; # significantly different from values observed after 30 min of post-exercise recovery.

Thr^{421}/Ser^{424} phosphorylation of S6K1 assessed with western blotting is shown in figure 7.3. Following resistance exercise, S6K1 phosphorylation was increased and was significantly higher 30 min post-exercise compared to basal resting values ($P<0.05$). Ser$^{235/236}$ phosphorylation of S6 assessed with western blotting is presented in figure 7.4. No significant differences in S6 phosphorylation were observed following exercise.

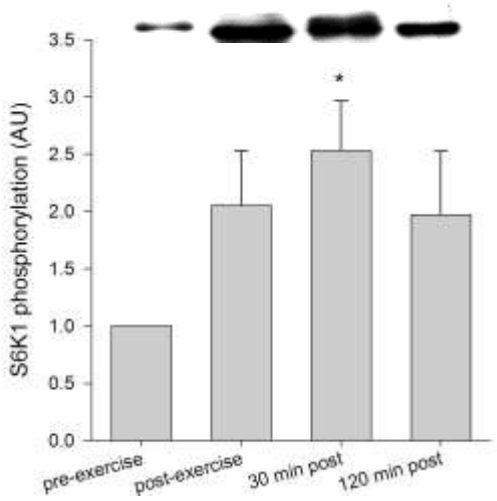

Figure 7.3 S6K1 phosphorylation at Thr^{421}/Ser^{424} in vastus lateralis muscle at rest, immediately post-exercise and after 30 and 120 min of post-exercise recovery. Representative immunoblots are shown (top). Values are means+SEM; * significantly different from resting values.($P<0.05$).

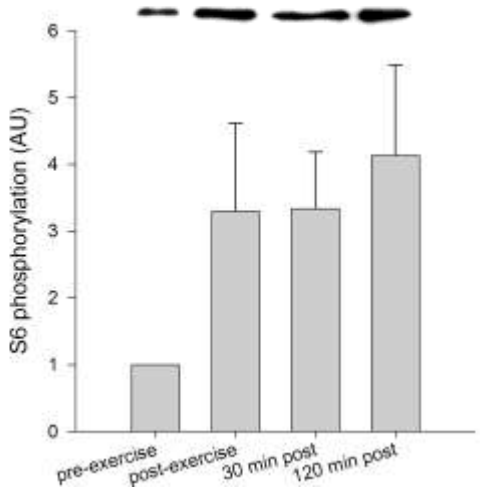

Figure 7.4 S6 phosphorylation at Ser$^{235/236}$ in vastus lateralis muscle at rest, immediately post-exercise and after 30 and 120 min of post-exercise recovery. Representative immunoblots are shown (top). Values are means+SEM. No differences were observed over time.

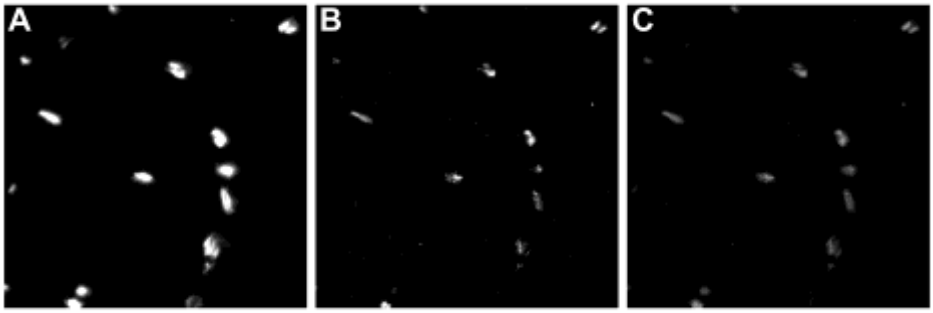

Figure 7.5 Immunofluorescent images (360x magnification) of muscle nuclei (A) and S6K1 (Thr^{421}/Ser^{424}) (B) in the pre-exercise muscle biopsies showing nuclear localization of phospho-S6K1 (C). The nuclei were stained using DAPI.

Immunohistological analyses

Western blotting with phospho-specific antibodies showed that Thr^{421}/Ser^{424} phosphorylation of S6K1 was increased following resistance exercise and was significantly higher 30 min post-exercise compared to the observed resting values. Using immuno-histochemical techniques we were able to quantify changes in localization and phosphorylation of S6K1 in a fiber-type specific manner. To determine the cellular localization of S6K1 in muscle fibers, we used immuno-fluorescence staining of the muscle membrane, nuclei and S6K1 (Thr^{421}/Ser^{424}). As presented in figure 7.5, staining for phospho-specific S6K1 in the pre-exercise muscle biopsies shows a predominant localization of phospho-S6K1 in the nuclei. However, after 2h of post-exercise recovery we show a marked increase in phosphorylated S6K1 present in the cytosol (figure 7.6). In figure 7.6, images of representative cross-sections of vastus lateralis muscle obtained before and 120 min after exercise with sections stained for phospho-S6K1 are presented. Phosphorylation of S6K1 was increased following resistance exercise in both the type I and type II fibers (figure 7.7; $P<0.05$). However phosphorylation of S6K1 increased to a greater extent in the type II fibers compared to the type I fibers (figure 7.7; $P<0.05$). At 30 min after the resistance exercise session nuclear phospho-S6K1 content was increased in the type II fibers only ($P<0.05$).
On average, muscle samples taken from the vastus lateralis muscle consisted for 53±2% of type I and 47±3% of type II muscle fibers. Type II muscle fibers had a significantly greater cross sectional area compared to the type I fibers (5243±331 vs. 4668±240 μm^2, $P<0.05$). No differences between fiber-types were observed in average number of myonuclei per muscle fiber cross-section (2.1±0.3 vs. 2.3±0.2 nuclei per fiber for type I and II muscle fibers, respectively; NS). In addition, nuclei area did not differ between type I and II muscle fiber (31.8±0.9 vs. 31.7±1.0 μm^2, respectively).

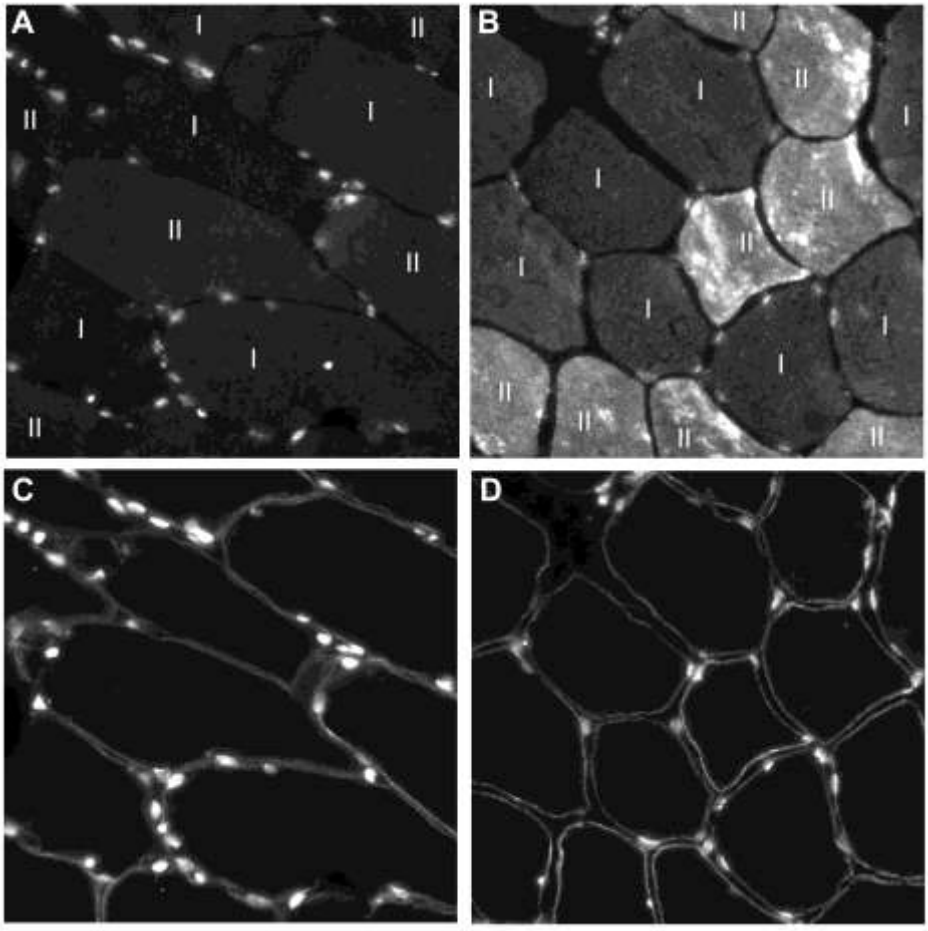

Figure 7.6 Images (240x magnification) of representative cross-sections of vastus lateralis muscle showing primarily nuclear localization of phospho-S6K1 in muscle samples obtain before exercise (A). Phospho-S6K1 is primarily located in the cytosol of type II muscle fibers after 2h of post-exercise recovery (B). Staining for caveolin-3 and nuclei are shown in panel C and D (showing the cell membranes in green and nuclei in blue). The muscle fibers are labeled as type I and type II.

Discussion

In the present study, we show that a single bout of resistance exercise leads to the phosphorylation of S6K1 in human skeletal muscle in a fiber-type-dependent manner, with the most pronounced phosphorylation being observed in the type II muscle fibers. The phosphorylation of S6K1 observed following exercise was not accompanied by a substantial activation of S6K1. The latter could, in part, be attributed to the observed immediate exercise induced increase in AMPK phosphorylation, which has been shown to induce reductions in 4E-BP1 phosphorylation and S6K1 activation (4).

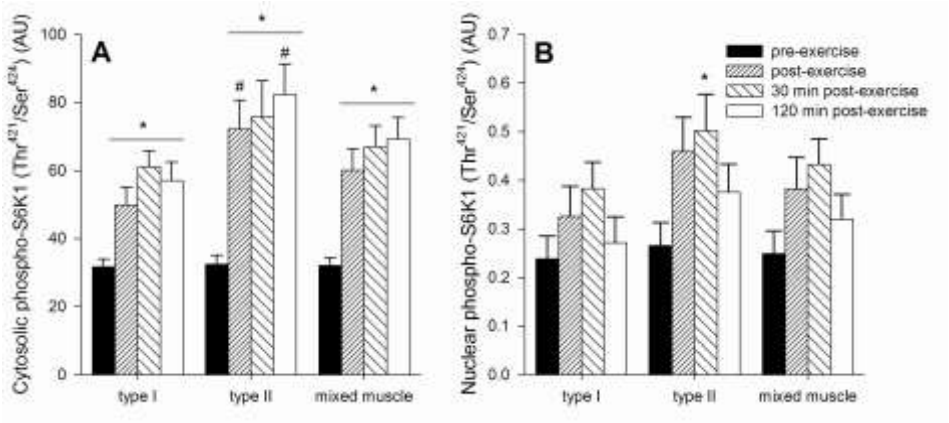

Figure 7.7 The effect of resistance exercise on the phosphorylation status of S6K1 in the cytosol (A) and nuclei (B). Mean fiber-type-specific intramyocellular phospho-S6K1 (expressed as staining intensity) and mean fiber-type-specific nuclear phospho-p70/p85-S6K (expressed as % area stained) before, immediately after exercise and following 30 and 120 min of post-exercise recovery as determined by fluorescence microscopy on stained muscle cross-sections. Data provided are means+SEM. * significantly different from pre-exercise values ($P<0.05$); # significantly different from values observed in type I fibers ($P<0.05$).

Though the metabolic response to resistance exercise has not been studied extensively, it has been shown that muscle lactate concentrations quickly increase up to ~17 mmol·kg^{-1} wet muscle weight, during a single session of resistance exercise training *in vivo* in humans (12, 31). Furthermore, we observed that skeletal muscle fiber glycogen content declines substantially in both type I and II muscle fibers following resistance type exercise tasks (19). In line with these findings, we observed a marked increase in the phosphorylation of AMP-activated protein kinase (AMPK) in the present study (figure 7.1). This indicates that resistance exercise leads to a reduction in the energy status of the muscle fiber, thereby stimulating the phosphorylation of skeletal muscle AMPK. As far as we know, this is the first study to report an increase in AMPK phosphorylation following resistance type exercise activities. In accordance, earlier studies have reported that high-intensity endurance exercise (6, 37) but not low or moderate intensity exercise (6, 34) augments AMPK activation. The latter requires AMPK phosphorylation on Thr^{172} by an AMPK kinase. The major AMPK kinase in skeletal muscle is LKB1 (29). In addition, AMPK phosphorylation has been associated with the concomitant decline in muscle glycogen content, as AMPK has been suggested to have a glycogen-binding domain (29), which would explain the observation that AMPK is activated to a greater extent when muscle glycogen concentrations are low (37). Though AMPK is generally regarded as a main energy sensor in the cell, being responsible for the regulation of skeletal muscle glucose and fatty acid uptake and oxidation (18, 27), it was recently proposed that the AMP-activated protein kinase might also play an important role in the regulation of muscle protein synthesis and/or degradation (4, 7).

Bolster and co-workers (4) were the first to show that AMPK activation in skeletal muscle tissue, by 5-aminoimidazole-4-caroxamide 1-β-D-ribonucleoside (AICAR) administration in rats, reduces protein synthesis rates. The latter was associated with a reduced phosphorylation state of mTOR at Ser^{2448}, S6K1 at Thr^{389} and 4E-BP1 at Thr^{37} (4). More recently, it was proposed that activation of AMPK results in mTOR phosphorylation at Thr^{2446} (7), which decreases mTOR phosphorylation at Ser^{2448}, thereby diminishing the ability of insulin (and other growth factors) to phosphorylate S6K1 (7). These observations are indicative of the presence of another metabolic master switch mechanism in which AMPK plays a key role by integrating signals associated with the energy state of the cell and growth factors associated with protein translation.

The present study shows that a single session of high-intensity resistance exercise *in vivo* in humans significantly increases AMPK phosphorylation and reduces the phosphorylation status of 4E-BP1 in skeletal muscle tissue immediately after cessation of exercise (figure 7.2). The exercise bout did not significantly increase S6 phosphorylation (figure 7.4), indicating that S6K1 activity was not substantially increased. The latter is in close agreement with observations *in vitro* in H2K myotubes (7) and *in vivo* in rats (4) showing AMPK phosphorylation to suppress S6K1 activity. The kinase activity of S6K1 is controlled by a series of phosphorylation steps at several Ser/Thr residues (11, 35). Phosphorylation of S6K1 in the carboxy terminal auto-inhibitory domain (at Ser^{411}, Ser^{418}, Thr^{421} and Ser^{424}) results in a conformational change in the protein allowing the phosphorylation of Ser^{404}, Thr^{389} in the linker domain and Thr^{229} in the catalytic domains, which activates the kinase (27, 35). Consequently, the ribosomal protein S6 on the 40S subunit is phosphorylated, which is thought to alter the interaction of the protein with the eIFs and the mRNA (7).

In the present study, we show that a single session of high-intensity resistance exercise was not sufficient to fully activate S6K1. The exercise effect of S6K1 was likely to be limited to Thr^{421}/Ser^{424}, as we did not observe a significant increase in the phosphorylation of S6. The extracellular signal-regulated kinase-1/2 ($ERK_{1/2}$) has been suggested to play an important role in phosphorylation of S6K1 on Thr^{421}/Ser^{424}. In accordance $ERK_{1/2}$ phosphorylation has been shown to be substantially increased immediately following exercise (16, 24). Our observations are in agreement with data reported by Karlsson et al. (16), showing that a single session of resistance exercise (4 sets, 10 repetitions of leg extension) does not fully activate S6K1. However, they showed that post-exercise ingestion of branched chain amino acids has a striking effect on S6K1, which was evident by site-specific phosphorylation at Thr^{389} (16). These data indicate that intake of an ample amount of protein/amino acids is needed to fully activate skeletal muscle translation initiation. In contrast to these and our observations, there are some studies in rodents that have reported an increase in S6K1 activity in response to either isometric or eccentric contractions (2, 15, 23). The apparent contradictory findings are attributed to the fact that maximal muscle contraction was induced through electrical stimulation of the nerve at 100 Hz. The latter results in the recruitment of all motor units (including all fast and slow twitch fibers), which is quite different

from voluntary muscle contraction *in vivo* (13). Therefore, in the present study we also aimed to study muscle fiber-type specific effects of resistance exercise on S6K1 phosphorylation.

Using immuno-histochemical techniques, we were able to quantify changes in phosphorylation of S6K1 at Thr^{421}/Ser^{424} in a fiber-type specific manner. Phosphorylation of S6K1 was increased following resistance exercise in both the type I and type II fibers (figure 7.6). However, phosphorylation of S6K1 increased to a greater extent in the type II fibers compared to the type I fibers ($P<0.05$). In accordance, the phosphorylation of PKB (or Akt), mTOR and S6K1 following resistance exercise in rats has been reported to be more pronounced in muscle groups containing a greater proportion type II vs. type I muscle fibers (e.g. m. tibialis anterior vs. m. soleus) (2, 25, 28). Moreover, by the application of immuno-histochemistry in rat m. tibialis anterior, it has recently been shown that mTOR phosphorylation is selectively increased in type IIa fibers several hours after a bout of resistance exercise (25). These observations are most likely due to specificity in muscle fiber-type recruitment. Interestingly, we have recently shown that a single session of resistance exercise results in a substantial reduction in muscle fiber glycogen content. Moreover, these changes are fiber-type specific and are closely related to fiber-type oxidative capacity. As such, net changes in muscle fiber glycogen content were more pronounced in type II fibers compared to type I muscle fibers, which can be attributed to the greater recruitment of these fibers during high-intensity resistance exercise (13). One could, therefore, speculate that AMPK phosphorylation will increase to a greater extent in these fibers, phosphorylating mTOR on Thr^{2446}, thereby decreasing the capacity of the kinase to phosphorylate S6K1 at Thr^{389}. This could explain why we did not observe a substantial increase in S6 phosphorylation during the 2h of post-exercise-recovery, while phosphorylation of S6K1 on Thr^{421}/Ser^{424} was increased.

The localization of proteins involved in the regulation of translation initiation is thought to play an important role in the modulation of protein synthesis (17). Proteins like mTOR and S6K1 have been reported to be localized both in the cytosol as well as in the nuclei of human embryonic kidneys (HEK) 293 cells (17). Interestingly, it has been demonstrated that *in vitro* nuclear import of mTOR enhances 4E-BP1 phosphorylation and S6K1 activation, whereas nuclear export of mTOR attenuated the phosphorylation/activation, suggesting that both activation of S6K1 and phosphorylation of 4E-BP1 require nuclear mTOR (17). Similar nuclear transport has also been reported for S6K1 (17). In the light of these observations, the present study also examined the sub-cellular location of S6K1, using immuno-fluorescence staining of the muscle membrane, nuclei and S6K1 (Thr^{421}/Ser^{424}). In the pre-exercise muscle biopsies we show a predominant nuclear localization of phospho-S6K1 (figure 7.5, 7.6 A/C), while following the resistance exercise session, phosphorylated S6K1 is primarily located in the cytosol (figure 7.6 B/D), especially in type II fibers. Interestingly, we show that 30 min after cessation of exercise nuclear phospho-S6K1 content was increased in the type II fibers. The latter could imply that the nucleus plays a key role in the activation of S6K1 and/or that S6K1 functions as a transcription factor. However, as the antibody

that was used recognizes both p70 and p85-S6K, we cannot differentiate between nuclear import of S6K1 and phosphorylation of p85-S6K, the latter of which is known for its unique nuclear localization (10, 21). More research is warranted to determine the role of sub-cellular location of S6K1 and its potential regulatory role.

We conclude that a single bout of resistance exercise *in vivo* in humans activates skeletal muscle AMPK, resulting in a decrease in the phosphorylation state of 4E-BP1. During recovery S6K1 phosphorylation is increased, and showed to be fiber-type specific, indicating that the signaling response to S6K1 is muscle fiber-type specific. However, in the absence of food intake, the phosphorylation status of S6K1 on Thr^{421}/Ser^{424} is not accompanied by a substantial increase in the phosphorylation of S6 under fasting conditions. As such, in the absence of food intake, exercise does not fully activate the translation initiation process in skeletal muscle tissue.

Acknowledgements

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8

General Discussion

Skeletal muscle plays a central role in the regulation of whole-body glucose metabolism. By virtue of its mass, skeletal muscle is the primary tissue responsible for insulin-dependent glucose clearance, accounting for up to 80% of whole-body glucose uptake after meal ingestion. In addition, the total amount of muscle mass is also one of the main determinants of basal metabolic rate (BMR). Aging is accompanied with a substantial loss of muscle mass, which consequently leads to muscle weakness and major disabilities in every day life. The latter is accompanied by a reduction in BMR, the loss of functional capacity and an increased likelihood of developing chronic metabolic diseases like obesity, type 2 diabetes and osteoporosis.

Various interventions have been proposed to reduce or reverse the loss of muscle mass and function. It has been suggested that physical activity (i.e. resistance exercise) and nutritional interventions (i.e. protein, amino acids) could represent effective strategies to reduce, or counteract the loss of muscle tissue that occurs with aging and disease. In this thesis we focus on the beneficial effects of resistance exercise and the potential of dietary supplementation with carbohydrate, protein and/or specific amino acids to enhance muscle protein anabolism. In chapter 2 we first show that resistance exercise is as powerful as endurance exercise in enhancing post-exercise insulin sensitivity. Both the amount of lipid and glycogen stored in muscle have been shown to play a role in the exercise induced increase in muscle insulin sensitivity. We, therefore, have investigated the use of intramuscular lipid and glycogen stores during resistance exercise in chapter 3. In chapter 4-6 the use of insulinotropic protein/amino acid mixtures to accelerate post-exercise (total muscle) protein synthesis has been investigated. In chapter 7, we looked at changes in the phosphorylation of 4E-BP1 and S6K1 following resistance exercise to get a better understanding of the upregulation of the molecular signaling mechanisms that control muscle protein synthesis.

In this final chapter, we will elaborate on some of the reported findings by discussing their significance and clinical relevance in the elderly population. In addition, we will discuss specific methodological issues that need to be considered in future studies. In the last paragraph, important aims for future research will be defined.

Resistance exercise and insulin sensitivity

Most exercise intervention programs solely use endurance exercise because of its acute enhancing effect on insulin stimulated glucose clearance. Interestingly, we have shown that a single bout of resistance exercise also improves whole-body insulin sensitivity by ~15% when measured 24h after cessation of exercise (chapter 2). This seems to be of a similar magnitude as the ~20% improvement that has been reported following an acute (~60 min) bout of endurance exercise (51, 59). As such, there should be no restriction in combining the benefits of both types of exercise in future lifestyle intervention programs. This could be of significant importance in frail and/or type 2 diabetic elderly, who are often in such a physical

state that generally does not allow them to perform endurance exercise. In that case, it would be helpful to first gain muscle strength and mass through resistance training.

The mechanisms by which resistance exercise stimulates insulin sensitivity have not yet been fully elucidated. It seems likely to assume that resistance exercise is followed by an attenuated muscle GLUT-4 translocation (78) and/or elevated GLUT-4 expression (45). Factors thought to play a major regulatory role in this process include AMPK activation (26, 28, 35, 101), Ca^{2+}/calmodulin-dependent kinases (108, 109), muscle glycogen content and subsequent activation of glycogen synthase activity (11, 23, 29, 55, 59, 102, 103). Prolonged moderate intensity endurance exercise does not seem to increase AMPK phosphorylation (20, 90) and does not affect signaling through the insulin signaling pathway (102). The latter is evidenced by unchanged phosphorylation of insulin receptor tyrosine kinase, Akt and GSK-3 (102). It is therefore thought that activation of Ca^{2+}/calmodulin-dependent kinases (22, 68) and the reduction in muscle glycogen content and subsequent activation of glycogen synthase are the main regulatory factors that modulate glucose uptake following endurance exercise (55). Interestingly, in contrast to moderate intensity endurance exercise, resistance exercise has been reported to lead to an increase in AMPK phosphorylation (chapter 7) and to activate signaling through the PI-3 kinase/mTOR pathway (36). Prolonged (16-24h) activation of PI-3 kinase, and several downstream effectors like S6K1 (36, 46), 4E-BP1 (46) and S6 (46) have been reported in rat skeletal muscle following resistance exercise (36). The reported activation of the PI-3 kinase/mTOR pathway is likely to play an important role in the augmented glucose uptake and protein synthesis rates following resistance exercise. Increased Akt signaling has been shown to inhibit GSK3, which leads to the activation of glycogen synthase and to GLUT4 translocation (71). In addition, resistance exercise can also substantially reduce muscle glycogen content (chapter 3, (24, 77)). This implies that resistance exercise has the ability to enhance muscle insulin sensitivity via several other potential mechanisms and might therefore represent a more powerful means to increase the blood glucose clearance capacity when compared to endurance exercise (18).

In contrast to endurance exercise, information on the metabolic demands imposed upon by resistance exercise (i.e. muscle glycogen and IMTG utilization) is scarce. Early reports suggested that the energy needs during resistance exercise are provided with only a minimal activation of glycogenolysis or lipolysis (39). In chapter 3 we, therefore, investigated fiber-type specific changes in skeletal muscle glycogen and lipid content following resistance exercise and subsequent recovery using histological techniques. The use of (immuno)histochemistry on skeletal muscle cross-sections prepared from muscle biopsy samples represent an effective strategy to assess fiber-type specific changes in intramyocellular lipid and/or glycogen content, without the methodological limitations generally associated with biochemical extraction analyses on skeletal muscle samples, like the contamination of skeletal muscle tissue with extramyocellular fat (37). In 2001, we introduced an optimized lipid staining protocol, permitting automated quantification of

intramyocellular lipid depositions in skeletal muscle sections together with immuno-localization of other myocellular elements by fluorescence microscopy. Lipid droplets were detected in skeletal muscle by oil red O (ORO) staining, which was modified to diminish background staining, to prevent crystallization of ORO and to optimize lipid retention in cryosections (43). Since then we have implemented this method in several studies to investigate the effects of exercise and dietary interventions on muscle fiber-type specific lipid content (85, 87, 89, 90). Moreover, we recently reported a good correlation between IMTG content as determined by ^{1}H-magnetic resonance spectroscopy (MRS) and fluorescence microscopy (86). The conventional periodic acid-Schiff reagent (PAS) reaction to stain for muscle glycogen content has been optimized in a similar manner as the oil red O staining (74). Strong correlations between data on (mixed-muscle) glycogen content obtained by the use of both biochemical and histochemical methods have recently been reported (74). These observations indicate that semi-quantitative microscopy on PAS or ORO-stained muscle cross-sections represent valid methods to determine fiber-type-specific changes in muscle glycogen and lipid content and can be used to create insight in differences between fuel storage between the different muscle fiber-types and, as such, in their subsequent role in skeletal muscle metabolism.

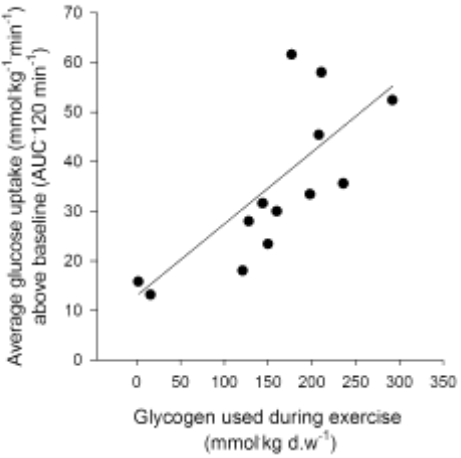

Figure 8.1 Correlation between the degree of glycogen depletion and the insulin-stimulated glucose uptake in thigh muscles of healthy men. Adapted from Richter et al. (66).

The applied methodology clearly shows that mixed muscle glycogen content is substantially decreased following resistance exercise, which is in line with previous studies applying biochemical analysis on mixed muscle tissue (24, 77). We extend on the earlier findings by showing that these changes in muscle glycogen content are fiber-type specific, and more pronounced in type IIx fibers when compared to type I muscle fibers. This is in contrast to observations following endurance

exercise, where glycogen stores are primarily reduced in type I fibers (unpublished observations, L. van Loon & R. Koopman). This apparent difference may be the result of the high rate of ATP production per second necessary to sustain the high power output during resistance exercise in type II fibers. Due to the observed low mitochondrial content in type IIx fibers, they must rely for the greater part on anaerobic glycogenolysis for ATP generation.

The exercise-induced reduction in skeletal muscle glycogen content has been associated with an increase in insulin sensitivity (11, 59, 102, 103) as shown in figure 8.1. In fact, skeletal muscle glucose uptake and glycogen synthase activity have been shown to strongly depend on muscle glycogen availability (56, 104). Interestingly, not only muscle substrate utilization has been shown to be fiber-type specific. AMPK activation, induced by AICAR, increases glucose uptake in rat fast-twitch muscle (109), but not in slow twitch soleus muscle (108). There are also indications that the activation of the insulin signaling cascade following resistance exercise is fiber-type specific. In chapter 7, we show that S6K1 phosphorylation following resistance exercise in humans is more pronounced in type II, compared to type I muscle fibers. In addition, the phosphorylation of PKB (or Akt), mTOR and S6K1 following resistance exercise in rats has been reported to be more pronounced in muscle tissue containing a greater proportion of type II muscle fibers (5, 58, 70), as opposed to those muscle groups containing more type I fibers (58). Moreover, by applying immunohistochemistry in rat tibialis muscle, it was recently shown that mTOR phosphorylation is selectively increased in type IIa fibers for up to several h after resistance exercise (58). This selective activation of the signaling through mTOR in fast-twitch muscle fibers may in part explain why endurance exercise, in contrast to resistance exercise, does not affect signaling through the insulin signaling pathway.

We have recently demonstrated a substantial (~60%) net reduction in type I muscle fiber lipid content following 2h of moderate intensity endurance exercise (86, 90). Moreover, it has been reported that it takes ~48h to fully replenish the IMTG stores following a 3 h endurance exercise bout (89). Interestingly, in chapter 3 we report a net decline (~27%) in IMTG content following resistance exercise in type I muscle fibers. Nevertheless, this effect was only temporary and not evident after 2h of post-exercise recovery. The absence of a prolonged net reduction in skeletal muscle lipid content implies that the reported stimulating effects of resistance exercise on whole-body insulin sensitivity are unlikely to be attributed to a reduction in intramuscular lipid content. However, it has been suggested that not IMTG content per se, but fatty acid intermediates like fatty acyl-CoA, ceramides and/or diacylglycerol modulate skeletal muscle insulin signaling. Increased intracellular concentrations of such fatty acid intermediates have been shown to increase PKC activation and enhance serine phosphorylation of the insulin receptor substrate-1 (IRS-1). The latter reduces its tyrosine phosphorylation, thereby suppressing PI-3 kinase activity, leading to a reduced GLUT-4 translocation to the sarcolemma. It could be speculated that acute changes in muscle fatty acyl-CoA, ceramides and diacylglycerol concentrations following resistance exercise are (partly) responsible for the observed effects of resistance

training on skeletal muscle insulin sensitivity (84, 85). However, more research is warranted to address the proposed relationship between exercise, fatty acid intermediates and skeletal muscle insulin sensitivity.

Nutritional interventions to promote muscle protein synthesis

Endurance athletes optimize carbohydrate availability before, during and after prolonged endurance exercise to replenish muscle and liver glycogen stores and, as such, to optimize endurance performance capacity. In addition, it has been suggested that the ingestion of carbohydrate reduces protein degradation (69). In line with these findings, we demonstrate in chapter 4 that net protein degradation does not increase during moderate intensity endurance exercise when carbohydrate is ingested. This implies that net protein breakdown and oxidation is of minor magnitude during endurance type activities, like (ultra)marathons and prolonged cycling races. However, when only carbohydrate is ingested during and after exercise, whole-body protein balance remains slightly negative during exercise and subsequent recovery (42). The latter indicates that the ingestion of protein/amino acids is needed to obtain a positive net protein balance. In accordance, we show that the combined ingestion of protein and carbohydrate improves net protein balance at rest, during prolonged moderate intensity exercise and subsequent recovery in elite endurance athletes. The latter may well play an important role in the facilitation of specific muscle adaptation following endurance exercise training. Specific muscle adaptation includes the increased expression of proteins instrumental in the processes involved in the mobilization and oxidation of endogenous fat and carbohydrate stores.

Optimizing protein metabolism following resistance exercise

In contrast to endurance exercise, resistance exercise training can result in profound increases in skeletal muscle mass and strength (25, 67, 99). Interestingly, after a single session of resistance exercise, both muscle protein synthesis and degradation rates are strongly elevated (60). As a result, net muscle protein balance will remain negative in the absence of food intake. The latter implies that skeletal muscle hypertrophy can only occur under conditions where food is ingested during the early phases of post-exercise recovery. Therefore, the combined ingestion of protein and carbohydrate represents an effective strategy to promote net muscle protein accretion, as it can stimulate protein metabolism in several ways. Besides providing amino acids as precursors for protein synthesis, combined ingestion of carbohydrate and protein/amino acids has been shown to elevate plasma insulin levels (41, 46, 63, 65, 66), which could further augment protein anabolism (6, 10, 27, 31, 32). Interestingly, a growing body of evidence indicates that amino acids can also function as potent nutritional signaling molecules, with an active regulatory function in muscle protein metabolism. It has been suggested that all essential amino acids (EAA) are responsible for the amino acid induced stimulation of muscle protein synthesis (92). Their presence stimulates protein synthesis, most

likely through stimulation of multiple steps in the mRNA translation process (3, 4). The branched-chain amino acids (30), and leucine in particular (3, 4), seem to have the highest potential to promote protein anabolism, by decreasing the rate of protein degradation and increasing muscle protein synthesis rates (54). The latter could partly be attributed to the insulinotropic potential of leucine, and the subsequent phosphorylation of key regulatory proteins that modulate protein synthesis (38, 48, 62). However, the anabolic effects of leucine have also been reported in the absence of elevated insulin concentrations. As such, leucine seems to be able to stimulate skeletal muscle protein synthesis through both insulin-dependent as well as insulin-independent mechanisms (3), which involve signaling through the mammalian target of rapamycin or mTOR (41). Despite many speculations and promising results from recent animal studies, there is hardly any evidence showing leucine supplementation to improve protein balance *in vivo* in humans.

Therefore, in chapter 5 and 6 we have investigated the potential of co-ingestion of leucine and protein with carbohydrate following resistance exercise or activities of daily living (ADL) to stimulate protein anabolism. Co-ingestion of leucine resulted in lower whole-body protein breakdown and oxidation rates and increased protein synthesis. The latter could partly be attributed to the higher plasma insulin concentrations following leucine co-ingestion. In line with previous studies (47, 52, 64), we showed that net protein balance can be improved by the ingestion of protein and carbohydrate in the post-exercise/ADL period. Moreover, in chapter 5 we have shown that the co-ingestion of leucine and protein with carbohydrate following resistance exercise resulted in higher muscle protein synthesis rates, compared to the ingestion of carbohydrate only. In addition, a greater whole-body protein balance was observed when compared to the ingestion of carbohydrate only and carbohydrate with protein (44). Nonetheless, muscle protein synthesis rates were similar with and without leucine when added to protein and carbohydrate. Although this study does not clearly demonstrate that leucine stimulates muscle protein synthesis *in vivo* in humans following resistance exercise, it demonstrates that protein anabolism following resistance exercise can be augmented with the ingestion of the leucine, protein and carbohydrate mixture.

As there is an overwhelming interest in the effects of leucine on muscle protein metabolism, numerous studies have been performed to investigate the pathways involved in (amino acid induced) protein synthesis and degradation. With the identification of leucine as a potent nutritional signaling molecule, most studies have focused on the effects of leucine to modulate these signaling events. However, it would be naive to assume that the regulatory role of leucine in protein metabolism is unique. Even though leucine seems to be most effective in stimulating protein anabolism (17), we hypothesize that most essential amino acids are likely to play a regulatory role in protein metabolism and that these are likely to be mutually synergistic in stimulating protein anabolism. Future research should define the most important essential amino acids with such a regulatory potential.

Optimizing protein anabolism in the elderly

Aging is associated with the progressive decline in skeletal muscle mass and function. The age-related changes in skeletal muscle mass are attributed to a disturbance in the regulation of muscle protein turnover, which results in a chronic imbalance between muscle protein synthesis and degradation. In chapter 6, we investigated the anabolic response to carbohydrate (CHO) ingestion following simulated ADL type activities *in vivo* in young (~20 y) and elderly men (~75 y). Consequently, we show that muscle protein synthesis rates are significantly lower in the elderly (~75 y) compared with the young (~20 y) under such conditions. The observed mixed muscle fractional synthetic rate (FSR) values following ADL activities were ~30% lower in the elderly compared with the young subjects. These observations are similar to previous reports showing muscle protein synthesis rates to be 20-30% lower in elderly humans (60-84 yrs) than in young adults in a basal, fasted state (34, 75, 100). Our findings on the muscle level are in line with our observations using the whole-body tracer methods showing whole-body phenylalanine and tyrosine turnover to be 20-25% lower in the elderly compared with the young. Thus, on a whole-body level, protein synthesis, oxidation and breakdown are reduced in the elderly compared with the young subjects. The reduction in whole-body protein turnover may indicate that tissue remodeling is impaired in the elderly. Muscle tissue remodeling, which involves the up- and/or down-regulation of specific sets of proteins (72, 73), is necessary to optimize muscle function for a new type of activity. The lower protein turnover rate in the elderly could indicate that the adaptive response to training is impaired. Consequently, it would be interesting to investigate whether there are differences in the specific sets of proteins that are up and/or down-regulated after exercise in young and elderly humans.

In accordance to our earlier findings (44), we observed that whole-body net protein balance remains negative when only carbohydrate is ingested. To our knowledge we are the first to show that muscle protein synthesis is reduced in the elderly under practical daily living conditions in which ADL activities are followed by ingestion of carbohydrate only. Interestingly, co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly men (chapter 6). This is in line with more recent observations, showing a similar response of muscle protein turnover to an amino acid load in healthy elderly and younger adults (57, 91, 94). However, our FSR data seem to be quite the opposite from data presented by Volpi et al (93), who showed that the response of muscle protein synthesis to the combined ingestion of amino acids and glucose is impaired in the elderly. The latter could be related to the blunted insulin response in the elderly compared with the young controls that were reported by Volpi et al (93). In contrast, we excluded subjects with impaired insulin sensitivity/glucose tolerance and we observed similar plasma insulin responses in the young and elderly volunteers following the co-ingestion of protein and leucine with carbohydrate. The observed differences might be attributed to the property of leucine to stimulate insulin secretion (88) and/or mRNA translation (2). In this study, we investigated the potential differences in the capacity to stimulate muscle

protein synthesis in the young and elderly following the ingestion of a theoretically optimal anabolic nutritional intervention strategy. The latter shows that the response to the intervention is of a similar magnitude in the young and elderly. However, our study does not demonstrate that leucine stimulates muscle protein synthesis *in vivo* in elderly humans. Whether additional leucine ingestion is more effective in promoting muscle anabolism than protein and carbohydrate only remains to be established in the elderly population. The latter will be addressed in one of our future studies.

Amino acids tracer considerations

Stable isotope amino acid tracers are widely used to study protein metabolism (96). With the continuous intravenous infusion of these tracers, and repeated sampling from the plasma pool, whole-body amino acid kinetics can be determined (i.e. the rate of appearance (Ra) and the rate of disappearance (Rd) of the amino acid of interest). In this model amino acid Rd from the blood compartment equals the rate of oxidation + protein synthesis, whereas amino acid Ra equals the rate of protein breakdown + the rate of appearance of amino acids from dietary protein from the gut (105).

The use of the (whole-body) tracer balance methodology to investigate the effect of post-exercise nutrition on protein metabolism has some important limitations as it is based on a few important assumptions. Two important assumptions include: 1) tissue amino acid pool size and enrichment are constant and 2) the amino acid tracer used is representative for the metabolism of the other amino acids. The first assumption indicates that the whole-body method (or any other compartmental analyses) is only valid when a steady state is present in the concentration and enrichment of the tracer amino acid in the plasma and muscle pool. However, after the oral ingestion of amino acids, it has been shown that amino acid concentrations increase substantially in the muscle free amino acid pool (15). The latter indicates that a significant portion of the plasma amino acid Rd results in muscle amino acid pool extension. This results in an overestimation of whole-body protein synthesis rates. Consequently, methods were introduced to measure tissue or protein-specific synthesis rates. In chapter 5 and 6, we have measured tracer incorporation in muscle protein as a means to directly measure mixed muscle protein synthesis rates.

The second assumption indicates that the whole-body method can only provide useful information upon whole-body protein turnover, when the applied amino acid tracer is a valid representative for the metabolic fate of all other amino acids. In early amino acid tracer studies, leucine was generally applied as a tracer (49, 53, 65, 76, 100, 106, 107). However, in chapter 4 we stress that care should be taken with the use of the leucine tracer to study protein metabolism during exercise. Leucine metabolism during exercise is not generally representative of the fate of the other amino acids that are present in proteins. During exercise, uptake (1, 82) and oxidation (82, 97, 98) of branched chain amino acids (leucine, isoleucine and valine) increase several fold in contracting muscle. The latter means that the

stochastic leucine model of whole-body protein metabolism (50) becomes invalid during exercise and cannot be used to estimate qualitative or quantitative changes in whole-body protein synthesis and/or degradation. Therefore, more recent studies from our (42, 44) and other laboratories (12, 60, 64, 80, 95) have used phenylalanine as a tracer to study protein metabolism. It has been shown that phenylalanine is not oxidized in rat skeletal muscle (19), which would mean that all phenylalanine that enters the muscle is used for muscle protein synthesis (7). This is one of the other basic assumptions of the three compartment model as proposed by Biolo et al (7). Interestingly, in chapters 5 and 6 we provide data that tend to proof this assumption wrong. In these chapters we infused labeled phenylalanine and measured phenylalanine and tyrosine enrichment in plasma and free muscle amino acid pool. Consequently, we observed ~60% higher tyrosine enrichments in the free muscle amino acid pool compared to values observed in plasma. This seems to suggest that phenylalanine is converted to tyrosine in skeletal muscle. The latter is in line with observations by van Hall et al. (83), showing that during one-legged exercise, tyrosine is released from the muscle in larger amounts than its relative occurrence in muscle protein and phenylalanine in smaller amounts. These and our findings may have major implications for the published data to date, as most tracer models are based on the assumption that phenylalanine is not broken down in the muscle. Consequently, these studies are likely overestimating muscle protein synthesis, and care should be taken with the interpretation of these data. The latter will be investigated in detail in future studies.

Regulation of protein synthesis: effects of exercise and nutrition

It has been shown that both muscle protein synthesis (8, 60, 79) as well as protein breakdown rates (8, 60) are accelerated following resistance exercise. Resistance exercise exerts its effect either via the increased local production of muscle specific IGF-1 or MGF (33) and/or other contraction induced signaling events. These factors can increase signaling of the PI-3 kinase/mTOR pathway resulting in an increase in muscle protein synthesis rates and glucose uptake. However, food intake is an essential factor to fully stimulate the activity of this signaling pathway. Although muscle protein synthesis is stimulated during the post-exercise recovery phase (60), net muscle protein balance remains negative in the absence of food intake (8, 60, 61). It has been shown that the administration of protein/amino acids with carbohydrate results in a rapid increase in muscle protein synthesis rates (chapter 5 and 6), whereas (whole-body) protein degradation rates are decreased (9, 15, 64, 79, 81).

Recent evidence suggests that the phosphatidylisositol 3-kinase (PI3-K) signaling pathway plays a key role in the integration of contraction mediated signaling (for example MGF) and signaling from nutritional factors (40, 70). The activity of the signaling cascade controlling muscle protein synthesis is largely determined by the phosphorylation of the mammalian target of rapamycin (mTOR) and its subsequent activation of the 70-kDa S6 protein kinase (p70-S6K) and the

eukaryotic initiation factor 4E-binding protein (4E-BP1) (40). In chapter 7, we report a marked increase in the phosphorylation of AMP-activated protein kinase (AMPK), indicating that resistance exercise leads to a reduction in the energy status of the muscle fiber, thereby stimulating the phosphorylation of skeletal muscle AMPK. AMPK activation in skeletal muscle tissue has been associated with a reduced phosphorylation state of mTOR at Ser^{2448}, p70-S6K at Thr^{389} and 4E-BP1 at Thr^{37} (13). More recently, it was proposed that activation of AMPK results in mTOR phosphorylation at Thr^{2446} (21), which decreases mTOR phosphorylation at Ser^{2448} (figure 8.2), thereby diminishing the ability of insulin (and other growth factors) to phosphorylate p70-S6K (21) during exercise. These observations are indicative of the presence of a metabolic switch mechanism in which AMPK plays a key role by integrating signals associated with the energy state of the cell and growth factors associated with protein translation.

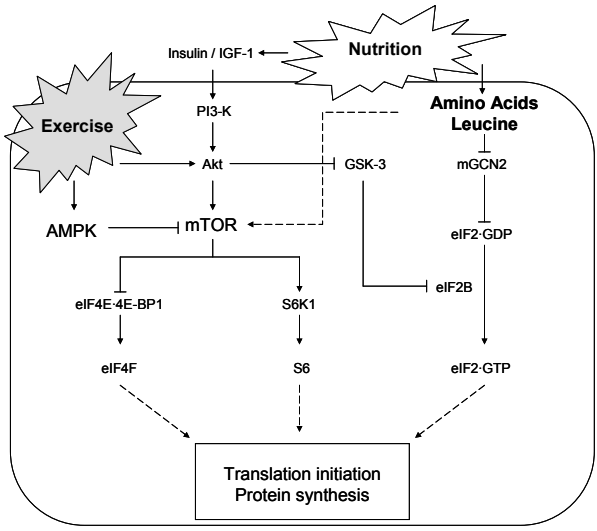

Figure 8.2 The integration of signals from exercise and nutritional factors. PI3-K, phosphatidylinositol-3 kinase; Akt, protein kinase B; AMPK AMP-activated kinase; mTOR, mammalian target of rapamycin protein kinase; eIF4E, eukaryotic initiation factor 4E; 4E-BP1, eIF4E binding protein 1; S6K1; p70/p85 ribosomal protein S6 protein kinase; eIF4F, eukaryotic initiation factor 4F; S6, ribosomal protein S6; GSK-3, glycogen synthase kinase 3; eIF2B, eukaryotic initiation factor 2B; eIF2·GDP, inactive eukaryotic initiation factor 2; eIF2·GTP, activated eukaryotic initiation factor 2.

Chapter 7 shows that a single session of high-intensity resistance exercise *in vivo* in humans significantly increases AMPK phosphorylation and reduces the phosphorylation status of 4E-BP1 in skeletal muscle tissue immediately after cessation of exercise. The exercise bout did not substantially increase S6 phosphorylation, indicating that p70-S6K activity was not increased to a large extent. Similar results were reported following resistance exercise in rats (14), and

S6K1 phosphorylation at Thr^{389} was shown to be unchanged. Our observations are also in agreement with data reported by Karlsson et al. (38), showing that a single session of resistance exercise (4 sets, 10 repetitions of leg extension) does not fully activate p70-S6K. However, they showed that post-exercise ingestion of branched chain amino acids has a striking effect on p70-S6K, which was evident by site-specific phosphorylation at Thr^{389} (38). These data indicate that intake of an ample amount of amino acids is essential to activate skeletal muscle translation initiation. Though translation initiation is likely to represent a main regulatory factor in modulating muscle protein synthesis, Proud and colleagues (16, 63) have shown that also the translation elongation process is under regulatory control by nutrients and hormones. The latter suggests that the anabolic response to exercise and/or nutrient intake can be controlled at various different levels (i.e. mRNA translation initiation, elongation, termination and post-translational modification). The relationship between activation of the molecular signaling pathways and *in vivo* muscle protein synthesis rates in response to exercise and nutrition clearly remains to be assessed *in vivo* in human studies.

In chapter 5 and 6 we investigated the effect of post-exercise intake of carbohydrate, protein and free leucine and showed that muscle protein synthesis rates were substantially increased when leucine was co-ingested with protein and carbohydrate when compared with the ingestion of carbohydrate only. Although these studies do not demonstrate that leucine specifically stimulates muscle protein synthesis *in vivo* in humans following resistance exercise/ADL activities, it demonstrates that protein anabolism following resistance exercise can be augmented with the ingestion of a leucine, protein and carbohydrate mixture. Studies in rats have increased the understanding of the mechanisms of nutritional regulation of protein synthesis at the molecular level and indicate that leucine ingestion could stimulate muscle anabolism in several ways, as it can further increase plasma insulin concentrations and also directly stimulate protein synthesis in an insulin independent manner. The stimulatory effect of leucine on protein synthesis occurs at the level of translation initiation and involves signaling through mTOR (41, 62), and subsequent phosphorylation and activation of 4E-BP1, S6K1 and ribosomal protein S6 (figure 8.2). The protein kinase mTOR is thought to serve as a convergence point for leucine- and insulin-mediated effects on translation initiation (41, 62). Furthermore, leucine has been shown to have the potential to affect muscle protein metabolism by decreasing the rate of protein degradation (54), most likely via an increase in circulating insulin (38, 48, 62). However, not only leucine has the potential to stimulate muscle protein synthesis, as intramuscular and plasma essential amino acid availability has been shown to play an important regulatory role in protein anabolism (12, 92). In addition to increased signaling through mTOR, an increase in essential amino acid availability results in a decreased phosphorylation of the eukaryotic initiation factor 2 (eIF2). The phosphorylation of the eIF2 (α-subunit) is mediated by the mammalian ortholog of the yeast general control nonderepressing kinase 2 (mGCN2), which responds to changes in (essential) amino acid availability. Decrease in phosphorylation of eIF2α leads to the dissociation and activation of the eukaryotic

initiation factor 2B (eIF2B), thereby increasing the translation of most mRNAs (Fig 2).
Data provided in chapters 4-6 in this thesis, show that the combination of exercise and nutrition can effectively stimulate (muscle) protein synthesis. There is an increasing body of evidence showing that the PI3-Kinase signaling pathway is likely to play a key role in the integration of contraction mediated signaling and signaling from nutritional factors. Most of the research has been performed using *in vitro* and *in vivo* animal models. In these models, exercise or electrical stimulation have been shown to increase signaling through the PI3-kinase/mTOR pathway resulting in increases in protein synthesis and glucose uptake. In addition to exercise, ingestion of amino acids, and leucine in particular, has been shown to effectively increase mTOR phosphorylation and the activation of translation. For now, there is no human data available to assess the proposed stimulating effects of leucine on skeletal muscle protein synthesis.

General conclusions

In this thesis, we focused on the beneficial effects of resistance exercise and the potential of dietary supplementation with carbohydrate, protein and leucine to enhance muscle protein anabolism. Resistance exercise was shown to be as powerful as endurance exercise in enhancing post-exercise insulin sensitivity. In addition, we reported that a single resistance exercise session substantially reduced intramyocellular glycogen content in a muscle fiber-type specific manner. The latter suggests that the acute improvements in whole-body insulin sensitivity following resistance exercise could, at least partly, be attributed to the net reduction in skeletal muscle glycogen content. Additionally, we showed that immediately after resistance exercise AMPK phosphorylation is increased. This could be the initial trigger to increase glucose uptake and to simultaneously inhibit mRNA translation initiation (and thus protein synthesis). Although S6K1 phosphorylation was increased in a muscle fiber-type specific manner following resistance exercise, the phosphorylation of S6 was not substantially increased under fasting conditions.
As such, in the absence of food intake, exercise does not fully activate the translation initiation process in skeletal muscle tissue. Therefore, the combined ingestion of protein and carbohydrate has been suggested as a more effective strategy to promote net muscle protein accretion, as it can stimulate protein metabolism by providing amino acids as precursors for protein synthesis and further elevate plasma insulin levels and reduce protein breakdown. We showed that prolonged exhaustive moderate intensity endurance exercise does not result in increased protein degradation and/or a decrease in protein synthesis compared with resting situations when carbohydrates are ingested. However, protein intake is necessary to increase protein synthesis and further decrease protein breakdown, resulting in a positive net protein balance.
Amino acids have more metabolic roles than to merely serve as building blocks for proteins. They function as potent nutritional signaling molecules, and have an

active regulatory role in modulating muscle protein metabolism. The branched-chain amino acid leucine seems to have the potential to promote protein anabolism. Despite many speculations and promising results from recent animal studies, there is hardly any evidence showing leucine supplementation to improve protein balance *in vivo* in humans. Therefore, we investigated the effect of post-exercise intake of leucine and showed that muscle protein synthesis rates were substantially increased when leucine was co-ingested with protein and carbohydrate compared with the ingestion of carbohydrate only.
These observations have lead to the hypothesis that physical activity followed by the ingestion of leucine with protein and carbohydrate could represent an effective strategy to reduce or counteract the loss of muscle tissue that occurs with aging. Consequently, we demonstrated that co-ingestion of protein and leucine with carbohydrate improves whole-body protein balance when compared with the ingestion of carbohydrate only in young and elderly men. Mixed muscle protein synthesis was shown to be reduced in the elderly when only carbohydrate was ingested. However, co-ingestion of protein and leucine substantially increases mixed muscle protein synthesis rates to the same level in the young and elderly, thereby improving whole-body protein balance. The present data indicate that the combined ingestion of carbohydrate, protein and leucine represents an effective strategy to increase muscle anabolism following resistance exercise or ADL activities in young and elderly humans.

Future research

The studies described in this thesis provide novel information on the beneficial effects of resistance exercise and the use of dietary supplements containing carbohydrate, protein and/or leucine as means to optimize post-exercise muscle protein anabolism *in vivo* in humans. However, there are many questions that still need to be addressed in future research:

* Is the combined ingestion of leucine and protein with carbohydrate more effective in stimulating protein synthesis compared to carbohydrate and protein ingestion only?
* Is phenylalanine converted to tyrosine in human skeletal muscle?
* Which proteins are synthesized at a higher rate following exercise and are there differences in the specific sets of proteins that are synthesized after resistance and endurance exercise as well as between young and elderly humans?
* What is the effect of protein ingestion following exercise on the specific sets of proteins that are synthesized and are there differences between young and elderly humans?
* Do different types of proteins differ in ability to stimulate muscle protein anabolism?
* What is the relationship between the activation of intracellular signaling and *in vivo* muscle protein synthesis?

* What signaling pathways that regulate muscle protein synthesis are impaired in the elderly?
* What is the time course of activation of the key kinases leading to protein anabolism and remodeling and does the activation window depend on the timing of nutrient intake and composition?

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Summary

Skeletal muscle is one of the major organs involved in metabolism. Aging and chronic metabolic diseases, like type 2 diabetes, are generally associated with a significant loss of skeletal muscle mass. Muscle wasting eventually leads to muscle weakness and major disabilities in day-to-day life. Therefore, various interventions have been proposed to reduce or reverse the loss of muscle mass and function. Most attention so far was paid to endurance type of exercise. In this thesis we focus on the beneficial effects of resistance exercise and the proposed importance of the ingestion of carbohydrate, protein and/or specific amino acids as a means to augment muscle protein anabolism.

In **chapter 2**, we report that a single resistance exercise session can improve whole-body insulin sensitivity by as much as 15% when measured 24h after cessation of exercise. The latter tends to be of a similar magnitude as the ~20% improvement that has been reported in literature following an acute (~60 min) bout of endurance exercise. The presented data indicate that there should be no restriction in combining the benefits of both types of exercise in future lifestyle intervention programs. The greater whole-body insulin sensitivity following endurance exercise has been associated with the reduction of muscle substrate sources following exercise. However, information on the metabolic demands of resistance exercise is scarce. In **chapter 3**, it is shown that mixed muscle glycogen content is substantially decreased following a single resistance exercise session. These changes in muscle glycogen are shown to be fiber-type specific, and tend to be more pronounced in type IIx muscle fibers compared to type I muscle fibers. Furthermore, intramyocellular lipid content is reduced in the type I muscle fibers only and returns to baseline values within 2h of post-exercise recovery. The absence of a prolonged net reduction in skeletal muscle lipid content implies that the reported stimulating effects of resistance exercise on whole-body insulin sensitivity is unlikely to be attributed to a temporary reduction in intramyocellular lipid content.

In **chapters 4, 5** and **6** the effects of exercise or activities of daily living and nutrition on protein metabolism are evaluated. In these studies, continuous infusions of labeled amino acids are applied to measure their rate of appearance and/or disappearance in blood. With this method we can estimate protein breakdown, synthesis, oxidation and net-balance on a whole-body level. In **chapters 5** and **6** we also measure the rate of incorporation of a labeled amino acid into skeletal muscle protein to directly assess mixed muscle protein synthesis rates.

In **chapter 4**, different tracer methods are compared with the intention to assess whole-body protein turnover, during and following 6h of prolonged endurance exercise when carbohydrate or carbohydrate and protein are ingested. Discrepancies are shown when applying [$^{2}H_{5}$]phenylalanine and/or [^{15}N]urea and the [1-^{13}C]leucine tracer methods during exercise. These discrepancies are likely attributed to the fact that during exercise the uptake and oxidation of leucine is increased several fold. Therefore, leucine is likely not representative of the fate of the other essential amino acids during exercise. With phenylalanine and urea as tracers it is demonstrated that net protein degradation does not increase during moderate intensity exercise when carbohydrate is ingested. However, under these

conditions, protein net balance remains negative. The combined ingestion of carbohydrate and protein is shown to improve net protein balance at rest, during exercise and post-exercise recovery.

In **chapters 5** and **6** the effects of amino acid and/or protein (hydrolysate) ingestion on muscle protein synthesis are investigated. The combined ingestion of carbohydrate, protein and leucine stimulates protein anabolism in several ways. It provides amino acids as precursors for protein synthesis and elicits a strong insulinotropic response. In addition, in animal models, leucine has been shown to stimulate protein synthesis by an insulin-independent mechanism. In **chapter 5**, it is shown that the combined ingestion of carbohydrate (0.3 $g \cdot kg^{-1} \cdot h^{-1}$), protein hydrolysate (0.2 $g \cdot kg^{-1} \cdot h^{-1}$) and leucine (0.1 $g \cdot kg^{-1} \cdot h^{-1}$) following resistance exercise substantially augments the plasma insulin response. The combined ingestion of this mixture reduces whole-body protein breakdown and oxidation rates and increases protein synthesis rates. The net protein balance is negative when only carbohydrate is ingested, positive with carbohydrate and protein and improves further following the combined ingestion of carbohydrate, protein and leucine. In addition, muscle protein synthesis rates are shown to be significantly higher when carbohydrate, protein and leucine are ingested. These data suggest that the additional ingestion of free leucine in combination with protein and carbohydrate likely represents an effective strategy to increase muscle protein anabolism following resistance exercise.

The progressive loss of skeletal muscle mass with aging, or sarcopenia, is partly attributed to a disruption in the regulation of skeletal muscle protein turnover. The anabolic effect of food intake on muscle protein synthesis seems to be blunted in the elderly. The latter has been proposed to play a key-role in the etiology of sarcopenia. In **chapter 6**, potential differences in the response to the ingestion of carbohydrate (0.49 $g \cdot kg^{-1} \cdot h^{-1}$) with or without protein hydrolysate (0.16 $g \cdot kg^{-1} \cdot h^{-1}$) and free leucine (0.03 $g \cdot kg^{-1} \cdot h^{-1}$) following simulated activities of daily living (ADL) on whole-body protein balance and mixed muscle protein synthesis rates are investigated in both young and elderly men. Whole-body phenylalanine and tyrosine fluxes are shown to be significantly higher in the young versus the elderly, indicative of a slower protein turnover rate in the elderly. Protein balance is shown to be negative when only carbohydrate is ingested, but positive when both protein and leucine are co-ingested in both groups. Muscle protein synthesis rates are shown to be substantially lower in elderly compared with young adults when only carbohydrates are ingested. Combined ingestion of protein hydrolysate and leucine with carbohydrate increases muscle protein synthesis rates and improves net protein balance to the same extent in young and elderly men. These data indicate that the anabolic properties of this nutritional intervention are equally effective in young and elderly men.

The rate of muscle protein synthesis is regulated in part by the process of mRNA translation initiation. The latter has been shown to be regulated by the posphatidylinositol 3 kinase signaling pathway, with major regulatory roles for the mammalian target of rapamycin (mTOR), S6 protein kinase 1 (S6K1) and the eukaryotic initiation factor 4E-binding protein (4E-BP1). In **chapter 7**, the effects

of a single session of resistance exercise on the phosphorylation status of several proteins involved in the regulation of muscle protein synthesis are assessed. In addition, it is observed that changes in the phosphorylation status are muscle fiber-type specific. Resistance exercise results in a strong activation of AMP activated kinase (AMPK), while 4E-BP1 phosphorylation is reduced, suggesting that translation initiation is blunted immediately following resistance exercise. During recovery, S6K1 phosphorylation increases but this is shown to occur predominantly in the fast twitch type II muscle fibers. The observation that S6 phosphorylation does not substantially change when subjects remain fasted implies that the ingestion of protein/amino acids is essential to fully activate the translational machinery.
In the final chapter, the significance and clinical relevance of the acquired data are discussed and important aims for future research are defined. In addition, specific methodological issues that need to be considered in future studies are also mentioned.

In summary, resistance exercise reduces muscle glycogen stores in a fiber-type specific manner, which could partly be responsible for the observed increase in insulin sensitivity following exercise. Although resistance exercise increases S6K1 phosphorylation in a fiber-type specific manner, S6 phosphorylation does not increase under fasting conditions. The latter indicates that food intake following exercise is needed to fully activate mRNA translation initiation and muscle protein synthesis. In contrast to common belief, endurance exercise does not increase protein degradation and oxidation when only carbohydrates are ingestion. However, the intake of protein/amino acids is necessary to attain a net positive protein balance. Amino acids do not only serve as building blocks for *de novo* protein synthesis. Amino acids, and leucine in particular, seem to be able to function as signaling molecules with an important regulatory role in muscle protein metabolism. The combined intake of leucine and protein hydrolysates with carbohydrate following resistance exercise can effectively stimulate muscle protein synthesis. These observations have lead to the hypothesis that physical activity followed by the ingestion of leucine with protein hydrolysate and carbohydrate could represent an effective strategy to reduce or counteract the loss of muscle tissue that occurs with aging. Co-ingestion of protein hydrolysate and leucine substantially increases mixed muscle protein synthesis rates to the same level in the young and elderly, thereby improving whole-body protein balance. The present data indicate that the combined ingestion of carbohydrate, protein and leucine represents an effective strategy to increase muscle protein anabolism following resistance exercise or ADL activities in young and elderly humans.

Samenvatting

De skeletspier is één van de belangrijkste organen die betrokken is bij de regulatie van het metabolisme. Veroudering en chronische metabole ziekten zoals diabetes zijn geassocieerd met een sterk verlies aan spiermassa. Dit heeft tot gevolg dat spierkracht en uithoudingsvermogen afneemt, waardoor de functionele capaciteit van de patiënt achteruit gaat. Om deze afname van de spiermassa tegen te gaan zijn verschillende interventies voorgesteld. Tot nu toe is er vooral aandacht geweest van de effecten van duurtrainingen. In dit proefschrift wordt aandacht besteed aan de voordelen van het uitvoeren van krachttraining en de mogelijkheden van de gecombineerde inname van koolhydraten, eiwithydrolysaten en leucine om de spieraanwas te stimuleren.

In **hoofdstuk 2** wordt aangetoond dat de insuline gevoeligheid, 24 uur na één enkele krachttraining met ongeveer 15% kan worden verbeterd. Dit is vergelijkbaar met de resultaten die gerapporteerd worden na het uitvoeren van één enkele duurtraining. De uitkomsten van deze studie geven dus aan dat er geen beperking behoeft te bestaan in het implementeren van beide vormen van inspanning in toekomstige interventieprogramma's. De verbeterde insuline gevoeligheid na inspanning wordt vaak geassocieerd met het gebruik van substraatvoorraden in de spier tijdens inspanning. Echter, er is eigenlijk nauwelijks iets bekend over hoeveel van die voorraden tijdens krachttraining gebruik wordt. In **hoofdstuk 3** wordt aangetoond dat de spierglycogeen voorraad sterk gedaald is na een krachttrainings-sessie. De verlaging in glycogeen voorraad blijkt daarnaast spiervezelspecifiek te zijn. De daling is namelijk groter in de snelle type IIx vezels in vergelijking met de langzamere type I vezels. Verder daalt ook de vetvoorraad in de spiervezels. Dit gebeurt echter selectief in de langzame vezels (type I) en 2 uur na inspanning is de vetvoorraad alweer aangevuld tot basale hoeveelheden. Het feit dat de vetvoorraden in de spier na krachttraining niet verlaagd worden voor langere tijd, geeft aan dat de gerapporteerde toename in insuline gevoeligheid na dit soort inspanning niet kan worden toegeschreven aan een daling in vetvoorraden in de spier.

In de **hoofdstukken 4, 5** en **6** zijn de effecten van fysieke inspanning en voedingsinterventies op het eiwitmetabolisme bestudeerd. In deze studies worden proefpersonen geïnfuseerd met gelabelde aminozuren. Op de wijze kan bepaald worden hoe snel aminozuren in het bloed verschijnen en hoe snel ze in weefsels verdwijnen. Met behulp van deze techniek kan op heel lichaamsniveau de eiwitaanmaak, afbraak, oxidatie en de netto balans uitgerekend worden. In **hoofdstuk 5** en **6** is ook nog gemeten hoe snel het gelabelde aminozuur wordt ingebouwd in spierweefsel om op die manier de snelheid te kunnen bepalen waarmee spiereiwit wordt aangemaakt.

In **hoofdstuk 4** worden verschillende gelabelde aminozuren (tracers) gebruikt om het eiwitmetabolisme voor, tijdens en na een 6 uur lange duurtraining in kaart te kunnen brengen terwijl de proefpersonen continue koolhydraten of koolhydraten en eiwitten innamen. Er bestaan duidelijke verschillen in de uitkomsten die verkregen zijn met de [$^{2}H_{5}$]phenylalanine en [^{15}N]ureum tracer vergeleken met de [1-^{13}C]leucine tracer tijdens inspanning. Dit verschil wordt waarschijnlijk veroorzaakt door het feit dat de leucine opname en oxidatie tijdens inspanning

sterk verhoogd wordt, terwijl dat met andere aminozuren niet gebeurd. Hierdoor is leucine niet representatief voor het lot van de andere essentiële aminozuren tijdens inspanning. Met de phenylalanine en ureum tracers is aangetoond dat tijdens ultra lange duurinspanning de eiwitafbraak en oxidatie niet verhoogd is wanneer koolhydraten worden ingenomen gedurende de inspanning. Echter onder deze condities is de netto eiwitbalans negatief. Uit deze studie blijkt dat de inname van een koolhydraat en eiwitmengsel ervoor zorgt dat de netto eiwitbalans sterk positief wordt.

In de **hoofdstukken 5** en **6** wordt het effect van de inname van eiwit/aminozuren op de spiereiwitsynthese bepaald. Deze inname van een mengsel met koolhydraten, eiwithydrolysaten en leucine kan op verschillende manieren de eiwitaanmaak stimuleren. Het levert bouwstenen (aminozuren) voor de aanmaak van eiwit en kan de insuline afgifte door de alvleesklier sterk verhogen. Verder blijkt uit dierenonderzoek dat het essentiële aminozuur leucine de unieke eigenschap heeft om direct de eiwitsynthese te stimuleren via een insuline onafhankelijk mechanisme. In **hoofdstuk 5** wordt aangetoond dat de gecombineerde inname van koolhydraten (0.3 g/kg/u), eiwit (0.2 g/kg/u) en leucine (0.1 g/kg/u) na een krachttraining leidt tot een sterke stijging in de plasma insuline concentratie, wanneer deze wordt vergeleken met de inname van koolhydraat met of zonder eiwit. De eiwitafbraak en oxidatie wordt verlaagd terwijl de eiwitaanmaak wordt verhoogd na de inname van de koolhydraat, eiwit en leucine drank. De netto eiwitbalans op heel lichaamsniveau is negatief wanneer alleen koolhydraten worden ingenomen, positief wanneer eiwithydrolysaat aan de drank wordt toegevoegd en nog verder verhoogd wanneer ook leucine wordt toegevoegd. De spiereiwitsynthese is significant hoger wanneer koolhydraat, eiwithydrolysaat en leucine wordt ingenomen vergeleken met de inname van koolhydraten alleen. Deze data lijkt te suggereren dat de inname van eiwithydrolysaat en leucine met koolhydraten een effectieve strategie is om de spiereiwitaanmaak te verhogen na krachttraining.

Het verlies van spiermassa dat optreedt naarmate men ouder wordt, ook wel sarcopenie genoemd, wordt gedeeltelijk veroorzaakt door een verstoorde regulatie van de spiereiwitsynthese en afbraak. Bijvoorbeeld, het stimulerende effect van voedselinname op de spiereiwitsynthese lijkt bij ouderen verminderd te zijn. Dit wordt gezien als een van de belangrijkste factoren in de etiologie van sarcopenie. In **hoofdstuk 6** wordt gekeken of er verschillen zijn in eiwit balans en spiereiwitsynthese na normale dagelijkse activiteiten met de inname van koolhydraten (0.49 g/kg/u) met of zonder eiwithydrolysaat (0.16 g/kg/u) en vrije leucine (0.03 g/kg/u) tussen oudere (± 75 jaar) en jongere (± 21 jaar) mensen. De flux van phenylalanine en tyrosine blijkt lager te zijn in de ouderen wat impliceert dat de eiwitturnover (aanmaak en afbraak) lager is in de oudere proefpersonen. Wanneer alleen koolhydraten worden ingenomen is de eiwitbalans op heel-lichaamsniveau negatief, terwijl hij positief is na de inname van leucine en eiwit met koolhydraten. De spiereiwitsynthese is significant lager in de oudere personen in vergelijking met de jongeren. De gecombineerde inname van eiwit en leucine met koolhydraten verhoogt de eiwitsynthese snelheid in zowel de jongeren als de

ouderen. Sterker nog, de spiereiwitaanmaak wordt net zo sterk gestimuleerd door de inname van deze drank in de ouderen als in de jongeren. Dit betekent dat de anabole capaciteit van deze voedingsinterventie net zo groot is in ouderen als in jongeren.
De snelheid van spiereiwitaanmaak wordt gereguleerd door een proces genaamd translatie initiatie dat ervoor zorgt dat begonnen kan worden met het aflezen van mRNA en daarna eiwit gevormd kan worden. Dit proces wordt gereguleerd door de phophatidylinositol-3 kinase signaalcascade. De belangrijkste enzymen/kinases in deze keten zijn de proteïne kinase mTOR, S6 proteïne kinase 1 (S6K1) en eukaryotische initiatie factor 4E-bindingsproteïne (4E-BP1). In **hoofdstuk 7** wordt het effect van één enkele krachttraining op de fosforylatiestatus van verschillende eiwitten gemeten die betrokken zijn in de regulatie van de spiereiwitsynthese snelheid. Het blijkt dat veranderingen in fosforylatie van S6K1 spiervezeltype afhankelijk zijn. Deze fosforylatie treedt vooral op in snelle type II spiervezels. De krachttraining resulteert in een sterke activering van de AMP geactiveerde kinase (AMPK), terwijl 4E-BP1 fosforylatie verlaagd is. Dit impliceert dat direct na inspanning translatie initiatie geremd is. Gedurende het herstel neemt de mate van S6K1 fosforylatie toe, terwijl de fosforylatie van S6 niet verandert wanneer proefpersonen nuchter blijven. Deze data suggereert dat de inname van voldoende eiwitten nodig is om de translatie initiatie optimaal te laten verlopen.
In het laatste hoofdstuk wordt het belang van de behaalde onderzoeksresultaten besproken en hun klinische relevantie voor de oudere populatie bediscussieerd. Verder worden belangrijke doelen voor toekomstig onderzoek gedefinieerd. Ook worden specifieke methodologische problemen waarmee rekening moet worden gehouden in toekomstig onderzoek aangehaald.

Samenvattend, krachttraining leidt tot een spiervezelspecifieke verlaging van de glycogeenvoorraad in de spier, wat gedeeltelijk verantwoordelijk is voor de verhoogde insuline gevoeligheid na inspanning. Hoewel krachttraining de fosforylatie van S6K1 verhoogt op een spiervezelspecifieke manier, blijft de fosforylatie van S6 onveranderd wanneer men gevast blijft. Dit geeft aan dat de inname van voedsel na het sporten essentieel is om de mRNA translatie initiatie en de aanmaak van spiereiwit volledig te activeren. Hoewel over het algemeen gedacht wordt dat duurtraining leidt tot een toegenomen eiwit afbraak en oxidatie, blijkt dit niet het geval te zijn wanneer koolhydraten worden ingenomen. Om een positieve netto eiwitbalans te verkrijgen moet men naast koolhydraten echter ook eiwitten/aminozuren innemen. Aminozuren vormen niet alleen de bouwstenen voor nieuwe eiwitten, ze kunnen ook functioneren als signaal moleculen die een belangrijke regulerende rol in het eiwitmetabolisme kunnen spelen. Dit geldt vooral voor het aminozuur leucine. De gecombineerde inname van leucine, eiwithydrolysaat en koolhydraten na een krachttraining blijkt effectief in het stimuleren van de spiereiwitsynthese. Deze observatie heeft tot de hypothese geleid dat fysieke activiteit gevolgd door de inname van leucine, eiwit en koolhydraten een effectieve strategie kan zijn om het verlies van spiermassa bij ouderen te verminderen of te voorkomen. Het blijkt dat de gecombineerde inname van

leucine, eiwit en koolhydraten de spiereiwit-aanmaak in jongeren en ouderen in dezelfde mate stimuleert, wat leidt tot een verbeterde netto eiwit balans. Deze data geven aan dat de gecombineerde inname van leucine, eiwit en koolhydraten een effectieve strategie is om de spiereiwit-aanmaak te stimuleren in de eerste uren na een krachttraining of in de periode na een verhoogd dagelijkse activiteiten patroon in jonge en oudere mensen.

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Curriculum Vitae

René Koopman was born on February 9th 1979 in Rotterdam, the Netherlands. In 1997, he completed his pre-university education at the Rijks Scholengemeenschap in Middelharnis. He started his study Health Sciences at Maastricht University in 1997. He graduated in the field of Movement Sciences in 2001 after an internship with Prof. Wagenmakers and Dr. van Loon at Maastricht University, after which he worked as a research assistant at the Department of Human Biology, Maastricht University. During this period he acquired practical experience with the use of (immuno)histochemical analyses.
In Februari 2002, he started as a PhD student at the department of Human Biology at Maastricht University. In 2005, he was awarded by the Nutrition Interest Group of the American College of Sports Medicine with the international research award for his work on nutritional interventions to promote post-exercise protein synthesis. In 2005 he was awarded with an International Training Fellowship Grant from the Nutricia Research Foundation to visit the Human Nutrition Laboratory (CRNH/INRA) in Clermont-Ferrand, France. There he will have the opportunity to acquire expertise in the use and application of molecular biology techniques and measurement of protein synthesis rates in different muscle protein fractions.
René Koopman is now appointed as a staff member at the Department of Movement Sciences. He continues his work on the stimulation of protein anabolism in the young and elderly by nutrition. Changes in protein metabolism are investigated on a whole-body, tissue and cellular level, with skeletal muscle as the main tissue of interest using a combination of stable isotope methodology, immuno-histochemistry and molecular biology methods.

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