NUTRITIONAL IMPACT ON MOLECULAR AND PHYSIOLOGICAL ADAPTATIONS TO EXERCISE

nutrition matters

Pim Knuiman

PROPOSITIONS

1. Carbohydrate availability is immaterial in the acute adaptive response to resistance.
   (this thesis)

2. Protein supplementation enhances molecular and physiological adaptation to endurance training.
   (this thesis)

3. Variability of endurance trainability weakens science-based endurance training recommendations.

4. The extrapolation of experimental findings from studies on acute effects of exercise to potential mechanisms of chronic adaptation requires caution.

5. Chronic testosterone administration should be advised by general practitioners for hypogonadal older male adults.

6. Scientific reviewers providing comments like "this finding is not interesting or surprising" should read crime novels instead of scientific papers.

7. Perseverance and risk taking are key to achieving success in science.

Propositions belonging to the thesis, entitled:

Nutritional Impact on Molecular and Physiological Adaptations to Exercise

Pim Knuiman
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Chapter 1

General introduction

Exercise training and skeletal muscle adaptation

Multiple exercise bouts within a certain period of time are generally referred to as exercise training. Exercise training can be simply divided into endurance- and resistance exercise. At the musculoskeletal level, endurance exercise training increases mitochondrial and capillary density together contributing to enhanced oxidative capacity (1). In contrast, general adaptations observed with resistance exercise training and anaerobic type of exercise such as high intensity power training are an increase in muscle fibre size, strength and power (2). In humans, approximately 40 - 50% of body mass consist of skeletal muscle tissue (3). Skeletal muscle is therefore the largest tissue in humans and likely the tissue that is most affected by contractile-activity stimuli such as exercise (4). The plasticity of skeletal muscle tissue in response to exercise training and altered nutrient availability is remarkable and demonstrated by its phenotypic adaptation over time (5, 6). Phenotypic adaptation can be defined as the changes in the body as a result of environmental changes such as exercise training. From a mechanistic perspective, the exercise-induced change in metabolic and mechanical factors, the so-called “acute exercise response” that disturb cellular homeostasis is likely explanatory for the phenotypic alteration. Cellular homeostatic disturbances begin with the onset of exercise and persist until the end of the post-exercise recovery phase (5). Furthermore, it is the current thought among molecular exercise physiologists that repeated exercise bouts causes a variety of biological events thereby affecting gene expression profiles and subsequently specific proteins required for phenotypic adaptation. This thought is substantiated by previous work demonstrating that structural and metabolic adaptations during exercise training is the outcome of the sum of the biological effects of each exercise bout (7-9).

Skeletal muscle adaptation and nutrition

The acute molecular responses during and after cessation of an exercise bout, such as changes in gene expression and protein levels and enzyme activity appears to be a fundamental process in the phenotypic adaptation to exercise training (1). At the molecular level, exercise affects gene expression profiles, augments signalling pathways thereby enhancing mRNA translation and the synthesis of particular proteins. The timespan of adaptive response ranges from acute (e.g. immediately during/after exercise) to more prolonged (e.g. weeks/months) (8), and can be influenced by nutrition (e.g. carbohydrates or proteins) **(Figure 1)**.

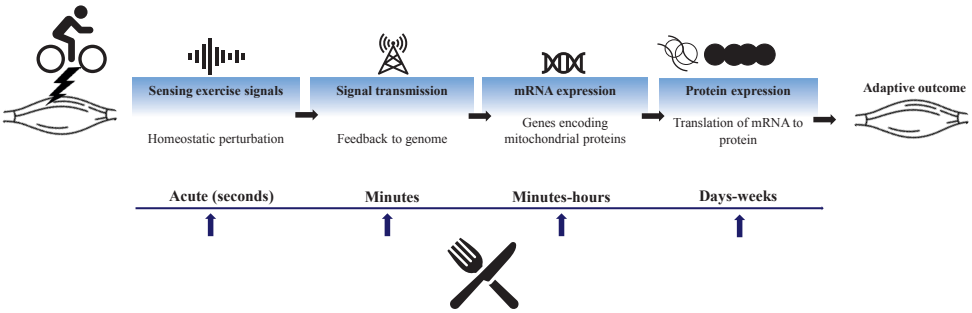

Figure 1. General model based on the signal transduction hypothesis for adaptation to endurance exercise training (1, 5, 10), highlighting the importance nutrition. Homeostatic perturbation: Ca2+, pO_2, AMP, glycogen; Signal transmission: ↑AMPf → AMPK, Ca^{2+} → CaMK, Ca^{2+} → p38/MAPK; mRNA expression: transcription factors and coactivators, genes encoding specific protein; Protein expression: translation of mRNA to protein, import and assembly and expansion of reticulum (enhanced capacity); Skeletal muscle adaptation: increase in mitochondrial density/function.

Carbohydrates and fat

It is widely accepted that nutrition plays a central role in mediating skeletal muscle adaptations with exercise training (11, 12). Carbohydrates and fat are recognized as the main substrates for facilitating energy requirements during endurance exercise (13). Carbohydrates are stored as glycogen in muscle and liver cells. The relative use of energy sources during exercise is mainly determined by the intensity and the duration of the exercise bout, as well as the athlete's training status (13). Fat as source of energy is relatively most dominant during low- to moderate intensity exercise (30-65% of VO_{2max}), whereas the relative contribution of carbohydrate oxidation to total energy expenditure becomes greater when exercise intensity increases, with muscle glycogen becoming the most important substrate source (14). Although the amount of liver and skeletal muscle glycogen is relatively small compared to stored fat, glycogen is recognized as the major source for fuel during prolonged moderate- to high intensity endurance exercise (15).

Protein

Skeletal muscle mass is maintained by the balance between muscle protein synthesis and muscle protein breakdown rates such that overall net muscle protein balance remains essentially unchanged over the course of the day. A prolonged imbalance between muscle protein synthesis and muscle protein breakdown results in a positive (hypertrophy: muscle protein synthesis > muscle protein breakdown) or negative net protein balance (atrophy: muscle protein synthesis < muscle protein breakdown). The two main potent stimuli

for muscle protein synthesis are food ingestion and exercise (16). Food ingestion (e.g. dietary protein) causes a transient stimulation of muscle protein synthesis, but cannot account for muscle protein accretion alone. Likewise, endurance and/or resistance exercise improves net protein balance, however, the ingestion of protein during the post-exercise recovery period is required to induce a positive net protein balance (17). Thus, both exercise and protein ingestion must be deployed in combination in order to create a positive net protein balance (16).

Empirical shortcomings: carbohydrate availability and adaptation to resistance exercise

It is well known that carbohydrate intake, and hence glucose availability, after exercise is of great importance for replenishing liver and muscle stores and subsequent exercise performance (18). Similarly, the ability of protein or amino acid intake to stimulate muscle protein synthesis, and to reduce, to a lesser extent, protein breakdown promotes net muscle growth (19, 20). The molecular events activated by exercise show some similarity with the molecular events that can be activated by altered nutrient availability. For instance, the phosphatidylinositol 3-kinase/Akt/mechanistic target of rapamycin signalling pathway (PI3k-Akt-mTOR) and 5'adenosine monophosphate-activated protein kinase (AMPK) are targets for both exercise and nutrient availability (5, 21). Yet, most studies in the field of exercise training and nutrient availability have largely focused on either carbohydrates with endurance exercise training or proteins with resistance exercise training. This scientific paradigm has resulted in empirical shortcomings in the field of nutrient availability and exercise training. Indeed, literature on the role of carbohydrates with resistance exercise training and protein with endurance exercise training is scarce (22-26).

Carbohydrate availability and resistance exercise

During the past decades, the role of carbohydrate availability with endurance training and performance has been intensively studied. Scientific consensus has been achieved on the facilitating role of carbohydrates as a key substrate during endurance exercise as well as a mediator for numerous molecular responses to a single bout of endurance exercise (12, 23). In addition, it is generally well accepted that high habitual carbohydrate intake is beneficial for endurance training performance, in particular that of prolonged and high-intensity (27, 28). In contrast, the role of carbohydrate availability with resistance exercise on cellular responses, phenotypic adaptation and performance has yet to be fully characterised. Theoretically, based on molecular events underpinning resistance exercise adaptation, it could be argued that carbohydrate intake may not be of great importance to maximise adaptation, as well as the importance of reduced muscle glycogen levels.

Indeed, current evidence indicates that the key kinase PI3k-Akt-mTOR signalling pathway is unaffected by limited carbohydrate availability or low skeletal muscle glycogen concentrations. A reasonable approach to tackle this empirical shortcoming is a shift in the scientific focus; more research focusing on carbohydrate availability with resistance exercise training.

Protein intake and endurance training

It is a widely held view that general recommendations for protein intake in individuals commencing endurance exercise training is sufficient to maximise adaptation to endurance training (29, 30). However, there is some evidence to suggest that additional dietary protein has the potential to further enhance endurance exercise training adaptations such as whole-body aerobic capacity in humans (31, 32), changes in body composition (32), muscle fibre type I hypertrophy (33, 34), upregulated expression profiles associated with skeletal muscle growth, oxidative muscle fibre remodelling, immunity and defence, energy metabolism (35) and, performance (36-38). Limited scientific insight on this topic might be the result of reasoning that carbohydrates and fat are the most important nutrients to facilitate energy requirements during endurance exercise (13). Furthermore, it is generally thought that endurance athletes already consume sufficient protein and that adding dietary protein therefore will not further enhance endurance training adaptations. The latter may have prevented scientists to fully scrutinise the topic. Future evidence must be derived from long-term endurance training studies that combine performance outcomes and biochemical/physiological endpoints.

Outline of this thesis

Studying exercise-nutrient interactions on skeletal muscle level is complex. A better understanding of the physiological and molecular responses can provide new insights into potential nutritional strategies to maximise skeletal muscle adaptation and ultimately performance. The aim of the research presented in this thesis is to study the role of nutrient availability with divergent types of exercise on molecular responses and physiological adaptations. Specifically, carbohydrate availability with resistance exercise and protein intake with endurance exercise. This thesis will shine new light on the empirical shortcomings pointed out in the introduction by conducting I) an acute exercise study on carbohydrate availability and the adaptive response with resistance exercise; II) a chronic training study on the effect of protein intake and endurance training adaptations.

In **chapter 2** an overview of the role of skeletal muscle glycogen with endurance and resistance exercise is given. In **chapter 3**, skeletal muscle glycogen levels and acute selective mRNA responses are used to study the effects of carbohydrate availability on the physiological and molecular

responses in the post-resistance exercise period. With this study, we aimed to explore the effects of differences in carbohydrates and fat availability on post-resistance exercise gene expression. After a glycogen depleting endurance exercise session in the morning subjects received an isocaloric mixed meal containing different amounts of carbohydrates and fat 2 hours before a resistance exercise session in the afternoon, while ample protein was provided throughout the day. We hypothesize that some of the post-resistance exercise selected mRNAs associated with substrate metabolism and mitochondrial biogenesis will differ between the nutritional conditions, without any changes in proteolytic genes. In **chapter 4,** from the same study, we analysed plasma cytokines to see whether these cytokines are affected by carbohydrate availability. In this study we aimed to determine the effects of a pre-resistance exercise carbohydrate meal versus fat meal on post-resistance exercise circulating plasma cytokine concentrations. We hypothesized that some of the selected cytokines will differ between nutritional conditions, but that the majority of the circulating cytokines with a possible role in skeletal muscle growth do not respond differently. Findings of **chapter 3 and 4** may call into question the prominence of carbohydrate intake with regard to resistance exercise training adaptations. In **chapter 5**, a perspective on the field of protein and the adaptive response with endurance training is given. In this perspective, the theoretical rationale why protein may be beneficial for individuals participating in endurance training programs is proposed. **Chapter 6** investigates the effects of protein supplementation on endurance training adaptations in healthy young males. The aim of the study was to determine the impact of protein supplementation on VO_{2max}, body composition and skeletal muscle adaptations with 10-weeks of vigorous endurance exercise training. In addition, to gain insight into temporary adaptations we measured adaptations after 5 and 10 weeks of endurance training. We hypothesized that protein supplementation further enhances gains in VO_{2max}, lean mass and skeletal muscle adaptations during 10-weeks of endurance training. **Chapter 7** reveals how the enhanced endurance training adaptations by protein supplementation could be explained by steady-state skeletal muscle transcriptional changes. These findings contribute to the view that protein supplementation with endurance exercise plays a major role in the process of adaptation with prolonged endurance training. Finally, **chapter 8** provides an overall discussion of the results presented in this thesis, as well as future perspectives.

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Chapter 2

Glycogen availability and skeletal muscle adaptations with endurance and resistance exercise

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Abstract

It is well established that glycogen depletion affects endurance exercise performance negatively. Moreover, numerous studies have demonstrated that post-exercise carbohydrate ingestion improves exercise recovery by increasing glycogen resynthesis. However, recent research into the effects of glycogen availability sheds new light on the role of the widely accepted energy source for adenosine triphosphate (ATP) resynthesis during endurance exercise. Indeed, several studies showed that endurance training with low glycogen availability leads to similar and sometimes even better adaptations and performance compared to performing endurance training sessions with replenished glycogen stores. In the case of resistance exercise, a few studies have been performed on the role of glycogen availability on the early post-exercise anabolic response. However, the effects of low glycogen availability on phenotypic adaptations and performance following prolonged resistance exercise remains unclear to date. This review summarizes the current knowledge about the effects of glycogen availability on skeletal muscle adaptations for both endurance and resistance exercise. Furthermore, it describes the role of glycogen availability when both exercise modes are performed concurrently.

Introduction

Roughly, exercise can be divided in endurance- and resistance exercise. Endurance exercise can be further subdivided in traditional -endurance exercise and high intensity interval training (HIIT). Traditional endurance exercise is characterized by continues submaximal muscular contractions aimed at improving aerobic power production. Whereas high intensity interval training primarily consists of brief, intermittent bursts of vigorous movements, alternated by periods of rest or low-intensity movements with the purpose to improve both aerobic and anaerobic power production (1). Resistance exercise, on the other hand involves short bursts of nearly maximal muscular contractions and primarily focuses on the development of muscle hypertrophy and/or muscular strength. The skeletal muscle adaptations are determined by the type, intensity and duration of the performed exercise. In short, endurance exercise training mainly results in mitochondrial biogenesis, increases capillary density and enzymes leading to enhanced skeletal muscle O_2 utilization capacity (2-4). In contrast, resistance exercise promotes skeletal muscle hypertrophy and strength through increases in myofibrillar volume predominantly in type II fibres (5, 6).

It is now widely accepted that nutrition plays an important role in mediating skeletal muscle adaptations (7). Carbohydrates and fat are recognized as the main substrates for powering prolonged muscle contractions during endurance exercise (8). Although carbohydrates are widely accepted as fuel for skeletal muscle both during (8) and following endurance exercise (8), recent investigations introduced a novel approach of exercising with reduced glycogen levels aimed to optimize skeletal muscle adaptations (9, 10). Indeed, several studies have reported that endurance exercise with low glycogen availability may be a strategy to augment the response in exercise-induced signalling associated with improved oxidative capacity (11-17), and potentially enhance exercise performance (17, 18). In contrast, the effects of low glycogen availability on muscular adaptations following resistance exercise remain somewhat unclear. A recent study revealed that performing resistance exercise with low glycogen could improve acute signalling processes that promote mitochondrial biogenesis to a larger extent compared to exercise with normal glycogen levels (19), whereas another study demonstrated that muscle protein synthesis following a single bout of resistance exercise appeared to be unaffected by the level of glycogen (20).

A literature review concerning the role of glycogen availability for both endurance- and resistance exercise on skeletal muscle adaptations is at this time absent. Therefore, the purpose of this review is to identify the effects of glycogen availability on skeletal muscle training adaptations and performance with both endurance- and resistance exercise. Firstly, the role of glycogen in local skeletal muscle fatigue and energy metabolism will be described.

Thereafter, the effects of glycogen availability on performance and markers of skeletal muscle adaptations are discussed. Finally, this review addresses the role of glycogen availability when both exercise modes are performed concurrently.

Glycogen content, location and skeletal muscle fatigue

In humans, most glycogen is made and stored in cells of the liver (~ 100 g) and muscles (~350 – 700 g; depending on training status, diet, muscle fibre type composition, sex and bodyweight) and can be reduced by fasting, low intake of dietary carbohydrates and/or by exercise. It seems that the critical level of muscle glycogen is approximately around 250-300 mmol · kg^{-1}dry weight (d.w.), levels below this amount have been associated with impaired sarcoplasmic reticulum function by diminishing vesicle Ca^{2+} release rate and reductions in peak power output (21). Glycogen is differently distributed within the muscle fibres (subsarcolemmal ~5-15%, intermyofibrillar ~75% and intramyofibrillar ~5-15%) (22). Moreover, it appears that subsarcolemmal, intermyofibrillar and intramyofibrillar glycogen powers different mechanisms in muscle contractions. It is thought that intermyofibrillar glycogen powers the release of sarcoplasmic stored Ca^{2+} and in this way activates the tropomyosin active sites. Intramyofibrillar glycogen is preferably depleted during high-intensity exercise and seems to power cross-bridge cycling (23). Moreover, depletion of this form highly correlates well with skeletal muscle fatigue (24). Reduction of intramyofibrillar glycogen might decrease Na, K-ATPase activity leading to decreased ATP cleavage, and subsequently a lower energy production to power cross-bridge cycling (22).

Moreover, Duhamel *et al.* (25) found that commencing an endurance type bout of exercise till fatigue with low glycogen availability resulted in earlier deteriorations in SR Ca^{2+} release. Specifically, their data indicated that a cycling session of 70% VO_{2peak} carried out at low glycogen levels causes faster reductions in SR Ca^{2+} uptake and Ca^{2+} release during exercise compared to high glycogen levels. Furthermore, it was found that reductions in SR Ca^{2+}-ATPase activity followed a similar time course as that of Ca^{2+} uptake suggesting a mediating role for SR Ca^{2+}-ATPase activity. In another study by Ortenblad *et al.* (21) it was shown that ingestion of carbohydrates during 4 h recovery following exercise markedly increases glycogen content and normalizes SR Ca^{2+} compared to the group who were omitted from carbohydrates during the recovery period. Based on SR vesicle experiments Ortenblad *et al.* (22) proposed that there is mechanistic role of glycogen on SR Ca^{2+} release. Moreover, Ortenblad *et al.* stated that the reduction in SR Ca^{2+} release by itself induces a diminution in tetanic intracellular free $[Ca^{2+}]_i$, which is in accordance with isolated fibres studies showing a faster decrease of tetanic $[Ca^{2+}]_i$ when glycogen content is reduced within these fibres. Taken together, the aforementioned findings at both the whole-body and organelle level suggest

that the location of the glycogen, especially the intramyofibrillar pool, is important to sustain repeated muscle contractions.

Glycogen and energetic demand with exercise

Glycogen is an essential substrate during high intensity exercise by providing a mechanism by which adenosine tri phosphate (ATP) can be resynthesized from adenosine diphosphate (ADP) and phosphate. The relative use of energy sources during exercise is mainly determined by the intensity and the duration of the exercise bout, as well as the athlete's training status (8). Fat as source of energy is relatively most dominant during low-moderate intensity (30-65% of VO_{2peak}), whereas the relative contribution of carbohydrate oxidation to total energy expenditure becomes greater when exercise intensity increases, with muscle glycogen becoming the most important substrate source (26). Although the amount of liver and skeletal muscle glycogen is relatively small compared to endogenously stored fat, glycogen is recognized as the major source for fuel during prolonged moderate- to high intensity endurance exercise (27). Therefore, glycogen availability is essential to power ATP resynthesis during high intensity exercise which relies heavily on glycogenolysis. Furthermore, it has been well documented that the capability of skeletal muscle to exercise is impaired when the glycogen store is reduced to a certain level, even when there is sufficient amount of other fuels available (28). Together, prolonged endurance exercise leads to muscle glycogen depletion, which is in turn linked to fatigue and makes it difficult to meet the energetic requirements of training and competition (22, 29).

Low glycogen and performance with exercise

Endurance training performance

Low-glycogen availability causes a shift in substrate metabolism during and after exercise (30, 31). In addition, low-glycogen availability induces an increase in systemic release of amino acids and simultaneously increases fat oxidation, and as a consequence exercise intensity drops (30). However, the low-glycogen approach seems to promote expression of genes that stimulate fat catabolism and mitochondrial biogenesis and as such improves oxidative capacity (10). To date, few studies have found an improved training-induced performance effect of conducting the exercise bouts with low glycogen levels compared with replenished glycogen levels (17, 18). Hansen *et al.* (17) were the first to show that training with reduced glycogen availability results in improved oxidative capacity. In their study seven untrained males completed a 10-week training program. The untrained subjects performed leg-knee extensor exercise for 5 $d \cdot wk^{-1}$. Although the total amount of work was the same for each leg, one leg was trained in a glycogen depleted manner, while the contralateral leg was trained with full glycogen stores. The finding of their study was a significant gain in endurance (time till exhaustion) in the low-

glycogen compared to normal glycogen levels. In addition, they found that low-glycogen improved oxidative capacity (citrate synthase activity) to a larger extent than commencing all exercise sessions with high-glycogen. The findings of Hansen *et al.* (17) were pioneering and in contrast with the studies reporting that glycogen content is a limiting factor when it comes to exercise adaptation and performance. Subsequently, other research groups tested the same hypothesis by using an alternative model with trained subjects (12, 16). Yeo *et al.* (16) demonstrated that subjects who undertook the exercise sessions in the low-glycogen state (~50% depletion) showed significantly lower performance during the sessions compared to the subjects that undertook the sessions with high-glycogen (16). Interestingly, following the 3-wk intervention period, several markers of training adaption were increased. However, 60-min time-trial performance was similar in both the low-glycogen and high-glycogen group. Although speculative, the similar effect in performance suggests that the low-glycogen group showed a greater training adaptation, relative to their level of training intensity. Hulston *et al.*(12) reported that lipid oxidation during the steady-state exercise at 70% $VO_{2max,}$ increased more in the low-glycogen group relative to the high-glycogen group, as a result of increased intramuscular triglycerides utilization. Moreover, this was accompanied by increases in oxidation of fatty acids, sparing of muscle glycogen, and greater increases in succinate dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase enzyme activity (12). However, with regard to performance, the training with low muscle glycogen availability was not more effective than training with high muscle glycogen levels (12). Together, low-glycogen availability affects substrate use during exercise by increasing fatty acid oxidation compared to training with normal glycogen levels; this effect is independent of the subject training status.

Recently, Cochran *et al.* (18) demonstrated that a two week high-intensity-interval-training (HIIT) protocol performed with low muscle glycogen levels, improved cycling time trial performance compared with high glycogen stores. Specifically, training sessions consisted of 5 times 4-min intervals at 60% W_{peak} (~95-100% of heart rate reserve) interspersed with 2 min of rest. Both groups trained on a total of 6 d over a 2-wk period, with a minimum of one day of rest between training days. Furthermore, subjects completed two identical HIIT sessions on each training day, separated by 3 h of recovery. After two weeks of HIIT, mean power output during a 250-kJ time trial increased to a greater extent in the low-glycogen group compared to the high-glycogen group (18). A novel aspect of their study was that the subjects performed whole-body exercise for a relatively short period of time (2 weeks), while the study of Hansen *et al.* (17) lasted 10 weeks and used an exercise protocol where upper leg muscles performed isolated knee extensions

Discrepancies between and limitations of the low-glycogen endurance exercise studies

A possible explanation for the different outcomes on performance between low-glycogen studies could be differences in the training status of the subjects. Hansen *et al.* (17) and Cochran *et al.* (18) used untrained subjects, whereas others tested well-trained subjects (12, 16). Indeed, it has previously been shown that the effectiveness of nutritional interventions is influenced by the subject training status (32), possibly because trained subjects depend less on carbohydrate utilization because they have greater metabolic flexibility. Another methodological issue is the selected test used to determine performance. In some studies, self-selected intensities were used, which could be influenced by carbohydrate manipulation. Cochran *et al.* (18) therefore prescribed and controlled power output during training to ensure that glycogen manipulation did not affect training intensity.

To summarize, although some studies reported that repetitive low-glycogen training leads to improved performance compared with high glycogen (17, 18), extrapolating these findings to sports-specific performance should be done with prudence. First, the study of Hansen *et al.* (17) used an isolated knee-extensor model ($5\ d \cdot wk^{-1}$) as training protocol and performance measurement. However, this model does not accurately reflects an athlete's performance in a real life sports event. Second, as suggested by Yeo *et al.* (16), athletes generally use multiple intensities, as well as progressive overload (33, 34), rather than a fixed submaximal exercise intensity as was used during the training sessions in the study of Hansen *et al.* (17). Lastly, chronic exercise sessions commencing in the low-glycogen state may enhance the risk for overtraining syndrome (35) which in turn may result in reduced training capacity (36).

Resistance exercise performance

Resistance exercise is typically characterized by short bursts of nearly maximal muscular contractions. When performing resistance exercise, glycogen is crucial to resynthesize the phosphate pool, which provides energy during high intensity muscle contractions (37). According to MacDougall *et al.* (37), the majority of ATP is derived from glycolysis (38). In line with this, a typical resistance exercise session has been shown to reduces glycogen levels by approximately ~24-40% (37, 39-41). This reduction in glycogen content during exercise is determined by the intensity and volume of the performed exercise bout. The largest reductions in glycogen are seen with high repetitions with moderate load training (40), an effect that mainly occurs in type II fibres (39). It has been demonstrated that a reduction of muscle glycogen affects both isokinetic torque (29) and isoinertial resistance exercise capacity negatively (42). However, this effect is not always evident (43) and is likely to be affected by the protocol used to induce glycogen depletion (44). Based on the

assumption that pre-exercise glycogen content can influence exercise performance, it seems that the pre-exercise carbohydrate ingestion requires particular attention (44).

Although it is widely accepted that carbohydrate ingestion before endurance exercise enhances work capacity (45, 46), carbohydrate ingestion before resistance exercise has not been studied to the same extent. The importance of carbohydrates for the resistance exercise-type athlete can be substantiated by the idea that glycogen plays a relatively important role in energy metabolism during resistance exercise. For example, it has been shown that pre-resistance exercise carbohydrate ingestion increases the amount of total work (47-49). In contrast, other reports show no benefit of carbohydrate ingestion on total work capacity (50, 51). To precisely determine the role of glycogen availability for the resistance exercise athlete more training studies that feature a defined area of outcome measures specifically for performance and adaptation are needed.

Mitochondrial biogenesis on low-glycogen regimes and molecular pathways involved

Endurance exercise

PGC-1α

Activity of the exercise-induced peroxisome proliferator-activated γ-receptor co-activator 1α (PGC-1α) has been proposed to play a key role in the adaptive response with endurance exercise (**Figure 1**). Endurance exercise leads to more PGC-1α and more active of PGC-1α which increases mitochondrial volume. Increased mitochondrial volume improves oxidative capacity through increased fatty acid *β*-oxidation and mitigating glycogenolysis (52). As a result, muscle glycogen can be spared which might delay the onset of muscle fatigue and enhances oxidative exercise performance. PGC-1α is responsible for the activation of mitochondrial transcription factors e.g. the nuclear respiratory factors (NRF-1 and -2) and the mitochondrial transcription factor A (Tfam) (53).

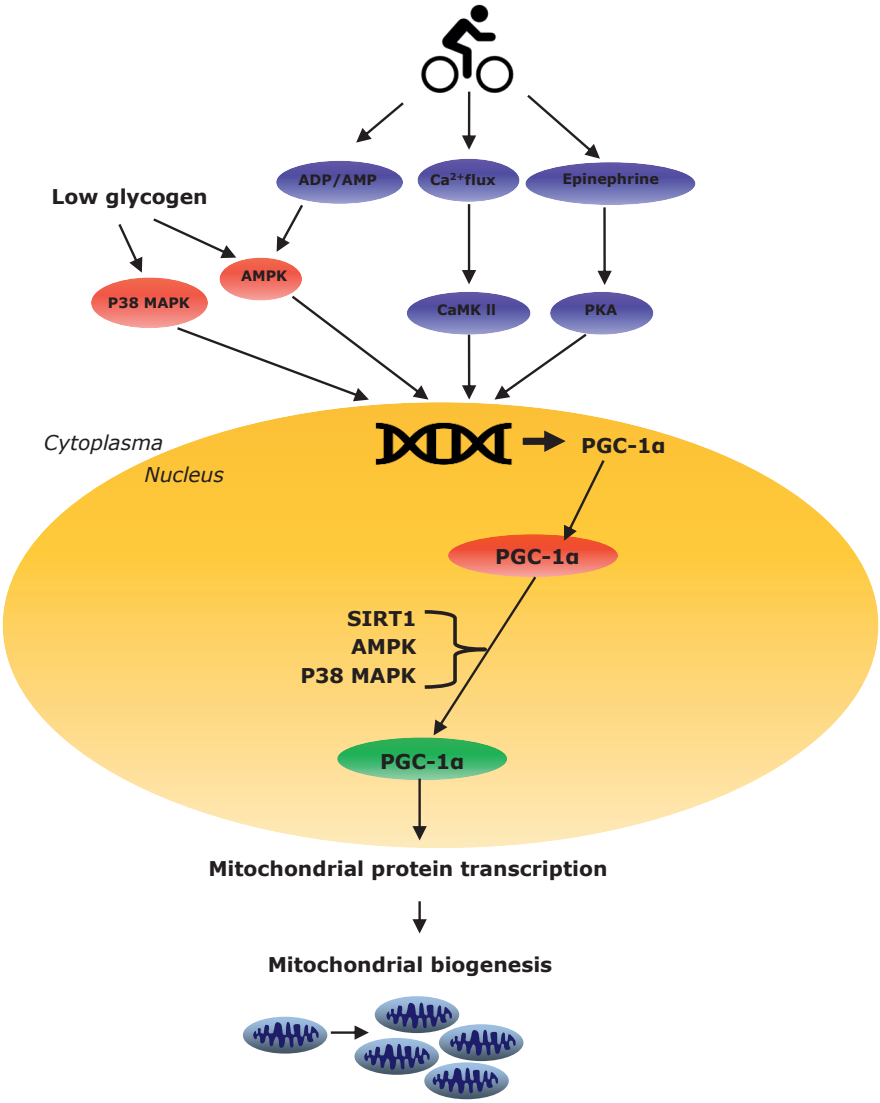

Figure 1. Schematic overview of training-induced mitochondrial adaptation in skeletal muscle. Schematic figure representing the regulation of mitochondrial biogenesis by endurance exercise. Endurance exercise acutely increases Ca^{2+}, ADP, AMP and epinephrine. In addition, exercise reduces skeletal muscle glycogen in the contracting muscles which in turn activates the sensing proteins AMPK and p38 MAPK. Especially elevated AMP and ADP trigger an increased phosphorylation of AMPK at Thr172 and the increased Ca^{2+} concentration via calmodulin causes CaMK II autophosphorylation. Both AMPK and p38 MAPK activate and translocate the transcriptional co-activator PGC-1α to the mitochondria and nucleus. The kinases AMPK , p38 MAPK and SIRT 1 then might phosphorylate PGC-1α and reduce the acetylation of PGC-1α, which increases its activity. Thus, endurance exercise leads to more PGC-1α which over time results in mitochondrial biogenesis.

PGC-1α and AMPK

Activation of PGC-1α is amongst others regulated by the major up-stream proteins 5' adenosine monophosphate-activated protein kinase (AMPK) (54). Prolonged endurance type exercise requires a large amount of ATP resulting in accumulation of ADP and AMP in the recruited muscle fibres (55). This activates AMPK with the purpose to restore cellular energy homeostasis (56, 57). A relative drop of ATP by less than 1% translates into a doubling or more of the ADP and AMP concentrations (58). The rise of ADP and AMP during prolonged endurance type exercise results in the phosphorylation of AMPK at Thr172, the active site on the AMPK α subunit (58-60). Canto and colleagues (2009) showed that AMPK action on PGC-1α transcriptional activity is partly regulated by SIRT1, a sirtuin family protein which deacetylates several proteins that contribute to cellular regulation (57). Furthermore, it was shown that the acute actions of AMPK on lipid oxidation alter the balance between cellular NAD1 and NADH, which acts as a messenger to activate SIRT1 (57).

PGC-1 α, AMPK and p38 MAPK in response to glycogen depletion

During prolonged endurance type exercise skeletal muscle glycogen reduces, this is sensed by the AMPK β subunit resulting in an activation of AMPK (Figure 1). The AMPK is then also activated through phosphorylation of Thr172 and this response is likely dependent on the rise of AMP and ADP during exercise. Chan et al (2004) suggested that low muscle glycogen availability associates with the phosphorylation of the nuclear P38 mitogen-activated protein kinases (p38 MAPK), rather than translocation of p38 MAPK to the nucleus per se (61). Accordingly, p38 MAPK particularly phosphorylate the expression of PGC-1α (53, 62), whereas AMPK could both phosphorylate and enhance expression of PGC-1α (53, 62). Restricted CHO availability during or after exercise has also been shown to augment phosphorylation of (i.e. activate) p38 MAPK (63) and AMPK (15). In another study by Mathai and colleagues (2008) it was shown that changes in muscle glycogen correlates with the changes in PGC-1α protein abundance during exercise and recovery (64). The majority of the studies show that the PGC-1α mRNA content increased during and directly after exercise and returned to resting levels by 24 h after exercise. However, the studies that measured both PGC-1α mRNA and PGC-1α protein after chronic or acute exercise failed to find increases in both (64). Therefore, changes of PGC-1α mRNA content are not necessarily compatible with changes in PGC-1α protein abundance following exercise (64).

Although these studies suggest that the signalling response to exercise is affected by CHO supply, it remains unclear whether exercise in a glycogen-depleted state can enhance the adaptive signalling response that is required for mitochondrial biogenesis. Thus, AMPK and MAPK 38 play a key role in the transcriptional regulation of mitochondrial biogenesis trough PGC-1α in response to stress. However, the precise role of potential regulators which are

responsive to glycogen availability, in the processes of mitochondrial biogenesis, needs to be further elucidated.

P53

Another described protein that regulates mitochondrial biogenesis is p53, which appears to be sensitive to changes in glycogen availability (65). Previous research has shown that p53 is phosphorylated by AMPK and p38 AMPK (66, 67). Furthermore, p53 is implicated in the stimulation of gene expression of mitochondrial function (66, 67). It has been demonstrated that commencing endurance exercise in a glycogen depleted state upregulates p53 to a larger extent than during exercise in a replenished glycogen state (68). However, the influence on PGC-1α mRNA expression is difficult to interpret because the subjects involved were not only on an exercise regime with low glycogen availability, but also on a calorie restricted diet. Accordingly, it remains unknown which potent regulator was responsible for the increase in mitochondrial biogenesis in this study. The precise role of both potential regulators in the processes of mitochondrial biogenesis needs to be further elucidated.

Resistance exercise

Although resistance exercise is mainly recognized as mechanical stimulus for increases in strength and hypertrophy, the aerobic effects following resistance exercise have also been studied. Early investigations have shown that skeletal mitochondrial volume (69) and oxidative capacity (70) are unaltered following prolonged resistance exercise. However, it has been recently reported that resistance exercise increases the activity of oxidative enzymes in tissue homogenates (19, 71) and respiration in skinned muscle fibres (72). Moreover, resistance training augmented oxidative phosphorylation in sedentary older adults (73) and respiratory capacity and intrinsic function of skeletal muscle mitochondria in young healthy men (74). In a recent investigation by Irving and co-workers (75) young and older adults performed 8 weeks of endurance training, resistance training or concurrent/combined training. Interestingly, following all exercise modalities, concurrent training induced the most robust improvements in mitochondrial related outcomes and mRNA expression (75). Notably, the improvements in mitochondria were independent of age. Therefore, exploring molecular processes regulating the metabolic and oxidative responses with resistance training may lead to a better understanding and eventually to optimized adaptations. Studies examining the effect of low glycogen availability on mitochondrial regulators largely centred on endurance training. However, Camera *et al.* (19) recently showed that both phosphorylation of p53 and mRNA abundance of PGC-1α increased during the early (4 h) post-exercise recovery period after resistance exercise undertaken with low glycogen availability. It appears that the level of glycogen acts as a

modulator of processes regulating mitochondrial biogenesis, independent of the nature of exercise stimuli. The supposed mechanism by which p53 is translocated from the nucleus to the mitochondria and subsequently enhances mitochondrial biogenesis is through its interaction with mitochondrial transcription factor A (Tfam) and also by preventing p53 suppression of PGC-1α activation in the nucleus (67). According to the findings of Camera *et al.* (19) and others (20, 76, 77) resistance exercise seems capable of increasing PGC-1 mRNA expressions and p53 phosphorylation that has the potential to stimulate mitochondrial adaptation. Moreover, the acute metabolic response to resistance exercise can be modulated in a glycogen-dependent manner. However, whether these acute alterations in regulators of mitochondrial biogenesis are sufficient to promote mitochondrial volume and function remains to be elucidated in future long-term training studies.

Low glycogen availability and protein metabolism

Endurance exercise

Skeletal muscle mass is maintained by the balance between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) rates such that overall net muscle protein balance (NPB) remains essentially unchanged over the course of the day. A prolonged imbalance between MPS and MPB results in a positive (hypertrophy: MPS > MPB) or negative NPB (atrophy: MPS < MPB). The two main potent stimuli for MPS are food ingestion and exercise (78). Nutrition, proteins in particular, induces a transient stimulation of MPS and is therefore in itself, i.e. in the absence of exercise, not sufficient to induce a positive NPB. Likewise, resistance exercise improves NPB, however, the ingestion of protein during the post-exercise recovery period is required to induce a positive NPB (79). Thus, both exercise and food ingestion must be deployed in combination in order to create a positive NPB (78).

To date, only a few studies examined the role of glycogen availability on protein metabolism following endurance exercise (30, 80, 81). It seems that glycogen availability mediates MPB. An early study from Lemon and Mullin showed that when exercise was performed with reduced glycogen availability nitrogen losses more than doubled, suggesting an increase in MPB and amino acid oxidation (80). Subsequently, two other studies (30, 81) used the arterial-venous (a-v) difference method to explore whether exercise in the low glycogen state affects amino acid flux and then estimated NPB. In both studies subjects performed an exercise session in the low-glycogen state, the researchers found a net release of amino acids during exercise indicating an increase in MPB. However, these studies may be methodologically flawed because the a-v balance method only allows for the determination of net amino acid balance. Conclusions about changes in MPS and MPB are therefore of a speculative nature (82). A more recent study by Howarth *et al.* (82) used the stable isotope tracer methodology and therefore enabled to determine the changes in MPS

and MPB. They found that skeletal muscle NPB was lower when exercise was commenced with low glycogen availability compared to the high glycogen group, indicating an increase in MPB and decrease in MPS during exercise. It appears that endurance exercise with reduced muscle glycogen availability negatively influences muscle protein turnover and impairs skeletal muscle repair and recovery from endurance exercise. As described previously, low glycogen could be used as a strategy to augment mitochondrial adaptations to exercise, however, protein ingestion is required to offset MPB and increase MPS. Indeed, recent evidence reported that protein ingestion during or following endurance exercise increases MPS leading to a positive NPB (83, 84).

Resistance exercise

Resistance exercise type muscular contraction and/or protein ingestion affect the complex of regulatory processes that determines the changes in MPB and MPS. The Akt-mTOR-S6K pathway that controls the process of MPS has been studied extensively (85, 86). However, the effects of glycogen availability with resistance exercise and its effects on these regulatory processes remains to be further scrutinized. It has been observed that low-glycogen availability (~ 160 mmol · kg^{-1}d.w.) elevates resting and exercise-induced AMPK activity compared to high-glycogen availability (~ 910 mmol · kg^{-1}d.w.) (87). Furthermore, work by Churchly *et al.* (88) demonstrated that low-glycogen availability (~ 193 mmol · kg^{-1}d.w.) did not enhance the activity of genes involved in muscle hypertrophy. Creer *et al.* (89), on the other hand, investigated the role of glycogen availability on two key pathways involved in cellular growth. Subjects performed three sets of 10 repetitions of bilateral knee extension exercise at 70% of 1-RM separated by a 2-min recovery period. Muscle biopsies were taken to determine the activation of extracellular signal-regulated kinase (ERK1/2) and Akt signalling pathways. They found an increase in both ERK1/2 and p90 ribosomal S6 kinase phosphorylation, but this effect was independent of the level of glycogen. Further, Akt phosphorylation was attenuated in the low glycogen (~ 175 mmol · kg^{-1}d.w.), whereas it was increased in the high glycogen (~ 600 mmol · kg^{-1}d.w.) group. mTOR phosphorylation was similar to that of Akt, however, the change was not significant. In a comparable study from Camera *et al.* (20) young healthy men performed 8 sets of 5 unilateral leg press repetitions at 80% 1RM. Muscle biopsies were taken at rest and 1 and 4 h after the single exercise bout. Although mTOR phosphorylation increased to a higher extent in the normal glycogen group, there were no detectable differences found in MPS suggesting that the small differences in signalling are negligible since MPS was unaffected. The result that MPS is not influenced by the level of glycogen was an interesting finding since it has been previously reported that energy deficit attenuates rates of mixed MPS by ~19% (90). However, it should be noted that being in an energy deficit state does not

necessarily reflects glycogen levels are low. Hence, the total energy available for the cell to undertake its normal homeostatic processes is less.
Summarized, it seems that glycogen availability had no influence on the anabolic effects induced by resistance exercise. However, aforementioned studies on the effects of glycogen availability on resistance exercise-induced anabolic response do not resemble a training volume typically used by resistance-type athletes. Future long-term training studies (~12 weeks) are needed to find out whether performing resistance exercise with low glycogen availability leads to divergent skeletal muscle adaptations compared to performing the exercise bouts with replenished glycogen levels.

Role of glycogen availability with concurrent training
To date, many sports such as soccer, volleyball, tennis or swimming require a phenotype which features a high oxidative capacity and a sufficient amount of strength/power in order to achieve maximal performance. To develop both endurance capacity and strength/power simultaneously, a targeted training model must include both endurance and resistance exercise, known as concurrent training. Concurrent training does improve both endurance capacity and muscular strength/power, although numerous studies have revealed that strength development is compromised with concurrent training compared to resistance exercise alone (91-94), a phenomenon known as the "interference" effect. Vice versa, the effect of resistance exercise on endurance performance and VO_{2max} appears to be marginal (95, 96). However, some studies reported compromised gains in aerobic capacity with concurrent training compared to endurance exercise alone (97, 98). Following the work of Hickson *et al.* (99), numerous studies have investigated the effect of concurrent training on skeletal muscle adaptations. Although the existing human data on acute molecular events is insufficient to entirely explain the impaired strength/power gains for the concurrent athlete compared with either exercise modality alone, it has been hypothesized that the exercise-induced signalling antagonism may play a role herein. Since a detailed analysis on the interference effect associated with concurrent training is beyond the scope of this review, we refer the reader to expert reviews on the interference effect seen with concurrent training (Baar *et al.* (2014b); Fyfe, Bishop, & Stepto (2014); Perez-Schindler *et al.*(2015) (100-102). Briefly, the most hypothesized mechanism blameworthy for the impaired strength/power gains seen with concurrent training is the impaired upregulation of the P13K-AKT → mechanistic target of rapamycin complex-1 (mTORC1) signalling pathway (89, 102, 103). It is thought that endurance exercise results in an activation of AMPK, which inhibits the mTORC1 signalling via tuberous sclerosis protein (TSC), and this will eventually suppress MPS resulting in a negative net protein balance. In addition, a higher contractile activity also results in a higher calcium flux, which decreases peptide-chain elongation via activation of eukaryotic

elongation factor-2 kinase (eEF2k) leading to a decreased MPS (89, 102, 103). However, whether the exercise-induced acute interference between AMPK and mTORC1 entirely explains the blunted strength gains seen with concurrent training is to date obscure.

To optimize skeletal muscle adaptations and performance, nutritional strategies for both exercise modes should differ. Indeed, it was recently proposed that, when practicing endurance and resistance exercise on the same day, the endurance session should be performed in the morning in the fasted state, with ample protein ingestion (102). While the afternoon resistance exercise session should be conducted only after carbohydrate replenishment with adequate post-exercise protein ingestion (102). Furthermore, whether such a nutritional strategy leads to improved performance compared to general recommendations for carbohydrate and protein intake remains elusive. Interestingly, it has been demonstrated that a resistance exercise session subsequently after low-intensity endurance, non-glycogen depleting session could enhance molecular signalling of mitochondrial biogenesis induced by endurance exercise (104). Furthermore it is currently unclear whether performing resistance exercise with low-glycogen availability affects the acute anabolic molecular events and whether the effects of these responses possibly result in improved or impaired training adaptation.

Furthermore, whether low-glycogen availability during the endurance bout amplifies the oxidative resistance exercise induced response remains to be investigated. It seems that both modes of exercise in a low glycogen state as part of a periodized training regime are interesting in terms of acute expressions of markers involved in substrate utilization and oxidative capacity. However, on the other hand, a sufficient amount of glycogen is essential in order to meet the energetic demands of both endurance and resistance exercise.

Most existing information on nutrition and concurrent training adaptation is derived from studies where subjects performed exercise in the fasted state (104-108). Coffey and colleagues investigated the effects of successive bouts of resistance and endurance exercise performed in different order in close proximity on the early skeletal muscle molecular response (76). Although the second exercise bout was performed with different levels of skeletal muscle glycogen content, the subsequent effects on Akt, mTOR and p70 signalling following the second exercise bout remained the same. Prospective long-term concurrent training studies may help to understand the complexity of the impaired adaptation with concurrent training and further determine to what extend the acute signalling antagonism contributes to this. Moreover, the role of nutritional factors in counteracting the interference effect remains to be further elucidated.

Concluding remarks and perspectives

In this review we summarized the role of glycogen availability with regard to performance and skeletal muscle adaptations for both endurance and resistance exercise. Most of the studies with low-glycogen availability focused on endurance type training. The results of these studies are promising if the acute molecular response truly indicates skeletal muscle adaptations over a prolonged period of time. Unfortunately, these results on low-glycogen availability may be biased because many other variables including training parameters (time, intensity, frequency, type, rest between bouts) and nutritional factors (type, amount, timing, isocaloric versus non-isocaloric placebo) varied considerably between the studies and it is therefore difficult to make valid inferences. Furthermore, the majority of the studies with low glycogen availability were of short duration (18) and showed no changes (11-17), or showed, in some cases decreases in performance (109). Nevertheless, reductions in glycogen stores by manipulation of carbohydrate ingestion have shown to enhance the formation of training-induced specific proteins and mitochondrial biogenesis following endurance exercise to a greater extent than in the glycogen replenished state (11-16, 18, 68).

For resistance exercise, glycogen availability seemed to have no significant influence on the anabolic effects induced by resistance exercise when MPS was measured with the stable isotope methodology. However, the exercise protocols used in most studies do not resemble a training volume that is typical for resistance-type athletes. Future long-term training studies (~12 weeks) are needed to investigate whether performing resistance exercise with low glycogen availability leads to divergent skeletal muscle adaptations compared to performing the exercise bouts with replenished glycogen levels.

The role of glycogen availability on skeletal muscle adaptations and performance needs to be further investigated. In particular researchers need to examine glycogen availability when endurance and resistance exercise are conducted concurrently, for example, on the same day or on alternating days during the week. To date, only a few studies have investigated the interactions between nutrient intake and acute response following a concurrent exercise model. We recommend that future research in this field should focus on the following questions:

- What is the impact of performing one of the exercise bouts (endurance or resistance) with low glycogen availability on response of markers of mitochondrial biogenesis of the subsequent (endurance or resistance) exercise bout?
- Does the resistance exercise bout need to be conducted with replenished glycogen stores in order to optimize the adaptive response when performed after a bout of endurance exercise?

- Is nutritional timing within a concurrent exercise model crucial to maximize skeletal muscle adaptations following prolonged concurrent training?

To conclude, depletion of muscle glycogen is strongly associated with the degree of fatigue development during endurance exercise. This is mainly caused by reduced glycogen availability which is essential for ATP resynthesis during high-intensity endurance exercise. Furthermore, it is hypothesized that other physiological mechanisms involved in excitation-contraction coupling of skeletal muscle may play a role herein. On the other hand, the low glycogen approach seems promising with regard to the adaptive response following exercise. Therefore, low glycogen training may be useful as part of a well-thought out periodization program. However, further research is needed to further scrutinize the role of low glycogen training in different groups (e.g. highly trained subjects) combined with different exercise protocols (e.g. concurrent modalities), to develop a nutritional strategy that has the potential to improve skeletal muscle adaptations and performance with concurrent training.

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Chapter 3

Select skeletal muscle mRNAs related to exercise adaptation are minimally affected by different pre-exercise meals that differ in macronutrient profile

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Abstract

Background Substantial research has been done on the impact of carbohydrate and fat availability on endurance exercise adaptation, though its role in the acute adaptive response to resistance exercise has yet to be fully characterised.

Purpose We aimed to assess the effects of a pre-resistance exercise isocaloric mixed meal containing different amounts of carbohydrates and fat, on post-resistance exercise gene expression associated with muscle adaptation.

Methods Thirteen young (age 21.2 ± 1.6 year), recreationally trained (VO_{2max} 51.3 ± 4.8 ml/kg/min) men undertook an aerobic exercise session of 90-min continuous cycling (70% VO_{2max}) in the morning with pre- and post-exercise protein ingestion (10g and 15g casein in a 500ml beverage pre- and post-exercise, respectively). Subjects then rested for two hours and were provided with a meal consisting of either 3207 kJ; 52g protein; 51g fat and 23g carbohydrate (FAT) or 3124 kJ; 53g protein; 9g fat; and 109g carbohydrate (CHO). Two hours after the meal, subjects completed 5 x 8 repetitions (80% 1-RM) for both bilateral leg press and leg extension directly followed by 25g of whey protein (500 ml beverage). Muscle biopsies were obtained from the *vastus lateralis* at baseline (morning) and 1 h and 3 h post-resistance exercise (afternoon) to determine intramuscular mRNA response.

Results Muscle glycogen levels were significantly decreased post-resistance exercise, without any differences between conditions. Plasma free fatty acids increased significantly after the mixed meal in the FAT condition, while glucose and insulin were higher in the CHO condition. However, PDK4 mRNA quantity was significantly higher in the FAT condition at 3 h post-resistance exercise compared to CHO. HBEGF, INSIG1, MAFbx, MURF1, SIRT1 and myostatin responded solely as a result of exercise without any differences between the CHO and FAT group. FOXO3A, IGF-1, PGC-1α, and VCP expression levels remained unchanged over the course of the day.

Conclusion We conclude that mRNA quantity associated with muscle adaptation after resistance exercise is not affected by a difference in pre-exercise nutrient availability. PDK4 was differentially expressed between CHO and FAT groups, suggesting a potential shift towards fat oxidation and reduced glucose oxidation in the FAT group.

Introduction

The fundamental phenotypic adaptation to repeated bouts of resistance exercise is an increase in muscle mass and strength. It is assumed that the cumulative effect of frequent resistance exercise sessions within a certain period of time modifies specific proteins, thereby enabling or enhancing the crucial biological processes required for adaptation [1-3]. Considerable attention has been paid to the time course of mRNA response in relation to exercise, and we now know that the exercise-induced changes in mRNA quantity peak immediately post exercise up to 12 hours and return to basal levels within 24 hours [4-7].

It is well known that manipulating nutrition affects the acute intramuscular response with exercise. For instance, post resistance exercise protein provision augments the AKT-mTOR-S6K signalling pathway to initiate translation [8, 9]. On the other hand, carbohydrate restriction with endurance exercise reduces muscle glycogen levels, which in turn affect mRNA levels of genes involved in mitochondrial biogenesis [10, 11]. In addition, undertaking resistance exercise with low skeletal muscle glycogen levels enhances PGC-1α mRNA quantity [12]. The mRNA response to resistance exercise has been examined under a variety of nutritional states (fasted vs fed; protein vs placebo) [1, 13], however, the role of pre-exercise fat and carbohydrate availability on the acute adaptive response remains to be examined. Additionally, most literature assessing low-carbohydrate diets [14-18] or low-carbohydrate availability with exercise [19-22] has focused on endurance exercise. Consequently, specific guidelines for daily carbohydrate intake and timing regarding resistance exercise training are primarily derived from endurance models and the available data on the acute resistance exercise response with different carbohydrate availability. However, to our knowledge, it remains unclear whether pre-resistance exercise carbohydrate or fat availability affects the post-resistance exercise mRNA response when matched for protein intake. Therefore, we aimed to explore the effects of differences in carbohydrates and fat availability on post-resistance exercise gene expression. After a glycogen depleting endurance exercise session in the morning subjects received an isocaloric mixed meal containing different amounts of carbohydrates and fat 2 hours before a resistance exercise session in the afternoon, while ample protein was provided throughout the day. We hypothesize that some of the post-resistance exercise selected mRNAs e.g. PDK4, PGC-1α and SIRT1 will respond differently to the nutritional conditions, without any changes in proteolytic genes.

Methods

Subject characteristics

All subjects were non-smokers, free of injury and not using any medication or nutritional supplements. All subjects provided a full-written informed consent. This study was carried out in accordance with the guidelines for human research of The Medical Ethical Committee of Wageningen University. The Medical Ethical Committee of Wageningen University approved all study procedures. Fourteen healthy physically-active males volunteered to participate in this study: age 21.2 ± 1.6 years, height 1.87 ± 0.05 cm, weight 76.7 ± 4.7) kg, leg extension 1-RM 111±11 kg, leg press 1-RM 266±30 kg and VO_{2max} 51.3±4.8 ml/kg/min^{-1} (**Table 1**). One subject dropped out after test day 1 because of discomfort of the muscle biopsies. Physical characteristics of 13 volunteers are shown in table 1.

Table 1. Physical characteristics of subjects.

	Mean± SE
Age (years)	21.2 ± 0.5
Height (m)	1.87 ± 0.0
Weight (kg)	76.7 ± 1.3
BMI (kg/m^2)	22.0 ± 0.2
W_{max} (W)	346 ± 7.6
VO_{2max} (mL/kg/min^{-1})	51.3 ± 1.3
Leg press 1-RM (kg)	266 ± 8.2
Leg extension 1-RM (kg)	111 ± 3.0

Study Design

This study used a crossover design with two experimental days. On both experimental days subjects completed the same protocol: an endurance exercise session in the morning (08.30 – 10.00 am) and a resistance exercise session in the afternoon (02.00 – 02.30 pm) with a resting period of 4 hours between sessions (**Figure 1**). The subjects received a mixed meal in between the exercise sessions (12.00 pm), which was either a high carbohydrate – low fat meal (CHO) or a low carbohydrate– high fat meal (FAT). Each trial was

separated by a minimum of 12 days (range: 12-30 days), during which time the subjects were instructed to maintain their habitual lifestyle.

Figure 1.

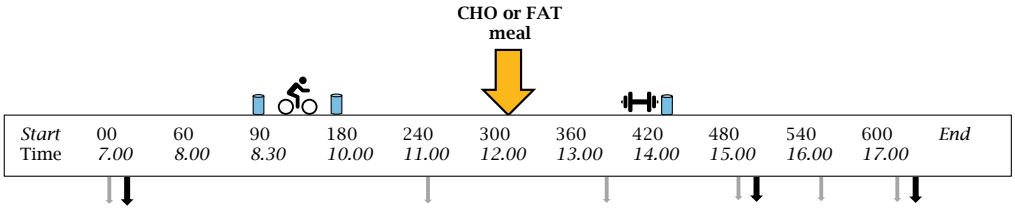

Figure 1. Schematic diagram of the experimental trials. This study adopted a counterbalanced crossover design where subjects completed both exercise trials with different nutritional treatments on separate occasions. Endurance exercise session: 90-min cycling at 70% VO_{2max}. Resistance exercise sessions 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1-RM. Blue containers indicates protein beverage (from left to right; 10g casein, 15g casein and 25g whey. Big orange arrow with black outline indicates lunch with carbohydrates or fat (nutritional intervention meal). Open white arrows indicates time point for blood sample; black arrow indicates sampling time point for muscle biopsy.

Preliminary testing

Maximal aerobic capacity (VO_{2max})

Preliminary testing was performed in the week prior to the start of the first experimental day. Following a 30-min rest, subjects performed a ramped VO_{2max} test on an electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). After a 5-min warm up at 50 W, the subjects started cycling at 100 W. Workload was progressively increased by 20 $W \cdot min^{-1}$ until the subject reached volitional exhaustion. The VO_{2max} test was considered to be valid when two out of three criteria were met: I) levelling of VO_2 with increasing workload; II) heart rate within 10 beats of the theoretically estimated maximum (220-age) and III) respiratory exchange ratio (RER) of ≥1.15. Oxygen consumption (VO_2) was measured through breath-by-breath sampling with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define maximal oxygen consumption (VO_{2max}). Subjects were asked to maintain a cadence between 80-100 $r \cdot min^{-1}$.

Maximal strength (1RM)

Subjects were asked to arrive fasted (at least 4 h) and to avoid any strenuous physical activity 48 h before the preliminary tests. Each subject performed a bilateral one-repetition maximum (1RM) using the leg press and leg extension machine (Technogym, Cesena, Italy). Subjects were familiarized with the movement and warmed up prior to testing by performing six repetitions (at

~40% of estimated 1RM) through a full range of motion with a 1-min rest. After each successful lift, the weight was increased until a failed attempt occurred with 3-min recovery between each attempt. The 1RM was attained within five attempts. Subjects performed a familiarization session for both the endurance and the resistance exercise session ~ one week before the first experimental day.

Diet and physical activity control

Subjects were instructed to avoid alcohol, caffeine and physical activity during 48 h prior to the two experimental days. Diet and physical activity levels were recorded for 24 h before the first experimental day and subjects were asked to replicate dietary intake and physical activity prior to the second experimental day. A standardized meal for the subjects was provided the night before each trial (standard deep-frozen meal and ice-cream dessert; 43.80 kJ/kg BW; 15 Energy% protein, 30 Energy% fat and 55 Energy% carbohydrate).

Experimental days

All subjects arrived in the laboratory at 07.00 being fasted since 10 pm the evening before. After changing into sport gear, a catheter was inserted into the antecubital vein of the left arm. Fifteen minutes after insertion of the catheter baseline blood samples were taken followed by a baseline muscle biopsy. Approximately 10 minutes after the baseline biopsy a protein beverage containing 10 gram of casein dissolved in 350 ml water was consumed (t = -05 min). Within five minutes after consumption of the protein beverage, the subjects started with 90 minutes cycling (t = 0 min) at submaximal intensity (70% VO_{2max}). If subjects could not sustain the intensity of at least 60 RPM, resistance was decreased with 20 W, and the same intensity protocol was repeated during the second training. All subjects performed same exercise intensity of both experimental days. Immediately after the endurance exercise bout, a beverage containing 15 gram of casein dissolved in 350 ml water was consumed (t = 90 min). A second blood sample (~25 ml) was taken 1 hour after the end of the endurance exercise bout (t = 240 min). Two hours after the endurance exercise bout (t = 300 min) subjects were randomly provided with either a carbohydrate rich low-fat meal or low-carbohydrate fat meal (see intervention meal for details). Ninety minutes after the meal (t = 390 min) a third blood sample (~25 ml) was taken. At t = 390 min subjects performed the resistance exercise bout that consisted of 5 x 8 (80% 1RM) repetitions of bilateral leg press and leg extension with two minutes of rest in between the sets. If subjects could not sustain due to fatigue, the selected weight was decreased with steps of 10 kg or 5 kg for the leg press and leg extension, respectively. Immediately after the resistance exercise bout (t = 420 min), a protein beverage containing 25 gram of whey protein dissolved in 350 ml water was consumed. One and three hours after the resistance exercise bout a second

(t = 480 min) and a third (t = 600 min) skeletal muscle biopsy was taken. All biopsies were taken from the same leg. Additional blood samples were taken at t = 480, 540 and 600 min. A timeline for the experimental day can be found in figure 1. The whole experimental protocol was repeated on the 2nd day, while the other meal was provided.

Nutritional intervention meal

Both meals were prepared by a research dietician of Wageningen University. Both the carbohydrate and fat meal consisted of commercially available meat, macaroni and vegetables with an energetic value of ~3200 kJ. The absolute macronutrient amounts can be found in table 2.

Table 2. Overview of the energy and macronutrient composition intervention meal.

Energy & Nutrient	Fat meal	Carbohydrate meal
Energy (kJ)	3207	3124
Protein (g)	52	52
Fat (g)	51	9
Carbohydrates (g)	20	110

Nutritional strategy

Although the findings on gene expression with exercise and carbohydrate restriction are inconsistent, carbohydrate restriction during and after endurance exercise may upregulate genes involved in mitochondrial biogenesis [23]. Therefore, we decided to provide our subjects ~2 h post-exercise with carbohydrates. The protein supplementation strategy used in our study was mainly based on muscle protein synthetic response findings after exercise. A substantial body of research suggests that post resistance exercise whey protein increases myofibrillar protein synthesis to a higher extend when compared with casein [24]. In contrast, little evidence exist for the optimal type of protein to maximise the mitochondrial protein synthetic response after endurance exercise. We therefore decided to provide our subjects with a slow digestible casein protein before and after the endurance exercise sessions. Our nutritional strategy has been recently proposed by others [25].

Muscle biopsies

Muscle biopsies were taken as described by Bergstrom [26]. Biopsies were taken under local anaesthesia (2-3 ml of 2% Xylocaine) using a 5-mm Bergstrom needle modified with suction. All three muscle biopsies per experimental day were taken from the vastus lateralis of the same leg, with separate incisions (~1-1.5 cm) apart and from distal to proximal direction. Muscle biopsies on the second test day were taken from the contralateral leg. Muscle biopsies were

immediately frozen (in 5–10 s) in liquid nitrogen and stored at −80°C for subsequent biochemical analysis, after being freed from visible fat, blood and connective tissue.

Muscle glycogen

Muscle tissue, ~30 mg wet weight, was freeze dried, after which collagen, blood, and non–muscle fibre materials were removed from the muscle fibres under a microscope. The isolated muscle fibre mass (~5–7 mg) was weighed, and 500 µL of 1 M HCl was added. After heating for 3 h at 100°C to hydrolyse the glycogen to glycosyl units and cooling down to room temperature, 500 µL of the solution was neutralized by adding 280 µL of Tris–KOH (Tris 119 mM, KOH 2.14 M) and centrifuged at 1000*g* and 4°C for 10 min. Thereafter, 150 µL of this solution was analysed for glucose concentration (Glucose HK CP A11A01667, ABX Pentra) with a COBAS FARA semiautomatic analyser (Roche).

Blood samples and analyses

All blood samples were immediately centrifuged at 1000g at 4°C for 10 minutes, after which plasma was stored in −80°C until further analysis. Blood samples were analysed for cortisol, creatine kinase, glucose and insulin (Gelderse Vallei hospital, Ede, NL). Cortisol was measured with immunometric chemilumiescence (sandwich) assay with Immulite XPi, (Siemens, the Netherlands). Creatine kinase was measured using Vista device (Siemens, the Netherlands). Free fatty acids were assessed using an enzymatic test kit according to the manufacturer's protocol (InstruChemie, Delfzijl, the Netherlands). Glucose was measured using absorption changes caused by the formation of NADH as a measure of glucose concentration and was measured bichromatic (340 and 383 nm) by means of an end point technique (Siemens, the Netherlands). Insulin was measured using an solid-phase enzyme-linked chemilumiescent immunometric assay with Immulite 2000 XPi (Siemens, the Netherlands).

RNA extraction and real-time quantitative PCR

For RNA isolation, muscle samples (27.8 ± 13.5 mg) were homogenized using an Ultra Turrax (Qiagen, Venlo, Netherlands, cat no: 9001272) and TRIzol-based kit according to manufacturer's guidelines (Fisher Thermo Scientific, Amsterdam, the Netherlands, cat no: 15596026,). After homogenisation in TRIzol, RNA isolation was performed using an RNeasy micro-kit according to the manufacturer's guidelines (Qiagen, Venlo, the Netherlands, cat no: 74004). Briefly, chloroform was added to homogenized samples before centrifuging for 15 minutes at 10.000 rpm. Afterwards the aqueous phase was transferred to the RNeasy micro column and was washed for several times to isolate all RNA. Yield was quantified with a NanoDrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, United States of America), and integrity

was measured with an Agilent 2100 Bioanalyzer with RNA 6000 Nano chips (Agilent Technologies, South Queensferry, United Kingdom).

cDNA synthesis and real-time quantitative polymerase chain reaction
RNA from all samples was reversely transcribed to cDNA using a Fermentas cDNA synthesis kit according to the manufacturer's protocol (Fischer Thermo Scientific, Amsterdam, the Netherlands, cat no: K1612). Briefly, 500 ng of isolated RNA was diluted in 10 µl of RNase-free water and afterwards a mix for cDNA synthesis was added. When cDNA was formed, quantitative real-time polymerase chain reaction (qPCR) was conducted using SYBR Green on a Bio-Rad CFX384 machine (Bio-Rad Laboratories BV, Veenendaal, the Netherlands). SensiMix (Bioline, London, United Kingdom) was used to carry out the PCR reaction. Primers for all genes, obtained from the Harvard PrimerBank [27], are shown in table 3. Samples were run in duplicate, and all samples from each subject were run on the same plate to allow direct relative comparisons. Relative changes in mRNA expression for the genes of interest were quantified using the relative standard curve method [28]. qPCR data were normalized using GAPDH as housekeeping gene for the human samples, since it has shown to be stable within skeletal muscle during exercise [29], and was stable between the time points (data not shown). The threshold cycle (Ct) values of the target gene were normalized to Ct values of the internal control GAPDH, and final results were calculated as relative expression against the standard curve as described previously [30]. Statistical analysis for all mRNA data was performed on the delta Ct values. The baseline muscle biopsy was given a value of 1, and fold changes at 1 h post RE and 3 h post RE were calculated for figure presentation.

Table 3. Primers used for qPCR.

Gene	Forward primer	Reverse primer	Amplicon size
HBEGF	ATCGTGGGGCTTCTCATGTTT	TTAGTCATGCCCAACTTCACTTT	86
FOXO3A	CGGACAAACGGCTCACTCT	GGACCCGCATGAATCGACTAT	150
IGF-1	GCTCTTCAGTTCGTGTGTGGA	GCCTCCTTAGATCACAGCTCC	133
INSIG-1	CCTGGCATCATCGCCTGTT	AGAGTGACATTCCTCTGGATCTG	103
MAFbx	GCCTTTGTGCCTACAACTGAA	CTGCCCTTTGTCTGACAGAAT	187
MURF1	CTTCCAGGCTGCAAATCCCTA	ACACTCCGTGACGATCCATGA	116
Myostatin	TCCTCAGTAAACTTCGTCTGGA	CTGCTGTCATCCCTCTGGA	127
PDK4	GGAGCATTTCTCGCGCTACA	ACAGGCAATTCTTGTCGCAAA	117
PGC-1α	TCTGAGTCTGTATGGAGTGACAT	CCAAGTCGTTCACATCTAGTTCA	112
SIRT1	TAGCCTTGTCAGATAAGGAAGGA	ACAGCTTCACAGTCAACTTTGT	160
VCP	CAAACAGAAGAACCGTCCCAA	TCACCTCGGAACAACTGCAAT	114

Statistics

Data was analysed using a two-way repeated measures ANOVA (two factor, time x treatment) from IBM SPSS version 23 statistical software (IBM, Armonk, NY). When a main effect of condition or time or interaction was identified, a pairwise multiple comparison with a Bonferroni correction was done to identify differences. Statistical significance was set at the $P < 0.05$ level, and values were expressed as means ± SEM or different when indicated.

Results

Endurance and resistance exercise performance

Two subjects performed the endurance exercise sessions with a reduced workload. For one subject the workload was reduced with 20 W after 20 minutes whereas for the other subject the workload was reduced with 20 W after 30 minutes. There was no further reduction in workload during the remaining part of the endurance session. Additionally, both subjects were able to repeat this on the second experimental day. All thirteen subjects performed the resistance exercise training with exactly the same amount of work on the two experimental days.

Muscle glycogen

There were no differences in baseline muscle glycogen between the conditions (CHO 380 mmol/kg dry weight (dw) versus FAT 441 mmol/kg dw; $P > 0.05$ **Figure 2**). As a result of exercise, muscle glycogen was significantly reduced compared to baseline in both conditions at 1 h and 3 h post-resistance exercise ($P < 0.05$) (carbohydrate 163 and 181 mmol/kg dw; fat 185 and 140 mmol/kg dw), without any significant differences between the conditions ($P > 0.05$).

Figure 2.

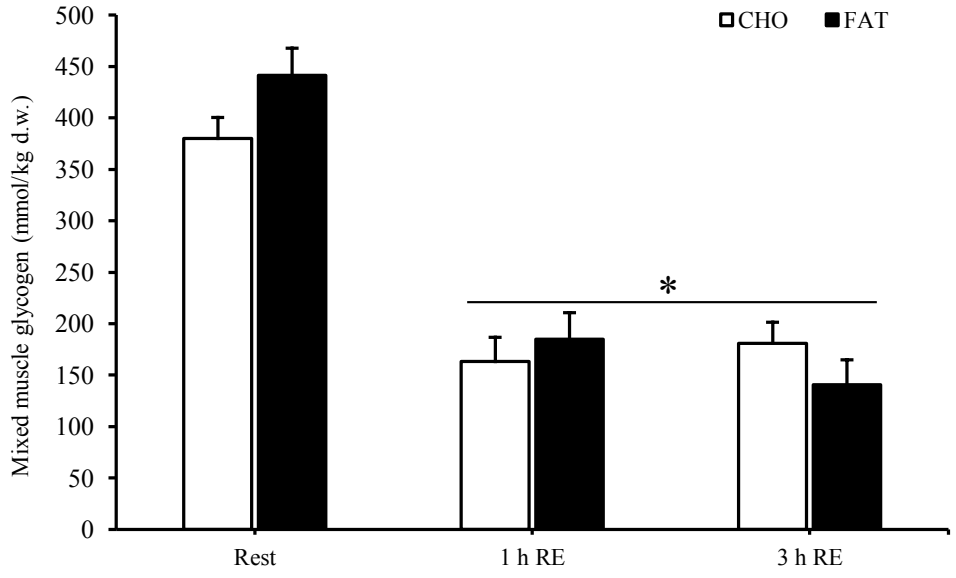

Figure 2. Mixed muscle glycogen at rest and 1h and 3h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM. Values are mean ± SEM. *Significantly different ($P < 0.05$) versus baseline. CHO = open bars , FAT = filled bars.

Free fatty acids, glucose and insulin

One hour post meal, plasma free fatty acid concentration was higher in the FAT condition compared with the CHO condition and this effect remained significant after 1 and 2 hours post-resistance exercise ($P < 0.05$) (**Figure 3**). Both glucose and insulin in the FAT condition were significantly lower at 1 h post meal, and at 1, 2 and 3 h post-resistance exercise compared with the CHO condition ($P < 0.05$) (**Figure 3**).

Figure 3.

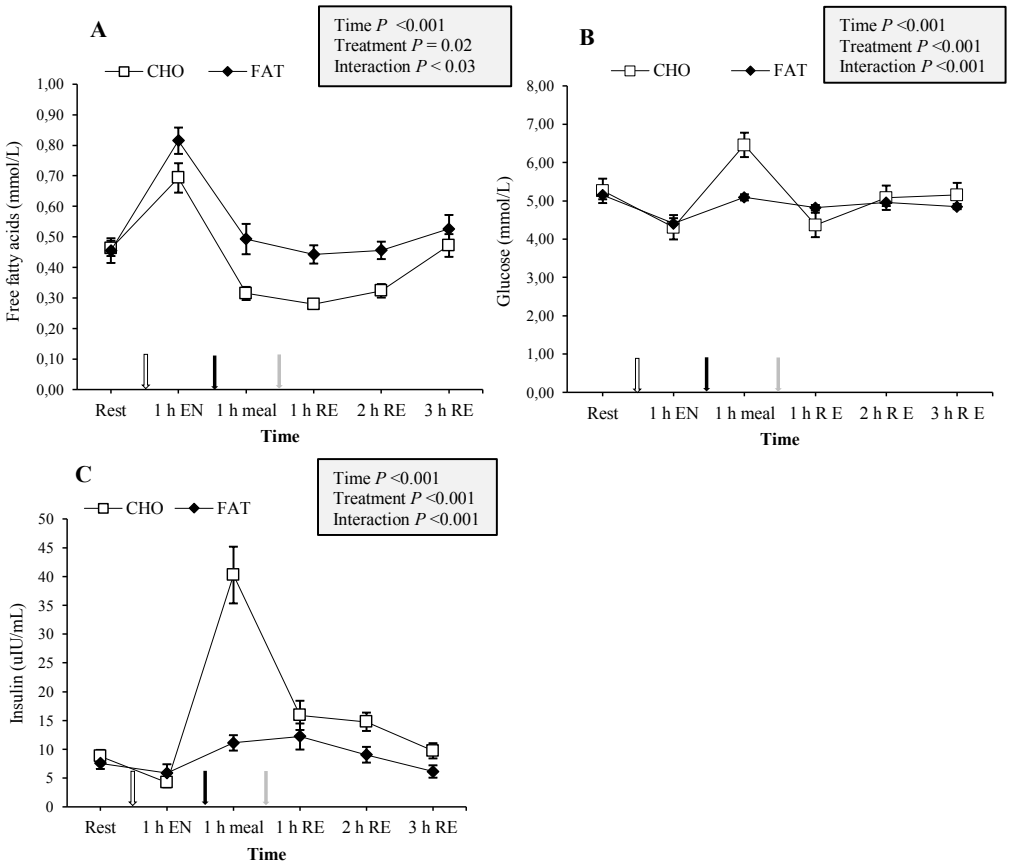

Figure 3. Plasma free fatty acids (A), glucose (B) and insulin (C) at rest and 1 hour after 90-min cycling 70% VO_{2max} (1h EN), 1 hour post meal (1 h meal) and 1h,2h, and 3h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM (1,2 and 3h RE) Values are mean ± SEM. White arrow: endurance exercise session; black arrow: nutritional intervention meal; grey arrow: resistance exercise session.

Cortisol and creatine kinase

Changes in both plasma creatine kinase and cortisol did not show any difference between the CHO and FAT condition (**Figure 4**). Compared with baseline and 1 h post endurance exercise cortisol plasma levels were lower at all other time points during the day ($P < 0.05$). Plasma creatine kinase levels were higher at all-time points compared with baseline with no differences between the CHO and FAT condition.

Figure 4.

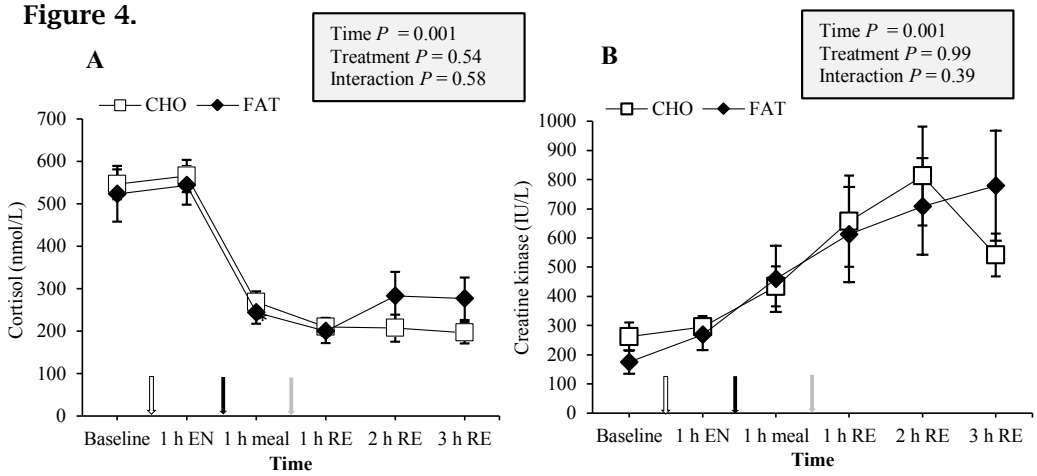

Figure 4. Plasma cortisol (A) and creatine kinase (B) at rest and 1 hour after 90-min cycling 70% VO_{2max} (1h EN), 1 hour post meal (1 h meal) and 1h,2h, and 3h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM (1,2 and 3h RE) Values are mean ± SEM. White arrow: endurance exercise session; black arrow: nutritional intervention meal; grey arrow: resistance exercise session.

Skeletal muscle gene expression

Substrate metabolism

There was a significant increase in HBEGF mRNA quantity at 1 and 3 hours post resistance exercise compared to baseline ($P < 0.05$), while no differences were observed between the treatments (**Figure 5a**). PDK4 mRNA was increased 1 h post resistance exercise ($P < 0.05$) in both conditions and significantly reduced at 3 h post resistance exercise (**Figure 5c**). Additionally, at 3 h post resistance PDK4 mRNA was higher in the FAT condition when compared with CHO ($P < 0.05$). The expression of INSIG1 increased at 1 h post resistance exercise in the FAT group, whereas the levels in the CHO group increased 3 h post resistance exercise compared to baseline (**Figure 5b**).

Figure 5.

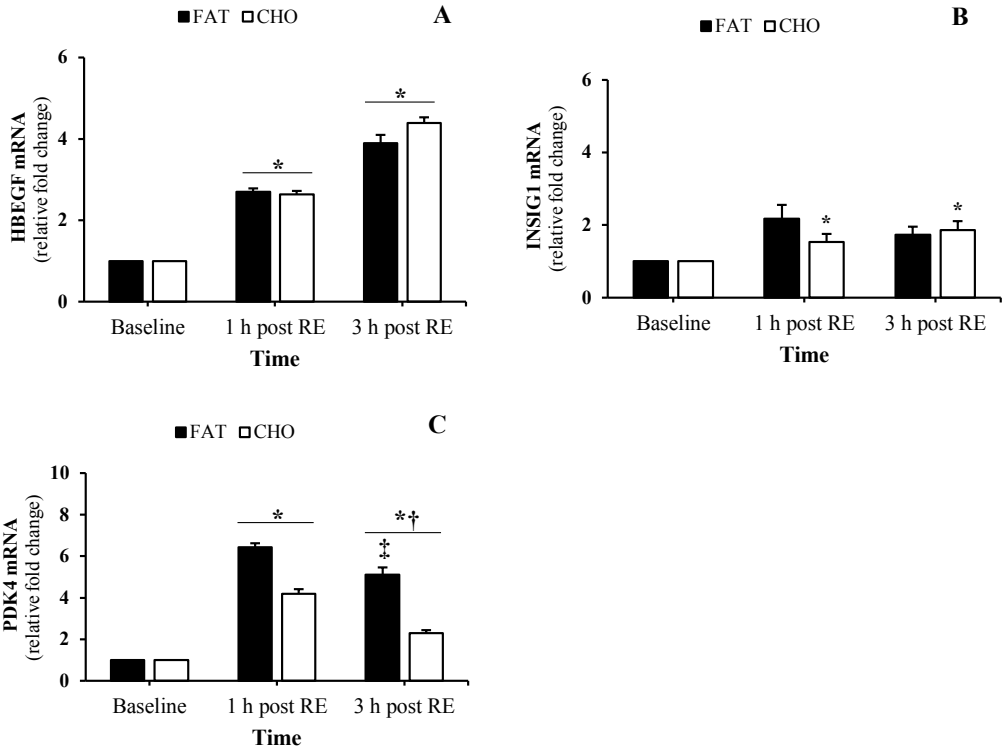

Figure 5. (substrate metabolism) HBEGF (A), INSIG1 (B) and PDK4 (C) mRNA quantity levels at rest and 1 h and 3 h post RE. Values are mean ± SEM. *Significantly different ($P < 0.05$) versus baseline. † Significantly different ($P < 0.05$) compared to previous time point. ‡Significant interaction ($P < 0.05$). CHO = open bars, FAT = filled bars.

Proteolytic genes

FOXO3A mRNA quantity did not change over the course of the day (**Figure 6a**). There was a reduction in both the CHO and FAT condition in MAFbx mRNA response at 3 h post-resistance resistance ($P < 0.05$) compared to baseline, however, no differences were found between the CHO and FAT condition at any time point (**Figure 6b**). MURF1 mRNA quantity increased in both conditions 1 h and 3 h post resistance exercise ($P < 0.05$) compared to baseline (**Figure 6c**). In addition MURF1 mRNA quantity decreased at 3 h post-resistance exercise when compared with 1 h post-resistance exercise ($P < 0.05$). No differences in MURF1 mRNA quantity was found between the FAT and CHO condition during the experimental days. VCP mRNA quantity was slightly higher at 3 h post resistance exercise in the FAT group ($P < 0.05$) (**Figure 6d**).

Figure 6.

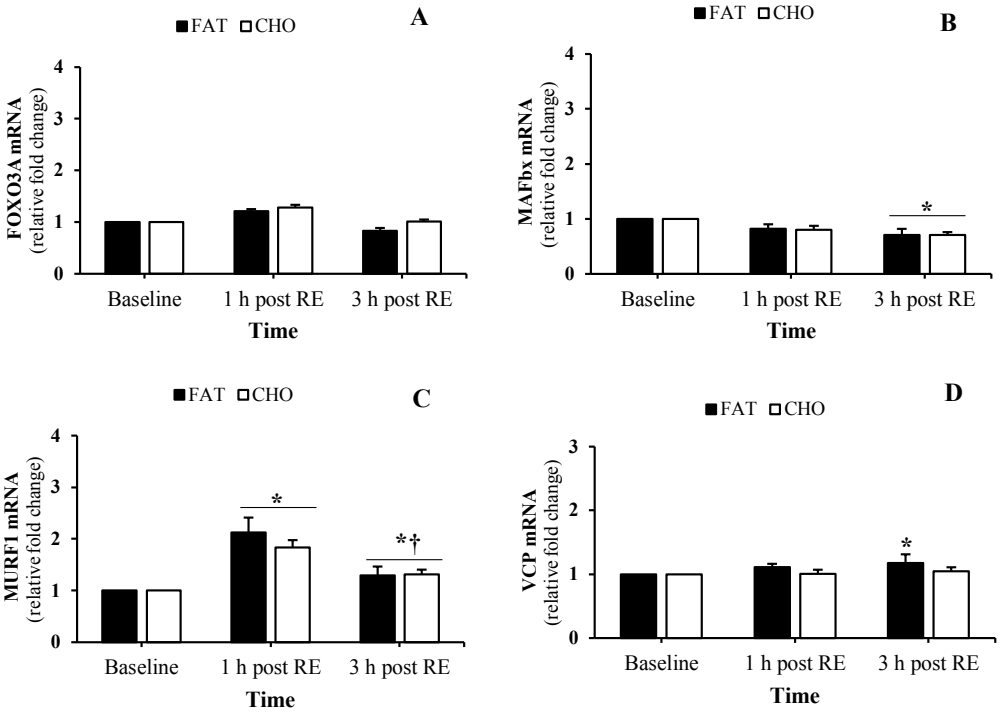

Figure 6. (protein degradation) FOXO3A (A), MAFbx (B), MURF1 (C), VCP (D) mRNA quantity levels at rest and 1 h and 3 h post RE. Values are mean ± SEM. *Significantly different ($P < 0.05$) versus baseline. † Significantly different ($P < 0.05$) compared to previous time point. ‡Significant interaction ($P < 0.05$). CHO = open bars, FAT = filled bars.

Mitochondrial biogenesis

There was no effect of exercise nor nutrition on PGC-1α mRNA quantity (**Figure 7a**). In contrast, SIRT1 mRNA levels increased at 1 h and 3 h post resistance exercise compared with baseline, no differences were found between the CHO and FAT condition in SIRT1 mRNA quantity (**Figure 7b**).

Figure 7.

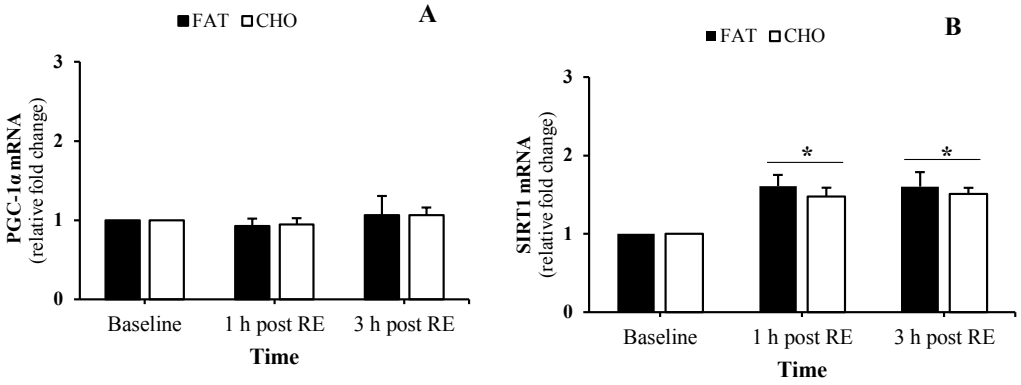

Figure 7. (mitochondrial biogenesis) PGC-1a (A) and SIRT1 (B) mRNA quantity levels at rest and 1 h and 3 h post RE. Values are mean ± SEM. *Significantly different ($P < 0.05$) versus baseline. † Significantly different ($P < 0.05$) compared to previous time point. ‡Significant interaction ($P < 0.05$). CHO = open bars, FAT = filled bars.

IFG-1 and myostatin

IGF-1 mRNA expression levels remained unchanged over the course of the day (**Figure 8a**). Myostatin mRNA decreased at 1 h and 3 h post resistance compared with baseline ($P < 0.05$) without any differences between the nutritional conditions (**Figure 8b**).

Figure 8.

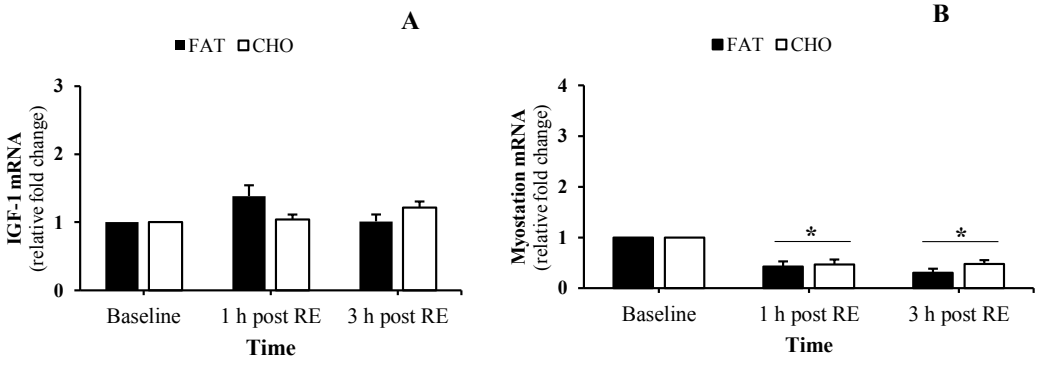

Figure 8. IGF-1 (A) and myostatin (B) mRNA quantity levels at rest and 1 h and 3 h post RE. Values are mean ± SEM. *Significantly different ($P < 0.05$) versus baseline. † Significantly different ($P < 0.05$) compared to previous time point. ‡Significant interaction ($P < 0.05$). CHO = open bars, FAT = filled bars.

Discussion

The gene expression profiles we investigated post-resistance exercise are of interest as they may play a role in mitochondrial biogenesis, protein degradation and substrate metabolism. When compared with the CHO-rich meal condition, we observed an increase in PDK4 mRNA quantity suggesting that pre-exercise FAT meal in combination with the glycogen depletion potentially induced a shift in fuel selection from carbohydrate to fat in the post-resistance exercise period. However, we observed that the genes with a proposed role in muscle adaptation, responded as a result of exercise without any differences between the CHO and FAT condition. Therefore, we suggest that the acute post-resistance exercise response of genes involved in muscle remodelling is not affected by a pre-resistance exercise mixed meal containing different amounts of carbohydrates and fat.

Since athletes commonly combine divergent exercise sessions on the same day, we decided to include both endurance and resistance exercise on the same day in our protocol. Additionally, it has been recently proposed that, when combining divergent exercise sessions within the same day, the endurance session should be performed in the morning in the fasted state, with ample protein ingestion, while the afternoon resistance exercise session should be conducted only after carbohydrate replenishment with adequate post-resistance exercise protein ingestion [25]. With our study we aimed to evaluate the effect of a pre-resistance exercise isocaloric (~3200 kJ) mixed meal containing different amounts of carbohydrates and fat, on post-resistance exercise gene expression after glycogen depleting exercise earlier that day.

Skeletal muscle glycogen

Our subjects performed an endurance exercise bout (90 minutes at ~70% VO_{2max}) in the morning. It is generally assumed that full skeletal muscle glycogen stores are sufficient to fuel endurance type activities for approximately 60-90 minutes [10]. Therefore, it can be assumed that the endurance exercise resulted in a significant reduction of the skeletal muscle glycogen levels post-endurance exercise. Two hours after the endurance exercise bout subjects were fed with either the CHO or FAT meal. Maehlum and Hermansen (1978) showed that omitting carbohydrates in the acute post-exercise period is accompanied with a skeletal muscle glycogen synthesis rate of only 1-2 mmol/kg wet weight (w.w.) of muscle/h through gluconeogenesis [31]. In contrast, when large amounts (~100 g of carbohydrates per hour) of carbohydrates are ingested in the post-exercise period the rate of liver and muscle glycogen synthesis can be up to 5-10 mmol/kg w.w./h [32]. This increased rate of synthesis is primarily explained by insulin-mediated activation of glycogen synthase [33], exercise induced increase in insulin sensitivity [34] and enhanced muscle cell membrane permeability to glucose [35]. With our approach, both at 1 and 3 h

post-resistance exercise, skeletal muscle glycogen stores were reduced compared with baseline. Although the amount of carbohydrates in the CHO condition (~1.44 g·kg^{-1} carbohydrates) meets the recommendation to facilitate post-exercise muscle glycogen resynthesis [36], no differences in muscle glycogen were observed in the post-resistance exercise period when compared with the FAT condition (~0.26 g·kg^{-1} carbohydrates). Since we have no data on the muscle glycogen levels prior to the resistance exercise bout, it remains elusive to what extent the meals affected the muscle glycogen stores. It also remains unclear to what extent the resistance exercise bout further reduced muscle glycogen stores. While the mixed meal with different amounts of carbohydrates and fat did not result in differences in skeletal muscle glycogen levels, significant differences in plasma glucose, free fatty acids and insulin were present. Therefore, we are convinced that the mixed meals resulted in a significant physiological difference with regards to macronutrient availability between the conditions.

PDK4 mRNA

PDK4 phosphorylates pyruvate dehydrogenase enzyme, the first component of the pyruvate dehydrogenase complex that contributes to the conversion of pyruvate into acetyl-CoA altering fuel selection from carbohydrate to fat [37]. Indeed, earlier work showed that PDK4 in human skeletal muscle is dependent on substrate availability rather than exercise-induced cellular perturbations [38, 39]. Likewise, exercise performed with normal skeletal muscle glycogen levels show higher levels of PDK4 mRNA quantity compared with low glycogen levels [39]. Since we observed no differences in post-resistance exercise skeletal muscle glycogen levels between the nutritional conditions, the exogenous provision of carbohydrates ostensibly reduced PDK4 mRNA expression. Interestingly, our finding that exogenous carbohydrate ingestion reduces PDK4 expression is in line with earlier investigations where PDK4 mRNA expression was reduced when exogenous carbohydrate was provided [38] or when skeletal muscle glycogen were normal compared to low [39].

PGC-1α mRNA

PGC-1α has been proposed as the master regulator of mitochondrial biogenesis. Furthermore, PGC-1α exists in different isoforms, which may have different roles in training adaptation. For instance, PGC-1α4 has been implicated to play a role in the regulation of skeletal muscle hypertrophy [40]. Unexpectedly, PGC-1α mRNA quantity remained unchanged during the post-resistance exercise period. This was somewhat surprising since work by Camera, Hawley and Coffey (2015) recently demonstrated that performing resistance exercise with low skeletal muscle glycogen levels amplifies intramuscular PGC-1α mRNA quantity when compared with normal glycogen

levels [12]. Notably, the time course of the muscle biopsies differed between the studies. Camera et al., (2015) took muscle biopsies 2 h and 4 h post resistance exercise and found enhanced expression of PGC-1α at the 4 h time point. We took biopsies 1 h and 3 h post resistance exercise and may therefore overlooked a potential effect of the intervention PGC-1α mRNA quantity. Furthermore, our subjects were provided we a mixed meal in the pre-resistance exercise period which makes is difficult to compare the divergent findings of the studies. Lastly, it has been reported that BCAA provision attenuates the resistance exercise induced elevation of PGC-1α4 mRNA, though, no effect of BCAA was found on PGC-1α1 and 2 mRNA [41]. Nevertheless, it may thus be possible that the post-exercise protein ingestion attenuated the PGC-1α mRNA response.

Gene expression of proteolytic genes

We also analysed a set of genes with a proposed role in protein degradation (FOXO3A, MAFbx, MURF1, Myostatin and VCP). Myostatin mRNA quantity decreased post-resistance exercise, which is in accordance with findings of others [42]. Muscle catabolism is a fundamental process of muscle remodelling ensuring that damaged proteins or misfolded proteins during exercise are adequately removed from the cell [43]. As expected, mRNA involved in both ubiquitin proteasome and autophagy-mediated protein degradation systems responded as a result of exercise, however without any effect of the pre-resistance exercise mixed meal. We found a higher plasma insulin response in the carbohydrate group. According to Abdulla et al., (2016) insulin reduces protein degradation rates thereby facilitating an overall net protein balance [44]. Mechanistically, insulin activates the PI3K-Akt pathway by binding to its transmembrane insulin receptor on the sarcolemma and initiates translocation of the proteins to the cell membrane [45]. Earlier work in animals [46] and C2C12 cell lines [47, 48] demonstrated that Akt inhibits the expression of FOXO3A mRNA, a transcription factor implicated with gene expression with the ubiquitin proteasome pathway. In our study, the mixed meal with the higher amount of carbohydrates resulted in higher insulin response post-resistance exercise, however, this effect did not translate into a difference in FOXO3A mRNA response between the CHO and FAT condition. The latter could be explained by the theory that the insulin mediated reduction in protein breakdown is more potent when amino acid availability is scarce [44, 49, 50]. This was not the case in our study since our subjects were provided with adequate protein ingestion post-resistance exercise [24]. Other reports confirm that when adequate protein is provided post-resistance exercise, the insulinogenic response seems to be redundant [24, 51].

Conclusion

In summary, resistance exercise with different carbohydrate/fat availability but ample protein provision did in general not influence intramuscular gene expression. Post-resistance exercise PDK4 mRNA quantity was higher in the CHO condition compared with the FAT condition suggesting a potential shift towards glucose oxidation. Furthermore, it appears that carbohydrate replenishment in between endurance and resistance exercise did not influence gene expression involved in the adaptive response after a subsequent bout of resistance exercise. Our findings support the view that pre-resistance exercise carbohydrate availability does not affect acute transcriptional responses associated with muscle recovery to resistance exercise.

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Chapter 4

Plasma cytokine responses to resistance exercise with different nutrient availability on a concurrent exercise day in trained healthy males

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Abstract

Carbohydrate availability is proposed as a potential regulator of cytokine responses. We aimed to evaluate the effect of a pre-resistance exercise carbohydrate meal versus fat meal on plasma cytokine responses to resistance exercise after an endurance exercise earlier that day. Thirteen young, healthy, recreationally active males performed two experimental days with endurance exercise in the morning and resistance exercise in the afternoon. Either a carbohydrate (110g carbohydrate, 52g protein, 9g fat; ~750 kcal) or an isocaloric fat meal (20 gr carbohydrate, 52g protein, 51g fat) was provided 2 h before resistance exercise. Blood was taken at baseline and at regular time intervals to measure circulating plasma cytokine levels (e.g. IL-6, IL-8, IL-10, IL-15, TNFα, ANGPTL4, Decorin and MCP-1). Plasma glucose and insulin were higher in the postprandial period before the start of the resistance exercise on the carbohydrate condition, while free fatty acids were reduced. At 2 h post resistance exercise, IL-6 concentrations were higher in the fat condition compared to the carbohydrate condition (P <0.05). In addition, in both conditions IL-6 levels were higher at all timepoints compared with baseline (P < 0.05). The pattern of increase in plasma IL-8 and IL-10 did not differ significantly between conditions (P > 0.05) . There were no differences between conditions on TNFα levels and levels remain constant when compared with baseline (P > 0.05). ANGPTL4, IL-15, Decorin and MCP-1 showed no differences between the fat and carbohydrate condition (P > 0.05). The composition of the pre-exercise meal did in general not influence cytokine responses in the post-resistance exercise period, except post-resistance exercise circulating plasma IL-6 levels being higher in the fat condition compared with carbohydrate. Our findings support the view that pre-resistance exercise carbohydrate availability does not have a major impact on acute responses of circulating plasma cytokines in humans.

Introduction

Cytokines are small proteins produced and released by several cells that play an integrative and regulatory role in local and systemic intercellular communication (1, 2). Up to now, many studies have assessed the effect of endurance and resistance exercise on circulating concentrations of cytokines (3). Even though the precise biological function of the majority of the cytokines is presently unclear, exercise-induced elevations of circulating cytokine concentrations such as interleukin 6 (IL-6), 8 (IL-8), 15 (IL-15), Decorin, monocyte chemoattractant protein-1 (MCP-1) and 4 (MCP-4) may play a role in adaptation of skeletal muscle (1, 4, 5). However, the proposed role of cytokines in skeletal muscle regulation is mainly derived from mechanistic cell and animal studies, as a consequence, data from human studies is highly limited.

It is well established that exercise parameters such as mode, intensity, duration and type of exercise largely modulate cytokine response following exercise (3). In addition, nutrition (e.g. carbohydrates) appears to play a role as well in the modulation of circulating cytokines following exercise, particularly IL-6 (1). For instance, circulating IL-6 concentrations decrease with glucose ingestion during endurance exercise (6, 7), whereas low pre-exercise muscle glycogen content augments these responses (8, 9). Furthermore, work by Nieman *et al* (2004) demonstrated that carbohydrate ingestion before and during a 2 h intensive resistance training did not alter circulating plasma levels of IL-1ra, IL-6, IL-8, IL-10 (10). Remarkably, the role of pre-exercise fat intake on plasma cytokines remains to be elucidated.

To our knowledge, not much work has been done on the effects of pre-exercise nutrient availability on circulating cytokine concentrations following resistance exercise in humans. Since some of these cytokines appear to play a role in skeletal muscle growth, we were specifically interested in the effects of nutrient availability on circulating cytokines that have been implicated to play a role in muscle reconditioning. In our study we aimed to evaluate the effects of a pre-resistance exercise carbohydrate meal versus fat meal on circulating cytokine concentrations to resistance exercise after an endurance exercise earlier that day. We designed an experimental protocol where endurance and resistance exercise were performed in the morning and afternoon respectively, with ample protein ingestion throughout the day. The nutritional difference between the conditions was induced by a pre-resistance exercise meal that differed in macronutrient content. Specifically, in one condition the subjects were provided with a meal rich in carbohydrate (CHO condition), whereas in the other condition an isocaloric meal high in fat (FAT condition) was provided. It is hypothesized that some of the selected cytokines will differ between nutritional conditions, but that the majority of the circulating cytokines with a possible role in skeletal muscle growth do not respond differently.

Methods

Subjects

Fourteen healthy recreationally-active males volunteered to participate in this study (age; 21.2 ± 0.5 years, height; 1.87 ± 0.0 m, mass; 76.7 ± 1.3 kg). One subject dropped out after test day 1 because of the discomfort of the muscle biopsies, therefore final analysis was performed on 13 subjects. All subjects were non-smokers, free of injury and not taking any medication or nutritional supplements. All subjects provided full-written informed consent. The Medical Ethical Committee of Wageningen University approved all study procedures.

Subject characteristics

Physical characteristics of 13 volunteers are shown in Table 1.

Table 1. Physical characteristics of 13 volunteers.

	Mean ± SE
Age (years)	21.2 ± 0.5
Height (m)	1.87 ± 0.0
Weight (kg)	76.7 ± 1.3
BMI (kg/m^2)	22.0 ± 0.2
W_{max} (W)	346 ± 7.6
VO_{2max} (ml/kg/min^{-1})	51.3 ± 1.3
Leg press 1-RM (kg)	266 ± 8.2
Leg extension 1-RM (kg)	111 ± 3.0

Study Design

This study used a randomised counterbalanced crossover design (Fig. 1). On both experimental days subjects completed the same exercise sessions: an endurance exercise session in the morning and a resistance exercise session in the afternoon with a resting period of 4 hours between sessions. The subjects received a nutritional treatment in between the exercise sessions (carbohydrate or fat meal). Each trial was separated by a minimum of 12 days (range: 12-30 days), during which time the subjects were instructed to maintain their habitual lifestyle.

Figure 1.

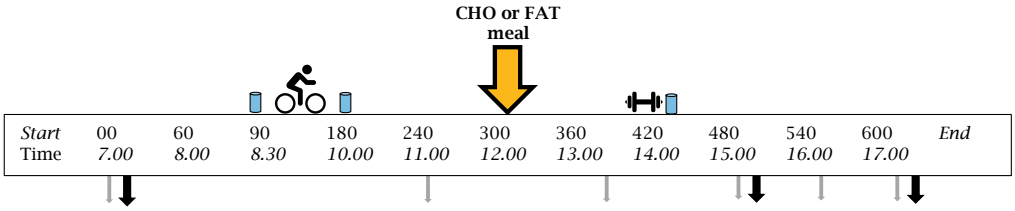

Figure 1. Schematic diagram of the experimental trials. This study adopted a counterbalanced crossover design where subjects completed both exercise trials with different nutritional treatments on separate occasions. Endurance exercise session: 90-min cycling at 70% VO_{2max}. Resistance exercise sessions 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1-RM. Blue containers indicates protein beverage (from left to right; 10g casein, 15g casein and 25g whey. Big orange arrow with black outline indicates lunch with carbohydrates or fat (nutritional intervention meal). Grey arrows indicates time point for blood sample; black arrows indicates sampling time point for muscle biopsy.

Preliminary testing

VO_{2max}

Maximal aerobic capacity (VO_{2max})

Preliminary testing was performed in the week prior to the start of the first experimental day. Following a 30-min rest, subjects performed a ramped VO_{2max} test on an electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). After a 5-min warm up at 50 W, the subjects started cycling at 100 W. Workload was progressively increased by 20 $W \cdot min^{-1}$ until the subject reached volitional exhaustion. The VO_{2max} test was considered to be valid when two out of three criteria were met: I) levelling of VO_2 with increasing workload; II) heart rate within 10 beats of the theoretically estimated maximum (220-age) and III) respiratory exchange ratio (RER) of ≥1.15. Oxygen consumption (VO_2) was measured through breath-by-breath sampling with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define maximal oxygen consumption (VO_{2max}). Subjects were asked to maintain a cadence between 80-100 $r \cdot min^{-1}$.

Maximal strength

Maximal muscle strength was determined by one-repetition maximum (1RM) strength tests on leg press and leg extension machine (Technogym, Cesena, Italy). Subjects were familiarized with the movement and warmed up prior to testing by performing six repetitions (at ~40% of estimated 1RM) through a full range of motion with a 1-min rest. After each successful lift the weight was increased until a failed attempt occurred with 3-min recovery between each attempt. The 1 RM was attained within five attempts. For each preliminary test,

subjects were fasted for at least 4 h and were instructed to avoid strenuous physical activity 48 h before.

Diet and physical activity control
Subjects were instructed to avoid alcohol, caffeine and physical activity during 48 h prior to the two main experimental trials. Diet and physical activity levels were recorded for 24 h before the first experimental trials and subjects were asked to replicate dietary intake and physical activity prior to the second experimental trial. Dietary intake records were analysed using Eetmeter Software 2005 (version 1.4.0;Voedingscentrum). A standardized meal for the subjects was provided the night before each trial (standard deep-frozen meal and ice-cream dessert; 43.80 kJ/kg BW; 15 Energy% protein, 30 Energy% fat and 55 Energy% carbohydrate).

Experimental days
All subjects arrived at 07.00 in the morning in the fasted state at the laboratory of our university. After changing into sport gear, a catheter was inserted into the antecubital vein of the left arm. Fifteen minutes after insertion of the catheter baseline blood samples were taken followed by a baseline muscle biopsy. Approximately 10 minutes after the baseline biopsy a protein beverage containing 10 gram of casein dissolved in 350 ml water was consumed (t = -05 min). Within five minutes after consumption of the protein beverage, the subjects started with 90 minutes cycling (t = 0 min) at submaximal intensity (70% VO_{2max}). If subjects could not sustain the intensity of at least 60 RPM, resistance was decreased with 20 W, and the same intensity protocol was repeated during the second training. All subjects performed the same exercise intensity of both experimental days. Immediately after the endurance exercise bout, a beverage containing 15 gram of casein dissolved in 350 ml water was consumed (t = 90 min). A second blood sample (~25 ml) was taken 1 hour after the end of the endurance exercise bout (t = 150 min). Two hours after the endurance exercise bout (t = 210 min) subjects were randomly provided with either a carbohydrate rich low-fat meal or low-carbohydrate fat meal (see intervention meal for details). Ninety minutes after the meal (t = 300 min) a third blood sample (~25 ml) was taken. At t = 330 min subjects performed the resistance exercise bout that consisted of 5 x 8 (80% 1RM) repetitions of bilateral leg press and leg extension with two minutes of rest in between the sets. If subjects could not sustain due to fatigue, the selected weight was decreased with steps of 10 kg or 5 kg for the leg press and leg extension, respectively. Immediately after the resistance exercise bout (t = 360 min), a protein beverage containing 25 gram of whey protein dissolved in 350 ml water was consumed. One and three hours after the resistance exercise bout a second (t = 480 min) and a third (t = 600 min) skeletal muscle biopsy was taken. All biopsies were taken from the same leg. Additional blood samples were taken

at t = 480, 540 and 600 min. A timeline for the experimental day can be found in figure 1. The whole experimental protocol was repeated on the 2nd day, while the other meal was provided.

Nutritional intervention

Both meals were cooked and prepared by a research dietician of Wageningen University. Both the carbohydrate and fat meal consisted of commercially available meat, macaroni and vegetables with an energetic value of ~3200 kJ. The absolute macronutrient amounts were chemically analysed as described previously (11) and can be found in table 2.

Table 2. Overview of the energy and macronutrient composition intervention meal.

Energy & Nutrient	Fat meal	Carbohydrate meal
Energy (kJ)	3207	3124
Protein (g)	52	52
Fat (g)	51	9
Carbohydrates (g)	20	110

Exercise protocol

Since athletes commonly combine divergent exercise sessions on the same day, we decided to include both endurance and resistance exercise on the same day in our protocol. Additionally, it has been recently proposed that, when combining divergent exercise sessions within the same day, the endurance session should be performed in the morning in the fasted state, with ample protein ingestion, while the afternoon resistance exercise session should be conducted only after carbohydrate replenishment with adequate post-resistance exercise protein ingestion (Perez-Schindler et al., 2015). With our study we aimed to evaluate the effect of a pre-resistance exercise isocaloric (~3200 kJ) mixed meal containing different amounts of carbohydrates and fat, on post-resistance exercise plasma cytokines after glycogen depleting exercise earlier that day.

Muscle biopsies

Muscle biopsies were taken as described by Bergstrom (12). Biopsies were taken under local anaesthesia (2-3 ml of 2% Xylocaine) using a 5-mm Bergstrom needle modified with suction. All three muscle biopsies on one experimental day were taken from the vastus lateralis of the same leg, with separate incisions (~1-1.5 cm) apart and from distal to proximal direction. Muscle biopsies on the second test day were taken from the contralateral leg. Muscle biopsies were immediately frozen (in 5–10 s) in liquid nitrogen and stored at −80°C for subsequent biochemical analysis, after being freed from visible fat, blood and connective tissue.

Muscle glycogen
Muscle tissue, ~30 mg wet weight, was freeze dried, after which collagen, blood, and non–muscle fibre materials were removed from the muscle fibres under a microscope. The isolated muscle fibre mass (~5–7 mg) was weighed, and 500 µL of 1 MHCI was added. After heating for 3 h at 100°C to hydrolyse the glycogen to glycosyl units and cooling down to room temperature, 500 µL of the solution was neutralized by adding 280 µL of Tris–KOH (Tris 119 mM, KOH 2.14 M) and centrifuged at 1000*g* and 4°C for 10 min. Thereafter, 150 µL of this solution was analysed for glucose concentration (Glucose HK CP A11A01667, ABX Pentra) with a COBAS FARA semiautomatic analyser (Roche).

Glucose, insulin and free fatty acids
Blood was collected in EDTA-containing tubes. The samples were immediately centrifuged at 1000g at 4°C for 10 minutes, after which plasma was stored in −80°C until further analysis. Blood samples were analysed for glucose and insulin (Gelderse Vallei hospital, Ede, the Netherlands). Free fatty acids were assessed using an enzymatic test kit according to the manufacturer's protocol (InstruChemie, Delfzijl, the Netherlands).

ANGPTL4 and Decorin
All plasma samples were kept on ice until centrifugation and stored at −80°C until further analysis. Angiopoietin-like 4 (ANGPTL4) and Decorin were assessed using human ELISA kits according to the manufacturer's protocol.

IL-6, IL-8, IL-10, IL-15,MCP-1 and TNF-α
Blood samples were taken at baseline, 1 h post endurance exercise, 1 h post meal, 1 h, 2 h and 3 h post resistance exercise. Participants were seated for 5 min after which blood samples were taken from the cephalic vein. Blood was collected in a 4 ml EDTA vacutainer (Becton-Dickinson, New Jersey, United States of America). The vacutainer was immediately put on melting ice water (4°C) and centrifuged at 1200g for 15 minutes at 4°C. Serum was transferred to polypropylene tubes and stored at -80°C until Cytokine analysis. We measured IL-6, IL-8, IL-10, IL-15, MCP-1 and TNF-α concentrations using the ultrasensitive MesoScale Discovery (MSD) QuickPlex SQ 120 Instrument with Multi-spot assay (Human Proinflammatory Panel 1, K15049D, MSD, Maryland, United States of America) according to the manufacturers' recommendations. The lower detection limit was 0.043-0.107, 0.039-0.059, 0.022-0.034, 0.46-0.54, 0.09-0.16 and 0.055-0.087 pg/ml, respectively, varying per plate. Precision of these validated kits was as follows: The intra-run % CV for the high-low controls were 3.6-4.5%, 2.7-3.0%, 2.6-3.7%, 3.3-4.1% and 2.7-3.4% for IL-6, IL-8, IL-10, IL-1β and TNF-α, respectively. The Inter-run % CV for the high-low controls were 5.2-7.3%, 5.0-7.1%, 5.7-10.1%, 5.5-7.7% and 6.1-10.1 % for IL-6, IL-8, IL-10, IL-1β and TNF-

α, respectively. With the high-low concentrations being as follows: 239-18.4 pg/ml, 166-12.5 pg/ml, 107-7.18 pg/ml, 152-11.2 pg/ml, and 75.5-4.45 pg/ml, for IL-6, IL-8, IL-10, IL-1β and TNF-α, respectively.

Statistics

Data was analysed using a two-way repeated measures ANOVA (two factor, time x treatment) from IBM SPSS version 23 statistical software (IBM, Armonk, NY). When a main effect of trial of time or interaction was identified, a pairwise multiple comparisons with a Bonferroni correction was done to identify differences. Statistical significance was set at the $P < 0.05$ level, and values were expressed as means ± SEM or different when indicated.

Results

Endurance and resistance exercise performance

Two subjects performed the endurance exercise sessions with a reduced workload. For one subject the workload was reduced with 20 W after 20 min whereas for the other subject the workload was reduced with 20 W after 30 min. There was no further reduction in workload during the remaining part of the endurance session. Additionally, both subjects were able to repeat this on the second experimental day. All 13 subjects performed the resistance exercise training with exactly the same amount of work on the 2 experimental days.

Muscle glycogen

There were no differences in baseline muscle glycogen between the conditions (carbohydrate 380 ± 20.4 mmol/kg dry weight (dw) versus fat 441 ± 26.3 mmol/kg dw; $P > 0.05$ **Figure 2**). As a result of endurance exercise, muscle glycogen was significantly reduced compared to baseline in both conditions at 1 h and 3 h post-resistance exercise ($P < 0.05$) (carbohydrate 163 ± 23.5 and 181 ± 20.3 mmol/kg dw; fat 185 ± 25.8 and 140 ± 24.4 mmol/kg dw), without any significant differences between the conditions ($P > 0.05$).

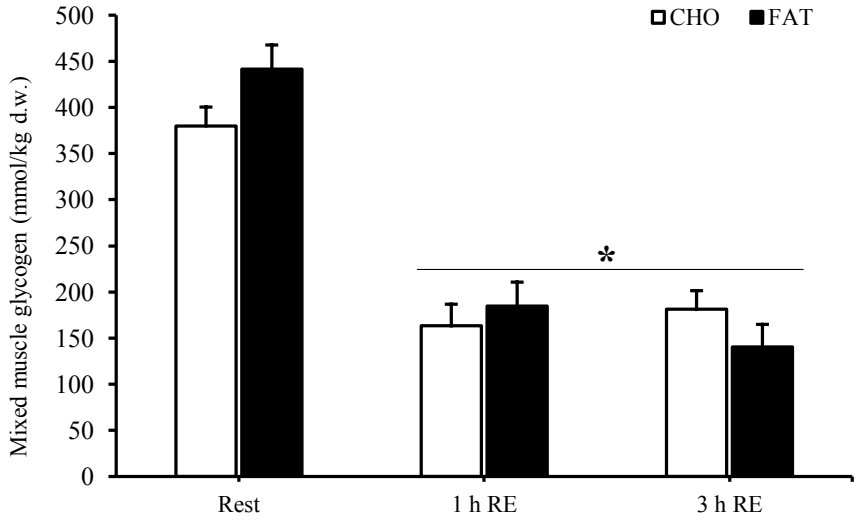

Figure 2. Mixed muscle glycogen at rest and 1h and 3h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM (1, 2 and 3h RE) Values are mean ± SEM.
*Significantly different ($P < 0.05$) versus rest. CHO = open bars, FAT = filled bars.

Free fatty acids, glucose and insulin

One hour post meal, plasma free fatty acid concentration was higher in the fat condition compared with the carbohydrate condition and this effect remained significant after 1 and 2 hours post-resistance exercise ($P < 0.05$) (Fig. 3). Both glucose and insulin in the fat condition were significantly lower at 1 h post meal, and at 1, 2 and 3 h post-resistance exercise compared with the carbohydrate condition ($P < 0.05$) (Fig. 3).

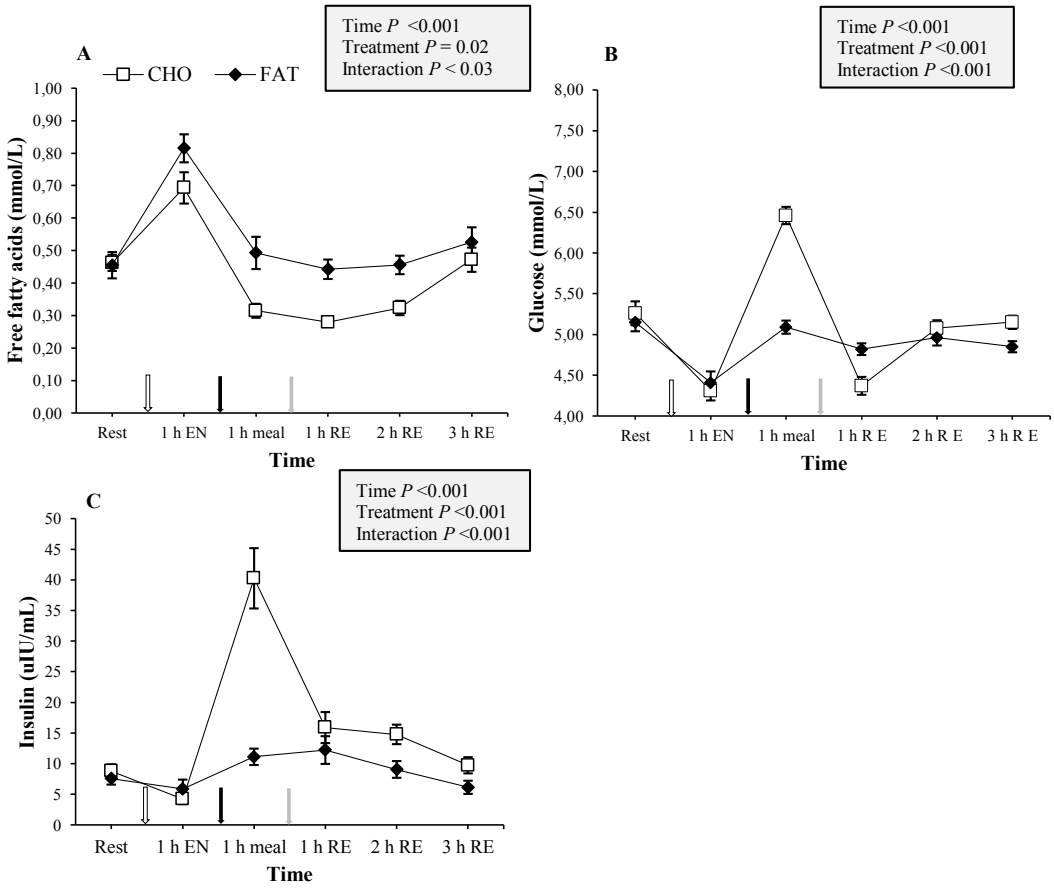

Figure 3. Plasma free fatty acids (a), glucose (b) and insulin (c) at rest and 1 hour after 90-min cycling 70% VO_{2max} (1 h EN), 1 hour post meal (1 h meal) and 1 h,2 h, and 3 h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM (1,2 and 3h RE) Values are mean ± SEM. White arrow: endurance exercise session; black arrow: nutritional intervention meal; grey arrow: resistance exercise session.

IL-6, IL-8, IL-10 and TNFα

At 2 h post resistance exercise, IL-6 concentrations were higher in the fat condition compared to the carbohydrate condition ($P < 0.05$) (Fig. 4). In addition, in both conditions IL-6 levels were higher at all timepoints compared with baseline ($P < 0.05$). The pattern of increase in plasma IL-8 and IL-10 did not differ significantly between conditions ($P > 0.05$) (Fig. 4). As a result of the endurance session in the morning, concentrations of both IL-8 and IL-10 were increased 1 h post endurance exercise ($P < 0.05$). Furthermore, compared to baseline, IL-8 was still increased 1 h post meal and 1 h post resistance exercise ($P < 0.05$), with no differences between meal conditions ($P > 0.05$). There were no differences between conditions on TNFα levels and levels remain constant when compared with baseline ($P > 0.05$).

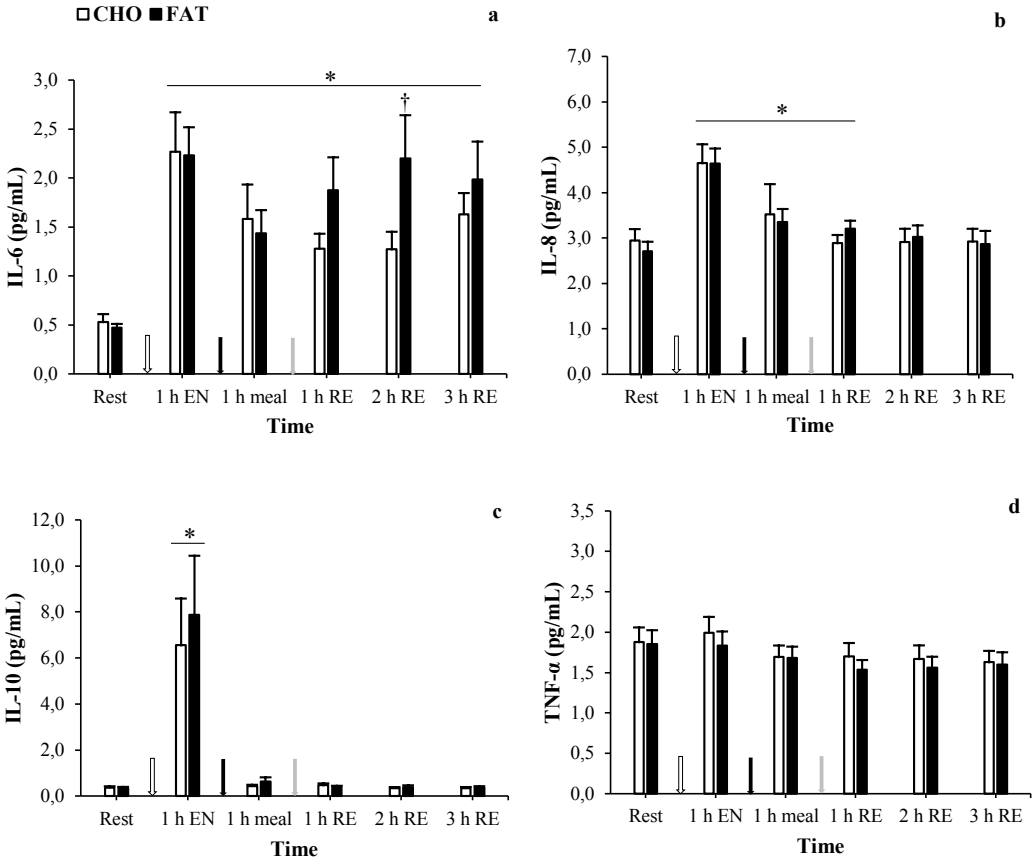

Figure 4. IL-6 (a), IL-8 (b), IL-10 (c) and TNFα (d) at rest and 1 hour after 90-min cycling 70% VO_{2max} (1h EN), 1 hour post meal (1 h meal) and 1 h,2 h, and 3 h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM (1,2 and 3h RE) Values are mean ± SEM. *Significantly different ($P < 0.05$) versus rest. †Significantly different ($P < 0.05$) between

conditions. White arrow: endurance exercise session; black arrow: nutritional intervention meal; grey arrow: resistance exercise session.

ANGPTL4, IL-15, Decorin and MCP-1

ANGPTL4, IL-15, Decorin and MCP-1 showed no differences between the fat and carbohydrate condition ($P > 0.05$) (**Figure 5**). ANGPTL 4 levels were higer compared to baseline in both conditions at all timepoints ($P < 0.05$). IL-15 was higher than baseline in the carbohydrate condition 1 h post meal and 1 h post resistance exercise, whereas in the fat condition an increase compared with baseline was found only 1 h post resistance exercise ($P < 0.05$). Decorin levels were not different from baseline at any time point for both conditions ($P > 0.05$). MCP-1 levels were higher than baseline in the fat condition at any time point except 2 hour post resistance exercise ($P < 0.05$). In the carbohydrate condition at 2 and 3 h post resistance exercise the MCP-1 levels were elevated compared to baseline ($P < 0.05$).

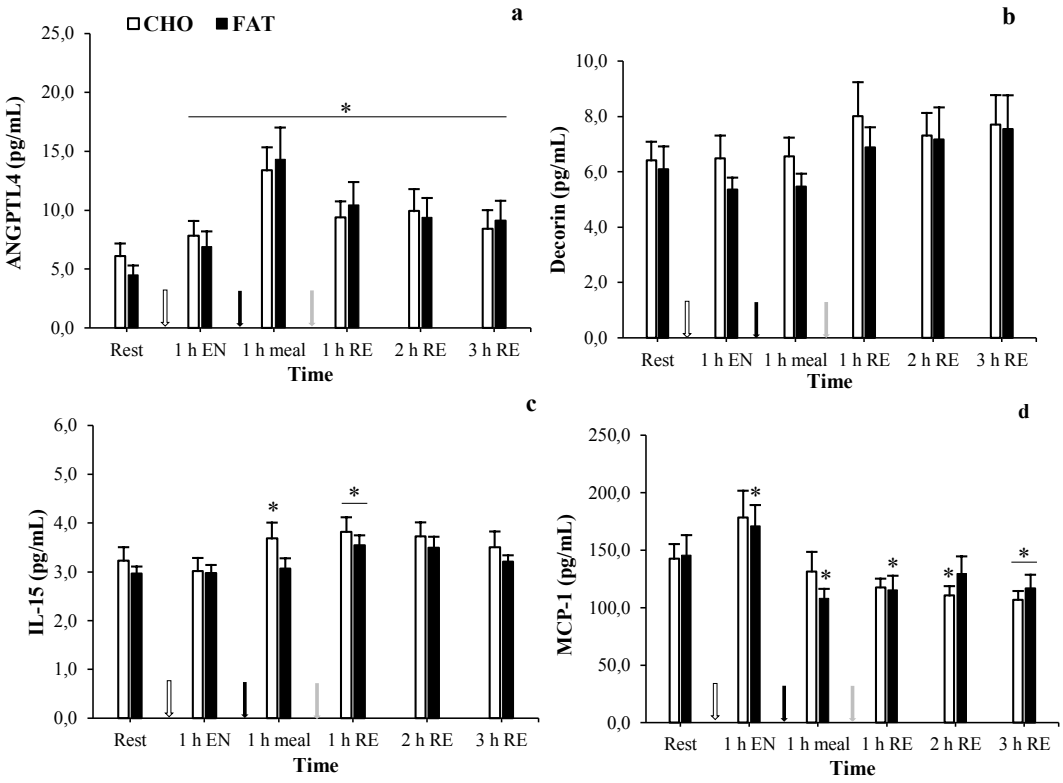

Figure 5. ANGPTL4 (a), Decorin (b), IL-15 (c) and MCP-1 (d) at rest and 1 hour after 90-min cycling 70% VO_{2max} (1 h EN), 1 hour post meal (1 h meal) and 1 h,2 h, and 3 h after 5 sets of 8 repetitions for both leg press and leg extensions at 80% 1RM (1,2 and 3h RE) Values are mean ± SEM. *Significantly different (P < 0.05) versus rest. White arrow: endurance exercise session; black arrow: nutritional intervention meal; grey arrow: resistance exercise session.

Discussion

In this study we investigated whether pre-exercise nutrient availability such as carbohydrates and fat influenced circulating cytokines concentrations with resistance exercise on a concurrent exercise day. We observed that the majority of the circulating cytokines (ANGPTL4, Decorin, IL-8, IL-10, IL-15, MCP-1 and TNF-α) are not affected by differences in pre-resistance exercise carbohydrate or fat availability during the early post-resistance exercise recovery period on a concurrent exercise day. We were especially interested in circulating IL-6, IL-8, IL-15, Decorin and MCP-1, since elevations of these cytokines may contribute or reflect adaptations of skeletal muscle (5, 13). The only cytokine of our selective set of circulating cytokines that responded differently between the nutritional conditions was IL-6.

Cytokines that have been implicated in skeletal muscle hypertrophy

IL-6 is one of the most discussed cytokines (1) that responds primarily to acute muscle contraction and peaks immediately after exercise (2), as was also observed in our study after the endurance session in the morning. In addition, it can be assumed that the significant reduction of hepatic and skeletal muscle glycogen levels post-endurance exercise contributed to the observed rise in plasma IL-6. Previous animal- and cell-culture work showed that IL-6 is probably an important regulator of satellite cell-mediated skeletal muscle hypertrophy (14). We found that two hours post-resistance exercise circulating IL-6 was significantly higher in the low carbohydrate condition compared with the high carbohydrate condition. Although we cannot exclude the possibility that this was partly due to a difference in muscle glycogen content during the resistance exercise bout, it is most likely due to the difference in carbohydrate availability in the pre-exercise meal since we found no differences in skeletal muscle glycogen in the post-resistance exercise period. Indeed, increased circulating levels of IL-6 have been found to be sensitive to changes in blood glucose levels (6), but also to pre-exercise muscle glycogen content (9). Likewise, pre-exercise reduced intramuscular glycogen augments IL-6 mRNA transcription factors and plasma IL-6 concentrations (8, 15), possibly explained through activation of AMPK (16) and p38MAPK (15). In addition, increased plasma IL-6 may act as a signal for hepatic glucose release to maintain blood glucose levels during exercise (1). Since we compared two isocaloric meals with different amounts of carbohydrates we cannot conclude whether the low carbohydrate availability augmented expressions of IL-6 plasma or that the high carbohydrate availability attenuated IL-6 plasma levels. In addition to circulating IL-6 we had particular interest in the responses of circulating IL-15 since this cytokine has been implicated in the regulation of skeletal muscle turnover (17-22). The responses of the circulating IL-15 with exercise vary between studies, but it seems that IL-15 primarily responds to

resistance exercise in both trained and untrained individuals (22, 23). Overexpression of IL-15 in cultured skeletal myotubes affects protein metabolism by stimulation of protein synthesis and inhibition of protein degradation, suggesting a possible role for IL-15 in muscle growth and wasting (20). Furthermore, it has been suggested that IL-15 plays a role in muscle adipose tissue interaction (24). Our plasma data of IL-15 showed an increase at 1 h post-resistance exercise without a difference between the carbohydrate and fat condition suggesting that circulating plasma IL-15 is not affected by the pre-resistance exercise difference in availability of carbohydrate or fat. The evidence that IL-15 plays an important role in skeletal muscle growth with exercise is mainly derived from in vitro and animal work and its relevance in terms of physiological adaptation in humans remains to be determined (18, 20, 25, 26).

The majority of the selected cytokines responded as a result of exercise, this was also the case for circulating Decorin. We found a mild increase, although not significant in plasma Decorin at 1, 2 and 3 h post-resistance exercise when compared to circulating levels of Decorin at baseline. However, no differences were found between the low and high carbohydrate condition. Decorin is a protein secreted by skeletal muscle cells, and promotes skeletal muscle hypertrophy by binding with myostatin (27). It has recently been demonstrated that plasma Decorin increases in response to acute resistance exercise (27). The somewhat moderate response in our study of plasma Decorin compared to the study of Kanzleiter *et al.* (2014) could be explained by exercise volume and time points of measurement. Indeed, Kanzleiter *et al.* used seven different exercises of three sets and measured plasma Decorin during and immediately after, every 30 minutes until 120 minutes post-resistance exercise (27). In their study, which clearly had a higher exercise load, plasma Decorin peaked immediately after exercise, a time point we are lacking in our study (27). The last cytokine we investigated with a possible role in skeletal muscle growth is MCP-1. Previous studies show that MCP-1 is both a cytokine and exercise factor. MCP-1 appears to be vital for muscle recovery and adaptation (28, 29). Divergent exercise modes increase skeletal muscle gene expression levels of MCP-1 (30-33). Yet, MCP-1 responded as a result of exercise while no differences were seen between the nutritional conditions.

ANGPTL4

In our nutritional intervention we compared two isocaloric pre-exercise meals that differ in macronutrient profile. Specifically, in the FAT condition, the carbohydrates were almost completely replaced by fat. Therefore, we also looked to the responses of ANGPTL4 since this protein appears to be sensitive to glucose ingestion (34). ANGPTL4 is found in both skeletal muscle and adipose tissue and is regulated by exercise via free elevated free fatty acids

(34). ANGPTL4 stimulates degradation of lipids and thereby the release of glycerol and free fatty acids to the circulation. Furthermore circulating plasma ANGPTL4 responds to exercise and previous work by Kersten *et al.* (2009) demonstrated that exercise-induced ANGPTL4 plasma responses are partly inhibited when subjects are given oral glucose (34). It is theorized that glucose inhibits ANGPTL4 possibly caused by the rise of insulin, via suppression of lipolysis and reduced plasma free fatty acids concentration (34, 35). Our data confirmed findings of others that plasma ANGPTL4 increases significantly after endurance exercise (34). The increase in ANGPTL4 was further elevated 1 h post meal possibly because of a positive feedback loop of plasma free fatty acids raising ANGPTL4 levels and ANGPTL4 promoting adipose tissue lipolysis, raising free fatty acids plasma levels (34). Despite a significant difference in circulating free fatty acids, glucose and insulin between feeding conditions, no differences in plasma ANGPTL4 were detected.

Study limitations

A limitation of the present study is that there was no muscle biopsy taken pre-resistance exercise and therefore the muscle glycogen content between the different nutritional conditions when commencing the resistance exercise bout remains unclear. Furthermore, our blood samples in the post exercise period were not immediately collected after the bout but at 1,2 and 3 h post exercise. Consequently, potential differences in cytokines between the nutritional conditions could have been overlooked since the established cytokines reach a peak during and immediately after exercise (2). In addition, the effects of the nutritional intervention are possibly obscured by the physiological effects of endurance exercise. Multiple time-points in a shorter period of time during and after exercise may reveal more additional insights about how nutrient availability influence cytokine responses. Another limitation is the intensity and duration of the resistance exercise bout, we cannot rule out the possibility that alternative resistance exercise bouts higher in volume and intensity with different carbohydrate or fat availability may affect the post-resistance exercise cytokine responses differently. Moreover, the post-resistance exercise plasma cytokine responses may be affected by the endurance exercise earlier that day, however, since we did not have a third condition without the endurance exercise we were not able to correct for this potential confounder. At last, another shortcoming of this study is that possible changes within the muscle such as mRNA quantity and protein content were not determined. Therefore, a possible effect of the intervention meal may have been overlooked.

Conclusion

In summary, resistance exercise with different carbohydrate/fat availability did not influence most plasma cytokine responses in the early post-resistance exercise period. Post-resistance exercise plasma IL-6 expression was higher in the low carbohydrate condition compared with high carbohydrate availability. Our findings support the view that the pre-resistance exercise carbohydrate availability does not affect acute cytokine responses to resistance exercise.

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Chapter 5

Protein and the adaptive response with endurance training: wishful thinking or a competitive edge?

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Abstract

The significance of carbohydrates for endurance training has been well established, whereas the role of protein and the adaptive response with endurance training is unclear. Therefore, the aim of this perspective is to discuss the current evidence on the role of dietary protein and the adaptive response with endurance training. On a metabolic level, a single bout of endurance training stimulates the oxidation of several amino acids. Although the amount of amino acids as part of total energy expenditure during exercise is relatively low compared to other substrates (e.g. carbohydrates and fat), it may depress the rates of skeletal muscle protein synthesis, and thereby have a negative effect on training adaptation. A low supply of amino acids relative to that of carbohydrates may also have negative effects on the synthesis of capillaries, synthesis and turn-over of mitochondrial proteins and proteins involved in oxygen transport including haemoglobin and myoglobin. Thus far, the scientific evidence demonstrating the significance of dietary protein is mainly derived from research with resistance exercise training regimes. This is not surprising since the general paradigm states that endurance training has insignificant effects on skeletal muscle growth. This could have resulted in an underappreciation of the role of dietary protein for the endurance athlete. To conclude, evidence of the role of protein on endurance training adaptations and performance remains scarce and is mainly derived from acute exercise studies. Therefore, future human intervention studies must unravel whether dietary protein is truly capable of augmenting endurance training adaptations and ultimately performance.

Introduction

Nutritional strategies to maximise recovery from exercise are widely used by recreational as well as elite athletes. Post-exercise carbohydrate ingestion is considered to facilitate muscle glycogen resynthesis (1), and that of proteins to repair the exercise-induced damage to the contractile proteins and for the de novo synthesis of proteins (2). Thus far, scientific evidence demonstrating the significance of dietary protein is mainly derived from research with resistance exercise training regimes (3). This is not surprising since the general paradigm states that endurance training has insignificant effects on skeletal muscle growth. This could have resulted in an underappreciation of the role of dietary protein for the endurance athlete. However, a recent review on endurance training and skeletal muscle hypertrophy revealed that both acute and chronic endurance training enhances muscle protein synthesis and skeletal muscle growth respectively (4). Particularly, they reported that eight out of nine studies on the effects of endurance training on skeletal muscle demonstrated significant muscle growth in both younger and older individuals (4). Further evidence for increased protein-needs of individuals participating in endurance training regimes comes from studies on amino acid oxidation during exercise (5) and hypothetically for capillarisation, synthesis and turnover of mitochondrial proteins and proteins involved in oxygen transport including haemoglobin and myoglobin. Just as with resistance exercise, exogenous essential amino acids are required to repair the endurance exercise-induced muscle damage. From these considerations it becomes clear that the role of dietary protein in optimising endurance training adaptations requires further study. Therefore, the aim of this perspective is to discuss the current evidence on the role of dietary protein and the adaptive response (e.g. biochemical and physiological endpoints) with endurance training. In addition, since the mechanisms underpinning these adaptations are not fully understood, we propose a novel hypothesis (**Figure 1**) based on our unpublished observations and the current literature why protein intake may potentially be advantageous for individuals participating in endurance training regimes.

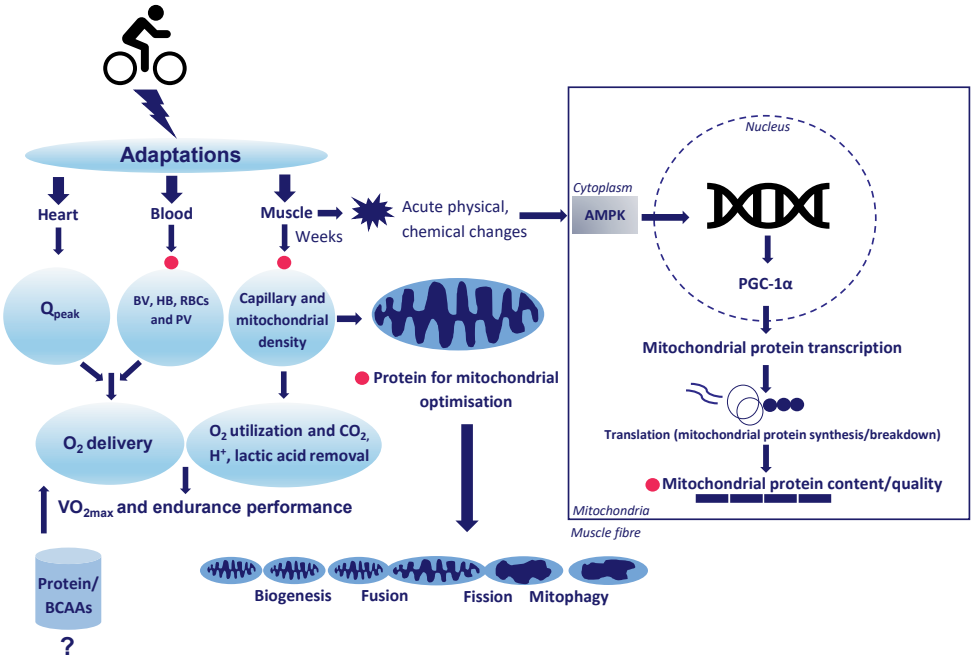

Figure 1. Simplified schematic figure representing the regulation of endurance training-induced adaptation and the hypothetical role of dietary protein herein. Changes in heart such as maximal cardiac output and blood e.g. blood and plasma volume, haemoglobin, red blood cell volume enhances O_2 oxygen-carrying capacity of the blood, whereas skeletal muscle adaptations potentially contribute to O_2 extraction. Pink circles indicates where dietary proteins might play a role in adaptation with endurance training. AMPK: 5'adenosine monophosphate-activated protein kinase; BV: blood volume; HB: haemoglobin; PGC-1α: peroxisome-activated receptor gamma coactivator 1-alpha; PV: plasma volume; Q_{peak}: peak cardiac output; RBCs: red blood cells.

Adaptation to endurance training

Endurance exercise performance roughly depends on three major aspects: I) maximal oxygen consumption (VO_{2max}), II) the percentage of VO_{2max} that can be sustained during endurance exercise, which in turn is largely dictated by the lactate threshold, and III) mechanical efficiency, defined as the energy cost to sustain a power output or velocity (6). Training these limiting factors may lead to an increase in oxidative capacity through haematological and metabolic adaptations and associated enhanced O_2 transport and utilization (7). O_2 transport is primarily regulated via cardiac (e.g. stroke volume) and circulatory (e.g. blood volume, O_2 carrying capacity) adaptations, while improvements in O_2 diffusion (involving capillaries) and utilization (involving mitochondria) are mainly the result of adaptations within skeletal muscle (e.g. mitochondrial capacity, capillary density, myoglobin, oxidative enzymes/proteins).

Protein requirements for the endurance athlete

Synthesis and degradation rates of skeletal muscle proteins are usually in balance, ensuring that the amount of skeletal muscle proteins remains unchanged in healthy individuals (8). However, acute changes in different intramuscular protein fractions (mitochondrial, myofibrillar, sarcolemma) could be training specific. It has been proposed that endurance training augments the concentration of mitochondrial proteins without any changes in muscle size (9). Based on this idea, one could expect that different types of training would stimulate the intramuscular protein fractions differently. However, various authors reported similar increases of the effect of different exercise modes (endurance, resistance or concurrent) on mitochondrial protein synthesis during the early post-exercise period (10-15). An increased need for dietary protein could partly arise from enhanced amino acid oxidation during endurance training (16, 17). Indeed, earlier studies have demonstrated increased amino acid oxidation rates through stimulation of protein breakdown rates (18-21). Additionally, it has been theorised that endurance training affects amino acid requirements for an increased need of enzymes, for capillarisation, and for haemoglobin and myoglobin synthesis (16). The current sport science consensus statements on nutrition and athletic performance advises 1.2-1.4 g protein·kg^{-1}·d^{-1} for endurance-trained athletes (22, 23). However, Kato *et al* (2016) studied the average protein requirements in endurance athletes during an acute 3-day training period using the indicator amino acid oxidation method and reported a recommended protein intake that is greater than the RDA (0.8 g · kg^{1}·d^{1}) and the current recommendations for endurance athletes (1.2-1.4 g·kg^{-1}·d^{-1}) (5). Moreover, they reported an estimated average requirement and a recommended protein intake of 1.6 of 1.8 g protein·kg^{-1}·d^{-1} respectively. Therefore, it remains to be established whether these recommendations are optimal for individuals participating in endurance training regimes and whether this is affected by the training phase of the individual and other training parameters including intensity, type and frequency.

Significance of protein ingestion before and during endurance training

Only a few investigations have addressed the role of protein ingestion before and during endurance training modalities. In line with previous work on protein ingestion prior to and during resistance exercise (24), Coffey and colleagues reported that protein ingestion before a single bout of repeated sprints increases post-exercise myofibrillar protein synthesis (13). However, short high-intensity endurance bursts such as repeated sprints differ both energetically and metabolically from prolonged continuous endurance training. For example, prolonged endurance training stimulates the oxidation

of amino acids, in particular isoleucine, leucine and valine, otherwise known as the branched chain amino acids (BCAAs). Even though mitochondria are capable of oxidizing a variety of amino acids, they preferentially oxidize BCAAs (25-27). These amino acids including BCAAs can be used as a substitute for carbohydrates and fat as fuel source for ATP resynthesis. When compared to carbohydrates and fat, leucine oxidation during endurance exercise is relatively low (16, 25, 26, 28), yet, the absolute leucine oxidation increases because of the increase in total energy demand during endurance exercise. Since leucine is an essential amino acid and considered to be important for its role in translational machinery (29), increased oxidation rates could depress the rates of skeletal muscle protein synthesis (30) and negatively affect protein requirements (16). Other work by Koopman and colleagues demonstrated that the combined ingestion of protein and carbohydrate throughout a prolonged endurance exercise bout (2.5 h cycling, 1 h of running and 2.5 h of cycling) improves whole body net protein balance at rest, as well as during exercise and post-exercise (31). The enhanced whole body net protein balance with protein ingestion may be partly explained by the diminished muscle protein breakdown during endurance exercise (32). Furthermore, the favoured enhanced net protein balance has been proposed as the theoretical basis for a potential ergogenic effect of protein ingestion during endurance exercise. Yet, findings of combined carbohydrate and protein ingestion during endurance exercise on performance outcomes are controversial (33). For instance, in a study by Saunders *et al* (2009), male cyclists performed two 60-km time trials with either carbohydrate or carbohydrate + protein beverage every 5 km (200 ml) and post-exercise (500 ml). No significant difference in 60-km total time between the conditions was found. In spite of that, the addition of protein hydrolysate to the carbohydrate beverage explained a significant amount of variance in performance times between conditions during the final stages (20 km and 5 km) of the time trial (34). The latter suggests a favourable effect of protein ingestion during exercise on endurance performance. Lastly, the addition of protein to a carbohydrate supplement consumed during exercise does not improve recovery or performance in elite cyclists despite high demands of daily exhaustive sessions during a one-week training camp (35). In summary, there is currently little evidence for improved endurance performance with protein intake before and during endurance exercise. Nevertheless, findings of abovementioned studies underline the importance of exogenous protein for remodelling/repair of the exercise-induced damaged protein.

Significance of protein ingestion after endurance exercise

Since most of the research so far has focused on protein ingestion with resistance exercise, these findings form generally the basis for protein ingestion recommendations for individuals participating in endurance-based programs. However, the skeletal muscle adaptive response during post-exercise recovery is strongly affected by food intake. Post-exercise supplementation in the form of protein after exercise has been the focus of many acute exercise interventions (13, 19, 36, 37). Only a few studies have examined the responses of dietary protein on mitochondrial protein synthesis after endurance exercise. Breen and colleagues (2011) examined the role of dietary protein on both mitochondrial and myofibrillar protein synthesis (12). In their study, trained healthy males cycled for 90 min at ~77% VO_{2max}. Such intensity (~77% VO_{2max}) can be considered as vigorous endurance exercise (38) and results in more mitochondrial mass and improved skeletal muscle oxidative capacity when applied chronically. Immediately and 30 min following the exercise bout, subjects ingested a carbohydrate beverage and in one condition a total of 20 g of whey protein was added. It was shown that the co-ingestion of whey protein with carbohydrate augments the myofibrillar protein synthetic response up to 4 h after exercise (12). Their finding, namely that endurance exercise with post-exercise dietary protein ingestion enhances myofibrillar protein synthesis, is in accordance with previous findings where subjects ingested protein after high-intensity sprint exercise (13). Noteworthy, the authors did not find a difference between the conditions on mitochondrial protein synthesis. It might be possible that the timing of the biopsy overlooked any potential increase in mitochondrial protein synthesis. Indeed, recent work by Hill *et al* (2013) demonstrated that the acute PGC-1α mRNA, considered as the master regulator of mitochondrial biogenesis, was enhanced after a 60-min endurance bout (~70% VO_{2max}) at 6 h when subjects were exposed to a 2-week dietary intervention with co-ingestion of carbohydrates + whey protein isolate (39). Their finding that carbohydrates + whey protein isolate enhanced a marker of mitochondrial recovery is in contrast with the findings of Breen et al (12). The different findings are possibly explained by the timing of the muscle biopsies and the applied nutritional strategy. For example, subjects in the study of Hill and colleagues were supplemented for two weeks with either carbohydrates or carbohydrates + whey protein isolate (39), whereas subjects in the study of Breen et al (2011) were merely supplemented post-exercise (12). Lastly, even though mitochondrial protein synthesis and PGC-1α mRNA can be both used as a marker for mitochondrial recovery, comparison of findings remains difficult.

Significance of protein ingestion during the prolonged recovery period after endurance exercise

At this moment, there is not much research on the effects of protein ingestion during the prolonged recovery period after endurance exercise (from 3 up to 12 h post-exercise). However, Areta and colleagues (2013) reported that the distribution of protein ingestion during the 12 h after resistance exercise affects the rates of myofibrillar protein synthesis (40). Specifically, in their study they compared three isocaloric timing strategies for protein ingestion during a 12 h period after resistance exercise: I) 2 x 40 g every 6 h (bolus); II) 4 x 20 g every 4 h (intermediate); and III) 8 x 10 g every 1.5 h (pulse). It was concluded that intermediate feeding was superior to either bolus or pulse feeding for stimulation of myofibrillar protein synthesis. Albeit somewhat speculative, it is likely that individuals participating in an endurance training regime also benefit from an intermediate protein ingestion strategy. Further on this notion, work from Breen and colleagues (2011) showed that the addition of protein to a carbohydrate drink in the early post-endurance exercise did not show increases in mitochondrial protein synthesis compared with a carbohydrate drink only (12). However, mitochondrial protein synthesis measurements were taken in the early post-exercise endurance period, since the latency of mitochondrial protein synthesis is currently unclear, it could be that a response at a later stage was overlooked.

Long-term endurance training and the role of protein

Unfortunately, to the best of our knowledge, the current literature still lacks studies exploring the role of dietary protein with a robust period of endurance training (> 6 weeks). The period of the endurance training intervention is an important aspect when looking at how skeletal muscle adaptations can be influenced by dietary protein. Indeed, recent work by Montero *et al* (2015) demonstrated that the increase in VO_{2peak} with six weeks of endurance training (3-4 endurance sessions per week at ~65% W_{max}) was primarily explained by an increase in peak cardiac output and oxygen-carrying capacity of the blood (7). Moreover, in their study, skeletal muscle adaptations related to muscle capillarisation and mitochondrial volume density did not substantially contribute to the improvements in VO_{2peak} following the six weeks of endurance training. It is therefore important to conduct endurance training intervention studies over a longer period (e.g. 8, 12 or 20 weeks), especially when the purpose of the study is to explore the role of protein within the entire spectrum of the physiological adaptive response to endurance training. Only a few studies investigated the effects of protein supplementation during "long-term" (4-6 weeks) endurance training in healthy young men (41) and older adults (42). In the study of Robinson *et al.* (2011) young and old participants performed 3 treadmill-based aerobic sessions weekly for 6 weeks (30 minutes;

intensity increased progressively ranging from ~65% – 85% HR_{max}) and were provided with a post-exercise beverage containing either carbohydrates or isocaloric protein. The absolute VO_{2max} increased in the protein group but not in the carbohydrate group following six weeks of aerobic training (42). The finding that protein supplementation improves endurance training-induced oxidative adaptations is supported by the study of Ferguson-Stegall *et al.* (2011). In this study it was demonstrated that 60 minutes of cycling, five times a week (~75%-80% VO_{2max}) for four weeks with intake of a post-exercise chocolate milk beverage, improves VO_{2max} and body composition to a greater extent compared to carbohydrates alone. Somewhat surprisingly, markers of mitochondrial adaptation such as citrate synthase activity, succinate dehydrogenase activity and PGC-1α increased as a result of training independent of the type of nutritional intervention (41). Finally, supplementation of a mixture BCAAs in mice increased mitochondrial biogenesis and whole body physical endurance as measured as the time till exhaustion in a treadmill test (43). Since sport specific performance outcomes were not included in aforementioned studies, it remains unclear whether the increase in VO_{2max} improved the performance.

The role of protein source

The superiority of one protein source over another in terms of exercise adaptation has not been convincingly demonstrated (44), though type and quality can influence bioavailability (22). The choice of protein source is of additional relevance to athletes given the environmental/ethical, overall health, and bioactivity differences which have been reported (45-48). In the context of this perspective, it is the bioactivity of different protein sources that is of primary consideration, given the potential beneficial effects on oxygen diffusion and utilization. The “food first” approach to sports nutrition is widely touted, but food structure can influence the suitability of protein sources via altered kinetics of amino acid availability (49, 50) and digestive discomfort (51). For these reasons as well as convenience, powder-form supplements are often recommended. Given the further geo-logistic, time, and dosage demands of endurance sport, tablet-form protein supplements may be of greater facility in some instances. On a metabolic level, the presence or absence of essential amino acids (52), leucine content (53), and type of protein (48) have been shown to alter the muscle protein synthetic response and body compositional changes, prompting the development of a number of assessment methods. Despite a range of assessment scales existing (22, 52), comparisons of protein sources have centred largely on biological value (proportion of nitrogen used for tissue formation) and digestion rate (50, 54). Multiple authors have reported differences in biological value between protein sources (52, 54). In addition, it has been demonstrated that more rapidly

digested sources (i.e. whey) seem to confer increased insulin response, post-exercise muscle protein synthesis, resting muscle protein synthesis and blood leucine enrichment in the first hour after consumption (48). However, the suitability of the existing assessment scales varies with respect to their relevance to endurance athletes (as does the use of an inappropriately short 1-hour assessment window). For instance, the Protein Efficiency Ratio (PER), representing mass gain per g of protein ingested, represents an inverse of suitability for most endurance athletes. Biological value too may be of limited use to endurance athletes as it considers only the tissue-related nitrogen use (thus omitting protein synthesis of oxidative enzymes and haematopoiesis, for instance). The Protein Digestibility Corrected Amino Acids Score (PDCAAS) is the most widely used assessment, but it too is limited by its lack of consideration of ileal digestibility, and the short-sightedness of determining protein quality based on the content of a single amino acid (which may be sufficiently abundant in the habitual diet). Endurance athletes demonstrate prolonged periods of increased muscle protein synthesis while still engaged in exercise , meaning protein requirements are elevated while in a state of compromised gastric function and reduced feeding opportunities (55). The PDCAAS may be of assistance to athletes engaging in ultra-endurance events, by informing a decision which should maximise essential amino acids per weight consumed, to minimise the risk of gastrointestinal distress. Coupled with personal experience of irritability triggers, this approach may be particularly practical for ultra-endurance runners and triathletes (events >3h duration).

Amino acid profile bioavailability

There is a lack of understanding concerning the practical relevance of established differences in source bioactivity on the adaptive response to endurance training, though some reports when combined suggest an adaptive advantage (4, 48). Unpublished data from our own study of supplementary effects of proteins on endurance training adaptation showed a response effect of habitual carbohydrate intake, before controlling for the expected variation due to casein supplementation. This suggests that the adaptation effect of supplementing even a robust bioavailable protein source is relatively low in the context of dietary effects on training outcomes, and so the importance of protein source is somewhat diminished.

Wolfe in his 2000 article raised the suggestion that endurance athletes' optimal protein intake may seek to maximise recovery (protein synthesis) while avoiding weight gain (protein deposition) (56). With this goal, one may seek to provide an endurance-tailored amino acid profile which avoids a hypertrophy-oriented profile (e.g. high in leucine) and instead provides an amino acid profile more specific to the requirements of oxygen utilisation. I.e. those

necessary for mitochondrial biogenesis (PGCs), fission (Fiss1, MPP, Drp1), mitophagy (Pink1, Parkin), fusion (Opa1, Mfn1/2), and the production of rate-limiting mitochondrial enzymes (pyruvate dehydrogenase, carnitine palmitoyl transferase). The amino acid composition of the parenthesised drivers of these processes may serve as a start-point for the elucidation of an endurance-specific ideal in terms of amino acid profile. However, the profile of downstream products, endogenous amino acid bioavailability, and the quantities involved ought to be considered. The leucine-induced increase in muscle protein synthesis is contested in humans, and high quantities may decrease autophagy (a vital aspect of endurance-specific adaptation) (Glynn et al., 2010). As such, the mechanisms of leucine's effects on body composition, muscle protein synthesis initiation factors (e.g. 4E-BP1) and autophagic regulators (e.g. ULK1) should be shown to be preferential in an endurance setting before disproportionate inclusion in supplements.

BCAAs, casein or whey

It has been reported that BCAAs are preferentially oxidised (ahead of un-branched amino acids) during endurance exercise (57, 58). Given that BCAAs are also essential and that muscle protein synthesis is elevated following but also during exercise (59), extended endurance exercise may evoke an environment of suboptimal BCAA availability. For this reason it may be beneficial to the overall adaptive response and/or performance (via substrate availability) to provide a source rich in BCAAs during extensive exercise (e.g. whey, soy or casein). When used concurrently with less intensive endurance training, high intensity and/or strength training will evoke a greater myofibrillar protein synthetic response (60, 61), which may improve performance adaptation (Howarth et al., 2009). The existing wisdom regarding protein supplementation is thus more likely to hold true; a readily digested source of protein with high leucine content (e.g. whey) may be preferable to maximise post-exercise muscle protein synthesis (48). In these situations protein supplementation may aid in satiety as well as achieving protein intake targets while total energy demands of training are likely decreased.

As described earlier in this paper, mitochondrial protein synthesis demonstrates a delayed response post-exercise when compared to myofibrillar protein synthesis (60, 62). It then follows that the acute protein requirement for endurance athletes immediately following exercise may be reduced, while the window of elevated utilisation may exist for a longer time-period compared to strength trainers. If true, the existing advice paradigm of rapidly absorbed protein to be ingested as soon as possible post-exercise may sub-optimally support the protein-synthetic adaptive response to endurance training. In consideration of this observation, protein with a slower digestion rate may be preferable. However, it has been suggested that essential amino acid content

and rapid digestion tend to coexist in protein sources (48). Furthermore, the greater insulin response reported to accompany these properties, which may contribute to digestion rate, is likely unavoidable in the context of post endurance exercise refuelling of muscle glycogen. Consumption of post-exercise protein in a whole-food form including dietary fibre to prolong the period of elevated amino acid availability is one simple solution. Division of the dose between two meals may also be necessary in order to optimally provide for protein availability over the 6 + hrs of elevated mitochondrial/cytosolic protein synthesis.

Conclusion

To summarize, evidence of the role of protein on endurance training adaptations and performance is scarce. Yet, a number of acute endurance exercise studies have raised interesting hypotheses. However, these hypotheses are mainly based on studies measuring muscle protein synthesis, physiological (e.g. amino acid oxidation) and biochemical (e.g. activation/phosphorylation of specific enzymes/proteins or mRNA profiles) endpoints which do not necessarily reflect an improved adaptation and performance. Even though the findings of acute exercise studies contribute to the understanding of the mechanisms that underpin adaptation with endurance training, it is no direct proof that individuals performing endurance training benefit from additional protein. Future evidence must be derived from long-term endurance training studies that combine performance outcomes and biochemical/physiological endpoints.

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Chapter 6

Protein supplementation elicits greater gains in maximal oxygen uptake capacity and stimulates lean mass accretion during prolonged endurance training: a double-blind randomized controlled trial

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Abstract

Background: Endurance training induces numerous cardiovascular and skeletal muscle adaptations thereby increasing maximal oxygen uptake capacity (VO_{2max}). Whether protein supplementation enhances these adaptations remains unclear.

Objective: This study was designed to determine the impact of protein supplementation on changes in VO_{2max} during prolonged endurance training.

Design: We used a double-blind randomized controlled trial with repeated measures among 44 recreationally active, young males. Subjects performed 3 endurance training sessions per week for 10-weeks. Supplements were provided immediately after each exercise session and daily before-sleep providing either protein (PRO; n=19; 21.5±0.4 y) or an isocaloric amount of carbohydrate as control (CON; n=21; 22.5±0.5 y). VO_{2max}, simulated 10-km time trial performance, and body composition (dual-energy X-ray absorptiometry) were measured before and after 5 and 10 weeks of endurance training. Fasting skeletal muscle tissue samples were taken before and after 5 and 10 weeks to measure skeletal muscle oxidative capacity, fasting blood samples were taken every two weeks to measure haematological factors.

Results: VO_{2max} increased to a greater extent in the PRO compared with CON group after 5 weeks (from 49.9±0.8 to 54.9±1.1 vs 50.8±0.9 to 53.0±1.1 $mL \cdot kg^{-1} \cdot min^{-1}$; p<0.05) and 10 weeks (from 49.9±0.8 to 55.4±0.9 vs 50.8±0.9 to 53.9±1.2 $mL \cdot kg^{-1} \cdot min^{-1}$; p<0.05). Lean body mass increased in the PRO group whereas the CON group remained stable the first 5 weeks of (1.5±0.2 vs 0.1±0.3 kg; p<0.05) and after 10 weeks (1.5±0.3 vs 0.4±0.3 kg; p<0.05). Throughout the intervention fat mass reduced significantly in the PRO with no changes in the CON group after 5 (-0.59±0.2 vs -0.10±0.2 kg; p=0.09) and 10 weeks (-1.24±0.4 vs -0.20±0.2 kg; p=0.02).

Conclusions: Protein supplementation elicited greater gains in VO_{2max} and stimulated lean mass accretion but did not improve skeletal muscle oxidative capacity and endurance performance during 10 weeks of endurance training in healthy young males.

Introduction

Endurance training represents an effective strategy to increase maximal oxygen uptake capacity (VO_{2max}) (1, 2). An increase in VO_{2max} is independently associated with a reduction in all-cause mortality, which emphasizes the important clinical benefits of endurance training (3). Increases in VO_{2max} as a result of endurance training can be attributed to adaptive responses of several organ systems involved in oxygen transport and utilization chain, from lungs, heart, vasculature to the mitochondria in muscle tissue (4-7). Impact of different endurance training regimes on both cardiovascular (8-11) and skeletal muscle (5, 12-15) adaptations have been thoroughly investigated. In contrast, few studies have determined the impact of protein supplementation on the adaptive response to endurance training, with conflicting findings (16-18). Additionally, these studies were either underpowered (17), not double-blinded (18), or exercise training sessions were not fully monitored by investigators (16), which makes it difficult to draw conclusions about the potential effectiveness of protein supplementation for endurance training adaptation. Clearly there is a strong need for well-powered double-blind randomized controlled trials to establish whether protein supplementation impacts the adaptive response to endurance training.

Therefore, we designed a double-blind randomized controlled trial with a supplementation strategy that theoretically should be most effective based upon previous post-exercise and before-sleep protein fractional synthetic rate measurements (19, 20). Our main purpose was to determine the impact of protein supplementation on changes in VO_{2max} following 10-weeks of endurance training. Secondarily, we were also interested in skeletal muscle oxidative capacity, endurance performance, haematological factors and body composition. To gain insight into the timespan of adaptations we assessed all outcome measures after both 5 and 10 weeks of training. We hypothesized that protein supplementation facilitates the adaptive response to endurance training.

Methods

Subjects

Forty-four young healthy males volunteered and gave full-written informed consent to participate in a 10-wk endurance training program, with or without additional protein supplementation. Primary inclusion criteria were non-smokers, free of injury and not using any medication or nutritional supplements. Additional exclusion criteria that would preclude successful participation in the training study included (diagnosed) lactose intolerance and/or dairy protein allergy, cardiorespiratory-related illness and musculoskeletal-related injuries impeding with the endurance training sessions. All subjects were physically active, performing sports on a non-competitive basis between one and four hours per week. None of the participants had a history of participating in any structured endurance training programs to improve performance over the past two years. This study was carried out in accordance with the guidelines for human research of The Medical Ethical Committee of Wageningen University. The Medical Ethical Committee of Wageningen University approved all study procedures and complied with the guidelines set by the Declaration of Helsinki of 1975 as revised in 1983. This trial was registered at clinicaltrials.gov as NCT03462381.

Experimental design

Subjects completed a progressive endurance training protocol with three exercise sessions per week for in total 10 weeks and consumed a post-exercise and pre-sleep drink providing either protein or an isocaloric amount of carbohydrate. The total study duration, including a series of mid-term and end measurements, was ~12 wk. After inclusion, subjects were randomly assigned to either a protein-supplemented (PRO) or carbohydrate-supplemented control (CON) group. Before (wk 0), during (wk 6), and after (wk 12) the exercise training program, anthropometric measurements (height, body mass, and waist circumference), VO_{2max} ramp tests, simulated 10-km time-trials, and DXA scans were carried out. In addition, muscle biopsy specimens, dietary intake records, and physical activity records were collected (**Figure 1**). Fasting blood samples were taken at week 0 (pre), 2, 4, 6 (mid), 8, 10, and 12 (end). The subjects were instructed to maintain their normal dietary habits and physical activity patterns throughout the intervention period. A standardized meal was provided the evening before each test day (standard deep-frozen meal and ice-cream dessert; 43.80 kJ/kg BW; 15 Energy% protein, 30 Energy% fat and 55 Energy% carbohydrate; Roerbaksensatie, Iglo, Utrecht, the Netherlands). Subjects refrained from continuous physical activity for at least 72 h before testing. At the different test days, subjects arrived at the laboratory after an overnight fast.

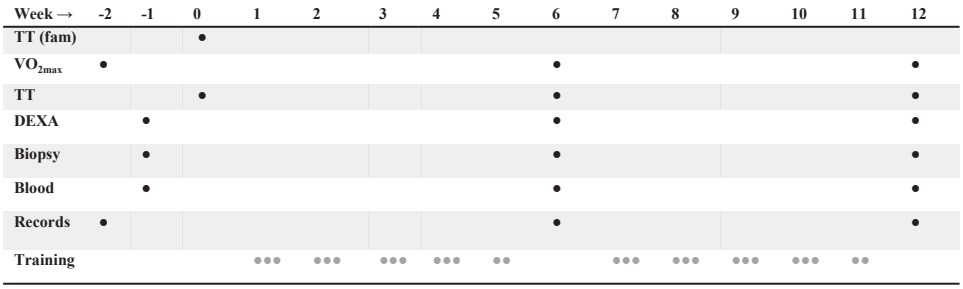


Figure 1. 40 subjects completed 10 weeks of exercise training while consuming either 29 g calcium casein protein or 29 g carbohydrates post-exercise and daily before-sleep. Measurements and biopsies were performed at Pre (week 0), Mid (week 6), and End (week 12), whereas blood samples were taken at week 0, 2, 4, 6, 8, 10 and 12. TT (fam): 10-km time-trial familiarization, TT: 10-km time-trial, VO_{2max}: maximal oxidative capacity exercise test. Black dots: measurement points, grey dots: exercise training sessions. TT fam and TT baseline were performed within the same week with 3-4 days between time trials.

Endurance training program

Training intervention was divided in two blocks of 5 weeks with one week (week 6) in between the blocks to perform the mid-term measurements, and a final week (week 12) to perform the end-measurements. The first four weeks subjects participated in 3 endurance exercise sessions/wk alternated with one day of rest between each session. The fifth week subjects performed 2 endurance exercise sessions. This was repeated in the second part of the study (week 7-11), see **Figure 1**. In total, subjects performed 28 endurance training sessions. After a 10-min warm-up on a cycle ergometer, the endurance training session consisted of 60-min continuous cycling. All training sessions were conducted under supervision of a researcher using indoor, mechanically braked spinning bikes (body bike smart®, Denmark) with participants free to adjust resistance and cadence as desired. Heart rate (HR) for each session was recorded (Polar Electro, USA), HR and rate of perceived exertion (RPE) were taken at the start and every 5 min thereafter by the supervisor. Music and verbal motivation were provided during training sessions which were conducted in ambient conditions at sea level at thermal-neutral conditions (21°C, 40% relative humidity). Each endurance training session ended with a 10-min cooling down period on the same cycle ergometer. Exercise intensity was determined using the Karvonen formula (21):

$$Intensity = HRreserve \cdot 0.85 + HRrest$$

Where *HRreserve* is the calculated difference between *HRmax* (determined during VO_{2max} ramp test) and *HRrest*. According to the American College of Sports Medicine, the selected exercise intensity can be considered as "vigorous" (22).

Protein and control supplementation

Subjects consumed a 250 mL beverage containing either a 29 gram of casein protein or an isocaloric amount of carbohydrate immediately after cessation of each exercise session (3 per wk) and every day before sleep (7 per wk). In addition, all subjects received two slices of gingerbread directly after every training session (total energy 280 Kcal; 63.2 g of carbohydrates; 1.4 g fat; 2.4 g protein). An overview of the energy and macronutrient composition of the beverages can be found in **Table 1**. Nutritional content of the supplements was analysed in duplicate by an independent laboratory (Nutrilab bv, Rijswijk, the Netherlands) and reported analogous nutritional values as given by the producer. The protein and control beverages were masked for taste and smell by adding several additives. In addition, beverages were masked for colour and produced in white non-transparent containers. Allocation to the PRO or CON group was done using block randomization (group 1, $n = 24$; group 2, $n = 20$) by an independent researcher not involved in the study. Study drink boxes/beverages were sequentially numbered on subject number.

Table 1. Nutritional composition of the intervention drinks (250 mL)

Energy & Nutrient	Control beverage (CON)	Protein beverage
Energy (Kcal)	~129	~127
Protein (casein) (g)	0.6	28.7
Fat (g)	2.4	0.3
Carbohydrates (maltodextrin and sucrose) (g)	26.3	2.7

Assessment of blinding success
Determination of blinding success was done using the blinding index method described by James, Bloch, Lee, Kraemer and Fuller (23). Following the intervention subjects were asked what treatment they thought they have had and could choose between *carbohydrate/protein/"I do not now"*. Subsequently, weights were given for subject's guess (*0 for correct guess; 0.5 for incorrect guess; and 1 for "I do not know"*).

Habitual dietary intake and physical activity
Over the course of the intervention period, subjects maintained their habitual dietary intake and physical activity pattern. Subjects recorded 3-d (days were randomized, but in general 2 weekdays and 1 weekend day) weighted dietary intake records to assess potential changes in daily food intake that might have occurred over the course of the intervention period (week 5), before the onset of the intervention (week 0) and at week 10 of the intervention. Dietary intake records were analysed using Compl-eat (Human Nutrition, Wageningen University & Research, the Netherlands). Habitual physical activity and supplemental exercise were assessed using the extended version of the International Physical Activity Questionnaires (IPAQ). The extended IPAQ is a self-administered 7-day physical activity recall questionnaire. Calculation and quantification of physical activity scores were determined according the guidelines for data processing and analysis of the international physical activity questionnaire, as described elsewhere (24).

Body composition
DXA measurements were carried out after an overnight fast using a Lunar Prodigy Advanced DXA scanner (GE Health Care, Madison, WI, United States of America). Each morning at the different test days, a quality assurance test was performed to ensure system suitability and precision of the scanner. Whole body scans were performed according to the manufacturer's protocol and identical scan protocols were used for all subjects. Subsequently, different regions for fat mass and lean mass were assessed. Anthropometrics were assessed using standardized procedures, body weight by digital scale to within 100 g, height by stadiometer to within 0.5 cm and waist circumference by tape measure to within 0.5 cm (SECA, Hamburg, Germany).

VO_{2max}
A ramped VO_{2max} test was performed at baseline (- 2 wk), mid-term and end (2-3 days after the last training sessions) between 9 am and 5 pm. Ninety minutes before each test, subjects consumed a standardized meal consisting of an

energy bar (3.7 g fat, 29.2 g carbohydrates, 2 g protein, 158.1 Kcal), an apple (granny smith) and 500ml water. Following a 30-min rest, subjects performed a ramped VO_{2max} test on an electrically braked cycle ergometer (Lode Excalibur, Groningen, the Netherlands). After a 5-min warm up at 50 W, the subjects started cycling at 100 W. Workload was progressively increased by 20 W·min−1 until the subject reached volitional exhaustion. The VO_{2max} test was considered to be valid when two out of three criteria were met: (I) levelling of VO_2 with increasing workload; (II) heart rate within 10 beats of the theoretically estimated maximum (220-age); and (III) respiratory exchange ratio (RER) of ≥1.15. Oxygen consumption (VO_2) was measured through breath-by-breath sampling with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define maximal oxygen consumption (VO_{2max}). Subjects were asked to maintain a cadence between 80 and 100 r·min^{-1}.

Endurance exercise performance (simulated 10-km time trial)

Familiarization was performed in the week after the start of the first experimental day (anthropometrics, DXA, blood and biopsy). Three or four days thereafter another time trial was conducted (baseline measurement) and this was repeated at mid-term and end. Subjects performed a simulated ~10-km cycling time trial. The data from the baseline VO_{2max} test was used for the amount of work to be performed and calculated as follows: total amount of work (J) = 0.85*Wmax*900 (s) (25). The ergometer was set in linear-mode so that 85% Wmax was achieved when subjects cycled at their preferred pedalling rate of 85 ± 7 rpm, as determined during familiarization. Subjects received no verbal or physiological feedback during the time-trial, and were only aware of the absolute (kJ) and relative (%) amount of work performed. Ratings of perceived exertion (RPE) were assessed after each 30-min submaximal exercise test and after the time-trial using the Borg 6–20 scale (Borg, 1982). All testing was performed under standardized conditions (21 ˚C, 40% relative humidity) on the same time of day, and the same day in the week.

Blood sampling & analysis
Resting blood samples were collected after an overnight fast at week 0, 2, 4, 6, 8, 10 and 12 in EDTA-coated Vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) by venipuncture. Whole blood was analysed for red blood cells, haemoglobin concentration and haematocrit.

Muscle Biopsies
Muscle biopsies were taken after an overnight fast 3-4 days prior the start of the first training session (pre), 5-7 days after the 14th (mid-term) and 28th (end) training session. Muscle biopsies were taken as described by Bergstrom (1974) (26). Biopsies were taken under local anaesthesia (2–3 mL of 2% adrenaline) using a 5-mm Bergstrom needle modified with suction. Biopsies were taken from the *vastus lateralis* of the same leg, with separate incisions (~1–1.5 cm apart) and from distal to proximal direction. Muscle biopsies were immediately frozen (within 5–10 s) in liquid nitrogen and stored at −80°C for subsequent biochemical analysis, after being freed from visible fat, blood, and connective tissue.

Oxidative enzymes
Wet muscle was used for determining indices of skeletal muscle oxidative capacity. In approximately 30 mg muscle tissue (maximal) citrate synthase (CS) and cytochrome C oxidase (CytC) activity were measured according to methods published previously (27). Both enzyme activities are expressed as micromoles of product (citrate and reduced cytochrome C for CS and CytC, respectively) generated per gram of wet muscle tissue per min during the assay ($\mu mol \cdot g^{-1} \cdot min^{-1}$).

Statistics
Power calculation: Sample size (*n*) was calculated with 90% power and type I error probability of 0.05 based on the primary outcome of VO_{2max} increase. An expected and/or relevant difference in VO_{2max} seen with endurance training is 0.32 ± 0.30 ($L \cdot min^{-1}$) (28). To demonstrate a statistically significant greater increase in VO_{2max} by a nutritional supplement 16 participants/group have been shown to be sufficient (18). Considering a drop-out rate of 20-30%, the final number of the participants included was 22/group.

General statistics: An independent *t* test was carried out to verify that groups were similar at baseline. Data were assessed for normality with the use of a Shapiro-Wilk test, and any non – normal data (time-trial, CS and CytC) were

corrected with the use of transformation with the type of transformation based on the nature of the skewedness of the data. Repeated measures of analysis of variance (two-way mixed ANOVA), was used to determine statistical significance for the dependent variables over time. The analysis of variance model for the dependent variables with three testing time points was described as $S_{40}\ x\ T_3\ x\ G_2$ as such that (S; number of subjects) are crossed with testing time (T; three testing times: Pre (week 0), Mid (week 6), and End (week 12) and group (G; CON and PRO), and for blood sample analysis $S_{40}\ x\ T_7\ x\ G_2$. Where two-way mixed ANOVA revealed significant interaction, a Tukey's post hoc test was conducted for multiple comparisons to further analyse within group effects and unpaired *t*-tests to compare between groups at specific time points. In case two-way mixed ANOVA revealed no significant interaction but significant main effects of time and/or group pairwise comparisons with Tukey's post hoc correction were done. Data management and statistical analysis were carried out using SPSS software version 23 (SPSS Inc., Chicago, IL). Statistical significance was declared when $p < 0.05$. Figures were prepared using GraphPad Prism 8.01 for Windows (San Diego, CA). All data are expressed as mean ± SEM.

Results

Baseline characteristics

Four subjects dropped out during the study, one because of relocation, one because of a hamstring injury, one because of a knee injury and one because of appendicitis. Final analysis was performed on the 40 subjects who completed the training program (CON: *n*=21 vs PRO: *n*=19) **(Figure 2)**.

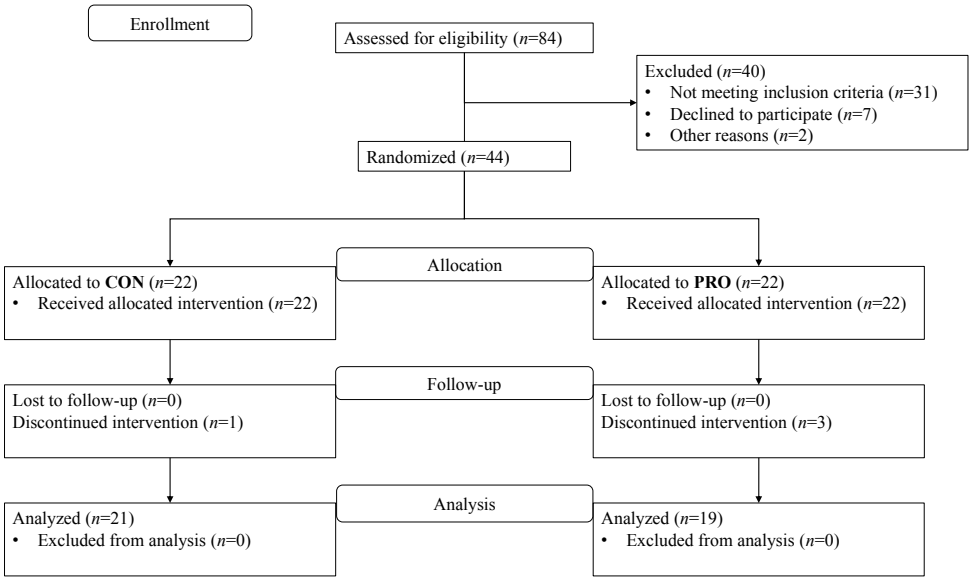


Figure 2. Subject recruitment and flow through the protocol. CON (carbohydrate supplementation, *n* = 21); PRO (protein supplementation, *n* = 19).

Baseline characteristics of both groups prior to the endurance training are summarized in **Table 2**. There were no differences at baseline between the CON and PRO groups in any of the variables of interest.

Table 2. Baseline characteristics

Characteristic	CON (*n* = 21)	PRO (*n* = 19)
Age (y)	22.5 ± 0.5	21.5 ± 0.4
Body mass (kg)	77.2 ± 1.6	76.3 ± 1.3
Height (m)	1.85 ± 0.0	1.85 ± 0.0
BMI ($kg \cdot m^{2}$)	22.4 ± 0.3	22.3 ± 0.4
VO_{2max} ($mL \cdot kg^{-1} \cdot min^{-1}$)	50.8 ± 0.9	49.9 ± 0.8

1 Values are means ± SEM. No significant differences were observed between groups. CON, carbohydrate-supplemented; PRO, protein -supplemented.

Endurance training adherence & supplement intake

All training sessions were performed between 9 am and 9 pm with no differences between groups. On average, subjects attended 98 ± 0.3% and 98.7% ± 0.3 of the sessions in the CON and PRO group, respectively, with no differences between groups. There was no significant time-by-treatment interaction for exercise intensity as measured by heart rate per min ($p = 0.37$) or rate of perceived exertion ($p = 0.78$) over the course of the training intervention. The post-exercise drinks were consumed under supervision resulting in a 100% compliance. The consumption of the pre-sleep drinks had an overall compliance of 98.3±0.3%, with no differences between groups. Blinding success was based on the upper bound of the confidence interval of the blinding index. Confidence interval was below 0.5 indicating there was insufficient evidence for unblinding (**Table 3.**)

Table 3. Assessment for success of blinding

Guess N (%)	CON	PRO	Do not know	Total	BI	95% CI
CON	7 (17)	6 (15)	8 (20)	21 (52)	0.5	0.32, 0.72
PRO	3 (8)	10 (25)	6 (15)	19 (48)	0.4	0.17, 0.62
Total	10 (25)	16 (40)	14 (35)	40 (100)		

1 Subject's guess, *n* (%). Determination of blinding success was done using the blinding index method described by James, Bloch, Lee, Kraemer and Fuller (23).

Habitual dietary intake and physical activity level

Statistical analysis revealed no differences in energy intake between the groups and/or over time (**Table 4.**). The carbohydrate supplement increased carbohydrate intake significantly over time on training days and non-training days, while carbohydrate intake remained stable in the PRO group. In contrast, the protein supplement increased protein intake significantly over time on training days (baseline: 1.3±0.1; mid-term: 1.8±0.1 end: 1.8±0.1 g · kg^{-1} · d^{-1}) and non-training days (baseline: 1.3±0.1; mid-term: 1.5±0.1 end: 1.7±0.1 g · kg^{-1} · d^{-1}), while protein intake remained stable in the CON group on training days (baseline: 1.3±0.1; mid-term: 1.2±0.1 end: 1.3±0.1 g · kg^{-1} · d^{-1}) and non-training days (baseline: 1.3±0.1; mid-term: 1.2±0.1 end: 1.3±0.1 g · kg^{-1} · d^{-1}). In addition to diet, habitual physical activity/exercise was also monitored. There were no differences in MET· min^{-1} ·wk^{-1} (excluding exercise sessions) between the groups at baseline, mid-term and end. Habitual physical activity tended to decrease after the onset of the exercise intervention in both the CON (baseline: 2415±248; mid-term: 1982±196 end: 2243±248 MET · min^{-1} · wk^{-1}) and PRO group (baseline: 2532±275; mid-term: 2169±246 end: 2233±305 MET · min^{-1} · wk^{-1}).

VO_{2max}

Statistical analysis revealed a significant time-by-treatment interaction for VO_{2max} (p = 0.033). VO_{2max} increased to a greater extent in the PRO compared with CON group after 5 weeks (from 49.9±0.8 to 54.9±1.1 vs 50.8±0.9 to 53.0±1.1 mL·kg^{-1}·min^{-1}; p = 0.017) and after 10 weeks (from 49.9±0.8 to 55.4±0.9 vs 50.8±0.9 to 53.9±1.2 mL·kg^{-1}·min^{-1}; p = 0.045) (**Figure 3a and 3b**). Changes in absolute VO_{2max}, absolute and relative maximal aerobic power (W) were all in line with the changes in VO_{2max} and are displayed in **Table 5**.

Table 4. Energy intake and macronutrient composition of the diet before, during and after 10 weeks of training in healthy young men who did receive carbohydrate or protein supplementation.

	CON group (n=21)			PRO group (n=19)			*P*		
	Pre	Mid	End	Pre	Mid	End	Training	Treatment	Interaction
Total energy, Kcal/d	2624 ± 96	2620 ± 99	2787 ± 98	2682 ± 103	2558 ± 106	2718 ± 105	NS	NS	NS
Fat, % of energy	34 ± 0.8	35 ± 1.1	34 ± 1.1	38 ± 0.9	34 ± 1.2	35 ± 1.3	NS	NS	NS
Alcohol, % of energy	2.4 ± 0.6	4.2 ± 0.9	5.1 ± 0.9	2.6 ± 0.7	3.1 ± 1.0	3.5 ± 0.9	NS	NS	NS
Carbohydrate, % of energy	47 ± 0.9	46 ± 1.0	47 ± 1.2	43 ± 1.1	47 ± 1.1	46 ± 1.3	NS	NS	=0.05
Carbohydrate, g/d	298 ± 12	295 ± 14	314 ± 12	282 ± 12	295 ± 15	299 ± 13	NS	NS	NS
Carbohydrate, $g \cdot kg^{-1} \cdot d^{-1}$	3.9 ± 0.2	3.8 ± 0.2	4.1 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	NS	NS	NS
Carbohydrate intake including supplement, $g \cdot kg^{-1} \cdot d^{-1}$ on non-training days	3.9 ± 0.2	4.1 ± 0.2	4.4 ± 0.2	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	=0.02	NS	NS
Carbohydrate intake including supplement, $g \cdot kg^{-1} \cdot d^{-1}$ on training days	3.9 ± 0.2	4.5 ± 0.2*	4.7 ± 0.2*	3.7 ± 0.2	3.8 ± 0.2	3.9 ± 0.2	<0.01	<0.01	=0.05
Protein, % of energy	15 ± 0.5	15 ± 0.7	16 ± 0.7	15 ± 0.5	15 ± 0.7	16 ± 0.7	NS	NS	NS
Protein intake, g/d	98 ± 3.7	92 ± 4.1	100 ± 4.3	97 ± 4.0	92 ± 4.3	101 ± 4.6	=0.03	NS	NS
Protein intake, $g \cdot kg^{-1} \cdot d^{-1}$	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	=0.02	NS	NS
Protein intake, including supplement $g \cdot kg^{-1} \cdot d^{-1}$ on non-training days	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1*	1.7 ± 0.1*†	<0.01	<0.01	<0.01
Protein intake, including supplement $g \cdot kg^{-1} \cdot d^{-1}$ on training days	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.8 ± 0.1*	1.9 ± 0.1*†	<0.01	<0.01	<0.01

1 Values are means ± SEM. NS, $P \geq 0.05$. *Significantly different compared with Pre. $P < 0.05$. †Significantly different compared with Mid, $P < 0.05$. CON, carbohydrate-supplemented; PRO, protein-supplemented

Table 5. Absolute VO_{2max} and Maximal aerobic power

	CON group (n=21)			PRO group (n=19)			*P*		
	Pre	**Mid**	**End**	**Pre**	**Mid**	**End**	**Training**	**Treatment**	**Interaction**
VO_{2max} ($L \cdot min^{-1}$)	3.87 ± 0.1	4.05 ± 0.1*	4.14 ± 0.1*	3.79 ± 0.1	4.21 ± 0.1*	4.24 ± 0.1*	< 0.0001	NS	= 0.008
Max aerobic power (W)	335 ± 6.9	366 ± 6.3	372 ± 6.7	324 ± 7.0	362 ± 7.5	377 ± 7.9	< 0.0001	NS	= 0.053
Max aerobic power ($W \cdot kg^{-1}$)	4.4 ± 0.1	4.8 ± 0.1*	4.9 ± 0.1*	4.3 ± 0.1	4.7 ± 0.1*	4.9 ± 0.1*†	< 0.0001	NS	= 0.016

1 Values are means ± SEM. NS, $P \geq 0.05$. *Significantly different compared with Pre. $P < 0.05$. †Significantly different compared with Mid, $P < 0.05$. CON, carbohydrate-supplemented; PRO, protein-supplemented.

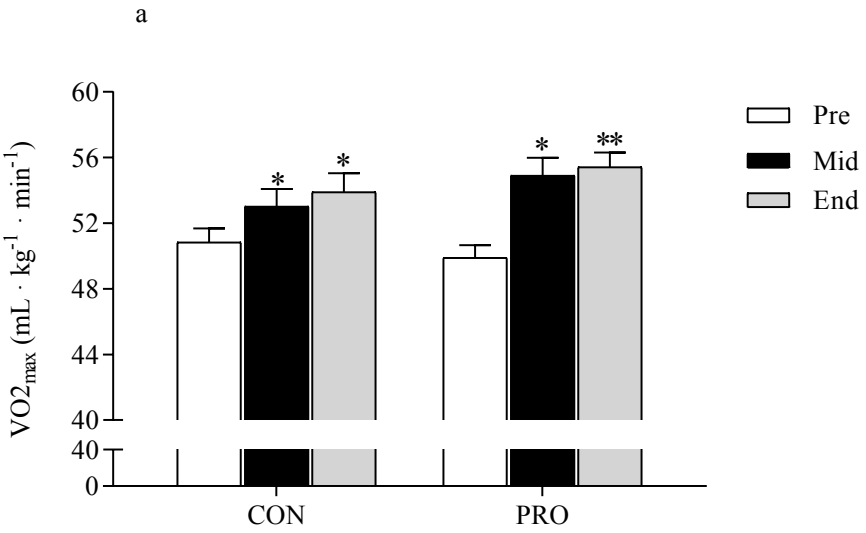


Figure 3a. Maximal oxygen uptake capacity throughout the intervention. VO_{2max} (mL · kg^{-1} · min^{-1}) during 10-weeks of endurance training (CON, n = 21; PRO, n = 19). Values are presented as group mean ± SEM. Data were analysed using two-way mixed ANOVA for time-by treatment interaction (P = 0.033). Tukey's post hoc test was conducted for multiple comparisons within each group. * = $P < 0.05$ significant main effect of time compared with Pre. ** = $P < 0.0001$ significant main effect of time compared with Pre

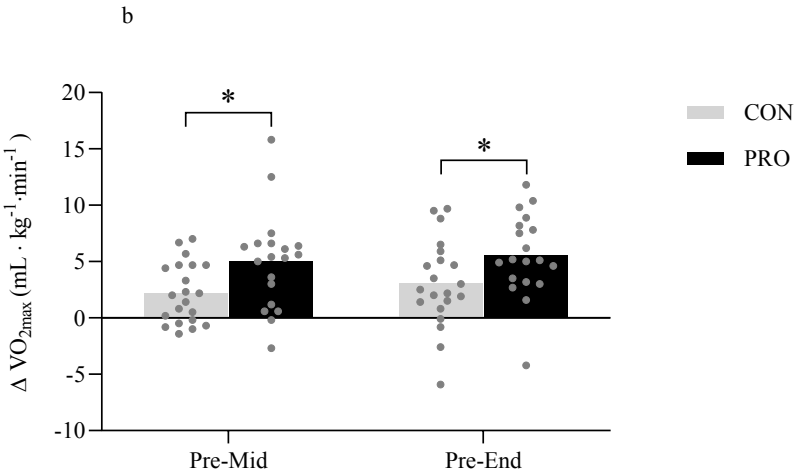


Figure 3b. Maximal oxygen uptake capacity change. Delta change in VO_{2max} (mL · kg^{-1} · min^{-1}) from Pre-Mid and Pre-End during prolonged endurance training (CON, n = 21; PRO, n = 19). Values are presented as mean group difference. Grey dots indicate individual subject responses. Data were first analysed using two-way mixed ANOVA to detect time-by treatment interaction (P = 0.033). Unpaired t-tests were used to compare delta changes between groups from Pre-Mid and Pre-End. * = $P < 0.05$ significantly different compared with CON.

10-km simulated time trial performance

As a result of endurance training, 10-km time trial performance improved in both groups ($p < 0.0001$). However, protein supplementation did not enhance the exercise-induced improvements in performance (time-by-treatment interaction $p = 0.64$) (**Figure 4**).

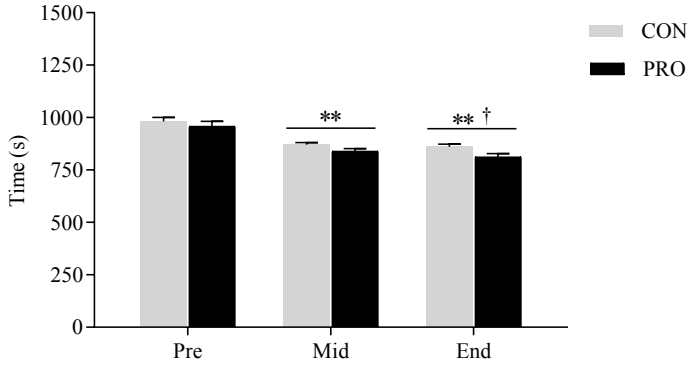


Figure 4. Endurance performance (seconds) as measured by a simulated 10-km time trial performance bicycle test during 10-weeks of endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as group mean ± SEM. Data were analysed using two-way mixed ANOVA for time-by treatment interaction ($P = 0.642$). Pairwise comparison with Tukey's post hoc correction were used to determine main effects of time and group. ** = $P < 0.0001$ significant main effect of time compared with Pre; † = $P < 0.05$ significant main effect of time compared with Mid.

Skeletal muscle mitochondrial enzyme activities

Endurance training increased maximal CS activity in the CON and PRO groups after 5 (21.7 ± 1.3 to 28.7 ± 1.1 vs 23.4 ± 1.4 to 31.9 ± 1.1 umol·g^{-1}·min^{-1}; $p < 0.05$) and 10 weeks (21.7 ± 1.3 to 29.8 ± 1.1 vs 23.4 ± to 33.9 ± 1.2 umol·g^{-1}·min^{-1}; $p < 0.05$) of training. However, the increases in CS were not statistically different between groups at mid-term ($p = 0.21$) and end ($p = 0.11$) (**Figure 5**). There were no differences in changes between the CON and PRO group in maximal CytC activity after 5 (24.8 ± 0.5 to 24.6 ± 0.4 vs 25.2 ± 0.7 to 24.0 ± 0.7 umol·g^{-1}·min^{-1}); $p > 0.05$) and 10 weeks (24.8 ± 0.5 to 23.9 ± 0.5 vs 25.2 ± 0.7 to 22.9 ± 0.7 umol·g^{-1}·min^{-1}); $p > 0.05$) of training (**Figure 5**).

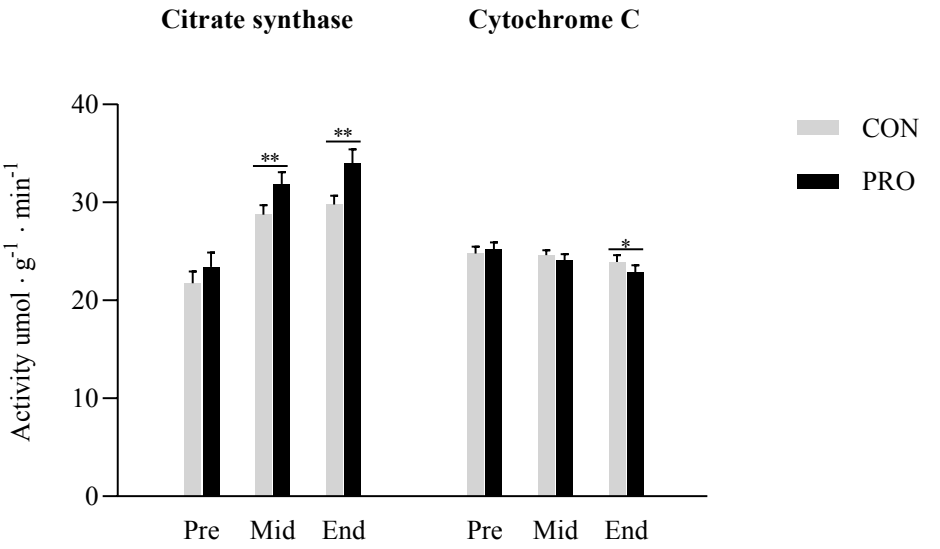
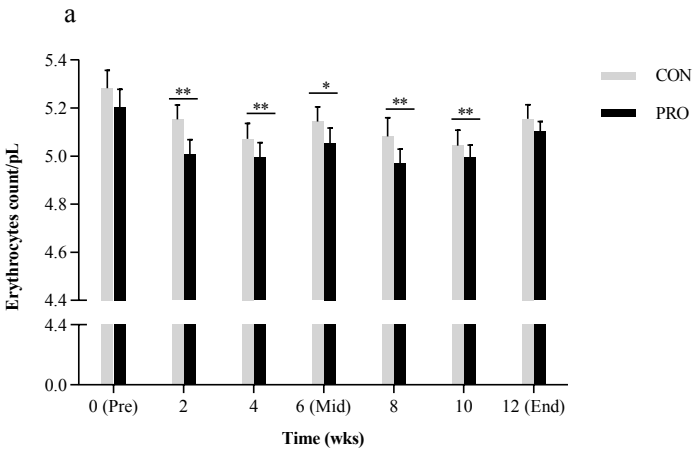


Figure 5. Skeletal muscle citrate synthase and cytochrome C oxidase activity during 10-weeks of endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as group mean ± SEM. Enzyme activities are expressed as micromoles of product (citrate and reduced cytochrome C) generated per gram of wet tissue per min during the assay ($\mu mol \cdot g^{-1} \cdot min^{-1}$). Data were analysed using two-way mixed ANOVA for time-by treatment interaction for citrate synthase ($P = 0.179$) and cytochrome C ($P = 0.292$). Pairwise comparison with Tukey's post hoc correction were used to determine main effects of time and group. * = $P < 0.05$ significant main effect of time compared with Pre; ** = $P < 0.0001$ significant main effect of time compared with Pre.

Haematological factors

Compared with baseline, endurance training caused significant reductions in erythrocytes, hemoglobin, hematocrit throughout the training period while all three factors returned to baseline levels after 10 weeks of training. There was no time-by treatment interaction for any of the haematological factors (**Figure 6**).



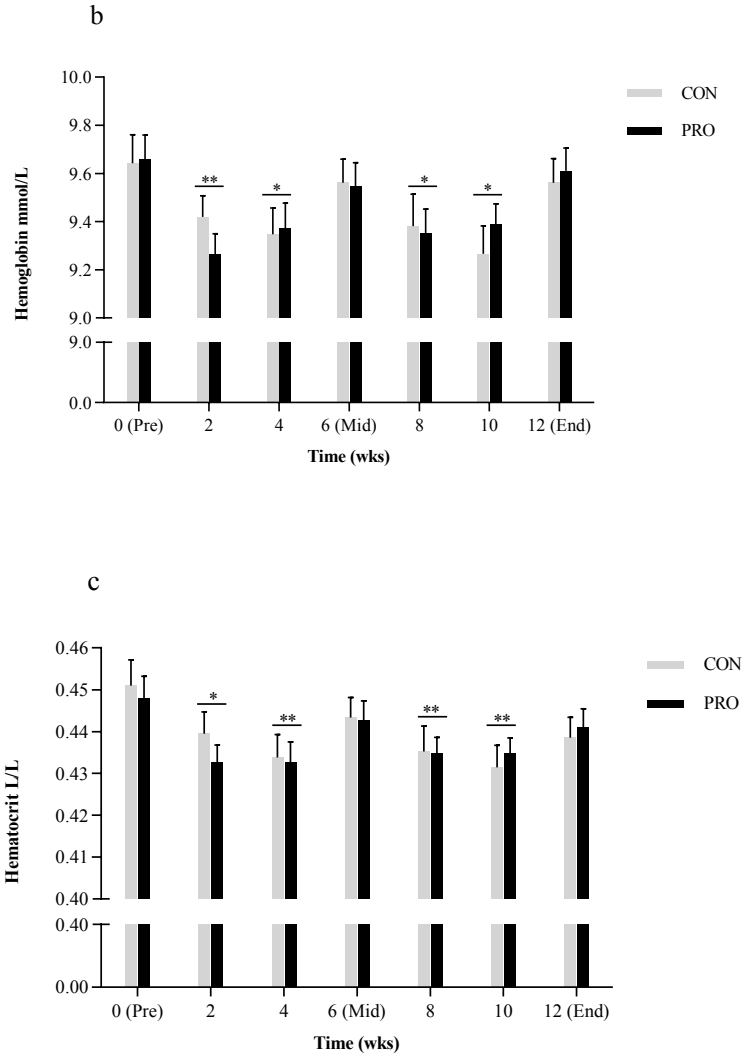


Figure 6. Haematological factors throughout the intervention. **a**)Erythrocytes (count/pL), **b**)hemoglobin (mmol/L) and **c**)hematocrit (L/L) during 10-weeks of endurance training (CON, n = 21; PRO, n = 19). Values are presented as group mean ± SEM. Data were analysed using two-way mixed ANOVA for time-by treatment interaction for erythrocytes (P = 0.771), hemoglobin (P = 0.483) and hematocrit (P = 0.699).. Pairwise comparison with Tukey's post hoc correction were used to determine main effects of time and group. * = $P < 0.05$ significant main effect of time compared with Pre; ** = $P < 0.0001$ significant main effect of time compared with Pre.

Body composition

There was time-by-treatment interaction (p = 0.049) for total body mass. Compared to baseline, body mass increased in PRO group after 5 weeks of training from 76.3 ± 1.3 to 77.2 ± 1.4 kg (p = 0.001) but returned to 76.5 ± 1.3 kg (p = 0.73) after 10 weeks of training. Body mass in the CON group remained stable over the course of the intervention (baseline: 77.2 ± 1.6; mid-term: 77.2 ± 1.6; end: 77.4 ± 1.6 kg) (p = 0.82). A significant time-by-treatment interaction (p = 0.001) for whole-body lean mass was observed (**Table 6**). Compared to CON, lean mass increases in the PRO group were significant at week 5 (p < 0.001) and week 10 (p = 0.0057) (**Figure 7**). Furthermore, there was a significant time-by-treatment interaction (p = 0.015) for fat mass. The decrease in fat mass between PRO and CON was statistically not different at wk 5 (p = 0.089) but reached statistical significance at wk 10 (p = 0.021) (**Figure 7**). Bone mineral density remained unchanged throughout the intervention in both groups.

Table 6. Body composition

	CON group (n=21)			PRO group (n=19)			*P*		
	Pre	**Mid**	**End**	**Pre**	**Mid**	**End**	**Training**	**Treatment**	**Interaction**
Lean mass, whole body (kg)	61.0 ± 0.9	61.1 ± 0.9	61.4 ± 1.0	60.1 ± 1.1	61.6 ± 1.2*	61.6 ± 1.2*	< 0.001	NS	= 0.001
Lean mass, trunk (kg)	28.1 ± 0.5	28.2 ± 0.5	28.4 ± 0.5	27.7 ± 0.5	28.3 ± 0.6	28.4 ± 0.5	= 0.001	NS	NS
Lean mass, legs (kg)	21.6 ± 0.4	21.5 ± 0.4	21.6 ± 0.4	20.9 ± 0.4	21.8 ± 0.5*	21.6 ± 0.5*	= 0.005	NS	= 0.002
Lean mass, arms (kg)	7.1 ± 0.2	7.2 ± 0.2	7.1 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	7.3 ± 0.2	NS	NS	NS
Fat mass, whole body (kg)	12.8 ± 1.0	12.7 ± 1.0	12.6 ± 1.1	12.8 ± 0.7	12.2 ± 0.7*	11.6 ± 0.8*†	< 0.001	NS	= 0.014
Fat mass, trunk (kg)	7.1 ± 0.5	7.1 ± 0.6	7.1 ± 0.6	7.0 ± 0.6	6.6 ± 0.6*	6.2 ± 0.6*	= 0.007	NS	= 0.010
Fat mass, legs (kg)	4.3 ± 0.3	4.1 ± 0.3	4.1 ± 0.3	4.3 ± 0.2	4.2 ± 0.2	3.9 ± 0.3	< 0.001	NS	NS
Fat mass, arms (kg)	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	NS	NS	NS
Bone mineral density (g/cm^2)	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	NS	NS	NS

1 Values are means ± SEM. NS, $P \geq 0.05$. *Significantly different compared with Pre. $P < 0.05$. †Significantly different compared with Mid, $P < 0.05$. CON, carbohydrate-supplemented; PRO, protein-supplemented.

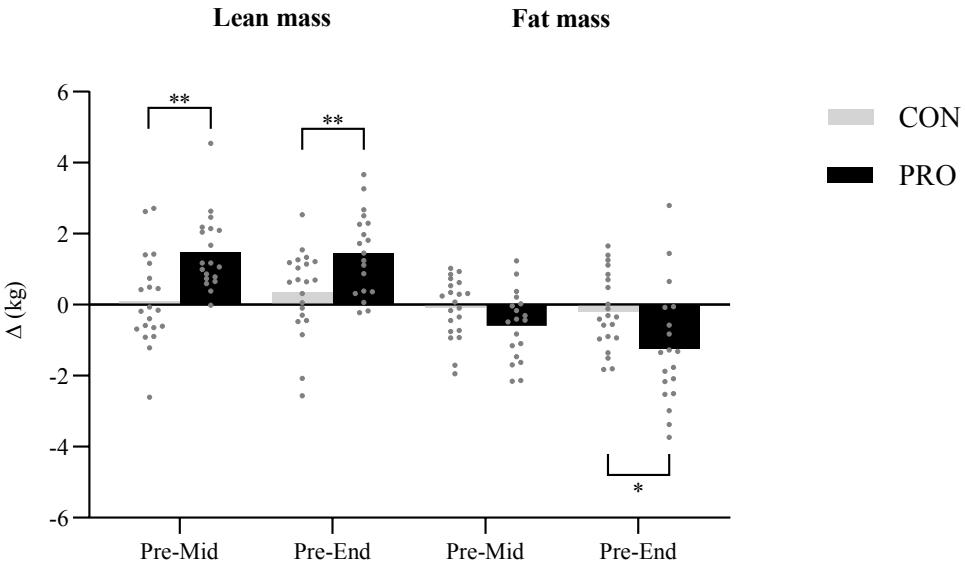


Figure 7. Delta changes in lean mass and fat mass from Pre-Mid and Pre-End during prolonged endurance training (CON, $n = 21$; PRO, $n = 19$). Values are presented as mean group difference. Grey dots indicate individual subject responses. Data were first analysed using two-way mixed ANOVA to detect time-by treatment interaction for lean mass ($P = 0.001$) and fat mass ($P = 0.015$). Unpaired *t*-tests were used to compare delta changes between groups from Pre-Mid and Pre-End. * = $P < 0.05$ significantly different compared with CON. ** = $P < 0.0001$ significantly different compared with CON.

Discussion

Ten weeks of endurance training increased VO_{2max}, skeletal muscle oxidative capacity, and 10-km time trial performance. Adding protein supplementation elicited greater gains in VO_{2max} and stimulated lean mass accretion, while promoting fat mass loss. However, protein supplementation did not improve skeletal muscle oxidative capacity and 10-km time-trial performance. Our findings confirm our hypothesis that protein supplementation facilitates the adaptive response during prolonged endurance training in young, healthy males.

It is well known that endurance training increases VO_{2max} (1). Our endurance training protocol substantially increased VO_{2max} after 5 and 10 weeks, which is in line with previous reports following 5-10 weeks of endurance training in healthy young males (29-31). However, after controlling for supplementation group, participants in the PRO group showed a greater increase in VO_{2max} compared to the CON group. The mechanism by which protein supplementation increased VO_{2max} is most likely explained by adaptive responses in both the cardiovascular and musculoskeletal system. Interestingly, in the PRO group, the increase in VO_{2max} during 5 weeks of training was accompanied by a substantial gain in lean mass, whereas lean mass remained unchanged in the CON group. In younger healthy individuals, lean mass has been well associated with VO_{2max} (32), as it was the case in our study (r^2=0.42:p<0.0001). Yet, the strongest correlation was found between leg lean mass and VO_{2max} at week 5 (r^2=0.59; p<0.0001). Changes in leg lean mass correlated mildly but significant with changes in VO_{2max} after 5 weeks of training (r^2 0.20; p=0.003). The greater increase in VO_{2max} we found may also relate to changes in skeletal muscle oxidative capacity such as mitochondrial density and/or function (33-37). Therefore, we measured CS and CytC activity on a skeletal muscle tissue level as proxy for mitochondrial content and function (33, 38). The increase in CS activity in skeletal muscle tissue tended to be greater in the PRO vs CON group, but this difference did not reach statistical significance. Because of the biological individual variation in CS it was not fully unexpected that we did not reach statistical significance between groups (38). Endurance training slightly decreased maximal CytC activity in the PRO group. This could be explained by the shorter half-life of CytC compared to CS (39). Specifically, muscle biopsies were taken eight days following the last exercise training session, considering the relatively short half-life of CytC it could be argued that its activity dropped to pre-training levels and that we may have missed peak levels of maximal activity of CytC throughout the training period (39, 40).

While a discussion on the main determinants of improvements in VO_{2max} falls outside the scope of this investigation, recent evidence suggests that

haematological adaptations may play a key role herein (28, 41). Yet, we found similar changes in concentrations of erythrocytes, hemoglobin and hematocrit between the PRO and CON group, suggesting that the differences in VO_{2max} between the groups cannot be explained by changes in oxygen-carrying capacity of blood. Adaptations in maximal cardiac output (e.g. stroke volume) and blood volume may also contribute to the greater increase in VO_{2max} in the PRO group, however, we were not able to measure this. Thus, based on our findings, the greater increase in VO_{2max} in the PRO group can be partly explained by changes in lean mass and skeletal muscle oxidative capacity.

It has previously been demonstrated that endurance training induces skeletal muscle hypertrophy (42-44). We found mild but non-significant increases in lean mass as a result of endurance training in the control group. In contrast, protein supplementation substantially increased lean mass after 5 weeks of endurance training. Our finding that lean mass had increased after 5 weeks of endurance training only when protein supplements were provided underlines the importance of adequate protein intake for skeletal muscle reconditioning during endurance training. Since endurance exercise increases muscle protein synthesis (45, 46), it is not surprising that studies have documented considerable muscle hypertrophy following prolonged endurance training (42, 44). The mechanism by which protein supplementation promotes lean mass gain is most likely the greater response following the exercise-induced stimulation in myofibrillar protein synthesis. Indeed, it has been previously shown that ingesting protein further stimulates myofibrillar muscle protein synthesis rates during recovery from a bout of cycling exercise (46). Furthermore, protein feeding following endurance exercise modulates mRNA-specific pathways involved in myogenesis and type-I fibre remodelling (47). In addition to the gain in lean mass, we also observed a substantial loss of fat mass in the PRO group (-1.2±0.4 kg). The loss of fat mass could have been resulted from the gain in lean mass through increased resting energy expenditure, thereby eliciting a caloric deficit. Furthermore, fat mass loss was inversely correlated with the gain in lean mass in the PRO group (r^2=0.5 , p=<0.001). The greater increase in VO_{2max} and improved body composition as a result of protein supplementation did not increase the exercise-induced improvements in 10-km time trial performance when compared with control. Acknowledging the inter-individual variation on the 10-km time trial, this study was probably underpowered and/or of too short duration to detect potential differences on endurance performance as a result of protein supplementation. Therefore, the benefits of protein supplementation during prolonged endurance training on exercise performance remain to be established and future work is needed to define the impact of the greater increase in VO_{2max} on various types of endurance performance, where duration and intensity may be of key importance.

The findings observed in this study mirror findings of some (17, 18) but not all (16) previous studies that have determined the impact of protein supplementation on changes in VO_{2max}, skeletal muscle oxidative capacity, endurance performance and body composition following prolonged endurance training. These discrepancies are most likely attributed to methodological differences between studies. For instance, aspects such as cohort size, applied exercise variables (e.g. type, duration, intensity), degree of monitoring, as well as the amount, type and timing of protein supplementation could largely affect study findings. We speculate that in our study the vigorous intensity of the exercise training performed in combination with the type of protein supplement and supplemental strategy may have contributed largely to the additional effects of protein supplementation. We decided to provide isocaloric supplements in both groups. Consequently, we provided the CON group with an isocaloric carbohydrate drink (28 g oligosaccharides). Hence, given that the carbohydrate beverage increased carbohydrate intake in the CON group it cannot be stated conclusively that merely the protein was responsible for the different responses between groups. For example, omission of post-exercise carbohydrate provision could have increased post-exercise molecular signalling responses thereby improving endurance training adaptations in the PRO group (48). However, it should be noted that all subjects received two slices of gingerbread (providing 2 x 28 g carbohydrate) immediately after every training session implying that protein (in)availability was the key factor responsible for the findings reported here. Furthermore, analysis of habitual macronutrient intake and physical activity levels revealed no differences between the PRO and CON groups.

This is to our knowledge the first double-blind randomized controlled trial with repeated measures providing a protein supplementation strategy designed to optimize protein availability to support training adaptation during prolonged endurance training. We chose to apply a supplementation strategy that theoretically should be most effective based upon previous post-exercise and before-sleep protein fractional synthetic rate measurements. Provision of 30 g calcium caseinate protein after each exercise session (19, 20) and daily before-sleep has previously been shown effective to increase gains in muscle mass and strength following prolonged resistance training (48). Though, it must be noted that subjects in our study were provided with a protein beverage post-exercise and before-sleep, based on the supplemental strategy utilized we cannot formulate inferences as to the effectiveness of the individual supplemental strategy, or potential superiority of combining post-exercise and before-sleep protein supplementation. Thus, combining the recent knowledge on the impact of protein ingestion during recovery from exercise and the impact on post-exercise muscle protein synthesis rates, we show that such a supplementation strategy effectively increases VO_{2max} and

stimulate lean mass gain thereby enhancing the efficiency of endurance training reconditioning.

In conclusion, protein supplementation elicited greater gains in VO_{2max} and stimulated lean mass accretion, while promoting fat mass loss during 10 weeks of endurance training in healthy, young males. Protein supplementation did not improve skeletal muscle oxidative capacity and endurance performance. Therefore, protein supplementation seems to form an effective dietary strategy to enhance the adaptive response to endurance training in healthy, young males.

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Chapter 7

Regulation of skeletal muscle transcriptome is increased by protein supplementation and reflects the extent of physiological adaptation to endurance training

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BMC Genomics (under review)

Abstract

Background: Protein supplementation has shown to enhance physiological adaptation to endurance training. Whether protein supplementation affect the adaptive skeletal muscle transcriptome response to endurance training remains unknown. The present analysis was executed to determine the impact of protein supplementation on changes in the skeletal muscle transcriptome following prolonged endurance training.

Methods: We used a double-blind randomized controlled trial among 44 recreationally active young males. Subjects performed three endurance training sessions per week for five weeks. Supplements were provided immediately after each exercise session and daily before sleep providing either protein (PRO; *n*=19; 21.5±0.4 y) or an isocaloric amount of carbohydrate as control (CON; *n*=21; 22.5±0.5 y). Fasting skeletal muscle tissue samples from the *vastus lateralis* were taken before and after five weeks to measure skeletal muscle transcriptome.

Results: Endurance training significantly induced gene expression related to extracellular matrix remodelling and oxidative phosphorylation. Gene expression changes as a result of endurance training was overall larger in the protein group than in the control group. Regulation of skeletal muscle gene expression transcripts related to extracellular matrix remodelling tended to be greater in the protein group than in control group.

Conclusions: Endurance training increased gene expression transcripts related to the extracellular matrix organization and oxidative phosphorylation. Transcriptional changes in skeletal muscle due to endurance training tended to be increased by protein supplementation. The subtle differing extracellular matrix expression profile may reflect the enhanced physiological adaptation as result of protein supplementation.

Background

Skeletal muscle is an extraordinary malleable tissue which is demonstrated by its rapid remodelling and adaptation to several kinds of stress, such as exercise (1, 2). Repetitive bouts of endurance exercise, e.g. endurance training, lead to various metabolic and morphological adaptations in skeletal muscle (3, 4). At the myocellular level, long term skeletal muscle adaptation is the result of repeated modifications in transcriptional and translational responses of each exercise bout thereby increasing the synthesis of specific proteins required for remodelling (5-8). Skeletal muscle transcriptome analysis provides an unbiased examination of the molecular alterations to exercise training, thereby potentially unravelling novel pathways involved in adaption to endurance training (9-11).

Protein feeding following endurance exercise has shown to affect mRNA-specific pathways involved in extracellular matrix, myogenesis, immunogenic response, and energy metabolism (12). We recently demonstrated that protein supplementation increased physiological adaptation to prolonged endurance training, where the major part of the increase in physiological variables was observed during the first 5 weeks of the 10 week training intervention (Knuiman et al., 2019, *in press*). Whether the beneficial effects of protein supplementation during prolonged endurance training are also pronounced at the skeletal muscle transcriptional level remains unknown. To this end, we assessed the impact of protein supplementation during 5 weeks of endurance training on changes in the skeletal muscle transcriptome. We hypothesize that protein supplementation elicits greater changes in the skeletal muscle transcriptome when compared to carbohydrate supplementation.

Methods

Subjects

Forty-four young healthy males volunteered and gave full-written informed consent to participate in a 10-wk endurance training program, with or without additional protein supplementation. Primary inclusion criteria were non-smokers, free of injury and not using any medication or nutritional supplements. Additional exclusion criteria that would preclude successful participation in the training study included (diagnosed) lactose intolerance and/or dairy protein allergy, cardiorespiratory-related illness and musculoskeletal-related injuries impeding with the endurance training sessions. All subjects were physically active, performing sports on a non-competitive basis between one and four hours per week. None of the participants had a history of participating in any structured endurance training programs to improve performance over the past two years. This study was carried out in accordance with the guidelines for human research of The Medical Ethical Committee of Wageningen University. The Medical Ethical Committee of Wageningen University approved all study procedures and complied with the guidelines set by the Declaration of Helsinki of 1975 as revised in 1983. This trial was registered at clinicaltrials.gov as NCT03462381 and adheres to CONSORT guidelines for clinical trials.

Experimental design

Subjects completed a progressive endurance training protocol with three exercise sessions per week for in total 10 weeks and consumed a post-exercise and pre-sleep drink providing either protein or an isocaloric amount of carbohydrate. The total study duration, including a series of mid-term and end measurements, was ~12 wk. After inclusion, subjects were randomly assigned to either a protein-supplemented (PRO) or carbohydrate-supplemented control (CON) group. Before (wk 0), during (wk 6), and after (wk 12) the exercise training program, anthropometric measurements (height, body mass, and waist circumference), VO_{2max} ramp tests, simulated 10-km time-trials, and DEXA scans were carried out. In addition, muscle biopsy specimens, dietary intake records, and physical activity records were collected (**Figure 1**). Fasting blood samples were taken at week 0 (pre), 2, 4, 6 (mid), 8, 10, and 12 (end). The subjects were instructed to maintain their normal dietary habits and physical activity patterns throughout the intervention period. A standardized meal was provided the evening before each test day (standard deep-frozen meal and ice-cream dessert; 43.80 kJ/kg BW; 15 Energy% protein, 30 Energy% fat and 55 Energy% carbohydrate; Roerbaksensatie, Iglo, Utrecht, the Netherlands). Subjects refrained from continuous physical activity for at least 72 h before

testing. At the different test days, subjects arrived at the laboratory after an overnight fast.

Endurance training program

Training intervention was divided in two blocks of five weeks with one week (week 6) in between the blocks to perform the mid-term measurements, and a final week (week 12) to perform the end-measurements. The first four weeks subjects participated in three endurance exercise sessions/wk alternated with one day of rest between each session. The fifth week subjects performed 2 endurance exercise sessions, see **Figure 1**.

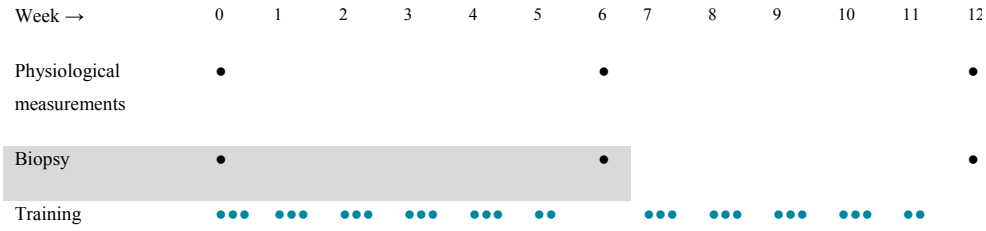

Figure 1. Schematic overview of the study protocol. 40 subjects completed 10 wk of progressive endurance training while consuming either 25 g carbohydrates or 25 g protein post-exercise and daily before sleep. All measurements were assessed before, midterm (week 6) and after (week 12). Strongest effect of protein supplementation was observed following 5 weeks of endurance training. To gain more insight into mechanisms underlying greater physiological adaptation as a result of protein supplementation we analysed skeletal muscle transcriptome data from baseline to midterm. Black dots: measurement points, bleu dots: exercise sessions. Grey part: contains physiological and microarray data analysed for this manuscript.

In total, subjects performed 28 endurance exercise training sessions. After a 10-min warm-up on a cycle ergometer, the endurance training session consisted of 60-min continuous cycling. All training sessions were conducted under supervision of a researcher using indoor, mechanically braked spinning bikes (Body Bike Smart®, Denmark) with participants free to adjust resistance and cadence as desired. Heart rate (HR) for each session was recorded (Polar Electro, United States of America), HR and rate of perceived exertion (RPE) were taken at the start and every 5 min thereafter by the supervisor. Music and verbal motivation were provided during training sessions which were conducted in ambient conditions at sea level at thermal-neutral conditions (21°C, 40% relative humidity). Each endurance training session ended with a

10-min cooling down period on the same cycle ergometer. Exercise intensity was determined using the Karvonen formula (13):

$$Intensity = HRreserve \cdot 0.85 + HRrest$$

Where *HRreserve* is the calculated difference between *HRmax* (determined during VO_{2max} ramp test) and *HRrest*. According to the American College of Sports Medicine, the selected exercise intensity can be considered as "vigorous" (14).

Protein and control supplementation

Subjects consumed a 250 mL beverage containing either a 29 gram of casein protein or an isocaloric amount of carbohydrate immediately after cessation of each exercise session (3 per wk) and every day before sleep (7 per wk). In addition, all subjects received two slices of gingerbread directly after every training session (total energy 280 Kcal; 63.2 g of carbohydrates; 1.4 g fat; 2.4 g protein). An overview of the energy and macronutrient composition of the beverages can be found in **Table 1**. Nutritional content of the supplements was analysed in duplicate by an independent laboratory (Nutrilab B.V., Rijswijk, the Netherlands) and reported analogous nutritional values as given by the producer. The protein and control beverages were masked for taste and smell by adding several additives. In addition, beverages were masked for colour and produced in white non-transparent containers. Allocation to the PRO or CON group was done using block randomization (group 1, $n = 24$; group 2, $n = 20$) by an independent researcher not involved in the study. Study drink boxes/beverages were sequentially numbered on subject number.

Table 1. Nutritional composition of the intervention drinks (250 mL)

Energy & Nutrient	Control beverage (CON)	Protein beverage
Energy (Kcal)	~129	~127
Protein (casein) (g)	0.6	28.7
Fat (g)	2.4	0.3
Carbohydrates (maltodextrin and sucrose) (g)	26.3	2.7

Body composition

DEXA measurements were carried out using a Lunar Prodigy Advanced DEXA scanner (GE Health Care, Madison, WI, United States of America). Each morning at the different test days, a quality assurance test was performed to ensure system suitability and precision of the scanner. Whole body scans were

performed according to the manufacturer's protocol and identical scan protocols were used for all subjects. Subsequently, different regions for fat mass and lean mass were assessed. Anthropometrics were assessed using standardized procedures, body weight by digital scale to within 100 g, height by stadiometer to within 0.5 cm and waist circumference by tape measure to within 0.5 cm (SECA, Hamburg, Germany).

VO_{2max}

A ramped VO_{2max} test was performed at baseline (- 2 wk), mid-term and end (2-3 days after the last training sessions) between 9 am and 5 pm. Ninety minutes before each test, subjects consumed a standardized meal consisting of an energy bar (3.7 g fat, 29.2 g carbohydrates, 2 g protein, 158.1 Kcal), an apple (Granny Smith) and 500ml water. Following a 30-min rest, subjects performed a ramped VO_{2max} test on an electrically braked cycle ergometer (Lode Excalibur, Groningen, the Netherlands). After a 5-min warm up at 50 W, the subjects started cycling at 100 W. Workload was progressively increased by 20 W·min−1 until the subject reached volitional exhaustion. The VO_{2max} test was considered to be valid when two out of three criteria were met: (I) levelling of VO_2 with increasing workload; (II) heart rate within 10 beats of the theoretically estimated maximum (220-age); and (III) respiratory exchange ratio (RER) of ≥1.15. Oxygen consumption (VO_2) was measured through breath-by-breath sampling with an Oxycon Pro (Jaeger, Hoechberg, Germany) to define maximal oxygen consumption (VO_{2max}). Subjects were asked to maintain a cadence between 80 and 100 r·min^{-1}.

Endurance exercise performance (simulated 10-km time trial)

Familiarization was performed in the week after the start of the first experimental day (anthropometrics, DEXA, blood and biopsy). Three or four days thereafter another time trial was conducted (baseline measurement) and this was repeated at mid-term and end. Subjects performed a simulated ~10-km cycling time trial. The data from the baseline VO_{2max} test was used for the amount of work to be performed and calculated as follows: total amount of work (J) = 0.85*Wmax*900 (s) (15). The ergometer was set in linear-mode so that 85% Wmax was achieved when subjects cycled at their preferred pedalling rate of 85 ± 7 rpm, as determined during familiarization. Subjects received no verbal or physiological feedback during the time-trial, and were only aware of the absolute (kJ) and relative (%) amount of work performed. Ratings of perceived exertion (RPE) were assessed after each 30-min submaximal exercise test and after the time-trial using the Borg 6–20 scale (Borg, 1982). All testing

was performed under standardized conditions (21 ˚C, 40% relative humidity) on the same time of day, and the same day in the week.

Muscle Biopsies

Muscle biopsies were taken after an overnight fast 3-4 days prior the start of the first training session (pre), 5-7 days after de 14th (mid-term) and 28th (end) training session. Muscle biopsies were taken as described by Bergstrom (1975) (16). Biopsies were taken under local anaesthesia (2–3 mL of 2% adrenaline) using a 5-mm Bergstrom needle modified with suction. Biopsies were taken from the *vastus lateralis* of the same leg, with separate incisions (~1–1.5 cm apart) and from distal to proximal direction. Muscle biopsies were immediately frozen (within 5–10 s) in liquid nitrogen and stored at −80˚C for subsequent biochemical analysis, after being freed from visible fat, blood, and connective tissue.

Sample preparation and microarray analysis

Total RNA was isolated from the skeletal muscle tissue by using Trizol reagent (Invitrogen, Breda, Netherlands). Thereafter, RNA was purified using the Qiagen RNeasy Micro kit (Qiagen, Venlo, the Netherlands), and RNA quality was checked using an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands). Total RNA (100 ng) was labelled using an Affymetrix WT plus reagent kit (Life Technologies, Bleiswijk, the Netherlands) and hybridized to human whole genome Genechip Human Gene 2.1 ST arrays, (Life Technologies, Bleiswijk, the Netherlands). Sample labelling, hybridization to chips, and image scanning were performed according manufacturer's instructions.

Statistics

Power calculation

Sample size (*n*) was calculated with 90% power and type I error probability of 0.05 based on the primary outcome of VO_{2max} increase. An expected and/or relevant difference in VO_{2max} seen with endurance training is 0.32 ± 0.30 ($L \cdot min^{-1}$) (17). To demonstrate a statistically significant greater increase in VO_{2max} by a nutritional supplement 16 participants/group have been shown to be sufficient (18). Considering a drop-out rate of 20-30%, the final number of the participants included was 22/group.

Physiological data

Data were assessed for normality with the use of a Shapiro-Wilk test, and any non – normal data were corrected with the use of transformation with the type of transformation based on the nature of the skewedness of the data. Repeated measures of analysis of variance (two-way mixed ANOVA), was used to determine statistical significance for the dependent variables over time. The analysis of variance model for the dependent variables with two testing time points was described as $S_{40}\ x\ T_2\ x\ G_2$ as such that (S; number of subjects) are crossed with testing time (T; three testing times: Pre (week 0) and Post (week 6) and group (G; CON and PRO). Where two-way mixed ANOVA revealed significant interaction, a Tukey's post hoc test was conducted for multiple comparisons to further analyse within group effects and unpaired *t*-tests to compare between groups at specific time points. In case two-way mixed ANOVA revealed no significant interaction but significant main effects of time and/or group pairwise comparisons with Tukey's post hoc correction were done. Data management and statistical analysis were carried out using SPSS software version 23 (SPSS Inc., Chicago, IL, United States of America). Statistical significance was declared when $p < .05$. Physiological data are expressed as mean ± SEM.

Microarray data

Statistical analysis of gene expression changes was performed using *limma* R library (19). Contrasts were set for endurance training effect in both groups and an interaction term was used to determine the effect of protein supplementation (protein group versus the control group). P-values were calculated using Intensity Based Moderated t-tests (IBMT) (20). Significant genes were first selected using the False Discovery Rate Adjusted F-statistic p-value <0.05. Unadjusted p-values below 0.01 for the contrasts were considered statistically significant within the genes that passed the F-test. Gene set enrichment analysis was done using pre-ranked lists ranked by the t-values from the *limma* contrasts (21, 22). We used the most recent library of canonical pathways from MsigDb (21). An adjusted p-value (q-value) of 0.10 was considered significant for the gene rest enrichment analysis results. Venn diagram and Heatmaps were made using the ComplexHeatmap library (23) and GraphPad Prism 8.01 for Windows (San Diego, CA, United States of America). EnrichR was used to determine differences in GO biological processes (24, 25).

Results

Baseline characteristics

Four subjects dropped out during the study, one because of relocation, one because of a hamstring injury, one because of a knee injury and one because of appendicitis. Final analysis was performed on the 40 subjects who completed the training program (CON: *n*=21 vs PRO: *n*=19) **(Figure 2)**. Baseline characteristics of both groups prior to the endurance training are summarized in **Table 2**. There were no differences at baseline between the CON and PRO groups in any of the variables of interest.

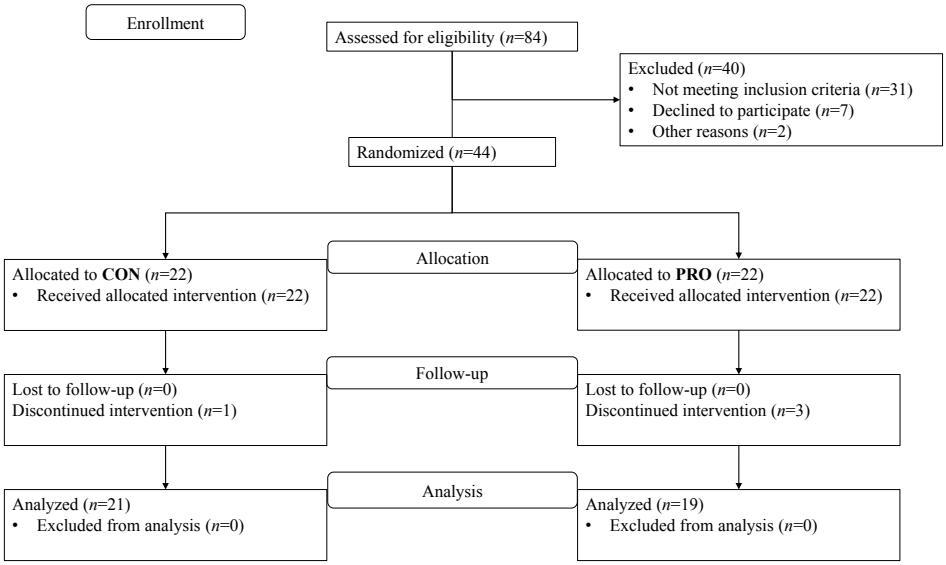

Figure 2. Subject recruitment and flow through the protocol. CON (carbohydrate supplementation, *n* = 21); PRO (protein supplementation, *n* = 19).

Endurance training effect

Five weeks of endurance training significantly increased maximal aerobic capacity and skeletal muscle oxidative capacity. VO_{2max} increased by 2.2 ± 2.6 $mL \cdot kg^{-1} \cdot min^{-1}$ and maximal activity of citrate synthase increased by 6.99 ± 3.50 $\mu mol \cdot g^{-1} \cdot min^{-1}$. These changes were accompanied by improved endurance performance, as revealed by a reduction in time on a simulated 10-km time trial of 111.2 seconds. Protein supplementation elicited a greater increase in maximal aerobic capacity and stimulated lean mass gain but did not improve skeletal muscle oxidative capacity and endurance performance (**Table 2**). A full discussion of the physiological effects of endurance training with or without protein supplementation can be found elsewhere (Knuiman et al., 2019, *in press*).

Table 2. Baseline characteristics and physiological effects of 5-weeks endurance training. Values are means ± standard deviation. P-values are from mixed model analysis. CON = control group. PRO = protein group.

	CON group	**(*n* = 21)**	**PRO group**	**(*n* =19)**	***P-values***		
	0 weeks	**5 weeks**	**0 weeks**	**5 weeks**	**Training**	**Treatment**	**Interaction**
Age (yr)	22.5 ± 2.3		21.5 ± 1.6				
Body mass (kg)	77.2 ± 7.2		76.3 ± 5.4				
Height (m)	1.85 ± 0.1		1.85 ± 0.1				
BMI (kg/m $^{-2}$)	22.4 ± 1.3		22.3 ± 1.5				
Lean mass (kg)	61.0 ± 4.2	61.1 ± 4.1	60.1 ± 4.8	61.6 ± 5.3	= 0.0001	= 0.9	= 0.000
Fat mass (kg)	12.8 ± 4.5	12.7 ± 4.6	12.8 ± 2.9	12.2 ± 3.1	= 0.02	= 0.8	= 0.089
VO_{2max} (L·min-^{1})	3.9 ± 0.3	4.1 ± 0.3	3.8 ± 0.4	4.2 ± 0.5	= <0.0001	= 0.7	= 0.004
VO_{2max} (mL·kg-1·min-1)	50.8 ± 3.9	53.0 ± 4.9	49.9 ± 3.4	54.9 ± 4.8	= <0.0001	= 0.7	= 0.016
Citrate synthase (µmol·g− 1·min− 1)	21.8 ± 5.4	28.7 ± 4.4	23.4 ± 6.2	31.9 ± 5.2	= <0.0001	= 0.1	= 0.206
Time-trial performance (seconds)	982.3 ± 86.1	871.1 ± 45.8	957.8 ± 106.5	839.1 ± 53.4	= <0.0001	= 0.1	= 0.796

Muscle transcriptome

Endurance training induced significant changes in the muscle transcriptome in both the CON and the PRO group. The activity of more genes was altered by endurance training in the PRO group than in the CON group (893 vs. 441, respectively, F-test q-value <0.05). **Table 3** shows the top 20 significant genes based on level of significance for both CON group and PRO group. Among the top 20 significant genes for the CON group are genes related to extracellular matrix organization including collagen type IV alpha chain (COL4A2), collagen type IV alpha 1 chain (COL4A1), laminin subunit alpha 4 (LAMA4), laminin subunit beta 1 (LAMB1) and alpha-2-macroglobulin (A2M). Top 20 significant genes for the PRO group were comparable with those of the CON group and relate to extracellular matrix organization including collagen type III alpha 1 chain (COL3A1), secreted protein acidic and cysteine rich (SPARC), collagen type IV alpha 2 chain (COL4A2), collagen type IV alpha 1 chain (COL4A1), laminin subunit alpha 4 (LAMA4), peroxidasin (PXDN), laminin subunit beta 1 (LAMB1), alpha-2-macroglbulin (A2M) and nidogen 1 (NID1).

Table 3. Top 20 significant genes in the CON and PRO group sorted on level of significance (F-test q-value<0.0001) in the CON (**A**) and PRO (**B**) group. Q-values for CON and pro group as well as the interaction effect of endurance exercise training with protein supplementation are adjusted IMBT p-values. FC is the signed fold change. CON is the change in the control group. PRO is the change in the protein group. Inter is the interaction effect between protein supplementation and endurance training.

A

Gene	FC CON	FC PRO	Q-val. CON	Q-val. PRO	P-val. Inter	Q-val. Inter
LAMA4	1.39	1.57	0.000	0.000	0.051	0.821
COL4A1	1.69	1.87	0.000	0.000	0.311	0.933
A2M	1.22	1.33	0.000	0.000	0.029	0.796
MYO1B	1.37	1.44	0.000	0.000	0.476	0.957
CD34	1.28	1.33	0.000	0.000	0.496	0.958
NFIX	-1.12	-1.09	0.000	0.001	0.288	0.926
THBS4	1.61	1.94	0.000	0.000	0.081	0.853
COL4A2	1.54	1.77	0.000	0.000	0.144	0.881
RYR3	1.42	1.12	0.000	0.336	0.003	0.647
FXYD1	-1.15	-1.13	0.000	0.000	0.612	0.974
COX4I1	1.19	1.15	0.000	0.001	0.441	0.952
TMEM159	-1.41	-1.23	0.000	0.026	0.083	0.854
SMTNL1	-1.55	-1.39	0.000	0.003	0.298	0.929
LAMB1	1.46	1.79	0.000	0.000	0.025	0.782
ALDH1B1	1.35	1.25	0.000	0.005	0.254	0.916
RHOJ	1.32	1.15	0.000	0.090	0.035	0.799
SMOC2	1.31	1.37	0.000	0.000	0.482	0.958
LXN	1.41	1.28	0.000	0.007	0.254	0.916
ANKRD29	1.42	1.16	0.000	0.198	0.020	0.756
DECR1	1.19	1.17	0.000	0.000	0.767	0.989

B

Gene	FC PRO	FC CON	Q-val. CON	Q-val. PRO	P-val. Inter	Q-val. Inter
LAMA4	1.57	1.39	0.000	0.000	0.051	0.821
A2M	1.33	1.22	0.000	0.000	0.029	0.796
LAMB1	1.79	1.46	0.000	0.000	0.025	0.782
COL4A1	1.87	1.69	0.000	0.000	0.311	0.933
THBS4	1.94	1.61	0.000	0.000	0.081	0.853
COL4A2	1.77	1.54	0.000	0.000	0.144	0.881
MYO1B	1.44	1.37	0.000	0.000	0.476	0.957
NID1	1.48	1.26	0.002	0.000	0.022	0.756
CD34	1.33	1.28	0.000	0.000	0.496	0.958
SPARC	1.45	1.29	0.000	0.000	0.075	0.848
COL15A1	1.43	1.25	0.001	0.000	0.045	0.816
UTRN	1.22	1.12	0.003	0.000	0.029	0.796
EDNRB	1.70	1.34	0.005	0.000	0.016	0.756
PXDN	1.75	1.48	0.000	0.000	0.107	0.863
ETS1	1.43	1.26	0.001	0.000	0.063	0.839
MXRA5	2.51	1.90	0.000	0.000	0.110	0.863
COL3A1	2.06	1.70	0.000	0.000	0.178	0.893
IGFBP7	1.35	1.26	0.000	0.000	0.233	0.914
ANXA5	1.35	1.10	0.268	0.000	0.001	0.558
CAPN6	1.83	1.46	0.003	0.000	0.060	0.836

Effect of protein supplementation

Figure 3 shows the number of genes regulated as a result of endurance training for each the CON group and the PRO group and the groups combined. After 5 weeks of endurance training, gene expression count was greater in the PRO group compared with CON. In addition, the top 20 and overall gene transcript change in muscle transcriptome was consistently greater in the PRO group when compared to the CON group (figure 3 & 4). The greater changes in the PRO group are visualized in figure 5.

Figure 3. Venn diagram showing the number of significantly changed genes per group. Selected genes (F-test q-value<0.05) for each the CON group and the PRO group and the groups combined (raw p-value<0.0001).

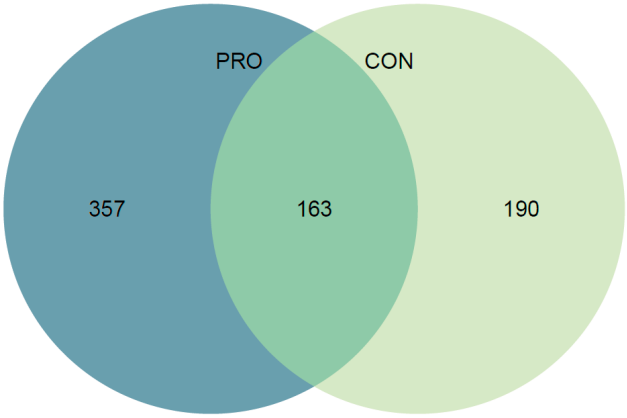

Figure 4. Heatmaps based on top 20 significant genes for each group. Genes are sorted on FC (F-test q-value<0.0001) in the CON (A) and PRO (B) group. Q-values for CON and PRO group are adjusted IMBT p-values. FC is the signed fold change. CON is the change in the control group. PRO is the change in the protein group.

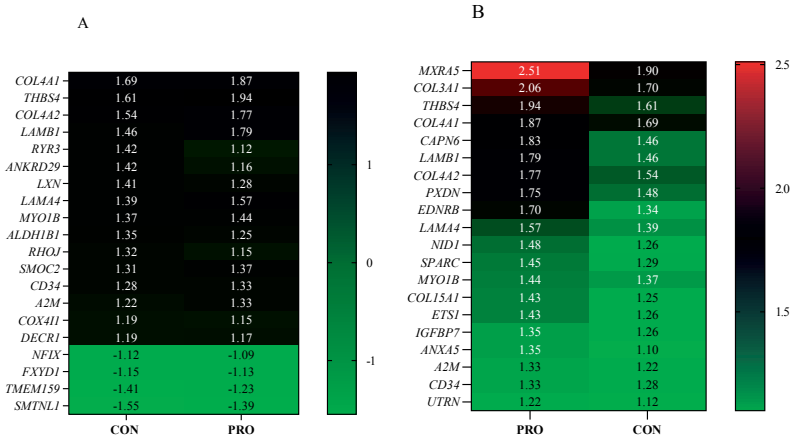

Figure 5. Scatterplots with line of identity to visualize the magnitude of change in muscle transcriptome per group. Figures A & B are based on the total number of genes changed per group (184 for CON (A) and 384 for PRO (B), F-test q-value<0.05). Figures C & D are based on the top 20 significant genes changes in the CON (C) and PRO (D) group.

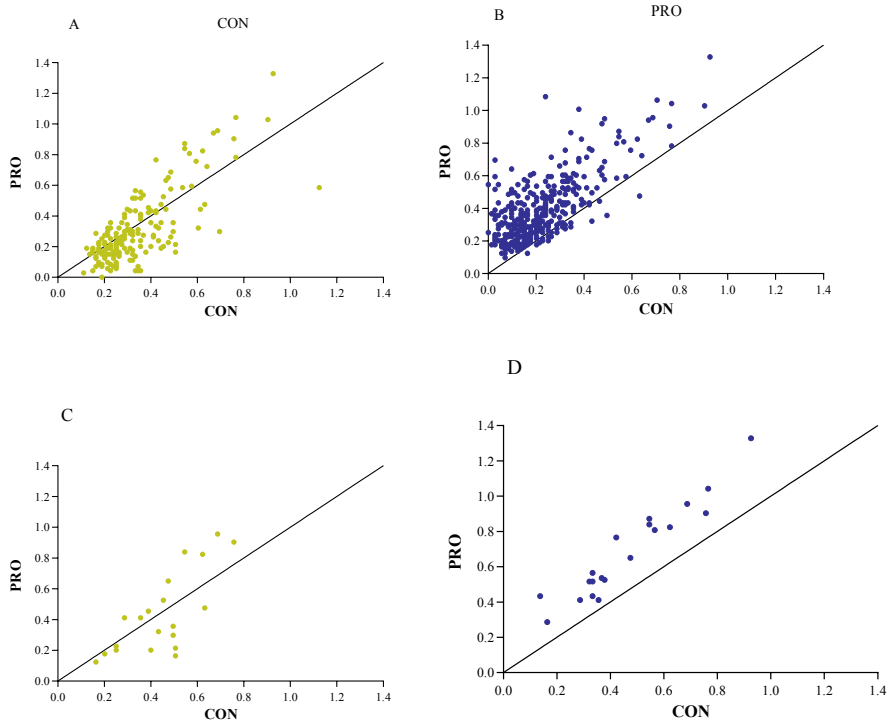

Figure 6. Heatmap of changes in gene expression per group. (F-test q-value<0.0001) in the CON (left) and PRO (right) groups.

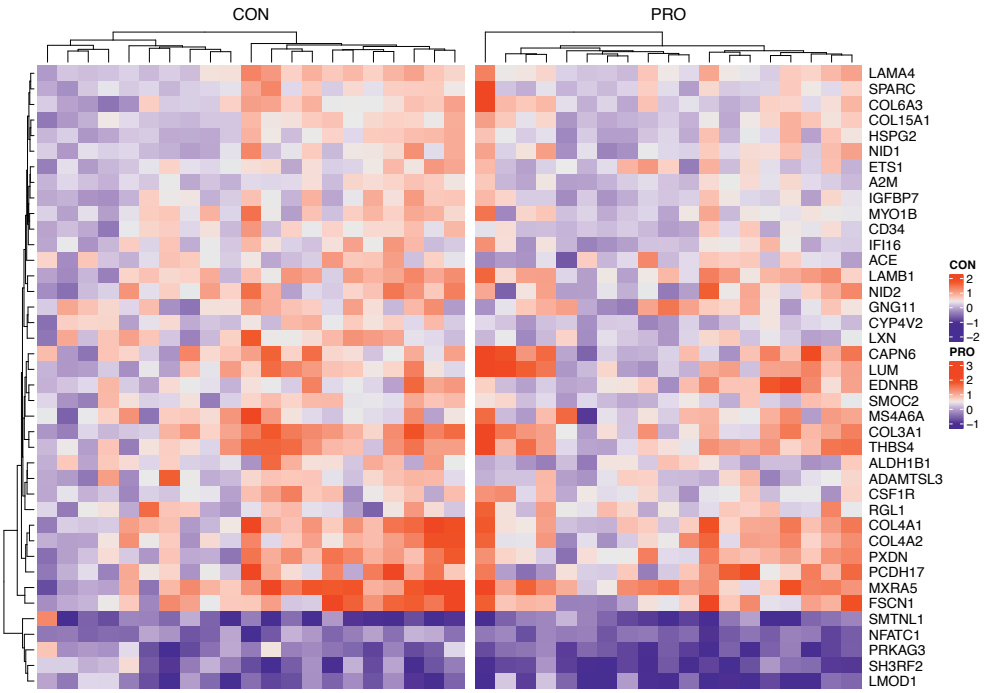

Figure 6 shows a heatmap of the genes that significantly changed by endurance training in both the CON group and PRO group (40 genes, F-test q-value<0.0001). The changes in gene expression following 5 weeks of endurance training did not markedly differ between the CON group and PRO group (time-by treatment interaction, F-test q-value>0.05). No major differences can be observed with regard to training response between the PRO and CON group. Gene-set-enrichment analysis showed a similar result, as gene sets that were significant for the CON group were generally also significant for the PRO group.

Biological processes

Based on all significant genes altered (F-test q-value<0.05) in each group, gene ontology biological processes revealed extracellular matrix organization as the process with the highest change in gene expression profile in both the CON group and the PRO group (table 3). In the CON group 25 genes were linked to extracellular matrix organization whereas 55 genes in the PRO group. Accordingly, gene set enrichment analysis (table 4) showed time-by treatment interaction for extracellular matrix organization processes such as extracellular matrix receptor interaction (q-value<0.001), extracellular matrix glycoproteins (q-value=0.006) and collagen formation (q-value=0.041). Gene set enrichment also showed significant increases in energy metabolism and oxidative phosphorylation with no clear differences between the CON group and the PRO group.

Table 4. Top 10 gene ontology biological processes from EnrichR regulated in the CON group (**A**) and PRO group (**B**) based on the total number of genes that was significantly regulated in the CON group (n = 440) and PRO group (n = 892) group (F-test q-value<0.05).

A	Name of biological process	Genes (n)	P-value	Q-value	Z-score
1	extracellular matrix organization	25	0.000	0.000	-1.65
2	sarcomere organization	7	0.000	0.002	-2.30
3	muscle contraction	19	0.000	0.000	-1.18
4	positive regulation of sprouting angiogenesis	7	0.000	0.001	-1.88
5	positive regulation of B cell differentiation	3	0.001	0.041	-3.10
6	regulation of angiogenesis	16	0.000	0.001	-1.77
7	mitochondrial ATP synthesis coupled proton transport	3	0.012	0.180	-4.85
8	regulation of release of sequestered calcium ion into cytosol	8	0.000	0.003	-1.89
9	actomyosin structure organization	11	0.000	0.000	-1.46
10	myofibril assembly	8	0.000	0.002	-1.81

B	Name of biological process	Genes (*n*)	P-value	Q-value	Z-score
1	extracellular matrix organization	55	0.000	0.000	-1.65
2	regulation of smooth muscle cell migration	8	0.001	0.000	-2.21
3	mitochondrial ATP synthesis coupled proton transport	5	0.002	0.075	-4.89
4	collagen fibril organization	11	0.000	0.000	-1.58
5	regulation of angiogenesis	25	0.000	0.000	-1.77
6	positive regulation of cell migration	27	0.000	0.001	-1.91
7	positive regulation of smooth muscle cell migration	4	0.001	0.049	-3.44
8	regulated exocytosis	19	0.000	0.005	-2.27
9	basement membrane organization	4	0.000	0.026	-2.81
10	cellular protein modification process	76	0.000	0.001	-1.73

Table 5. Top 10 significant enriched gene sets in both the CON group and the PRO group (interaction effect). CON is the training, Inter is the interaction effect. ES is the enrichment score.

GSEA	Name of biological process	ES CON	ES Inter	q-value CON	q-value Inter
1	Kegg ECM receptor interaction	0.70	0.58	0.000	0.000
2	Naba core matrisome	0.73	0.47	0.000	0.000
3	Naba ECM glycoproteins	0.73	0.46	0.000	0.006
4	Pid integrin1 pathway	0.69	0.54	0.000	0.007
5	Reactome integrin cell surface interactions	0.63	0.51	0.000	0.012
6	Biocarta RHO pathway	0.81	0.62	0.000	0.019
7	Pid TCR pathway	0.64	0.51	0.000	0.032
8	Reactome collagen formation	0.60	0.52	0.000	0.041
9	Pid syndecan 1 pathway	0.73	0.53	0.000	0.051
10	Pid integrin 3 pathway	0.53	0.54	0.000	0.051

Discussion

Five weeks of endurance training increased maximal aerobic capacity. Adding protein supplementation elicited a greater increase in maximal aerobic capacity and stimulated lean mass gain. At the skeletal muscle transcriptional level, endurance training caused relatively small (FC < 2) but consistent and statistically robust changes in the skeletal muscle transcriptome. Furthermore, changes in the skeletal muscle transcriptome tended to be greater in the protein group as compared to the control group. However, the differences in gene regulation between the two groups are far less clear. This lack of clear differences in skeletal muscle gene expression transcripts between the protein and control group is likely due to timing of muscle tissue sampling, low sample size and high inter-individual variation.

In this study we demonstrated that the physiological adaptive response to endurance training was accompanied by significant changes in the skeletal muscle transcriptome. Gene set enrichment analysis showed that endurance training caused significant changes in gene expression transcripts involved in extracellular matrix and oxidative phosphorylation, which is in line with previous reports that investigated changes in skeletal muscle transcriptome following prolonged endurance training (10, 11). There is currently an incomplete understanding of the molecular mechanisms that regulate physiological adaptation to endurance training. Several upregulated genes among the top 20 genes are involved in extracellular matrix organization, including COL4A2, COL4A1, LAMA41, LAMB1 and A2M . The results of gene-ontology biological processes and gene set enrichment analysis are consistent with the top 20 genes, showing increased extracellular matrix remodelling. The observed changes in gene expressions transcripts related to extracellular matrix remodelling tended to be more pronounced in the protein group than the control group. The latter suggests that the greater changes in skeletal muscle transcriptome, in particular the extracellular matrix, may reflect the greater physiological adaptations observed in the protein group.

The extracellular matrix is composed of collagen, glycoproteins and proteoglycans (26). Moreover, extracellular matrix remodelling is a primary adaptation to endurance training (4). The extracellular matrix is important for muscle cell development, structure maintenance, force transmission, and tissue remodelling through the modulation of growth factors and extracellular molecule interactions (27). Extracellular matrix degradation is an important morphological adaptation by allowing growth of new capillaries from existing ones in response to endurance training (28-33). Whether the exercise-induced growth of capillaries was further stimulated by protein supplementation and contributed to the larger increase in maximal aerobic capacity cannot be concluded from these data.

Our observation that protein supplementation increases extracellular matrix remodelling to endurance training is new and further elaborates on previous work, which demonstrates that addition of protein to post-exercise carbohydrate-lipid nutrition differentially alters the transcriptome involved in tissue structure and remodelling through regulation of extracellular matrix (12). General skeletal muscle adaptations to exercise training include regulation of angiogenesis, mitochondrial biogenesis, myogenesis and alterations in structural support such as the extracellular matrix (34, 35). There is surprisingly little known about the role of the extracellular matrix in response to endurance training. Our data show that the gene expression transcriptional response to endurance training in skeletal muscle is related to extracellular matrix components and that protein supplementation tended to enlarge this adaptive response. In this study, it could be that the extent in which the extracellular matrix remodelled reflects the degree of muscle growth. Lean mass substantially increased in the protein group and this was accompanied by stronger regulations in gene expression transcripts related to extracellular matrix remodelling. Previous research postulated that remodelling of the extracellular matrix is required for exercise-training induced muscle growth (36).

In contrast to the observed effect of protein supplementation on physiological adaptation, we were unable to find a clear additional effect of protein supplementation on the skeletal muscle transcriptome besides the extracellular matrix. It is possible that the effects of protein supplementation already started to manifest during the early hours of recovery from exercise, when mRNA abundance generally peaks (8, 37). Although the precise mechanisms by which protein supplementation elicited a greater increase in maximal aerobic capacity to endurance training cannot be derived from this analysis, it is likely that protein supplementation enhanced the gene/protein expression changes after each exercise session thereby improving skeletal muscle tissue adaptation, resulting in cumulatively meaningful changes in recovery and phenotypic adaptation over a prolonged period of time.

In conclusion, five weeks of endurance training changed expressions of genes involved in extracellular matrix organization and oxidative phosphorylation. Changes in extracellular matrix organization tended to be greater in the protein group than in the control group and these greater transcriptional changes may reflect the enhanced physiological adaptation as a result of protein supplementation.

Perspectives and significance

Thus far, much attention has been given to the acute molecular responses to a single bout of exercise, and the current theory suggests that acute signals predict/drive phenotypic adaptation over time. For example, the AMP-

activated protein kinase and peroxisome proliferator-activated receptor-γ coactivator-1α, have been proposed as primary regulators of muscle tissue adaptation in response to endurance training (38-41). Our transcriptomic analysis revealed that the extracellular matrix may be an important factor for skeletal muscle adaptation in response to prolonged endurance training. The current data suggest/demonstrate that protein supplementation enhances physiological adaptation and elicit greater changes in skeletal muscle gene expression transcripts related to the extracellular matrix. These changes in extracellular matrix gene expression may reflect modulation of the physiological adaptation to endurance training (4), leading to enhanced cumulative effects with regard to endurance performance and muscle size.

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Chapter 8

General discussion

The aim of this thesis was to study the nutritional impact on physiological and skeletal muscle adaptation to exercise. The first part (chapters 2 – 4) focussed on carbohydrate availability with resistance exercise, whereas the second part (chapters 5 - 7) specifically addresses the effects and potential of protein supplementation with endurance training. As a start, **Chapter 2** reviewed the existing knowledge on glycogen and carbohydrate availability on the adaptive response to endurance and resistance exercise. **Chapter 3** addresses the results of a study on the effects of carbohydrate availability during resistance exercise on selected mRNA profile changes in skeletal muscle. We were able to demonstrate that a selection of muscle mRNAs related to mitochondrial biogenesis, muscle degradation and muscle hypertrophy was minimally affected by different pre-exercise meals that differ in macronutrient profile. **Chapter 4** discusses the relationship between carbohydrate availability and plasma cytokine response with resistance exercise from the same study. Our main conclusion was that pre-exercise meal in general not influenced plasma cytokine responses in the post-resistance exercise period.

Shifting our focus to proteins, we first reviewed effects and possible underlying physiological mechanisms of protein supplementation on the adaptive response to endurance training in **Chapter 5**. To further explore these insights, we performed a double-blind randomised controlled trial with repeated measures to determine whether protein supplementation impacts adaptations to prolonged endurance training. The results of this trial as presented and discussed in **chapter 6** indicated that protein supplementation can be beneficial in improving adaptation to endurance training. Finally in **chapter 7**, we also analysed the skeletal muscle transcriptome from the intervention study aiming to reveal mechanisms underlying the effect of protein supplementation on the adaptive processes to endurance training.

Carbohydrate availability and resistance exercise

Macronutrients represent potent modulators of acute exercise response and chronic adaptation, due to their effects on substrate use during rest and exercise, gene expression and physiological changes (1, 2). Carbohydrates are mainly known for their role as fuel during endurance exercise, as well as potential modulator of the molecular response to endurance training (3, 4). It is well established that moderate to high daily intake of carbohydrates (5 and-12 g/kg bodyweight/day) is beneficial for prolonged high-intensity endurance exercise (5, 6). However, resistance exercise and endurance exercise involve metabolically different processes, while also inducing different adaptive responses at a cellular level (7). The resistance exercise-specific adaptation is primarily induced by mechanical stress (8, 9), whereas the adaptation to endurance exercise training mainly occurs as a result of metabolic stress (10).

Therefore, carbohydrate availability may affect both exercise modalities differently. In spite of this notion, not much research has been done with regard to carbohydrate availability and resistance exercise. In addition, specific carbohydrate intake recommendations for individuals participating in resistance exercise training regimes are scarce (11, 12). Most recommendations are general and usually in a range between 3 and 7 g/kg bodyweight/day (13, 14). However, there is also evidence that lower intakes of carbohydrates do not interfere with resistance exercise performance (15, 16) **(Table 1.1)**.

Table1.1 Overview of studies assessing carbohydrate (CHO) intake and resistance exercise performance (22).

Authors	Participants	CHO intervention	Performance outcomes
Dipla et al.**(17)**	10 active ♀	7 d of 30% CHO 7 d of 55% CHO	↔ Knee flexion (fatigue) ↔ Knee extension (fatigue)
Hatfield et al. **(18)**	8 resistance trained ♂	4 d of 50% CHO 4 d of 80% CHO	↔ Squat jump (peak power)
Leveritt & Abernathy **(19)**	6 active ♂+♀	Glycogen depleted protocol + 2 d of 1.26 g/kg per d of CHO	↓ Squats (failure) ↔ Knee extension (torque)
Mitchell et al.**(15)**	11 trained ♂	Glycogen depleted protocol + 2 d of 0.4 g/kg per d of CHO	↔ Back squats (total work) ↔ Leg press (total work) ↔ Knee extension (total work)
Paoli et al.**(20)**	9 athletes ♂	30 d 4.5% CHO vs. 30 d 46.8% CHO	↔ Squat jump (height) ↔ CMJ (height) ↔ Reverse grip chin up (failure) ↔ Push ups (failure) ↔ Dips (failure)
Sawyer et al. **(16)**	31 resistance trained ♂+♀	7 d of 40.7% CHO, vs. 7 d of 5.4% CHO	↑ Back squat (1-RM) ↔ Bench press (1-RM) ↔ Upper body (max power) ↑ CMJ (height) ↔ Wingate peak power ↑ Handgrip strength
Van Zant et al. **(21)**	6 aerobic trained ♂ 6 resistance trained ♂ 6 sedentary ♂	3 weeks of 42% CHO vs. 62% of CHO	↔ Knee extension (total work) ↔ Bench press (1-RM)

In **chapters 3** and **4** we have shown that carbohydrate availability during a single bout of resistance exercise has minimal impact on skeletal muscle gene expression and plasma cytokine response in the post-exercise period. Moreover, there were only mild but non-significant differences between pre-exercise fat or carbohydrate feeding on post-resistance exercise muscle glycogen levels, suggesting a relatively small pre-exercise meal effect in terms of macronutrient composition on muscle glycogen resynthesis. In theory, it could have been expected that low carbohydrate availability during resistance exercise would be associated with lower muscle glycogen levels, thereby inducing AMPK activity, which in turn regulates several processes involved in adaptation. Interestingly, our findings are in line with several other studies also reporting that the acute adaptive response to resistance exercise remains

unaffected by differences in carbohydrate availability (22-25), including translation initiation and the myofibrillar protein synthetic response (22, 23) **(Table 1.2**).

Table 1.2 A summary of studies assessing carbohydrate (CHO) intake and acute resistance exercise muscular response (22).

Authors	Participants	CHO intervention	Resistance exercise	Pre -exercise glycogen concentrations	Post-exercise cellular response
Camera et al. **(22)**	16 active ♂	1 g/kg CHO evening meal following glycogen depletion protocol	8 x 5 unilateral leg press 80% 1-RM (fasted)	184 mmol/kg DM Vs. 382 mmol/kg DM	↔Myofibrillar protein synthesis 1h and 4h
Churchley et al. **(24)**	7 resistance trained ♂	1 g/kg CHO evening meal following glycogen depletion protocol	8 x 5 unilateral leg press 80% 1-RM (fasted)	193 mmol/kg DM Vs. 435 mmol/kg DM	↔mRNA responses at 3h
Creer et al. **(23)**	7 cyclists ♂	2% CHO vs. 80% CHO	3 x 10 bilateral knee extension 70% 1-RM (fasted)	174 mmol/kg DM Vs. 591 mmol/kg DM	↔Protein signalling
Knuiman et al. **(26, 27)**	14 active ♂	Glycogen depletion protocol followed by 1.5 g/kg of CHO vs. 0.3 g/kg of CHO before resistance exercise	8 x 5 bilateral leg press and extension 80% 1-RM **(fed state)**	380 mmol/kg DM Vs. 441 mmol/kg DM	↔mRNA responses at 1h and 3h ↔plasma cytokines

At the transcriptional level we found that PDK4 was the only gene in muscle tissue that was differently expressed between the carbohydrate and fat condition. An increase in PDK4 mRNA would be in line with a shift towards fat oxidation and reduced glucose oxidation, hence simply reflecting a shift in substrate use due to substrate availability. In addition, plasma insulin was higher in the carbohydrate condition. However, an increase in plasma insulin elicited by carbohydrates does not further stimulate the muscle protein synthetic response (28), and thus does not affect muscle anabolism negatively *per se*. Additionally, the increased insulin response in the carbohydrate condition is not necessarily required to stimulate or augment the muscle protein synthetic response when amino acid availability is sufficient (e.g. reduce protein breakdown) (28-30), which was the case in our study. From the plasma cytokine analysis (**chapter 4**), IL-6 was the only cytokine that responded mildly different to differences in carbohydrate availability. This finding was not surprising since IL-6 has been found to be sensitive to changes in blood glucose levels (31), but also to pre-exercise muscle glycogen content (32).

Findings of **chapter 3** and **4** contribute to the view that carbohydrate availability during resistance exercise is of minor importance when aiming for

an anabolic skeletal muscle adaptive response. At present it might be premature to change carbohydrate recommendations for individuals performing resistance exercise. However, data from our study do call into question the importance of carbohydrates as both substrate for resistance exercise training and as modulator of the skeletal muscle response that underlies adaptation.

Protein supplementation and endurance training

In **chapter 6** we were able to demonstrate a proof-of-concept that protein supplementation elicits greater increases in VO_{2max} and stimulated lean mass gain in response to prolonged endurance training. To our knowledge, this was the first double-blind randomised controlled trial with repeated measures showing that protein supplementation enhances the adaptive response to endurance training. These remarkable effects of protein on VO_{2max} that were observed give rise to questions regarding their underlying mechanisms. Interestingly, the greater increase in VO_{2max} elicited by protein supplementation the first 5 weeks was accompanied by a substantial gain in lean mass. Based on this finding we questioned to what extent the lean mass gain contributed to the greater increase in VO_{2max}. To this end, we analysed the associations between these two variables. Overall, lean body mass and leg lean mass were well associated with absolute VO_{2max} (**Figure 1.1**), but the strongest correlation was found between leg lean mass and VO_{2max} at week 5 (r^2=0.59; p<0.0001). Changes in lean body mass and leg lean mass correlated mildly but significant with changes in absolute VO_{2max} after 5 weeks of training (**Figure 1.2**).

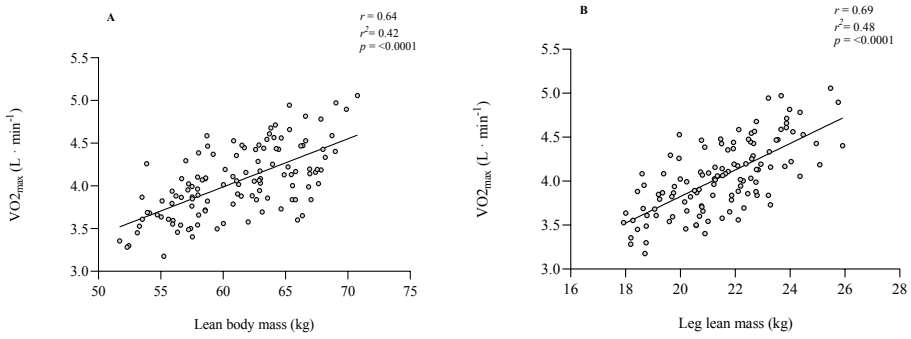

Figure 1.1 Scatter plot of the correlation between absolute VO_{2max} ($L \cdot min^{-1}$) and (**a**) lean body mass (kg) or (**b**) leg lean mass (kg). Merged data (control and protein supplemented group) from Pre (0 weeks), Mid (5 weeks) and End (10 weeks), (n = 120).

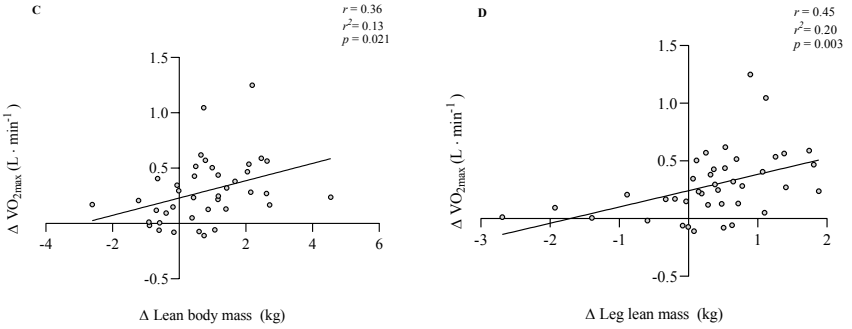

Figure 1.2 Scatter plot of the correlation between change in absolute VO_{2max} ($L \cdot min^{-1}$) and (**c**) change lean body mass (kg) or (**d**) change in leg lean mass (kg) in the protein supplemented group (n = 19) following 5 weeks of endurance training.

Thus, the correlation found between (leg) lean mass and VO_{2max} is interesting and based on this finding we decided to focus on the mechanisms by which protein supplementation enhanced the skeletal muscle adaptive response to endurance training.

Potential mechanisms underlying

Throughout the years, numerous studies have investigated the mechanisms by which endurance training increases VO_{2max} (33-41). Endurance training-associated increases in VO_{2max} are considered to result from central and peripheral adaptations (42, 43). Central adaptations include cardiac hypertrophy, increased plasma and red blood cell volume (44, 45), whereas peripheral adaptations include vascular resistance (46, 47), as well as skeletal muscle adaptations such as capillarisation (48), and increased mitochondrial content and function (41, 49). Previous research postulated that chronic endurance training alters the skeletal muscle transcriptome by down- and upregulating numerous pathways involved in adaptation (50, 51). Transcriptome analysis has previously only been used to a limited extent in the study of exercise and nutrition, notwithstanding its substantial impact for nutrition science in general. Most studies examining skeletal muscle phenotypic regulation at the mRNA level are hypothesis-driven in nature, and suppose predetermined single genes/myocellular pathways, trying to connect specific regulatory pathways to specific types of skeletal muscle adaptation responses (52-59). Consequently, such studies are unlikely to reveal yet unknown molecular effects and pathways such as redundant and oppositely acting mechanisms. Thus, skeletal muscle transcriptome analysis holds many

promises to the field by unravelling mechanisms underlying phenotypic adaptations to exercise training and the subtle effects of nutrition.

In **chapter 7** we aimed to unravel mechanisms by which protein supplementation elicited a greater increase in VO_{2max}. To this end, we analysed the muscle transcriptome to gain insight into changes in the steady-state gene expression. Although the endurance training clearly induced changes in the skeletal muscle transcriptome, its quantitative effects in terms of signal changes were relatively small. These relatively small changes can be explained by the time the muscle tissues were taken in our study (6-8 days after the last exercise session). Generally, greatest increases in mRNA expression are observed at 3-12 hr after ending exercise and return to baseline levels within 24 hr (7, 60). Moreover, long-term training adaptation is the result of the expression of a selection of genes after each acute exercise bout that subsequently increases the type and amount of proteins that drive the phenotypic alteration. As protein half-lives are usually much longer than those of mRNA, changes in protein content are more readily observed than changes in transcript expression in response to training as opposed to acute exercise (7) (**figure 2**).

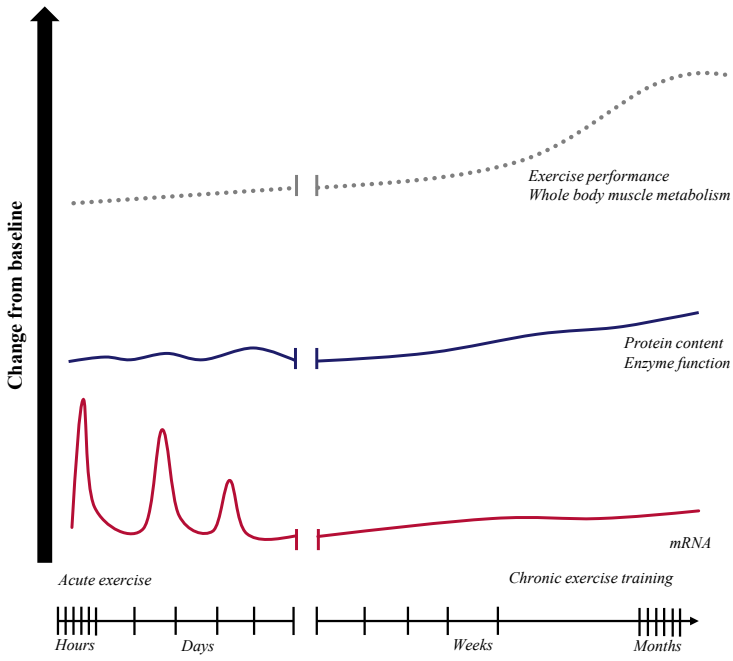

Figure 2 General model based on the signal transduction hypothesis for adaptation to exercise training (2), highlighting the importance of timing for sampling. Model adapted from Egan and Zierath (2013) (7).

Using analysis of skeletal muscle transcriptome we were not able to bring out clear mechanisms by which protein supplementation enhanced the skeletal muscle adaptive response in response to prolonged endurance training. Yet, we did find that protein supplementation induced greater changes in gene expression transcripts related to the extracellular matrix organisation. The extracellular matrix is composed by a variety of proteins including elastin, collagens, glycoproteins and matricellular proteins (61). In addition to its role in physical and mechanical properties of tissue, it appears that extracellular matrix remodelling is a vital adaptation in providing protection against exercise-induced skeletal muscle injury (62). Previous research has demonstrated that modulation of extracellular matrix genes reflects the magnitude of physiological adaptation to prolonged endurance training in humans (63). Our findings complement previous findings that the extent to which the extracellular matrix adapts reflects to a certain extent the degree of physiological adaptations. Additionally, we were also able to demonstrate a subtle effect of protein supplementation on top of the endurance training effect at the skeletal muscle transcriptional level. Thus, in **chapter 7** we demonstrated that prolonged endurance training changed expression of genes involved in extracellular matrix organisation, energy metabolism and oxidative phosphorylation. Changes in extracellular matrix organization tended to be greater in the protein group than in the control group and these greater transcriptional changes may reflect the enhanced physiological adaptation as a result of protein supplementation. Future research is needed to identify the specific role of the extracellular matrix within physiological adaptation to endurance training.

Future perspectives

Findings of **chapter 6 and 7** could also have potential for ageing and diseased populations. An increase in VO_{2max} has been well associated with an improved endurance exercise performance (70). From a clinical perspective, an increase in VO_{2max} is independently associated with a reduction in all-cause morbidity and mortality, which emphasizes the important clinical benefits of endurance training (71). During aging there is a linear decline in VO_{2max} (**Figure 3**) (72). Lower levels of VO_{2max} contribute to loss of independence, increased incidence of disability, and reduced quality of life in older people. Endurance training is a well-established strategy to improve VO_{2max} and offers a wide range of health benefits to older adults. Whether an increased intake of dietary protein enhances cardiovascular and skeletal muscle adaptive response to endurance training in the elderly population presents an interesting question for future research.

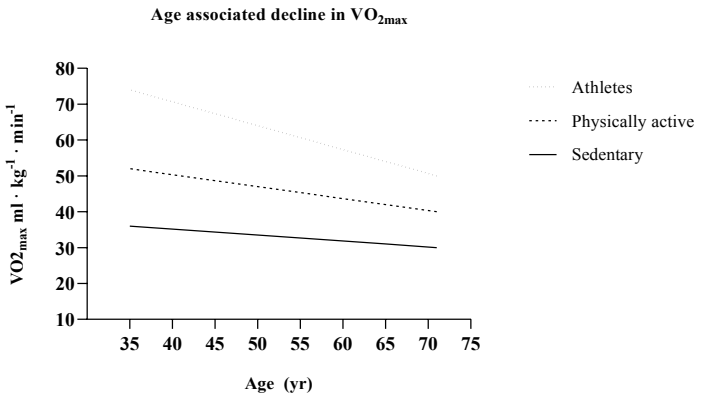

Figure 3 Simplistic model showing the age associated decline in VO_{2max}, adapted from Strasser and Burtscher (2018) (73).

Prerehabilitation

Findings of **chapter 6 and 7** may also have potential as part of a prerehabilitation intervention. Prerehabilitation is an intervention to enhance functional capacity in anticipation of a forthcoming physiological stressor, such as surgery (74). Evidence indicates that preoperative exercise training can improve postoperative outcome in patients undergoing major elective major surgery. Preoperative endurance training increases VO_{2max}, and a higher preoperative VO_{2max} has been shown to be effective in reducing the incidence of postoperative complications, decreasing the length of hospital stay (e.g. days of intensive care unit stay), disease and death after major abdominal, cardiac and vascular surgery (74, 75). Additionally, high-intensity endurance training appears feasible and safe in elderly and/or multimorbid candidates undergoing major surgery (75). Thus, our findings highlight the potential clinical importance of dietary protein intake with endurance training and may have considerable potential as part of a prerehabilitation program for patients undergoing major elective surgery.

Perspectives mechanisms of adaptation

Thus far, much attention has been given to the acute molecular responses to a single bout of exercise, and the current theory suggests that acute signals predict/drive phenotypic adaptation over time. For example, the AMP-activated protein kinase and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1 alpha), has been proposed as primary regulators of muscle tissue adaptation in response to endurance training (64-67). Findings of **chapter 7** demonstrate that the extracellular matrix may be an important

factor for skeletal muscle adaptation in response to prolonged endurance training. Our findings suggest that protein supplementation enhances physiological adaptation and elicit greater changes in skeletal muscle gene expression transcripts related to the extracellular matrix. These changes in extracellular matrix gene expression may reflect modulation of the physiological adaptation to endurance training (69), leading to enhanced cumulative effects with regard to endurance performance and muscle size. More experimental work is warranted to further scrutinize this topic.

Final conclusions

In this thesis we have studied the role of nutrition on the skeletal muscle adaptive response with exercise. We conclude that the role of carbohydrate availability on the adaptive response with resistance exercise requires more scientific attention (**chapter 2**). Taking **chapter 3** and **4** together, we conclude that carbohydrate availability has only a minor effect on the acute adaptive response to resistance exercise. This conclusion is based on an experimental study where we assessed skeletal muscle gene expression (**chapter 3**) and plasma cytokine responses (**chapter 4**). In this study we have shown that a pre-exercise meal low in carbohydrates did not impair acute gene expression profiles or plasma cytokine responses during the post-resistance exercise period. Next, we hypothesized how protein supplementation may affect the adaptive response to prolonged endurance training from a physiological perspective, and conclude that randomised controlled trials are needed to test this hypothesis (**chapter 5**). Taking **chapter 6** and **7** together, we conclude that dietary protein facilitates the adaptive response to endurance training. We were able to show that protein supplementation further enhances VO_{2max}, and stimulates lean mass gain (**chapter 6**). However, the improved physiological adaptation as a result of protein supplementation was partly explained by changes in the steady-state skeletal muscle transcriptome (**chapter 7**).

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Chapter 9

Summary
Acknowledgements
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Training activities

Summary

Skeletal muscle responds to exercise by a diversity of processes that collectively contribute to short-term and structural adaptations to the demanded performance capacities. There is common consensus that, in general, adequate nutrient availability during and after exercise is important to maximise skeletal muscle adaptation and ultimately performance. At the same time, several knowledge gaps remain regarding the precise mechanisms underlying these effects on adaptation, the most optimal nutrient composition in relation to type of exercise, optimal timing etc.

This dissertation addresses some of these unsolved issues by studying the role of carbohydrates and proteins during adaptation following different forms of exercise. The first part (chapters 2 – 4) focusses on carbohydrate availability with resistance exercise, whereas the second part (chapters 5 - 7) specifically addresses the effects and potential of protein supplementation with endurance training. In **chapter 2** we reviewed the existing literature regarding the role of skeletal muscle glycogen with endurance and resistance exercise. Based on this review we concluded that the role of muscle glycogen levels and/or carbohydrate availability on the skeletal muscle adaptive response to resistance exercise requires further scientific attention. To experimentally explore this, we assessed the impact of a pre-exercise meal that differed in macronutrient content on skeletal muscle glycogen levels and acute transcriptional level analysing specific mRNAs in the post-resistance exercise period in **chapter 3**. Specifically, after a glycogen depleting endurance exercise session in the morning, subjects received an isocaloric mixed meal containing different amounts of carbohydrates and fat 2 hours before a resistance exercise session in the afternoon, while ample protein was provided throughout the day. We hypothesized that some of the selected mRNAs associated with substrate metabolism and mitochondrial biogenesis would differ between the nutritional conditions, without any changes in proteolytic genes. The findings described in chapter 3 demonstrated that muscle mRNA responses related to exercise adaptation were minimally affected by the pre-exercise meals that differed in macronutrient composition. In **chapter 4,** derived from the same study, we describe the analysis of a number of plasma cytokine patterns during the day to investigate whether these mediators were affected by carbohydrate availability. We hypothesized that some selected cytokines would differ between nutritional conditions, whereas other circulating cytokines suggested to be involved in skeletal muscle adaptation would not respond differently. Our main finding was that a pre-exercise meal in general did not influence plasma cytokine responses in the post-resistance exercise period. Findings of **chapter 3** and **4** contribute to the view that carbohydrate availability during resistance exercise is of minor importance

when aiming for an acute positive skeletal muscle adaptive response. In addition, our data question the importance of carbohydrates as both substrate for resistance exercise and as modulator of the skeletal muscle response that underlies adaptation. Yet, at present it might be premature to change carbohydrate recommendations for individuals performing resistance exercise. Shifting our focus to proteins, we first reviewed the effects and possible underlying physiological mechanisms of protein supplementation on the adaptive response to endurance training in **Chapter 5**. To further explore these insights, we performed a double-blind randomised controlled trial with repeated measures to determine whether protein supplementation impacts the adaptive response to endurance training. In **chapter 6** we provide proof-of-concept that protein supplementation elicited greater increases in VO_{2max} and stimulated lean mass gain in response to prolonged endurance training. To our knowledge, this was the first double-blind randomised controlled trial with repeated measures showing that protein supplementation enhances the adaptive response to endurance training. These remarkable effects of protein on VO_{2max} that were observed give rise to questions regarding their underlying mechanisms. To this end, we analysed the muscle transcriptome to gain insight into changes in the steady-state gene expression. In **chapter 7,** we demonstrated that prolonged endurance training changed expression of genes involved in extracellular matrix organisation, energy metabolism and oxidative phosphorylation. Changes in extracellular matrix organisation tended to be greater in the protein group than in the control group and these greater transcriptional changes may reflect the enhanced physiological adaptation as a result of protein supplementation.

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List of publications

Knuiman P, Hangelbroek R, Hopman MTE, Mensink M. Regulation of skeletal muscle transcriptome is increased by protein supplementation and reflects the extent of physiological adaptation to endurance training (*under review*)

Knuiman P, van Loon L, Wouters JA, Hopman MTE, Mensink M. Protein supplementation elicits greater gains in maximal oxygen uptake capacity and stimulates lean mass accretion during prolonged endurance training in healthy young men. *The American Journal of Clinical Nutrition (2019)*

Knuiman P, Hopman MTE, Verbruggen C, Mensink M. Protein and the Adaptive Response With Endurance Training: Wishful Thinking or a Competitive Edge? *Front Physiol. (2018)*

Knuiman P, Hopman MTE, Hangelbroek R, Mensink M. Plasma cytokine responses to resistance exercise with different nutrient availability on a concurrent exercise day in trained healthy males. *Physiol Rep. (2018)*

Hangelbroek RWJ*, **Knuiman P***, Tieland M, de Groot LCPGM. Attenuated strength gains during prolonged resistance exercise training in older adults with high inflammatory status. *Exp Gerontol. (2018)*

Knuiman P, Hopman MTE, Wouters JA, Mensink M. Select Skeletal Muscle mRNAs Related to Exercise Adaptation Are Minimally Affected by Different Pre-exercise Meals that Differ in Macronutrient Profile. *Front Physiol. (2018)*

Knuiman P, Hopman MT, Mensink M. Glycogen availability and skeletal muscle adaptations with endurance and resistance exercise. *Nutr Metab (Lond). (2015)*

Knuiman P, Kramer IF. Contributions to the understanding of the anabolic properties of different dietary proteins. *J Physiol. (2012)*

*equal contribution

About the author

Pim Knuiman was born on March 27, 1984 in Wageningen, the Netherlands. He started his academic career at HAN University of Applied Sciences where he received his bachelor's degree (*cum laude*) in Exercise and Health in 2010. During his bachelor he performed an internship at the Netherlands Institute for Sports and Physical Activity, where he studied the impact of playgrounds on physical activity behaviour in young children. He then performed his thesis at HAN University lectorate of Sport & Nutrition where he determined the impact of different exercise modalities on lactate metabolism. His thesis was awarded for best thesis of HAN University of Applied Sciences. He continued with the master program Human Movement Sciences with the specialisation Biology of Human Performance and Health at Maastricht University where he received his master's degree in 2012. During this master he performed an internship at the Muscle Metabolism research group at Maastricht University, assessing the impact of protein intake on immobilization induced skeletal muscle loss. Also, during this internship he contributed two several other human metabolic trials and wrote his first peer-reviewed article. Following completion of his master's degree, he worked three years as a lecturer in exercise physiology and nutrition & metabolism at two different universities. In March 2015, Pim started his PhD trajectory entitled "Nutritional Impact on Molecular and Physiological Adaptations to Exercise" under the supervision of Dr Marco Mensink and Professor Maria Hopman within the Division of Human Nutrition at Wageningen University & Research. Pim completed his PhD in October 2019. From September 2019 Pim is appointed as postdoctoral research fellow at the University of Leeds in England where he will work with Dr. Carrie Ferguson. During this project Pim will focus on the physiological mechanisms of exercise intolerance in patients with chronic heart failure.

Completed training activities

Discipline specific activities

Name	Organiser	Year
Eat2Move meetings	Eat2Move	2015-2019
Invited presentations	Universities	2015-2019
Nutritional science days	NWO	2015-2019
Live session sport & nutrition	Eat2Move	2015
Annual ECSS congress	ECSS	2015
NZO symposia	Dairy association	2016
Annual BASES conference	BASES	2016
Annual ECSS congress	ECSS	2018
Annual BASES conference	BASES	2019

General courses

Name	Organiser	Year
PhD week	VLAG	2015
Nutriscience week	VLAG	2015
Scientific writing	VLAG	2016
Essentials of scientific writing	VLAG	2017
Writing grant proposals	VLAG	2018

Optional

Name	Organiser	Year
Adjusting PhD proposal	WUR	2015
Research group meetings	WUR	2015-2019
Muscle meetings	WUR	2015-2019

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