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The effect of protein ingestion immediately following
exhaustive endurance exercise on muscle glycogen
resynthesis and performance

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Summary

Introduction: Endurance athletes often train two times at the same day. Energy intake during recovery may influence glycogen resynthesis and subsequent performance. The aim of the present study was to compare glycogen synthesis and performance 5 hours (h) after a bout of exhaustive exercise when carbohydrate (CHO) or an isocaloric amount of carbohydrate + protein (CHO+Pro) was ingested during the first 2 h after exercise.

Methods: The study was performed with a double-blinded crossover design. Nine male subjects completed two dietary intervention days consisting of an initial glycogen depleting exercise to exhaustion at a workload corresponding to 70 % $\text{VO}_{2\text{peak}}$, followed by a performance test after 5 h of recovery. The first 2 h after exhaustion, either CHO ($1.2 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) or CHO+Pro (0.8 g CHO and $0.4 \text{ g Pro} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was consumed in a randomized order. Energy intake was standardised and similar between CHO and CHO+Pro the remaining 3 h. Performance was tested by time to exhaustion (TtE) at the same workload as during the initial glycogen depleting exercise. Biopsies were harvested from vastus lateralis after the initial glycogen depleting exercise, the 5 h recovery period and after TtE. Performance was only assessed with five subjects.

Results: Glycogen resynthesis during recovery was similar between CHO ($41.3 \pm 5.8 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$) and CHO+Pro ($46.8 \pm 23.9 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$). After the performance test, muscle glycogen was equally reduced in CHO and CHO+Pro. Nitrogen balance based on urine was positive in CHO+Pro ($19.6 \pm 7.6 \text{ mg N} \cdot \text{kg}^{-1}$, $p < 0.05$) and higher than CHO ($-10.7 \pm 6.3 \text{ mg N} \cdot \text{kg}^{-1}$, $p < 0.05$). TtE was only assessed with five subjects, and was improved by CHO+Pro ($54.6 \pm 11.0 \text{ minutes (min)}$) compared to CHO ($46.1 \pm 9.8 \text{ min}$) ($p < 0.01$).

Conclusion: CHO+Pro, compared to the isocaloric CHO diet, improved performance after 5 h recovery from an exhaustive exercise. The study shows that CHO+Pro intake immediately after exercise accelerates recovery of endurance capacity compared to CHO. There was no difference in rate of glycogen resynthesis, and we suggest that there are other mechanisms explaining the enhanced performance after intake of CHO+Pro compared to CHO. Increased protein synthesis is a possibility, as nitrogen balance was positive during CHO+Pro.

Key words: Muscle glycogen synthesis, carbohydrate, protein, nitrogen balance, endurance capacity

Preface

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1. Introduction

Endurance athletes often train or compete two times at the same day. Intensity during training and competition is usually very high, often pushing the metabolic processes in the muscle cell to the limit. Muscle glycogen is important fuel during prolonged activity with high intensity (Abbiss & Laursen, 2005), and low muscle glycogen content may cause exhaustion (Hermansen, Hultman, & Saltin, 1967). In a rested state, muscle glycogen concentration is approximately 320-600 mmol·kilogram (kg) dry weight (dw)⁻¹ (Jensen, Rustad, Kolnes, & Lai, 2011), and may be reduced to 14 mmol·kg dw⁻¹ after exhaustive exercise (Hermansen et al., 1967). By increasing muscle glycogen prior to training, subjects are able to sustain for a longer time period at high intensities (Bergström, Hermansen, Hultman, & Saltin, 1967). Muscle glycogen may thus, at least under some conditions, determine performance.

The diet during the period following exercise is of importance for effective recovery, and energy intake is central for the athlete who wants to perform at the best. Optimal glycogen synthesis rate is achieved at a carbohydrate (CHO) ingestion rate of 1.2 g CHO·kg⁻¹·hour (h)⁻¹ during the first 2-4 h of recovery (Jentjens & Jeukendrup, 2003; Betts & Williams, 2010), and at least 8-9 g CHO·kg⁻¹ during 24 h. Some have reported improved rate of glycogen resynthesis after ingesting proteins in combination with carbohydrates (CHO+Pro) compared to CHO (Zawadzki, Yaspelkis, & Ivy, 1992; Ivy et al., 2002). CHO ingestion stimulates insulin secretion, as does some amino acids, and an additive effect of CHO and protein has been shown on insulin secretion (Zawadzki et al., 1992; Jentjens & Jeukendrup, 2003). Insulin stimulates glucose transport to the muscles and activates glycogen synthase (Jensen et al., 2011).

Protein synthesis is influenced by the diet after exercise (Børsheim, Aarsland, & Wolfe, 2004b; Howarth, Moreau, Phillips, & Gibala, 2009). Energy intake after prolonged exercise may slow muscle protein degradation (Børsheim et al., 2004a), but protein intake is essential to increase muscle protein synthesis (Howarth et al., 2009). One mechanism behind the reduced muscle protein degradation following CHO intake is insulin secretion, as insulin reduces muscle protein degradation (Biolo, Williams, Fleming, & Wolfe, 1999). Protein degradation is counterbalanced by protein synthesis,

and protein intake *per se* activates the protein synthesis via the mTOR signalling pathway (Blomstrand, Eliasson, Karlsson, & Kohnke, 2006). The protein synthesis is important for recovery after exhaustive cycling, and increased expression of mitochondrial proteins could lead to improved performance and oxidative capacity (Ferguson-Stegall et al., 2011a; Rowlands et al., 2011). Rowlands et al. (2011) found increased expression of metabolic enzymes when CHO+Pro ingestion was compared to an isocaloric CHO diet after an acute training. Ferguson-Stegall et al. (2011a) reported more improved aerobic capacity when CHO+Pro was compared to no energy and isocaloric CHO intake after intermittent exercise five days per week for five weeks. Thus, both an acute and chronic effect of protein ingestion has been reported on recovery.

Several studies have reported improved recovery of performance when a CHO+Pro diet compared to isocaloric CHO diets has been ingested after an initial exercise (Berardi, Noreen, & Lemon, 2008; Ferguson-Stegall et al., 2011b; Thomson, Ali, & Rowlands, 2011). Two previous studies at the Norwegian School of Sport Sciences have also reported this effect of protein intake on recovery of endurance capacity and performance 18 h after exercise (Rustad, 2011; Sollie, 2013). The mechanism for this effect on performance is not known, but enhanced muscle glycogen synthesis rate following intake of CHO+Pro compared to CHO is proposed (Ivy et al., 2002). However, others have suggested that enhanced fat oxidation during exercise is the reason for enhanced performance after CHO+Pro compared to CHO intake during recovery (Berardi et al., 2008).

Performance during exercise until exhaustion is under some conditions determined by muscle glycogen (Bergström et al., 1967). Though, the mechanism for muscular fatigue is not completely understood. Hermansen et al. (1967) showed an association between low muscle glycogen content and exhaustion, which could be caused by reduced sarcoplasmic reticulum function (Ørtenblad, Nielsen, Saltin, & Holmberg, 2011; Ørtenblad, Westerblad, & Nielsen, 2013). Protein intake during the immediate recovery has in several studies improved recovery of performance (Berardi et al., 2008; Ferguson-Stegall et al., 2011b; Rustad, 2011; Sollie, 2013), and some of these speculate that muscle glycogen is not the mechanism behind the improved performance. Though, to my knowledge only one study measured this specifically (Berardi, Price, Noreen, &

Lemon, 2006). Interestingly, Berardi et al. (2006) reported higher glycogen resynthesis after CHO+Pro intake compared to CHO, and no effect on performance. It therefore remains unclear if improved recovery of performance after protein intake is related to glycogen resynthesis.

1.1 Hypotheses

The purpose of this study was to compare performance and glycogen synthesis 5 h after an exhaustive exercise when CHO+Pro or an isocaloric CHO diet was ingested the first 2 h after exercise. We tested the following hypotheses:

1. Recovery of time until exhaustion 5 h after a glycogen depleting exercise is improved following CHO+Pro ingestion compared to CHO.
2. Muscle glycogen resynthesis during 5 h recovery after a glycogen depleting exercise is higher when CHO+Pro compared to CHO is ingested the first 2 h.

2. Theory

This chapter will first focus on the carbohydrate metabolism and importance of muscle glycogen as energy substrate during exercise. Then, recovery of muscle glycogen and protein synthesis following exercise after carbohydrate and protein feeding will be presented. Finally, I present studies upon impact of carbohydrate and protein ingestion on recovery of endurance performance.

2.1 Carbohydrate metabolism

CHO can be large molecules consisting of many monosaccharide molecules linked together as starch, or smaller molecules with few monosaccharide molecules linked as in sucrose. The energy content in CHO is $4 \text{ kcal} \cdot \text{g}^{-1}$ (Frayn, 2010). When CHO is ingested, it is cleaved to monosaccharides and transported from the intestine to a portal vein via glucose transport proteins (GLUT) (Frayn, 2010; Harvey & Ferrier, 2011). A common form of monosaccharide is glucose. The portal vein transports glucose to the liver, where it either is taken up or it continues into the circulation.

In the human body, glucose is stored as glycogen mainly in the liver and in skeletal muscles. The liver contains about 80-120 g of glycogen in a fed state. Lactate, glycerol and amino acids from venous blood may function as source of glucose in the liver, being substrate for gluconeogenesis and form glucose (van Hall, 2010).

Glycogen concentration¹ in the skeletal muscle is normally between 320-600 $\text{mmol} \cdot \text{kg dw}^{-1}$ in rested, well fed situations (Jensen et al., 2011). In total, this equals approximately 500 g muscle glycogen in a 70 kg person. Muscle glycogen primarily functions as energy supply during work with intensities above 65 % of maximal oxygen consumption ($\text{VO}_{2\text{max}}$) (Romijn et al., 1993; van Loon, Greenhaff, Constantin-Teodosiu, Saris, & Wagenmakers, 2001), lasting 90 minutes (min) at 77 % of $\text{VO}_{2\text{max}}$ (Hermansen et al., 1967). Though, the energy is limited as ten 6'x0 seconds (s) sprint intervals may

¹ Studies reporting muscle glycogen relative to wet weight has been recalculated to dry weight assuming 75 % water in the muscle biopsy, unless otherwise stated.

deplete as much as 50 % of the muscle glycogen (Thomson, Green, & Houston, 1979; Abernethy, Thayer, & Taylor, 1990). Glycogen resynthesis is therefore an important part of the recovery process (Hawley, Burke, Phillips, & Spriet, 2011).

2.1.1 Muscle glycogen synthesis

Muscle glycogen synthesis is the process where glucose is stored as glycogen, beginning with the transport of blood glucose into the muscle cell (Rose & Richter, 2005; Jensen et al., 2011). Transport of glucose from the blood into the muscle cell occurs via the GLUT-4 (Jensen et al., 2011). Unstimulated, GLUT-4 is stored in vesicles inside the muscle fibre. Both muscle contraction and insulin stimulates translocation of GLUT-4 to the plasma membrane (Etgen, Memon, Thompson, & Ivy, 1993). Figure 2.1 shows the mechanism behind insulin-stimulated GLUT-4 translocation and glycogen synthase activation. Insulin activates protein kinase B (PKB) via phosphatidylinositol 3-kinase (PI3K) (Jensen et al., 2011). PKB may in turn phosphorylate the two downstream targets Akt substrate of 160 kDa (AS160) and TBC1D1. Activity of the two latter targets allows GLUT-4 translocation to the muscle cell membrane. Contraction may also cause GLUT-4 translocation (Etgen et al., 1993; Lai, Zarrinpashneh, & Jensen, 2010). The mechanism behind this contraction mediated GLUT-4 translocation is thought to be via AMP-activated protein kinase (AMPK) and Ca^{2+} /Calmodulin dependent protein kinase (CaMK). AMPK and CaMK phosphorylate AS160 and TBC1D1 (Lai et al., 2010; Jensen et al., 2011).

Glucose is rapidly phosphorylated to glucose 6-phosphate (G6P) upon entrance into the muscle cell. This happens with the action of hexokinase and the reaction is irreversible (Rose & Richter, 2005). Inside the muscle cell, glucose may either be stored as muscle glycogen, or be used for glycolysis immediately (Harvey & Ferrier, 2011). When glucose is stored as glycogen, G6P is converted to glucose 1-phosphate (G1P). Then G1P is converted to UDP-glucose. Afterwards, the glucose subunit of UDP-glucose is attached to a glycogen particle. The latter reaction is catalysed by glycogen synthase (GS).

GS is a rate-limiting enzyme in the glycogen synthesis, as shown by studies inhibiting or overexpressing the protein (Manchester, Skurat, Roach, Hauschka, & Lawrence, 1996; Pederson, Wilson, & Roach, 2004). In general, GS activity is reduced when

phosphorylated (Lawrence & Roach, 1997). The enzyme activity is regulated by phosphorylation at nine different phosphorylation sites (Lawrence, Skurat, Roach, Azpiazu, & Manchester, 1997; Lai et al., 2010). Lai et al. (2010) reported a good correlation between phosphorylation at GS^{ser641} with GS activity (R=-0.94), while phosphorylation at GS^{ser7} only showed a good correlation when combined with phosphorylation at GS^{ser10} (GS^{ser7,10}, R=-0.89). This shows that some sites are potent to individually regulate GS activity, and that different combinations of phosphorylation regulate GS activity.

Insulin and exercise may activate GS (Cohen, 1999; Lai et al., 2010; Jensen et al., 2011). Insulin activates GS via PI3K and PKB inhibition of glycogen synthase kinase 3 (GSK3) (fig. 2.1) (Cohen, 1999; Jensen et al., 2011). In its active form GSK3 phosphorylates and inhibit GS. GS is also activated by contraction. This was shown in a study by Lai et al. (2010), where rat muscles was electrically stimulated with or without insulin. They reported active GS when muscles were electrically stimulated without insulin. The study also reported an additive effect of contraction and insulin on GS activity when muscle glycogen content was low. The mechanism behind the contraction stimulated GS activation is unclear, however, Lai et al. (2010) found reduced GS^{ser7,10} phosphorylation by contraction, but not insulin stimulation.

Muscle glycogen concentration may also regulate GS activity (Danforth, 1965; Jensen et al., 2006; Lai et al., 2010). Jensen et al. (2006) manipulated muscle glycogen in rats to obtain high, normal or low muscle glycogen. They showed that muscle glycogen resynthesis and GS fractional activity was higher when muscle glycogen was low compared to both normal and high glycogen concentration. This has later been supported by Lai et al. (2010).

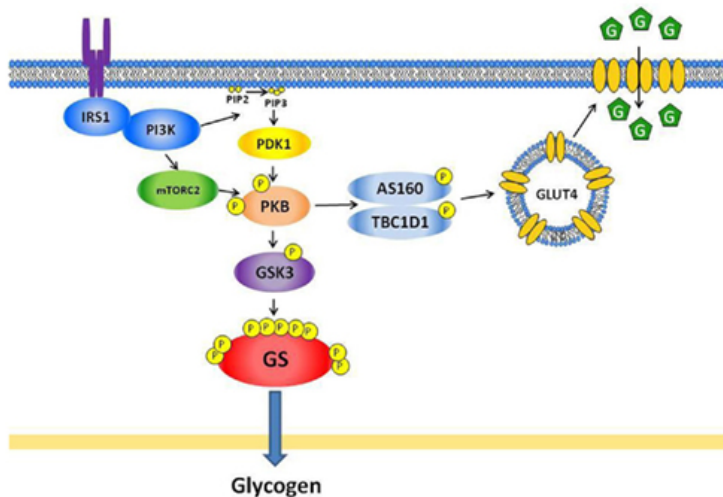


Figure 2.1: Insulin signalling pathway leading to GLUT-4 translocation and activation of GS in skeletal muscle. From Jensen et al. (2011).

2.1.2 Glycogen synthesis following exercise

The glycogen synthesis following a glycogen depleting exercise follows two phases. First, an initial rapid insulin independent glycogen synthesis phase. Thereafter, an insulin dependent glycogen synthesis phase (Price et al., 1994; Jentjens & Jeukendrup, 2003; Millard-Stafford, Childers, Conger, Kampfer, & Rahnert, 2008; Betts & Williams, 2010). A study by Maehlum, Hostmark, and Hermansen (1977) was the first to demonstrate this. In this study, type 1 diabetic and non-diabetic subjects ingested a CHO rich diet following an intense exercise. Glycogen resynthesis rate was $25.6 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ in the diabetic subjects taking their normal insulin doses, and $28.8 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ in the non-diabetic controls. The study showed that muscle glycogen resynthesis during recovery from exercise continues at the same rate between diabetic patients taking their regular medicine and non-diabetic controls. The year after, the same group investigated the effect of insulin deprivation on muscle glycogen synthesis (Maehlum, Hostmark, & Hermansen, 1978). In this study, type 1 diabetic performed the same exercise and ingested the same diet as in the previous study of Maehlum et al. (1977), but no insulin was taken after exercise. Muscle glycogen resynthesis was $18.4 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ the first 4 h of recovery. Thus, muscle glycogen resynthesis was active even in the absence of insulin. Supporting this is a study by Price et al. (1994), who compared glycogen synthesis after exercise in normal or decreased insulin conditions. The first hour after exercise, glycogen resynthesis was similar. After this, muscle glycogen content continued to increase in the normal insulin condition. In the

reduced insulin condition, muscle glycogen content stopped to increase after 60 min. The mechanism behind the insulin independent period is the contraction induced GLUT-4 translocation and GS activity (Lai et al., 2010).

2.2 Energy utilization during exercise

CHO and fatty acids (FA) are the most common sources for energy (McArdle, Katch, & Katch, 2014). The relative contribution between them is dependent on intensity of the work (Romijn et al., 1993; van Loon et al., 2001), training level of the subjects (Hickner et al., 1997) and substrate availability (Coyle, Coggan, Hemmert, & Ivy, 1986), and may be calculated based on a respiratory exchange ratio (RER) (Peronnet & Massicotte, 1991). When CHO is fully combusted, six oxygen (O_2) molecules are used and six carbon dioxide (CO_2) molecules produced. Thus, the ratio between O_2 and CO_2 (CO_2/O_2) gives an RER of 1.00. When FA is oxidized, this ratio is 0.70. An RER between 1.00 and 0.70 reflects that both fat and CHO contribute in the energy release (Peronnet & Massicotte, 1991). This ratio is based on the assumption that protein oxidation is small. Protein oxidation may be calculated afterwards from nitrogen excretion, and its contribution has been reported between 1-10 % during endurance exercise (Tarnopolsky, 2004).

The workload during exercise predicts the energy contribution from oxidation of FA and CHO. Romijn et al. (1993) showed that RER increased from 0.73 ± 0.01 to 0.83 ± 0.02 and 0.91 ± 0.01 when biking at 25, 65 and 85 % of VO_{2max} . The higher RER at 65 and 85 % compared to 25 % of VO_{2max} was caused by enhanced glucose uptake and whole body glucose oxidation (fig. 2.2). Figure 2.2 shows that muscle glycogen contribution was absent at 25 % of VO_{2max} . Then, at 65 % of VO_{2max} , muscle glycogen contributed 20 % of the total energy expenditure. The contribution increased to 59 % of the total energy expenditure when biking at 85 % of VO_{2max} . The findings of Romijn et al. (1993) have later been supported by van Loon et al. (2001).

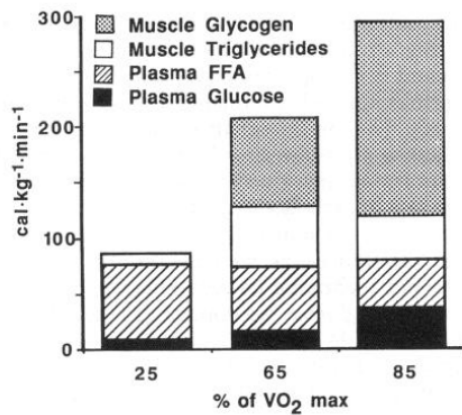


Figure 2.2: Energy substrate utilization while biking 30 min at 25, 65 and 85 % of VO_{2max} . From Romijn et al. (1993).

2.3 Exercise induced fatigue

Fatigue may appear at different levels during exercise (fig.2.3). Figure 2.3 illustrates potential origins where fatigue may occur, which could be separated in groups of peripheral and central fatigue. Peripheral fatigue is defined as reduction of neural transmission or a reduction in the muscles response to a nerve signal (Gandevia, 2001). Central fatigue is defined as a reduction in nervous signals from the central nervous system (CNS) to activate the motor unit. Central fatigue is further divided into spinal and supraspinal fatigue (Gandevia, 2001). Supraspinal fatigue is decreased ability to generate outputs from the motor cortex, and spinal fatigue is caused at the motor neuron. The "border" between peripheral and central fatigue is thus at the muscle membrane and signal transmission from the neuromuscular junction to the muscle cell.

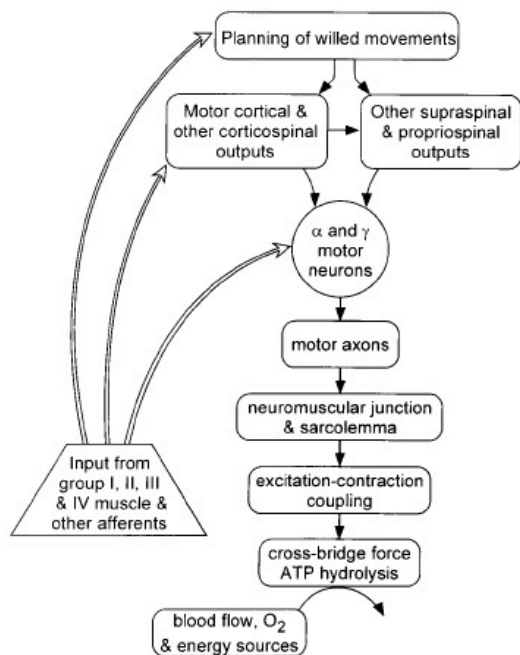


Figure 2.3 Potential origins for fatigue and sensory input. From (Gandevia, 2001).

2.3.1 Peripheral fatigue

Muscle contraction depends on adenosine tri-phosphate (ATP), which only is depleted in *rigor mortis*. The muscle fibres have a protective mechanism that prevents a large decrease in ATP, and peripheral fatigue may therefore be result of other mechanisms than lack of ATP. One potential mechanism is reduced calcium²⁺ (Ca²⁺) release from sarcoplasmic reticulum (SR) (Ørtenblad et al., 2011). Ca²⁺ release the actin complex from a troponin inhibition, allowing the myosin head to interact with the actin complex (Dahl, 2008).

Muscle glycogen may interact with SR, and it has been proposed that muscle glycogen also regulate SR activity (Ørtenblad et al., 2013). Ørtenblad et al. (2011) investigated muscle glycogen content and sarcoplasmic reticulum function, and found reduced SR Ca²⁺ release in muscles containing reduced muscle glycogen. Thus, low muscle glycogen concentrations may reduce Ca²⁺ release and cause peripheral fatigue.

Hermansen et al. (1967), studied untrained (VO_{2max} : $3.4 \text{ l O}_2 \cdot \text{min}^{-1}$) and trained (VO_{2max} : $4.6 \text{ l O}_2 \cdot \text{min}^{-1}$) subjects cycling at 77 % of VO_{2max} until exhaustion. Before

exercise, muscle glycogen concentration was $374.0 \pm 5.9 \text{ mmol} \cdot \text{kg dw}^{-1}$ in the untrained subjects, and $400.0 \pm 7.1 \text{ mmol} \cdot \text{kg dw}^{-1}$ in the trained subjects. Duration during the exhaustive exercise was shorter for the untrained (85 min) compared to the trained subjects (90 min). In both groups, muscle glycogen concentration gradually decreased until exhaustion. Muscle glycogen concentration at exhaustion was very low at $14.4 \pm 2.3 \text{ mmol} \cdot \text{kg dw}^{-1}$ for the untrained, and $28.4 \pm 3.6 \text{ mmol} \cdot \text{kg dw}^{-1}$ for the trained subjects. The study also showed that carbohydrate combustion was related to muscle glycogen utilization (fig 2.4.A), and concluded that glycogen depletion caused exhaustion.

Bergström et al. (1967) studied the role of initial muscle glycogen on performance. In this study, cycling to exhaustion was performed at three occasions. Muscle glycogen concentration before the three occasions was manipulated to obtain normal, low or high glycogen. With normal glycogen concentrations, subjects cycled for $113.6 \pm 5.3 \text{ min}$ before exhaustion. After this exhaustive exercise, subjects ate a high fat diet for three days and the glycogen concentration remained low. At the exercise test with low glycogen concentration, subjects cycled significantly shorter before exhaustion ($56.9 \pm 0.3 \text{ min}$) compared to the day with normal glycogen concentration. After this exercise, subjects ate a high CHO diet and the glycogen concentration increased. When glycogen concentration was high, subjects cycled significantly longer before exhaustion ($166.5 \pm 17.8 \text{ min}$) compared to both normal and low glycogen days. The study found a good correlation between initial muscle glycogen concentration and work time during a time until exhaustion test (fig 2.4.B). They concluded that initial muscle glycogen concentration was a determinant for capacity to perform prolonged heavy exercise.

Large variability in muscle glycogen content after exhaustive exercise protocols is not unusual, and concentrations from 14 to $190 \text{ mmol} \cdot \text{kg dw}^{-1}$ has been reported (Betts & Williams, 2010). In the study of Bergström et al. (1967), muscle glycogen at exhaustion was higher at the high glycogen day compared to low and normal glycogen days. This implicates that other mechanisms than low glycogen concentrations may cause fatigue.

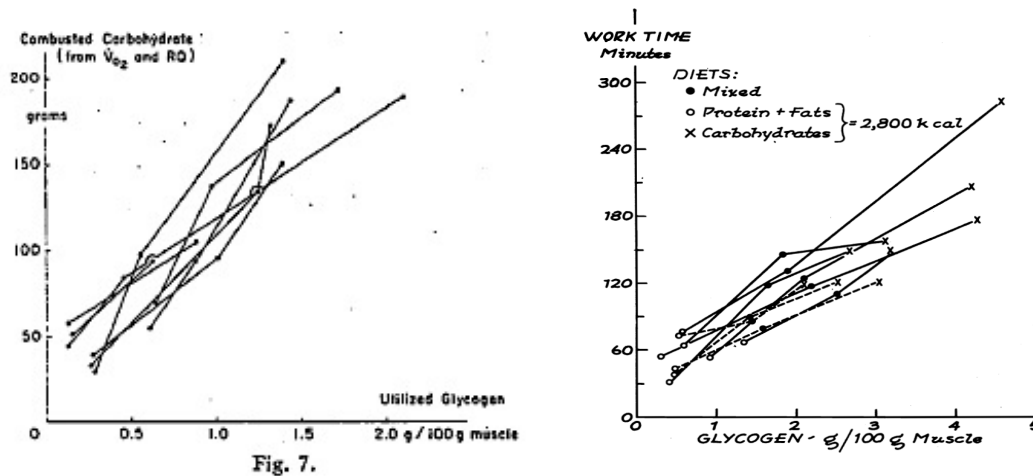


Figure 2.4: *A: The relationship between muscle glycogen utilization and CHO combustion during 60 min of biking at approximately 25, 50 and 75 % of VO_{2max} . Derived from Hermansen et al. (1967). B: The relationship between initial muscle glycogen and duration during a time until exhaustion test. From Bergström et al. (1967).*

2.3.2 Central fatigue

Central fatigue can be defined as a reduction in signalling from the CNS to the motor units (Gandevia, 2001). Many models have been proposed to explain the mechanism behind central fatigue (Abbiss & Laursen, 2005), and one of them is the central governor model of fatigue. The central governor model of fatigue states that performance is not limited by failure of homeostasis in the brain, heart, muscle or other tissues, but a reduced rate of central activation as a result of the anticipated task to be done (Noakes, 2012). The central activity is regulated by feedback mechanisms between the peripheral tissue and the CNS to keep the body in homeostasis. According to this model, exercise begins with a feed forward activation of the motor units, and self-selected pace is regulated according to afferent information.

The feedback mechanism in the central governor model is not well defined. It could be molecules released by the tissues, signals following depletion of peripheral energy stores such as muscle glycogen, afferent sensory nerve signals, or most likely a combination (Noakes, 2012). In a study by Rauch, St Clair Gibson, Lambert, and Noakes (2005), muscle glycogen was manipulated to obtain normal or high glycogen concentration before a performance test. When the initial muscle glycogen

concentration was high, subjects selected a higher workload compared to the day when muscle glycogen was normal. After exercise, muscle glycogen content was almost equal between trials. It was concluded that the intensity was adjusted according to muscle glycogen, and they suggest that the findings could support the central governor model.

It is well documented that blood glucose fuels brain function (Dahl, 2008). Exercise may reduce blood glucose, resulting in decreased central drive and motivation. In a study by Nybo, Moller, Pedersen, Nielsen, and Secher (2003), subjects performed three hours of cycling at 60% of VO_{2max} while supplemented with CHO or no energy (placebo (PLA)). CHO kept subjects in a euglycaemic state and rate of perceived exertion (RPE) remained stable. When no energy was ingested during exercise, hypoglycaemia caused RPE to increase. The study shows that an increase in RPE could be a result of low blood glucose.

Depletion of muscle glycogen could cause both peripheral and central fatigue. Some argues that impaired SR function is the mechanism behind this. Others argue that feedback mechanism between the muscle and the brain reduces central activation of the motor neurons, thereby causing central fatigue. Hypoglycaemia has also been shown to cause increased RPE. The mechanism behind fatigue during prolonged exercise is not clear, and more research is needed to further elucidate this.

2.4 Carbohydrates and glycogen resynthesis

Resynthesis of muscle glycogen following exercise is an important factor for recovery (Ivy, 2004; Betts & Williams, 2010). Exercise acutely increases insulin sensitivity and glycogen synthesis (Betts & Williams, 2010; Lai et al., 2010; Jensen et al., 2011).

Therefore, amount, type and timing of CHO ingestion are of importance for optimally feeding the glycogen synthesis (Ivy, 2001; Beelen, Burke, Gibala, & van Loon, 2010; Betts & Williams, 2010).

2.4.1 Amount of CHO ingestion

The amount of CHO needed to saturate muscle glycogen resynthesis is of importance when designing an optimal recovery diet. Betts & Williams (2010) reviewed the literature on reported rates of glycogen resynthesis caused by different amounts of CHO ingestion after exercise. They concluded that ingestion of $1.0 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ is

sufficient for optimal muscle glycogen synthesis (fig.2.5). Figure 2.5 shows large variation in the reported glycogen resynthesis rates. This could be because of the muscle glycogen concentration after exercise (as indicated by the dotted lines in fig 2.5), or training level of the subjects included to the studies. Other reviews have concluded that muscle glycogen resynthesis peaks at $1.2 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (Ivy, 2001; Jentjens & Jeukendrup, 2003; Beelen et al., 2010). Therefore, the recommended ingestion rate of CHO is between $1.0\text{-}1.2 \text{ g} \cdot \text{CHO}^{-1} \cdot \text{h}^{-1}$. During 24 h, athletes are recommended to eat $8\text{-}9 \text{ g CHO} \cdot \text{kg}^{-1}$.

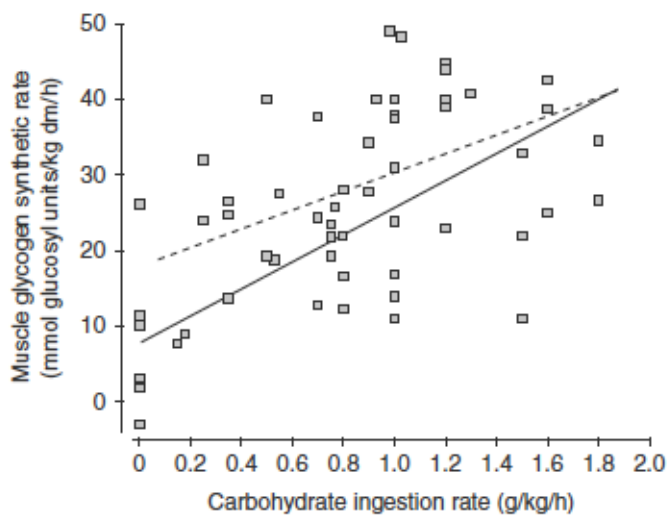


Figure 2.5: Reported values of glycogen synthesis after different rates of CHO ingestion over a 2-6 h recovery period. Solid trend line represent correlation coefficient when muscle glycogen was $>110 \text{ mmol glucosyl units} \cdot \text{kg dw}^{-1}$ after exercise. Broken trend line represents correlation coefficient when muscle glycogen was $<110 \text{ mmol glucosyl units} \cdot \text{kg dw}^{-1}$ after exercise. From Betts & Williams (2010).

Looking at the original articles, which form the background for recommendations (Ivy, 2001; Beelen et al., 2010; Betts & Williams, 2010), enables us to understand some of the reasons behind the variation of reported glycogen resynthesis rates. Ivy, Lee, Brozinick, and Reed (1988b) compared the glycogen resynthesis when no energy, a low CHO or high CHO diet was ingested after exercise. When no energy was provided the first 2 h after exercise, rate of glycogen resynthesis remained low ($2.0 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$). Increasing CHO ingestion rate to $0.75 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ caused glycogen resynthesis to increase ($18.4 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$). However, no further increase in muscle glycogen

resynthesis was reported ($20.6 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{h}^{-1}$) when they increased the CHO ingestion rate to $1.5 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$.

An increase in glycogen resynthesis after high compared to low CHO feeding was however shown by van Loon, Saris, Kruijshoop, and Wagenmakers (2000). van Loon et al. (2000) compared 0.8 and $1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ intake on glycogen resynthesis after an exhaustive exercise. During 5 h recovery, muscle glycogen resynthesis was greater in the high CHO diet ($44.8\pm 6.8 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{h}^{-1}$) compared to the low CHO diet ($16.6\pm 7.8 \text{ mmol}\cdot\text{kg dw}^{-1}\cdot\text{h}^{-1}$). The differences in the findings between Ivy et al. (1988b) and van Loon et al. (2000) could be initial exercise and the ingestion intervals of the CHO. In the study of van Loon et al. (2000), subjects were exhausted and ingested supplementation in 30 min intervals. In the study of Ivy et al. (1988b) subjects were not exhausted and ingested the CHO in 2 h intervals.

The findings from the study of van Loon et al. (2000) opens the question if a further increased glycogen resynthesis is possible by increasing the CHO ingestion rate above $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. Howarth et al. (2009) compared ingestion of 1.2 g CHO and $1.6 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ after exercise, and found no changes on the glycogen resynthesis rate. This does not rule out the possibility of further increasing glycogen resynthesis when ingesting more than $1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. Endurance exercise improves the ability to resynthesize glycogen in the muscle cell (Greiwe et al., 1999), and it is well known that trained subjects are able to store more glycogen than sedentary controls (Hickner et al., 1997). Chances are that extremely well trained subjects are able to further elevate the rate of glycogen resynthesis after exercise when ingesting CHO at a rate above $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$.

2.4.2 Composition of CHO ingestion

Carbohydrate molecules come in different forms, and can be divided by groups of high or low glycaemic index (GI) (Betts & Williams, 2010). CHO with high GI induce a rapid increase in blood glucose and insulin response. It has been reported greater glycogen resynthesis when a high GI diet was ingested after exercise compared with a low GI diet (Blom, Hostmark, Vaage, Kardel, & Maehlum, 1987). The diet immediately following exercise should therefore contain CHO with high GI if rapid glycogen

resynthesis is preferred. Glucose and the glucose polymer maltodextrin have a high GI, and a combination of these may thus be beneficial for glycogen resynthesis.

2.4.3 Timing of CHO ingestion

Timing of CHO intake after exercise is important for the glycogen resynthesis rate. Ivy, Katz, Cutler, Sherman, and Coyle (1988a) compared the glycogen resynthesis when CHO was ingested immediately or 2 h following exercise. When CHO was ingested immediately after exercise, muscle glycogen increased to $238.8 \pm 6.2 \text{ mmol} \cdot \text{kg dw}^{-1}$ after 4 h recovery. When CHO ingestion was delayed, muscle glycogen concentration increased only to $177.2 \pm 3.7 \text{ mmol} \cdot \text{kg dw}^{-1}$ after the 4 h recovery. When CHO was ingested immediately after exercise, blood glucose and plasma insulin increased rapidly. When CHO was ingested 2 h after exercise, blood glucose and plasma insulin stayed low until feeding, and increased rapidly after feeding. The study shows that CHO should be administered immediately following exercise to optimize the glycogen resynthesis.

High frequency of CHO feeding leads to a higher glycogen resynthesis rate than low frequency of CHO feeding (Betts & Williams, 2010). Muscle glycogen resynthesis rates between 35 and $45 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ have been reported when CHO has been administered with high frequent intervals (van Loon et al., 2000; Jentjens, van Loon, Mann, Wagenmakers, & Jeukendrup, 2001; Wallis et al., 2008). The mechanism behind this is not clear. Less frequent ingestion of high CHO doses leads to a rapid insulin secretion (Ivy et al., 2002). As compared, more frequent feeding of less CHO leads to a slower insulin secretion (van Loon et al., 2000). Thus, the slower glycogen synthesis seen when CHO is provided less frequent is not a result of limited insulin availability. One reason for the slower glycogen resynthesis could be oxidation of the glucose molecule as it enters the muscle cell. Glucose can also be stored in other tissue than the examined muscle, for example the liver.

Timing of CHO ingestion seems not to be of importance when recovery time is 24 h or longer. A study by Parkin, Carey, Martin, Stojanovska, and Febbraio (1997) investigated rate of glycogen synthesis when CHO was ingested immediately or 2 h after exercise. Muscle glycogen concentration was not different between the two treatments after 24 h of recovery.

To summarize, the optimal rate of CHO ingestion after exercise is $1.0\text{-}1.2\text{ g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. CHO with high GI should be ingested to rapidly after exercise providing substrate for glycogen synthesis. Furthermore, recovery supplementation should be provided immediately after exercise in order to utilize the rapid phase of insulin independent glycogen synthesis.

2.5 Protein and protein synthesis

Proteins are key molecules that define a cells function (Alberts, 2010). Amino acids are the building blocks of proteins. A protein is synthesised or activated in a manner that suits a cells need, and training increases protein degradation (Frayn, 2010; Harvey & Ferrier, 2011; Egan & Zierath, 2013). Proteins are continuously broken down, and there is a constant need of amino acids as building blocks for the next protein to be made. Our body is able to synthesise some of these amino acids, however, essential amino acids have to be provided endogenously (Alberts, 2010). Endurance athletes require more daily protein than sedentary, and are recommended to eat $1.2\text{-}1.6\text{ g}$ protein per kg bodyweight during 24 h (Olympiatoppen, 2014).

2.5.1 Protein balance and requirements for the endurance athlete

Proteins consist of about 16 % nitrogen on average. When a protein is broken down, nitrogen is mainly excreted to urine in the urea cycle (Frayn, 2010). Nitrogen is also excreted to sweat and feces. Relatively, 77 % of total nitrogen is excreted to urine, and 23 % to sweat ant feces (Tarnopolsky, MacDougall, & Atkinson, 1988). Nitrogen balance is a common method of determining the protein balance (Tipton & Wolfe, 2004). The net nitrogen balance is the difference between ingested and excreted nitrogen, as dietary nitrogen content can be calculated from the amount of ingested protein. A negative nitrogen balance is defined as when more nitrogen is excreted than ingested in the diet, and this indicates protein degradation.

Endurance athletes require more dietary protein than sedentary to be in nitrogen balance (Tarnopolsky et al., 1988). In the study of Tarnopolsky et al. (1988), positive nitrogen balance was maintained when endurance athletes ingested $1.7\text{ g}\cdot\text{kg}^{-1}$ during 24 h. For sedentary, positive nitrogen balance was maintained when $1.1\text{ g Pro}\cdot\text{kg}^{-1}$ was ingested during 24 h. The nitrogen balance is influenced by factors such as sufficient energy intake and exercise volume (Tipton & Wolfe, 2004). As a result of this, the

recommended daily protein ingestion for an endurance athlete is 1.2-1.6 g·kg⁻¹, with higher ingestion rates at days and periods with higher training volume (Tarnopolsky, 2004).

2.5.2 Exercise and protein synthesis

Even a single bout of exercise could increase the muscle protein synthesis (Lemon, Dolny, & Yarasheski, 1997). However, exercise also enhances muscle protein degradation (Børsheim et al., 2004a). The net muscle protein balance is the difference between protein degradation and protein synthesis, and remains negative if no protein is consumed after exercise (Børsheim et al., 2004a). The study of Børsheim et al. (2004a) compared protein synthesis and degradation when CHO or no energy was ingested after exercise. When no energy was ingested, muscle protein degradation remained high and muscle protein balance negative. CHO ingestion gradually reduced protein degradation throughout the recovery period, but the net muscle protein balance was still negative after 3 h. The study shows that CHO may reduce the muscle protein degradation, but that CHO feeding alone is not sufficient for a positive muscle protein balance during 3 h of recovery.

Composition of the diet after exercise influence the muscle protein synthesis, and play an important part in turning the muscle to an anabolic state (Børsheim et al., 2004a; Blomstrand et al., 2006; Howarth et al., 2009; Hawley et al., 2011). Protein ingestion is required to activate the muscle protein synthesis after exercise (Blomstrand et al., 2006). Howarth et al. (2009) investigated the effect of protein ingestion on muscle protein synthesis after exercise. Subjects ingested 1.2 g CHO·kg⁻¹·h⁻¹ and 0.4 g Pro·kg⁻¹·h⁻¹ (CHO+Pro), 1.6 g CHO·kg⁻¹·h⁻¹ (isocaloric CHO) or 1.2 g CHO·kg⁻¹·h⁻¹ (content matched CHO) after exercise. Nitrogen balance was positive after 4 h of recovery when CHO+Pro was ingested, but not after intake of either the content or caloric matched CHO diets. Amino acid concentration in the muscle was also higher after CHO+Pro intake compared to both CHO diets. Additionally, protein ingestion caused higher fractional muscle protein synthesis compared to the two CHO controls. The study of Howarth et al. (2009) showed that protein feeding after exercise caused positive nitrogen balance, which was related to improved amino acid concentration in the muscle cell and increased protein synthesis.

One mechanism behind enhanced protein synthesis following CHO+Pro feeding may be activation of the mammalian target of rapamycin (mTOR) signalling pathway (Kimball, Farrell, & Jefferson, 2002). mTOR regulates protein synthesis via p70 ribosomal S6 kinase (p70s6k). Proteins upstream for mTOR are PI3k and PKB. In addition, amino acids may also regulate mTOR directly (Karlsson et al., 2004; Blomstrand et al., 2006). Ivy, Ding, Hwang, Cialdella-Kam, and Morrison (2008) investigated the effect of CHO+Pro feeding with no energy after exercise on phosphorylation of these proteins. They concluded that CHO+Pro increased activation of protein synthesis via phosphorylation of mTOR, PI3K, and p70s6k. This has also been shown in studies comparing CHO+Pro with isocaloric CHO intake (Ferguson-Stegall et al., 2011b; Rowlands et al., 2011). In the study of Rowlands et al. (2011), gene expression and enzyme phosphorylation was investigated after 105 min of interval cycling when either CHO ($1.6 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) or CHO+Pro ($1.2 \text{ g CHO} + 0.4 \text{ g Pro} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was ingested after exercise. Protein intake improved activation of protein synthesis after 3 h of recovery compared to CHO. This was further reflected to gene expression count, which was higher after CHO+Pro compared to CHO.

Mitochondria biogenesis is a key process of protein synthesis that endurance athletes aim to activate. Increased mitochondrial biogenesis leads to either increased size or amount of mitochondria, and exercise may activate mitochondrial biogenesis (Safdar et al., 2011). A central protein involved in this process is the peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α). Expression and activity of PGC-1 α has been shown to be upregulated after exercise when CHO+Pro has been provided, as compared to CHO ingestion (Rowlands et al., 2011; Hill, Stathis, Grinfeld, Hayes, & McAinch, 2013). This could indicate that protein ingestion may cause increased mitochondrial biogenesis.

2.5.3 Protein and muscle glycogen resynthesis

The combination of CHO and protein ingestion may influence rate of glycogen resynthesis. One mechanism behind this can be insulin secretion, as glucose and some amino acids are known to stimulate this (Frayn, 2010).

The first study to show an effect of CHO+Pro on muscle glycogen resynthesis after exercise was conducted by Zawadzki et al. (1992). After a glycogen depleting exercise,

subjects ingested either 0.8 g CHO, 0.4 g Pro or 0.8 g CHO + 0.4 g Pro·kg⁻¹·h⁻¹ for 2 h. Muscle glycogen resynthesis during the 4 h recovery was greater when subjects ingested CHO+Pro compared to both CHO and protein. The study did not include an isocaloric trial, and the higher glycogen resynthesis rate in CHO+Pro compared to CHO could have been a result of the extra amount of energy provided in the diet.

When comparing CHO+Pro with isocaloric CHO ingestion, some studies reported no effect on muscle glycogen resynthesis (van Hall, Shirreffs, & Calbet, 2000; van Loon et al., 2000; Howarth et al., 2009; Ferguson-Stegall et al., 2011b; Rowlands et al., 2011), while others reported improved glycogen resynthesis after CHO+Pro intake (Ivy et al., 2002; Berardi et al., 2006). van Loon et al. (2000) compared ingestion of 0.8 g CHO, 1.2 g CHO and 0.8 g CHO+0.4 g Pro·kg⁻¹·h⁻¹ after a glycogen depleting exercise. By doing this, they were able to compare the effect of ingesting CHO+Pro on glycogen resynthesis with both CHO content and energy-matched controls. Muscle glycogen resynthesis was higher after CHO+Pro intake compared to the content matched CHO diet, supporting the findings from Zawadzki et al. (1992). When comparing CHO+Pro with the isocaloric CHO diet, muscle glycogen resynthesis was not affected by protein ingestion. However, Berardi et al. (2006) compared isocaloric diets, and found muscle glycogen resynthesis to be improved by CHO+Pro (0.8 g CHO+0.4 g Pro·kg⁻¹·h⁻¹) intake compared to CHO (1.2 g CHO·kg⁻¹·h⁻¹) after exercise. One reason for the different findings may be the frequency of supplementation. van Loon et al. (2000) provided supplementation every 30 min, while Berardi et al. (2006) provided supplementation every 60 min.

To summarize, endurance athletes performing at high level undertakes considerable amounts of training hours. To maintain protein balance, they are therefore recommended to ingest 1.2-1.6 g Pro·kg⁻¹ during 24 h. A single bout of exercise leads to muscle protein degradation, and ingesting amino acids is of essential value to maintain a positive muscle protein balance. Protein intake after endurance exercise activates enzymes involved in the protein synthesis. One protein of particular interest for the endurance athletes is PGC-1 α , which is involved in the mitochondria biogenesis (Safdar et al., 2011). PGC-1 α activity may increase by protein intake after exercise. Muscle glycogen resynthesis seems not to be affected when CHO+Pro is ingested in 15-30 min intervals the first 2-3 h following exercise, compared to isocaloric CHO intake.

2.6 Recovery of exercise performance

Carbohydrate ingestion has been shown to be important for glycogen resynthesis, and an additive effect of protein intake has been reported. Dietary protein may further affect muscle protein synthesis. An adequate diet following exercise may therefore exert important effects on recovery of performance. I will in the following present studies investigating the effect of CHO and CHO+Pro intake upon subsequent performance.

2.6.1 Effect of CHO ingestion on recovery of performance

CHO feeding before an acute bout of exercise may improve performance (Ormsbee, Bach, & Baur, 2014). This has however not always been reported when CHO is ingested after an exercise and the athlete is to perform within 5 h (table 2.1). Table 2.1 show some studies upon energy intake and subsequent performance. From a total of five studies, three reported improved performance when CHO was ingested compared to no energy. Wong, Williams, and Adams (2000) found improved recovery of endurance capacity when CHO was ingested after an initial exercise, as compared to PLA. Subjects ingested $1.41 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ or PLA during 3 h after exercise. After 4 h of recovery, time until exhaustion (TtE) at 70 % of $\text{VO}_{2\text{max}}$ was 24 min improved after CHO compared to placebo. In a study by Casey et al. (2000), subjects ingested $1.0 \text{ g CHO} \cdot \text{kg}^{-1}$ or no energy immediately after exercise. TtE performance 4 h later was not affected by CHO intake compared to no energy. This shows that recovery of performance could be sufficient even in the absence of energy when the recovery period is short.

Increasing the amount of CHO intake have either caused improved (Betts et al., 2005; Betts, Williams, Duffy, & Gunner, 2007), or unaffected recovery of performance (Fallowfield & Williams, 1997). In the study of Betts et al. (2007), subjects ingested either $0.8 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ or $1.1 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ after exercise. TtE performance at 70 % of $\text{VO}_{2\text{max}}$ was tested after 4 h, and was 16.2 min improved after high CHO intake compared to low CHO. Fallowfield and Williams (1997) compared intake of 0.5 g CHO with $1.5 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ after 90 min running at 70 % of $\text{VO}_{2\text{max}}$ on performance after a 4 h recovery period. No effect of increasing CHO intake was reported on TtE at 70 % of $\text{VO}_{2\text{max}}$. Increasing CHO intake could improve subsequent performance, however, this is not always the case as some also find unaffected performance when comparing high to low CHO intake during recovery.

2.6.2 Effect of Protein and CHO ingestion on performance

Protein intake stimulates protein synthesis and may turn the muscle cell to a positive protein balance. CHO intake accelerates the rate of glycogen resynthesis. The combination of CHO and protein intake could therefore have an additive effect on recovery of performance. Many studies have investigated this, and table 2.2 summarizes some of them. Table 2.2 shows an equivocal effect of protein intake during recovery compared to CHO. Some has found improved (Williams, Raven, Fogt, & Ivy, 2003; Saunders, Kane, & Todd, 2004; Karp et al., 2006; Betts et al., 2007; Berardi et al., 2008; Rowlands et al., 2008; Thomas, Morris, & Stevenson, 2009; Ferguson-Stegall et al., 2011b; Rustad, 2011; Thomson et al., 2011; Lunn et al., 2012; Sollie, 2013), while others found no effect of CHO+Pro on recovery of performance compared to CHO intake (Betts et al., 2005; Millard-Stafford et al., 2005; Berardi et al., 2006; Romano-Ely, Todd, Saunders, & Laurent, 2006; Betts et al., 2007; Rowlands, Thorp, Rossler, Graham, & Rockell, 2007; Rowlands et al., 2008; Pritchett, Bishop, Pritchett, Green, & Katica, 2009; Rowlands & Wadsworth, 2011; Goh et al., 2012; Nelson et al., 2012; Hall, Leveritt, Ahuja, & Shing, 2013).

Looking into the studies that form the background of table 2.2, an association appear between outcome and type of performance test used. Out of 20 studies, ten reported a positive effect of protein ingestion on performance evaluated within 24 h. In seven of these, a positive outcome was reported when a TtE test was administered (Williams et al., 2003; Saunders et al., 2004; Karp et al., 2006; Betts et al., 2007; Thomas et al., 2009; Rustad, 2011; Lunn et al., 2012). The remaining 3 used a prolonged time trial (TT) with total duration above 30 min (Berardi et al., 2008; Ferguson-Stegall et al., 2011b; Sollie, 2013).

Table 2.1 Studies upon effect of energy intake on performance.

Ref.	Initial exercise	Treatment Intake (g·kg⁻¹·h⁻¹)	Supplementation period	Recovery period	Performance test	Outcome
Fallowfield, Williams, and Singh (1995)	Non exhaustive running - CON	Pla: 0.0 CHO: 1.0	2 h	4 h	TtE (70 % VO _{2max})	↑
Wong et al. (2000)	Non exhaustive running - CON	Pla: 0.00 CHO: 1.41	3 h	4 h	TtE (70 % VO _{2max})	↑
Casey et al., (2000)	Non exhaustive cycling - CON	Pla: 0.0 CHO: 1.0	1 h	4 h	TtE (70 % VO _{2max})	↔
Ivy et al., (2003)	Non exhaustive cycling - INT	During: Pla: 0.00 CHO: 0.31	0 h	0 h	TtE (85 % VO _{2max})	↑
Ferguson-Stegall et al., (2011b)	Non exhaustive cycling - CON	Pla: 0.00 CHO: 1.34 ^f	2 h	4 h	TT (40 km)	↔

↑ = Increased effect of treatment. ↔ = No treatment effect. CON = Running/cycling continuously. INT = Intervals. TT = Time trial. TtE = Time until exhaustion. F: Fat was also provided.

Table 2.2 Studies upon effect of protein intake on performance.

Ref.	Initial exercise	Treatment Intake ($\text{g}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) (CHO \pm Pro)	Supplementation period	Recovery period	Performance test	Outcome
Williams et al. (2003)	Not exhaustive cycling - CON	1: CHO: 0.15 2: CHO+Pro: 0.40 + 0.10	2 h	4 h	TtE (85 % $\text{VO}_{2\text{max}}$)	↑
Saunders et al. (2004)	TtE 75 % $\text{VO}_{2\text{max}}$ cycling	During: 1: CHO: 0.48 2: CHO+Pro: 0.48 + 0.12	-	-	Initial exercise	↑
Betts et al. (2005)	Not exhaustive running - CON	1: CHO: 0.70 2: CHO+Pro: 0.70 + 0.20	0.5 h	12-15 h	TtE 85 % of $\text{VO}_{2\text{max}}$	↑
		1: CHO: 1.2 2: CHO+Pro: 1.2 + 0.2	4h	4 h	TtE (85 % $\text{VO}_{2\text{max}}$)	↔
Betts et al. (2005) (Same study as above)	Not exhaustive running - CON	1: CHO: 0.80 2: CHO+Pro: 0.80 + 0.13	4h	4 h	TtE (85 % $\text{VO}_{2\text{max}}$)	↔
Millard-Stafford et al. (2005)	Exhaustive running	1: CHO: 0.6 2: CHO: 1.0 3: CHO+Pro: 0.8 + 0.2	2h	2h	TtE (90 % $\text{VO}_{2\text{max}}$)	↔ ↔↔↔
	Exhaustive running	1: CHO: 0.6 2: CHO: 1.0 3: CHO+Pro: 0.8 + 0.2	2h	24 h	TT (5 km)	↔ ↔↔↔
Berardi et al. (2006)	Non exhaustive cycling - TT	1: CHO: 1.2 2: CHO+Pro: 0.8 + 0.4	2h	6h	TT (1 h)	↔
Karp et al. (2006)	Exhaustive Cycling	1: CHO: 0.41 2: CHO+Pro ^{fiso} : 0.96 + 0.25 3: CM ^{fiso} : 0.96 + 0.26	2 h	4 h	TtE (70 % $\text{VO}_{2\text{max}}$)	↓ ↔↑

Continued

Ref.	Initial exercise	Treatment Intake (g·kg ⁻¹ ·h ⁻¹) (CHO±Pro)	Supplementation period	Recovery period	Performance test	Outcome
Romano-Ely et al. (2006)	Exhaustive Cycling	During: 1: CHO ^f : 0.88 2: CHO+Pro ^f 0.66 + 0.17				↔
	Exhaustive Cycling	1: CHO ^f : 2.17 2: CHO+Pro ^f : 1.65 + 0.43	0.5h	24 h	TtE (80 % VO _{2max})	↔
Betts et al. (2007)	Non exhaustive Running - CON	1: CHO: 0.8 2: CHO: 1.1 3: CHO+Pro: 0.8 + 0.3	4 h	4 h	TtE (70 % VO _{2max})	↑ ↑↔
Rowlands et al. (2007)	Non exhaustive Cycling - INT	1: CHO+Pro ^{ef} : 2.35 + 0.12 2: CHO+Pro ^{ef} : 1.60 + 0.80	4 h	12-15 h	Repeated sprint	↔
Berardi et al. (2008)	Non exhaustivte Cycling - INT	1: CHO: 1.2 2: CHO+Pro: 0.8 + 0.4	2h	6h	TT (1 h)	↑
Rowlands et al. (2008)	Non exhaustive cycling - INT	1: CHO ^{ef} : 2.1 2: CHO+Pro ^{ef} : 1.4 + 0.7	4 h	15 h	Repeated sprints	↔
	Non exhaustive cycling - INT	1: CHO ^{ef} : 2.1 2: CHO+Pro ^{ef} : 1.4 + 0.7	4 h	60 h	Repeated sprints	↑
Thomas et al. (2009)	Exhaustive cycling	1: CHO: 0.42 2: CHO+Pro ^{fiso} : 1.0 + 0.26 3: CM ^{fiso} : 0.86 + 0.20	2 h	4 h	TtE (70 % VO _{2max})	↔ ↑↑
Ferguson-Stegall et al. (2011b)	Not exhaustive cycling - CON	1: CHO ^{ef} : 1.34 2: CHO+Pro ^{ef} : 1.02 + 0.32	2 h	4 h	TT (40 km)	↑
Rustad (2011)	Exhaustive cycling	1: PLA: 0 2: CHO: 1.2 3: CHO+Pro: 0.8 + 0.4	2 h	18 h	TtE (72 % VO _{2max})	↑ ↑↑

Continued

Ref.	Initial exercise	Treatment (g·kg ⁻¹ ·h ⁻¹) (CHO±Pro)	Supplementation period	Recovery period	Performance test	Outcome
Thomson et al. (2011)	Non exhaustive cycling - INT	1: CHO+Pro ^{ef} : 1.6 + 0.1 2: CHO+Pro ^{ef} : 1.2 + 0.5	1.5 h	39 h	Repeated sprints	↑
Rowlands and Wadsworth (2011)	Not exhaustive cycling - INT	1: CHO+Pro ^{ef} : 2.1 + 0.1 2: CHO+Pro ^{ef} : 1.4 + 0.7	4 h	15 h	Repeated sprints	↔
	Not exhaustive cycling - INT	1: CHO+Pro ^{ef} : 2.1 + 0.1 2: CHO+Pro ^{ef} : 1.4 + 0.7	4 h	45 h	Repeated sprints	↔
Nelson et al. (2012)	Not exhaustive cycling - INT	1: CHO ^{ef} : 1.60 2: CHO+Pro ^{ef} : 1.20 + 0.40	1 h	4 d	Repeated sprints	↔
	Not exhaustive cycling - INT	1: CHO ^{ef} : 1.6 2: CHO+Pro ^{ef} : 1.2 + 0.4	3h	2 d	Repeated sprints	↔
Lunn et al. (2012)	Non exhaustive running - CON	1: CHO: 1.00 2: CHO+Pro: 0.78 + 0.22	1 h	3 h	TtE (VO _{2peak})	↑
Goh et al. (2012)	Non exhaustive Cycling - INT	1: CHO: 0.51 2: CHO+Pro ^f : 0.05 + 0.38 3: CHO+Pro ^f : 0.31 + 0.17	2 h	4 h	TT (20 km)	↔ ↔↔
Sollie (2013)	Exhaustive Cycling	1: CHO: 1.2 2: CHO+Pro: 0.8 + 0.4	2 h	18 h	TT (KJ)	↑

↑ = Increased effect of treatment. ↔ = No effect between treatment. ↓ = Reduced effect of treatment. One arrow: comparison with treatment above. Two arrows: First arrow compares to treatment 1, second arrow compares to treatment 2. CON = Running/cycling continuously. INT = Intervals. TT = Time trial. TtE = Time until exhaustion. f: Fat was also provided. ef: Equal amounts of fat were also provided. Iso: isocaloric diets. CM: Chocolate milk.

Williams et al. (2003) compared ingestion of 0.15 g CHO with 0.40 g CHO + 0.10 g Pro·kg⁻¹·h⁻¹ on recovery of performance 4 h after 105 min of cycling at 65-75 % of VO_{2max}. TtE performance was improved when CHO+Pro was ingested (31.1±3.2 min) compared to CHO (20.4±2.0 min). Muscle glycogen resynthesis during the recovery was higher when CHO+Pro was ingested (33.7±4.5 mmol·kg dw⁻¹·h⁻¹) compared to CHO (17.4±5.2 mmol·kg dw⁻¹·h⁻¹), which led to 128 % higher glycogen concentration. Improved performance after CHO+Pro compared to CHO was likely caused by more muscle glycogen. However, this study did not compare isocaloric trials, and the enhanced performance and glycogen resynthesis may be caused by the extra amount of energy provided in CHO+Pro compared to CHO.

Saunders et al. (2004) compared the effect of ingesting 0.12 g CHO or 0.12 g CHO with 0.03 g Pro·kg⁻¹ every 15 min during exercise on TtE at 75 % of VO_{2max}. When CHO+Pro was ingested during exercise, TtE was 29 % improved as compared to CHO. At exhaustion, subjects ingested either 0.70 g CHO or 0.70 g CHO and 0.20 g Pro·kg⁻¹. A second TtE at 85 % of VO_{2max} was performed the next day, and CHO+Pro improved TtE with 40 % compared to CHO. CHO+Pro was not isocaloric with CHO, and the enhanced performance may be a result of the extra amount of energy provided.

In a study by Berardi et al. (2006), ingestion of 1.2 g CHO·kg⁻¹·h⁻¹ during recovery was compared with 0.8 g CHO + 0.4 g Pro·kg⁻¹·h⁻¹ on glycogen resynthesis and performance after 6 h of recovery. After CHO+Pro, muscle glycogen resynthesis was higher compared to CHO. However, CHO+Pro did not affect 1 h TT performance compared to the isocaloric CHO ingestion. Heart rate (HR) during the TT was similar between CHO+Pro and CHO, and they argue that the subjects did not pace themselves enough during the TT for muscle glycogen to cause an effect on performance. Two years later, Berardi et al. (2008) used the same protocol, but with more feedback during the performance test. In this study, CHO+Pro intake compared to CHO improved recovery of performance. Fat was oxidized with a higher rate during the TT in CHO+Pro compared to CHO, which could have a glycogen sparing effect. In addition, using an identical feeding protocol and almost identical exercise protocol as in Berardi et al. (2006), the authors speculated that more available muscle glycogen was resynthesized during recovery. They concluded that the enhanced recovery of performance after

CHO+Pro intake was a result of improved fat oxidation as a glycogen sparing energy source and more available muscle glycogen compared to CHO.

A study by Ferguson-Stegall et al. (2011b) also found improved performance after CHO+Pro compared to isocaloric CHO intake. In this study, subjects first performed a glycogen depleting exercise for 90 min at 70 % of VO_{2max} . Then, either CHO ($1.34 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) or CHO+Pro ($1.02 \text{ g CHO} + 0.32 \text{ g Pro} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was ingested for 2 h. After 4 h of recovery, 40 km TT performance was completed faster in CHO+Pro (79.4 min) compared to CHO (85.7 min). During recovery, muscle glycogen resynthesis was not different between CHO+Pro ($23.6 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$) and CHO ($30.6 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$). The improved performance after protein intake was therefore not a result of muscle glycogen resynthesis during recovery. Interestingly, protein ingestion caused increased phosphorylation of the proteins mTOR, PKB and rps6 was after 45 min of recovery, indicating activated protein synthesis. Increased activation of muscle protein synthesis could explain the improved performance in CHO+Pro compared to CHO.

Previous studies at the Norwegian School of Sport Sciences have reported improved recovery of performance 18 h after an exhaustive exercise when CHO+Pro or isocaloric CHO was ingested the first 2 h of recovery (Rustad, 2011; Sollie, 2013). In the study of Rustad (2011), subjects cycled to exhaustion three times before CHO+Pro, an isocaloric CHO diet or no energy was ingested for 2 h. Performance was tested as TtE at 73 % of VO_{2max} 18 h later, and was improved by CHO+Pro ($63.5 \pm 4.4 \text{ min}$) compared to CHO ($49.8 \pm 5 \text{ min}$). Both CHO+Pro and CHO improved TtE performance compared to no energy intake ($42.8 \pm 5.1 \text{ min}$). Sollie (2013) compared the effect of CHO+Pro with isocaloric CHO on elite cyclists using a TT performance test. The initial exhaustive exercise and diet the following 2 h were identical to Rustad (2011). CHO+Pro improved TT performance 18 h after the initial exercise by 4 min compared to CHO. The studies show that CHO+Pro intake compared to CHO during the first 2 h after exercise improves next day performance in terms of enhanced capacity (TtE) and workload (TT).

When recovery is less than 24 h, high protein intake in combination with CHO after exercise does not seem to recover repeated sprint performance better than isocaloric CHO diets, or isocaloric low protein high CHO diets. According to table 2.2, 4 studies did not report any effect of CHO+Pro compared to high CHO intake on recovery of

repeated sprint ability conducted within 24 h after an initial exercise (Rowlands et al., 2007; Rowlands et al., 2008; Rowlands & Wadsworth, 2011; Nelson et al., 2012). In two of these studies, a small proportion of protein was given in the high CHO diets (Rowlands et al., 2007; Rowlands & Wadsworth, 2011). In the study of Rowlands et al. (2007), repeated sprint ability was investigated 12-15 h after 2.5 h of interval exercise. The first 4 h during recovery, subjects either ingested 2.35 g CHO+0.12 g Pro·kg⁻¹·h⁻¹ (control diet), or 1.60 g CHO+0.40 g Pro·kg⁻¹·h⁻¹ (high protein diet). Diets were energy matched with fat. During ten sprints mean power output was 328 W after the control diet, and not affected by the high protein diet. Repeated sprint ability is limited by other factors than prolonged endurance performance, and one factor is muscle power (Mendez-Villanueva, Hamer, & Bishop, 2008). Muscle power recovery may be time-consuming, and a possible beneficial effect of protein ingestion could be delayed. A delayed effect of protein intake on repeated sprint performance was demonstrated in two studies (Rowlands et al., 2008; Thomson et al., 2011). The study of Rowlands et al. (2008) used a similar initial exercise and performance test as in Rowlands et al. (2007). In this study, protein intake was reserved for the CHO+Pro diet, and the control diet consisted only of CHO. They showed no effect of protein intake on performance after 15 h of recovery. However, 60 h after the initial exercise, CHO+Pro improved repeated sprint ability compared to CHO. Thomson et al. (2011) reported improved recovery of performance 39 h after an initial exercise when a CHO+Pro diet was compared to a high CHO diet with a small proportion of protein. This shows that protein ingestion after exercise could have a postponed effect on recovery of performance, and that the effect may be dependent on the amount of protein ingested.

Other groups also reported no effect on recovery of performance when CHO+Pro intake after exercise was compared to isocaloric CHO ingestion (Millard-Stafford et al., 2005; Betts et al., 2007). In the study of Millard-Stafford et al. (2005), subjects were exhausted by running at 70 % of VO_{2max} before 0.8 g CHO + 0.2 g Pro, 0.8 g CHO or 1.0 g CHO·kg⁻¹·h⁻¹ was ingested for 2 h. After 2 h of recovery, TtE at 90 % of VO_{2max} was not affected by protein intake compared to both CHO trials. Additionally, no effect of protein intake was seen on 5 km TT performance after 24 h of recovery. Betts et al. (2007) also compared recovery of running performance when CHO+Pro was compared with content matched and isocaloric CHO diets. TtE performance at 70 % of VO_{2max} was tested after 4 h of recovery from 90 min running at the same intensity. Supporting

Millard-Stafford et al. (2005), no effect of CHO+Pro compared to the isocaloric CHO intake was reported. However, CHO+Pro and high CHO intake improved performance compared to the low CHO intake during recovery.

Increasing the ingestion rate of CHO above $1.1 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ seems to have no effect on subsequent performance. This is also in accordance with the recommendations for the CHO ingestion rate for achieving an optimal glycogen resynthesis. The effect of protein intake on performance seems equivocal. When the performance test is of prolonged and exhaustive nature, an effect of protein intake is usually found. However, protein intake does not seem to affect repeated sprint performance within 24 h after an initial exercise or running performance.

3. Methods

3.1 Subjects

Nine male subjects were recruited for the study (table 3.1). The subjects were trained athletes in triathlon (n=2) or terrain biking (n=7). All subjects met the inclusion criteria of training with a bicycle more than twice a week for the last six months. All subjects met the inclusion criteria of a $VO_{2max} \geq 50 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$.

The subjects were individually informed about the study and biopsies both written and orally. After the information was given, subjects signed the informed consent. The study was approved by the Regional Ethical Committee of Midtjylland, Denmark and conducted in accordance to the principles from the declaration of Helsinki. Each subject was given an economical compensation of 4000 Danish kroner for his participation.

Table 3.1: Anthropometric data of the subjects.

Age (yr)	Weight (kg)	Height (cm)	HR _{peak} (beats·min ⁻¹)	VO _{2peak} (ml·kg ⁻¹ ·min ⁻¹)	Muscle glycogen* (mmol·kg dw ⁻¹)
26.7±1.7	76.4±3.2	182.4±2.2	188.0±2.2	58.1±1.7	535.7±30.3

Data are mean (SEM). N=9. * Fasted muscle glycogen. VO_{2peak} = peak oxygen consumption

3.2 Study design

The study was performed as a randomized study with a double-blinded crossover design. Subjects reported to the laboratory five times. These five days consisted of pretesting, fasted baseline biopsy and familiarization, before the two dietary intervention days (fig 3.1). In total, the study duration was approximately 18 days for each subject.

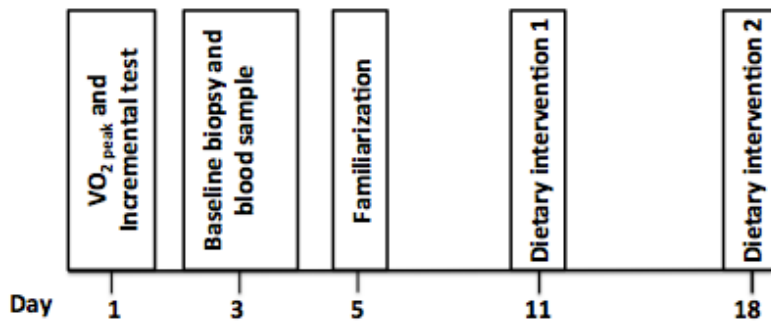


Figure 3.1: Schematic view of the study progress.

3.3 $VO_{2\text{ peak}}$ and incremental test

At the first arrival, subjects did an incremental test and a peak oxygen consumption ($VO_{2\text{ peak}}$) test.

The incremental and $VO_{2\text{ peak}}$ testing were done on an SRM cycle ergometer (SRM, Jülich, Germany), connected to a computer and controlled by software included from the factory (SRM training system). Cadence was digitally visualized with Powercontrol IV (SRM training system, Jülich, Germany) mounted on the steering. The bike was adjusted to the subjects before the incremental test, and the same settings were used during all subsequent testing. The subjects used their own shoes and pedals, and were able to bring their own saddle if desired. The bike adjustment and use of the riders' private gear were done to reduce possible uncomfortable factors that may have led to reduced compliance.

Oxygen consumption (VO_2) was measured on line with AMIS 2001 (Innovation, Odense, Denmark). AMIS was set for ambient conditions before each test. O_2 and CO_2 were calibrated to a known concentration of 16.5 % O_2 and 4.0 % CO_2 . Volume was calibrated manually with a 3 l Hans Rudolph syringe (Shawnee, USA) at three different flow rates; low, medium and high. An error of ± 2 % was accepted from the volume calibration. Prior to each test, a Hans Rudolph mask (Shawnee, USA) was adjusted to the subjects and connected to an inspiration and expiration tube. Expired air was collected in a 15 l mixing bag (Kruuse, Denmark, Langeskov), from which a mixed sample of air was analysed. Volume was calculated from inspired air. Gas concentration and airflow were paired with a 5 s delay, giving the VO_2 measurements. Sampling was done as an average for 10 s.

During laboratory visits, ambient conditions were 21.9 ± 0.2 °C and 41.6 ± 1.2 % humidity.

3.3.1 Incremental test

The relationship between workload and VO_2 was established with an incremental test. During a short (3-5 min) warm-up at 100 Watt, subjects selected a cadence between 90 and 100 revolutions per min (RPM). This self-selected RPM was used in all testing throughout the study. The incremental test started at 125-175 Watt depending on training condition, and increased 25 Watt every 5th min. VO_2 was measured during the last 90 s at each intensity.

Blood lactate was measured after 4 min at each intensity. Before a lactate sample was taken, the skin was disinfected and wiped, then a press needle (Accu-Check, Safe-T-Pro Plus; Mannheim, Deutschland) was used to puncture the skin and capillaries. The first blood drop and sweat drops were wiped away before a micro haematocrit tube (55 μl , Radiometer, Copenhagen, Denmark) was filled. 15 μl was then immediately pipetted into YSI (Yellow spring instruments 1500 SPORT, Ohio, USA) for analysis. The test terminated when the blood lactate concentration was higher than $4 \text{ mmol} \cdot \text{l}^{-1}$. YSI was calibrated with a standard of $5 \text{ mmol} \cdot \text{l}^{-1}$ lactate each day. An error of ± 2 % was accepted.

HR was measured during all testing with a Polar RS 800-CX (Kempele, Finland). The record rate was set to 1 s.

3.3.2 Testing of peak oxygen consumption ($\text{VO}_{2\text{peak}}$)

After the incremental test, subjects were allowed 5-10 min rest before a $\text{VO}_{2\text{peak}}$ test was performed. The $\text{VO}_{2\text{peak}}$ test started at the last workload at the incremental test with blood lactate below $4 \text{ mmol} \cdot \text{l}^{-1}$. The load was increased by 25 watts every 60 s until exhaustion. $\text{VO}_{2\text{peak}}$ was estimated based on the average of the two highest 30 s measurements. The highest HR from this test was considered HR_{peak} .

A linear regression equation established the relationship between workload and corresponding VO_2 measurements from the incremental test. Combined with the subjects $\text{VO}_{2\text{peak}}$, the intensities for subsequent testing were calculated.

3.4 Diet and training before baseline biopsy and interventions

Subjects were told to keep a normal diet, but sustain from any protein supplementation the last 24 hours prior to the baseline biopsy. Training was allowed, but restricted to easy endurance exercise with duration no longer than 60 min. Both the diet and training were noted. Subjects had an overnight fast until meeting at the lab. Before the overnight fast, the last meal was consumed 9.00 P.M. at latest. If the subjects lived a distance further than 2 km away from the laboratory, they were instructed to come by car or public transport. The test leader met the subjects when they arrived at the building. Together they took the elevator to the fourth floor, where the measurements took place.

The subjects were told to follow the same diet and training before both intervention days.

3.5 Baseline biopsy

Reporting to the laboratory at 8.00 A.M., a resting biopsy was taken after an overnight fast. A venous blood sample was taken and capillary blood glucose analysed (Hemocue Glucose, 201⁺, HemoCue AB, Norway).

The blood sample was taken from an underarm vein (v. basilica) while the subjects rested in a supine position. After the skin was disinfected, a butterfly needle (Terumo-Surflo, winged infusion set, Leuven, Belgium) was inserted, and an 8 ml lithium heparine tube was filled. The sample was kept on ice until it was centrifuged for 10 min at 4 °C and 1300 G. The supernatant was then stored at -80 °C until further analysis.

When the capillary blood glucose sample was taken, the skin was disinfected and wiped before a press needle (Accu-Check, Safe-T-Pro Plus; Mannheim, Deutschland) was used to puncture the skin and capillaries. The first blood drops were wiped away before a micro cuvette (HemoCue Glucose 201) was filled. The sample was then immediately analysed (HemoCue Glucose 201⁺, Angelholm, Sweden).

The muscle biopsy was taken from vastus lateralis after the blood sample (fig.3.2). After removing hair with a razor, the skin was disinfected with Klorhexidine (0.5% SAiD). Then, 2 ml Lidokanin (10 mg·ml⁻¹) was injected in the skin, subcutaneous, above and beneath the muscle fascia. A small incision (5 mm) was made in the skin and muscle fascia with a scalpel. Bleeding was stopped by pressure on the wound for ≈ 5 min. Biopsies were taken with a Bergström needle modified for suction. The tissue was quickly examined on a clean laboratory plate before frozen on liquid nitrogen (-196 °C) and stored at -80 °C until further analysis.



Figure 3.2: Pictures from the biopsy procedure. **A:** Preparations. **B:** Incision in the skin and muscle fascia. **C:** Pressure on the wound to stop bleeding. **D:** Locating the incision in the muscle fascia. **E:** Biopsy taken by suction. **F:** Examination of the muscle biopsy before it was frozen on liquid nitrogen.

3.6 Familiarization trial

A familiarization trial was performed to confirm correct workload for exercise during the dietary intervention days, and to habituate the subjects for riding on the SRM. The trial started with a standardised warm-up with three sets of 4 min biking at a workload

corresponding to 50, 55 and 60 % of $\text{VO}_{2\text{peak}}$. The same warm up was used in all subsequent testing throughout the project. After warm up, the workload was set to the estimated workload corresponding to 70 % of $\text{VO}_{2\text{peak}}$ from pretesting. VO_2 was measured after 4 min over 90 s. If the VO_2 was more than $1 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ from the calculated 70 % of $\text{VO}_{2\text{peak}}$, workload was adjusted accordingly and VO_2 was again measured 4 min later. The subjects were allowed a 15 min break when the workload was confirmed. Afterwards they biked 30 min while VO_2 , capillary blood samples and RPE were taken every 10 min. This was done to drill the subjects to keep a stable cadence while samples were taken.

After the familiarization at the workload corresponding to 70 % of $\text{VO}_{2\text{peak}}$, subjects were habituated to a 10 s sprint (10-s sprint). The 10-s sprint was used to evaluate maximal power generated during the dietary intervention days. The last min before the sprint, the subjects were told to accelerate the pedals slowly and progressively to an RPM of 95. The sprints were performed on 100 RPM, and in a seated position. This was standardized for all 10-s sprints during the dietary intervention days. For familiarization, four 10-s sprints were performed with 2 min rest in between.

The familiarization trial was completed at least three days after the resting biopsy.

3.7 The dietary intervention days

Subjects reported to the laboratory at 7:30 A.M. on the dietary intervention days (fig. 3.3). The day consisted of 10 min resting samples, an exhaustive glycogen depleting exercise starting at 8:00 A.M., 5 h of recovery with the diet intervention, and a performance test after 5 h of recovery. Muscle biopsies, blood and urine samples were collected. The diet was controlled throughout the period.

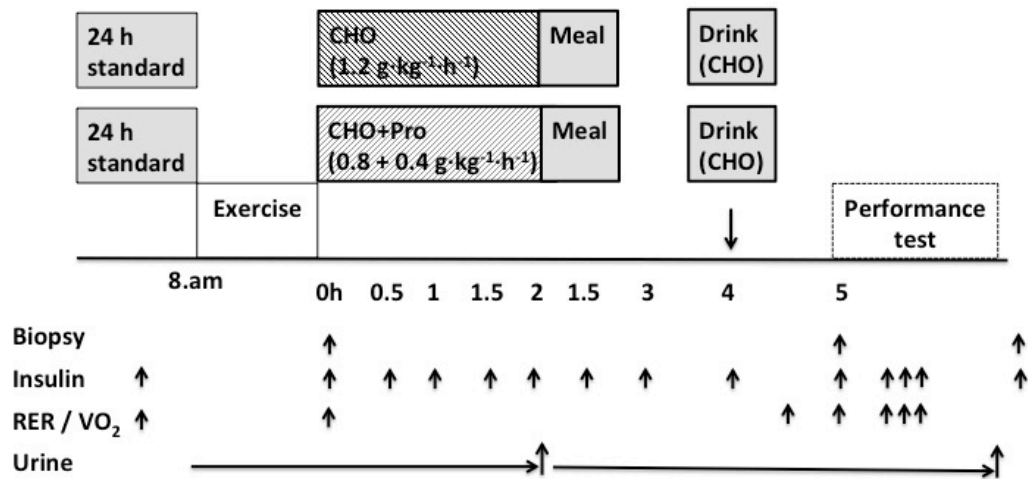


Figure 3.3: Schematic view of the dietary intervention days.

3.7.1 Resting samples

The subjects reported to the laboratory at 7:30 A.M. A breathing mask and the heart rate monitor were mounted to the subjects before the sampling started. VO_2 , RER and HR measurements were done over 10 min in a supine position on a bed, and presented as average over the last 5 min. No one was allowed to enter the room during these measurements.

After the 10 min rest, a venous blood sample, capillary blood lactate and glucose were taken. The subjects were then asked to urinate before they got ready for warm up. This was done in order to ensure that the bladder was empty before urine collecting started.

3.7.2 Initial glycogen depleting exercise

The initial glycogen depleting exercise began at 8:00 A.M., and aimed to deplete the muscle glycogen while biking at a predefined workload corresponding to 70 % of VO_{2peak} (fig.3.4). The exercise started with the standardized warm up. 5 min after the warm up, a 10-s sprint was performed². 5 min later, the subjects started biking at the workload corresponding to 70% of VO_{2peak} . The exercise was divided in bouts separated by 5 min breaks. The first bout lasted 30 min, and all subsequent bouts for 20 min. The subjects were reminded to drink water every 10 min of biking. The exercise terminated

² Data from the 10-s sprints are not presented in this thesis.

at voluntarily exhaustion (hereafter; exhaustion³). VO_2 and RER were measured over 90 s after 3.5 min of the first bout, at the end of each bout and at least 60 s before exhaustion. Following VO_2 and RER measurements, subjects were asked of RPE before blood lactate and glucose samples were taken. HR was measured throughout the whole performance test, and is presented as average over 60 s on time periods corresponding with the VO_2 . While biking to exhaustion, a music-list provided from the subjects was played with songs appearing in random order.

5 min after exhaustion, a series of 1 min sprints at a workload corresponding to 90% of $\text{VO}_{2\text{peak}}$, interspersed with a 1 min break, were performed until the subjects could not keep their predefined cadence. Then, after a 5-min break, a second 10-s sprint was performed in the same manner as after the warm-up (fig. 3.4).

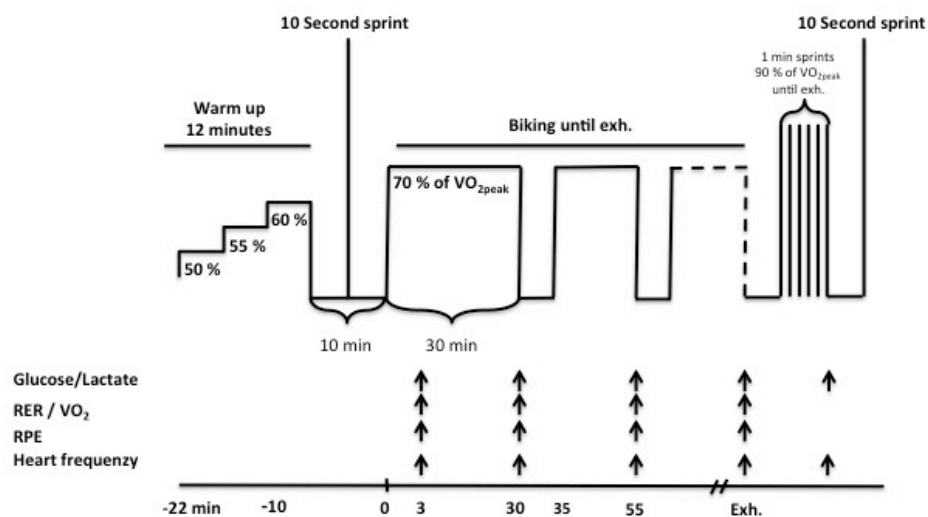


Figure 3.4: Schematic view of the initial glycogen depleting exercise. Exh = Exhaustion.

3.7.3 Tissue sampling during recovery

Blood samples

After the initial glycogen depleting exercise, a veneflon (BD veneflonTM Pro, Helsingborg, Sweden) connected to a three-way valve (BD ConnectaTM, Helsingborg, Sweden) was inserted in an underarm vein before a blood sample taken. A total of nine venous blood samples were collected during recovery: after 0, 30, 60, 90, 120, 150, 180,

³ Exhaustion was in this thesis defined as task failure, and occurred as the subjects could not keep a self-selected cadence despite great encouragement.

240 and 300 min (fig. 3.3). All venous blood samples were taken on lithium heparine tubes and treated in the same manner as the resting sample. To keep the veneflon patent, a saline solution was used to flush the tubes following each sample.

Muscle biopsies

After the first blood sample was taken, the subjects vastus lateralis was prepared for the muscle biopsy procedure as described for the resting biopsy. The recovery period started when the biopsy was taken \approx 15 min after the exhaustive exercise protocol (fig.3.3).

A second biopsy was taken from the other leg after 5 h of recovery. The procedure was the same.

Blood glucose and lactate

Blood glucose and lactate were measured from capillary blood every 30 min during recovery. The samples taken at exhaustion are presented as the post exercise sample.

Urine

Urine was collected in two periods (fig.3.3). From the beginning of the initial glycogen depleting exercise until 120 min of recovery, and from 120 min of recovery until the performance test was completed.

3.7.4 Heart rate and resting samples during recovery

HR was measured during the 5 h recovery period. Each HR plot was digitally visualized (Polar Pro trainer 5, Kempele, Finland), and any obvious error was excluded for HR analysis. Data are presented as means of the first 2 h and the last 3 h of recovery.

Resting metabolism was measured after 4.5 h of recovery. VO_2 , RER and HR were measured in a supine position over 10 min, and data are presented as mean of the last 5 min. No one was allowed entering the room while the measurements were done.

3.7.5 Recovery diet

The recovery supplementation during the first 2 h following exhaustion and biopsy procedures was, in a randomized order, either carbohydrate (CHO) or an isocaloric

carbohydrate with protein (CHO+Pro) drink. Supplementation was given after the first biopsy, and again after 30, 60 and 90 min of recovery (fig.3.3). Tissue and blood sampling was always completed before the subjects drank any beverage.

CHO

The concentration of carbohydrates in CHO was $170 \text{ g}\cdot\text{l}^{-1}$ (17 %). The carbohydrate was a mixture of $85 \text{ g}\cdot\text{l}^{-1}$ (50 %) glucose and $85 \text{ g}\cdot\text{l}^{-1}$ (50 %) maltodextrin. Glucose was bought from Merck (Darmstadt, Germany) and the maltodextrin from WWR (Herlev, Denmark). Subjects were given $0.6 \text{ g CHO}\cdot\text{kg}^{-1}$ in each drink. Thus, $1.2 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$ was ingested the first 2 h of recovery.

CHO+Pro

The CHO+Pro was isocaloric with the CHO drink. The concentration of CHO and protein was 113.7 and $56.3 \text{ g}\cdot\text{l}^{-1}$, respectively ($170\text{g}\cdot\text{l}^{-1}$). Drinks consisted of $57 \text{ g}\cdot\text{l}^{-1}$ glucose, $57 \text{ g}\cdot\text{l}^{-1}$ maltodextrin and $57 \text{ g}\cdot\text{l}^{-1}$ whey protein. Thus, the drink was a solution of 66.7 % carbohydrates, and 33.3 % proteins. The protein was whey isolate protein (Lacprodan, SP-9225 Instant), provided by ARLA (Aarhus, Denmark). Subjects were given $0.4 \text{ g}\cdot\text{kg}^{-1}$ of CHO and $0.2 \text{ g}\cdot\text{kg}^{-1}$ of Whey protein in each drink. In total, $0.8 \text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$ and $0.4 \text{ g protein}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$ was ingested the first 2 h of recovery.

The subjects and test manager were blinded for which of the two diets being served. All supplementation was served on opacified bottles. To make the drinks comparable on taste, a non-caloric lemonade (FunLight) in a 1/10 relationship and 0.7 mg/L sodium chloride was added to the drinks.

3.7.6 Additional food and recovery supplementation

A lunch was served after 2 h of recovery in both CHO and CHO+Pro. Normalized by bodyweight, the lunch contained minced meat, pasta and tomato sauce (table 3.2).

After 4 h of recovery, a carbohydrate drink was given in both CHO and CHO+Pro. This was the same drink as the CHO. A total of $1.2 \text{ g CHO}\cdot\text{kg}^{-1}$ was served.

Table 3.2: Intake of carbohydrate (CHO), protein and fat in the lunch and standard sport drink on both dietary intervention days.

	CHO ($g \cdot kg^{-1}$)	Protein ($g \cdot kg^{-1}$)	Fat ($g \cdot kg^{-1}$)
Lunch	1.7	0.5	0.2
Standard drink	1.2	-	-

3.7.7 Performance test

The performance test consisted of biking until exhaustion at a predefined workload corresponding to 70 % of VO_{2peak} (fig. 3.5). After the standardized warm up and 5 min rest, the 10-s sprint was done in the same manner as before the initial glycogen depleting exercise. 5 min later, subjects started biking at the workload corresponding to 70 % of VO_{2peak} without any pause until exhaustion. Subjects were blinded for duration, and reminded to drink water every 10 min. VO_2 and RER were measured after 3.5 min every 15th min for 90 s and at least 60 s before exhaustion. After breathing measurements, subjects were asked for RPE before blood lactate, blood glucose and venous blood samples were taken. HR was monitored throughout the performance test, and presented as average of 60 s on time periods corresponding with the VO_2 measurements. During the performance test, the same music list as during the exhaustive exercise was played, with songs appearing in a randomized order. To keep the air circulated around the subjects, a fan was placed at the contralateral side for the veneflon.

5 min after exhaustion, a 10-s sprint was performed (fig.3.5). Then, a third biopsy of the day was taken. This biopsy was taken from the same leg as the first biopsy of the day.

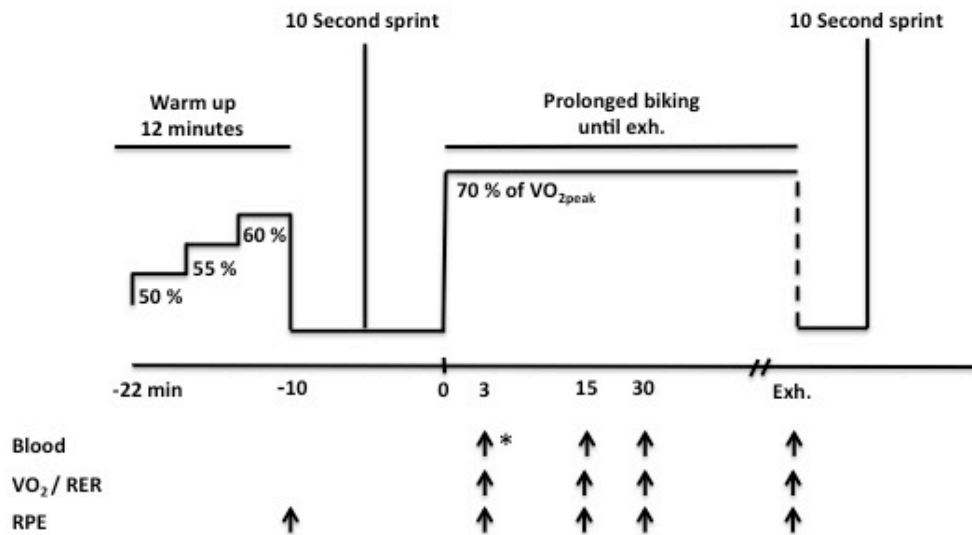


Figure 3.5: Schematic view of the performance test. * Only capillary blood sample was taken. Exh = exhaustion.

3.8 Analysis of blood, muscle and nitrogen balance

3.8.1 Insulin

Insulin was analysed from the venous blood samples. Samples were taken on containers containing lithium heparine (LH) before they were centrifuged for 10 min. Then the plasma was pipetted to eppendorph containers and stored at -80 °C until further analysis. Samples taken before the initial glycogen depleting exercise, immediately after and throughout the recovery period were analysed according to protocols described elsewhere (Mortensen et al., 2009).

3.8.2 Muscle biopsies

Preparation

About 30 mg muscle was freeze-dried with Christ Alpha 1-2 Lo plus (SciSqip, Shorpsire, United Kingdom). Christ alpha was set to a gas pressure of ≤ 0.04 mbar, giving an air temperature of ≤ -50 °C, and moisture was removed by suction for 2.5 h. Following drying, the samples were kept 10 min in a hexicator and scaled. About 5 mg dw was then prepared for homogenizing. The samples were homogenized 1:100 in an ice-cold homogenizing buffer (pH 7.4 in room temperature) with Retsch MR400 (Haan, Germany). The MR 400 was programmed to shake the muscles and buffer with a

frequency of 30Hz and duration of 3 · 30 s interspersed with 5 s. A metal ball ($\phi=5$ mm) was used to dissolve the muscle. See appendix 1 for further description of the homogenizing buffer.

Protein measurements

Measurements of protein content was done with Omega Fluostar (IBMG, Ortenberg, Germany) on a 96 well micro plate. In preparation of the protein measurements, 30 μ l of the homogenate was diluted with 200 μ l deionized H₂O (dH₂O). Further, 75 μ l seronorm (70 mg · ml⁻¹) was diluted with 5000 μ l dH₂O. A standard curve from six standards containing 0, 0.125, 0.25, 0.50, 1.00 and 1.50 μ g · μ l⁻¹ of protein was used to calculate protein concentrations. Triplets of 5 μ l of the sample dilution, seronorm and standards were mixed with 25 μ l reagent A and 200 μ l of reagent B. Protein measurements were done after 15 min incubation at 25 °C. Based on the protein measurements, each homogenate was diluted to 2 μ g protein · μ l⁻¹.

Muscle glycogen

The glycogen content was measured fluorometrically in an enzymatic reaction couple to NADHP formation with Omega Fluostar (IBMG, Ortenberg, Germany). Analyses were performed on a 96 well micro plate. The glycogen in the crude homogenate (50 μ l) was added to 100 μ l 1.8 N HCl, and incubated at 3 h at 100 °C. A 5 mM glycogen control (100 μ l) and homogenizing buffer (100 μ l) were hydrolysed in 200 μ l 1.8 N HCl and used as glycogen control and blank, respectively. While incubating, the samples were mixed every 0.5 hr. Following the incubation, samples were cooled to room temperature, spun at 1000 G for 2 s and neutralized with 6 N NaOH, then stored at -20 °C until further analysis.

All reagents were prepared daily before glycogen analysis. Briefly, the analysis buffer was diluted in dH₂O and was a solution of glucose 6-phosphate dehydrogenase (G6PDH), Tris (1 M, pH 8.1), MgCl₂, DTT (0.5 mM), ATP (100 mM), NADP⁺ (50 mM) and BSA (1 %); see appendix 2 for further information. The Hexokinase buffer contained 1500 U · ml⁻¹ Hexokinase, Tris (1 M pH 8.1) and BSA (1 %). The hydrolysis buffer was a solution of 1 N HCl and 0.2 N NaOH.

Six standards were prepared daily to get a standard curve. The standard curve was basis for measuring glycogen content of the hydrolysates. The standards had a concentration of 0.00, 1.15, 2.29, 4.56, 7.92 and 10.14 μM glucose, and were prepared from a 9.91 mM glucose stock solution.

10 μl standards, blank, glycogen controls and samples were diluted in 200 μl analysis buffer on a microplate, shaken and pre-incubated for 15 min at 25 °C. Following pre-incubation, a measurement was done and considered as baseline. Then, 5 μl hexokinase was injected to each sample followed by 30 min incubation at 25 °C, before fluorescence in the samples was measured again.

Glycogen concentration was calculated in Excel (Version 11 for Mac 2011 based on the standard curve, Microsoft) and presented as $\text{mmol}\cdot\text{kg dw}^{-1}$.

Western Blotting

A description of all the buffers used in the western blot procedure is presented in appendix 1. 100 μl of the homogenate was incubated for 1 h with 25 μl 5 X SDS sample buffer and 7.5 μl 5 M DTT before the western blot procedure was performed. 12-16 μl^4 of each sample was pipetted onto a gel (Miniprotean TGX 4-15%, BioRad). Proteins were separated by electrophoresis at 200 V for 45 min in an electrophoresis buffer. Proteins were then transferred to a PVDF-membrane (Millipore, 0.45 μM , Rule, Immobilon-P Millipore) in an ice-cold transfer buffer. The membrane was blocked by 2 h incubation in a 5 % skim milk blocking buffer. After blocking, membranes were incubated overnight at 4 °C in primary antibody. Next day, membranes were incubated 1 h in room temperature in a secondary antibody bound to a horseradish peroxidase. After the incubation in secondary antibody, an ECL substrate (Clevelands reagent) was applied. Binding of the secondary antibody was then detected by chemiluminescence and evoked digitally (Chemidoc, MP).

The membrane was washed in a PBS-tween 20% buffer solution for 6 · 10 min between transfer and blocking. After blocking, the membrane was washed for 2 · 30 s. After the overnight incubation in primary antibody, the membrane was washed for 6 · 10 min.

⁴ Same amounts was pipetted within the same gel.

After the incubation in secondary antibody, the membrane was washed for 6 · 10 min. Incubation and washing were always done on a rocking table.

Primary antibodies (dilution) used were GS^{ser641} (1:7500), AS160^{ser588} (1:1000) and PKB^{ser473} (1:1000). Secondary antibody for all primary antibodies was anti-rabbit (1:20 000). Antibodies for GS^{ser641} and PKB^{ser473} were bought from Cell Signalling Technology (Beverly, Ma, USA). Antibody for AS160^{ser588} was a kind gift from dr. Birgitte Nelleman. Western blot analysis for GS and PKB were presented relative to a rat gastrocnemius standard. Analysis for AS160 were presented relative to basal.

3.8.3 Urine nitrogen balance

Nitrogen balance was calculated based on ingested proteins and nitrogen concentration from urine. Nitrogen concentration in the urine was analysed with the Kjeldahl method. Tarnopolsky et al (1988) estimated that 76.76 and 77.11 % of total nitrogen loss is extracted in urine when fed with normal diet or high protein diet, respectively. Hair loss, feces and sweat accounts for the remaining 23 %. Only urine was analysed in this thesis, and thus, total nitrogen loss estimated assuming that 77.11% was excreted in urine.

3.9 Inclusion for statistical analysis

Technical issues struck the SRM during testing of subjects 6-9. The power-control unit failed to work following the first dietary intervention day for these volunteers. A solution in order to finish the testing was to get a replacement unit, which was calibrated according to the bike with guidance from SRM. This replacement was done before testing subject eight on day two. The testing was continued assuming the new power-control unit worked in a similar manner after as the original. However, during the initial glycogen depleting exercise and testing subject number eight, an unknown error was detected. Time values, VO₂ and HR during the exhaustive exercise were all different compared to dietary intervention day number one. Subject number eight was the first of subjects number six to nine to perform dietary intervention day number two; the workload for the following subjects were adjusted according to VO₂ and HR in an attempt to replicate the initial glycogen depleting exercise during dietary intervention day one.

While blinded for the randomization, my supervisors (one not blinded) and I discussed which subjects to include for the statistical analysis of performance and whom to include for the biochemical analysis of muscle glycogen, protein phosphorylation, urine nitrogen and insulin. The uncertainty with the workload during the initial glycogen depleting exercise and performance test for subjects 6-9 led to exclusion of these subjects for performance analysis. However, as the subjects were exhausted during the initial glycogen depleting exercise, all were included for the analysis of the biopsies during recovery. Subject number eight experienced a much greater workload during both sessions on day two compared with day one, and cycled much shorter during the initial glycogen depleting exercise on day two (104 min at the first test versus 60 min at the second). Therefore, this subject was excluded for the analysis of the biopsy taken following the performance test.

3.10 Statistical analysis

The statistical analysis was done with Sigma Plot Statistics version 20 (IBM). Data were tested for normality and are presented as mean \pm SEM if not otherwise stated. A between group difference was tested with a two tailed students T-test between group. Analysis of variance (ANOVA) for repeated measurements were used to establish group x time effects. Significant difference was considered with p-value < 0.05. A holm sidak post hoc test was performed to detect the specific differences.

4. Results

Technical issues struck the SRM ergometer as described in the methods (section 3.9), and intensity was adjusted to VO_2 and HR at the last test. These issues led to 14.2 min longer duration of the exhaustive exercise protocol on day two compared to day one ($n=4$; 2 subjects in CHO group, 2 subjects in CHO+Pro group). VO_2 and HR were higher (though not significant) after 5 min on day two (table 4.1). Based on VO_2 and HR measures, the subjects were included for analysis of blood, urine and muscle biopsies. However, the fourth subject did not adequately complete the performance test like the remaining three, and was also excluded for the muscle biopsy taken after the performance test.

Table 4.1: VO_2 , HR and duration during the initial glycogen depleting exercise protocol from day 1 and 2 for subjects 6-9.

	5 min	30 min	Exhaustion
VO_2 ($\text{ml}\cdot\text{min}^{-1}$)			
Day 1	2468.8 \pm 177.1	3226.9 \pm 198.4	3533.4 \pm 101.3
Day 2	2896.6 \pm 162.4	3317.4 \pm 175.7	3495.9 \pm 198.4
HR ($\text{beats}\cdot\text{min}^{-1}$)			
Day 1	133.9 \pm 11.3	159.3 \pm 5.6	174.0 \pm 5.9
Day 2	139.5 \pm 15.9	160.5 \pm 5.2	171.5 \pm 7.2
Duration (min)			
Day 1			95.2 \pm 5.1
Day 2			109.5 \pm 17.7

N=4. Data are mean \pm SEM.

4.1 Fasted samples

A baseline biopsy was taken on a separate day after overnight fasting. Muscle glycogen concentration was 535.7 \pm 30.3 $\text{mmol}\cdot\text{kg dw}^{-1}$.

Fasted blood glucose was 4.8 \pm 0.14, 5.1 \pm 0.23 and 4.7 \pm 0.14 $\text{mmo}\cdot\text{l}^{-1}$ before the baseline biopsy, CHO and CHO+Pro, respectively. Fasted blood lactate was 0.71 \pm 0.08 and 0.88 \pm 0.14 $\text{mmo}\cdot\text{l}^{-1}$ before CHO and CHO+Pro, respectively. Either fasted blood glucose or lactate was not different before CHO and CHO+Pro ($p>0.05$).

Resting VO_2 , RER and HR were measured in a supine position. VO_2 was 227.6 ± 15.0 and $241.4 \pm 15.0 \text{ ml O}_2 \cdot \text{min}^{-1}$ before CHO and CHO+Pro, respectively. The corresponding RER values were 0.96 ± 0.03 and 0.96 ± 0.03 . HR was 48.8 ± 3.3 and $50.7 \pm 3.0 \text{ beats} \cdot \text{min}^{-1}$ before CHO and CHO+Pro, respectively. No differences in the resting measures were observed between trials ($p > 0.05$).

4.2 Initial glycogen depleting exercise

Time until exhaustion in the morning exercise session prior to ingestion of supplements was 107.0 ± 5.6 and $101.7 \pm 9.0 \text{ min}$ for CHO and CHO+Pro, respectively. During the exercise, VO_2 increased gradually and was higher after 30, 50 min and exhaustion compared to after 5 min (table 4.2). After 5 min of biking VO_2 was higher during CHO+Pro than CHO ($p < 0.05$), subsequent samples were not significantly different. HR increased in a similar manner as VO_2 . No changes in RER were observed during the initial glycogen depleting exercise. RER did not differ between CHO and CHO+Pro at 5, 30, 50 min or at exhaustion. RPE increased gradually from 11.3 ± 0.8 and 12.0 ± 0.8 at 5 min, to 19.0 ± 0.3 and 19.1 ± 0.3 at exhaustion in CHO and CHO+Pro, respectively ($p < 0.05$).

Table 4.2: VO_2 , RER, HR and RPE measured at 5, 30, 50 min and at exhaustion during the initial glycogen depleting exercise.

	5 min	30 min	50 min	Exhaustion
VO_2 ($\text{ml} \cdot \text{min}^{-1}$)				
CHO	$2811.8 \pm 182.5^*$	$3170.6 \pm 105.5^\dagger$	$3209.4 \pm 113.9^\dagger$	$3426.5 \pm 137.1^\dagger$
CHO+Pro	2990.4 ± 158.8	3185.7 ± 136.3	$3339.1 \pm 121.0^\dagger$	$3448.3 \pm 104.9^\dagger$
RER ($\text{VCO}_2 \cdot \text{VO}_2^{-1}$)				
CHO	0.94 ± 0.02	0.96 ± 0.01	0.95 ± 0.02	0.96 ± 0.02
CHO+Pro	0.92 ± 0.02	0.94 ± 0.01	0.95 ± 0.01	0.94 ± 0.01
HR ($\text{beats} \cdot \text{min}^{-1}$)				
CHO	141.7 ± 5.7	$158.0 \pm 3.7^\dagger$	$159.3 \pm 3.9^\dagger$	$166.11 \pm 4.0^\dagger$
CHO+Pro	145.1 ± 5.9	$159.6 \pm 3.4^\dagger$	$161.4 \pm 3.5^\dagger$	$167.11 \pm 4.6^\dagger$
RPE (Borg scale)				
CHO	11.3 ± 0.8	$15.6 \pm 0.5^\dagger$	$15.8 \pm 0.5^\dagger$	$19.0 \pm 0.3^\dagger, \ddagger$
CHO+Pro	12.0 ± 0.8	$15.1 \pm 0.6^\dagger$	$15.9 \pm 0.7^\dagger$	$19.1 \pm 0.2^\dagger, \ddagger$

* Significant difference between CHO and CHO+Pro ($p < 0.05$). † = significant different from 5 min ($p < 0.05$). ‡ = Significant higher than 30 and 50 min ($p < 0.05$). N=9. Data are mean \pm SEM.

Blood glucose concentration fell during exercise (table 4.3). At exhaustion and after the 1-min sprints, blood glucose was lower than after 5 and 30 min in both trials. Further, a decrease in blood glucose from 50 min to exhaustion was seen in the CHO trial. Blood glucose was not different between trials at any time points during the initial glycogen depleting exercise. No between group differences were seen in blood lactate between CHO and CHO+Pro after 5, 30 or 50 min. Blood lactate at exhaustion and after the 1-min sprints were higher in CHO+PRO compared to CHO ($p<0.05$).

Table 4.3: Blood glucose and lactate after 5, 30, 50 min, exhaustion and after the 1-min sprints at 90 % of VO_{2peak} during the initial glycogen depleting exercise.

	5 min	30 min	50 min	Exhaustion	1 min-sprints
Blood glucose ($\text{mmol}\cdot\text{l}^{-1}$)					
<i>CHO</i>	4.7±0.1	4.6±0.2	4.0±0.1	3.3±0.2†,‡	3.4±0.2†,‡
<i>CHO+Pro</i>	4.8±0.1	4.7±0.1	4.2±0.2	3.8±0.3†,‡	3.6±0.3†,‡
Blood lactate ($\text{mmol}\cdot\text{l}^{-1}$)					
<i>CHO</i>	3.21±0.43	2.69±0.26	2.61±0.32	3.01±0.42	3.42±0.62
<i>CHO+Pro</i>	3.10±0.67	2.96±0.67	3.04±0.50	3.58±0.47*	4.42±0.74*

* Significant difference between trials ($p<0.05$). †= Significant different from 5 min ($p<0.05$). ‡= Significant different from 30 min ($p<0.05$). ‡= Significant different from 30 and 50 min ($p<0.05$). N=9. Data are mean±SEM.

4.3 Recovery period

Following exercise and a hypoglycaemic state, blood glucose concentration increased rapid the first 30 min following exercise (fig.4.1). In CHO+Pro, blood glucose peaked after 30 min at $7.4\pm 0.3 \text{ mmol}\cdot\text{l}^{-1}$. In CHO, blood glucose peaked at $9.1\pm 0.4 \text{ mmol}\cdot\text{l}^{-1}$ after 60 min. During the drink intervention, blood glucose was higher in CHO compared to CHO+Pro after 60 and 90 min ($p<0.05$). In CHO, blood glucose decreased during the subsequent 3 h of standardized recovery. In CHO+Pro, blood glucose decreased until 240 min before it increased after a standardized sports drink was ingested. Blood glucose was higher in CHO at 150 and 210 min compared to CHO+Pro. After 270 min, blood glucose was higher in CHO+Pro compared to CHO.

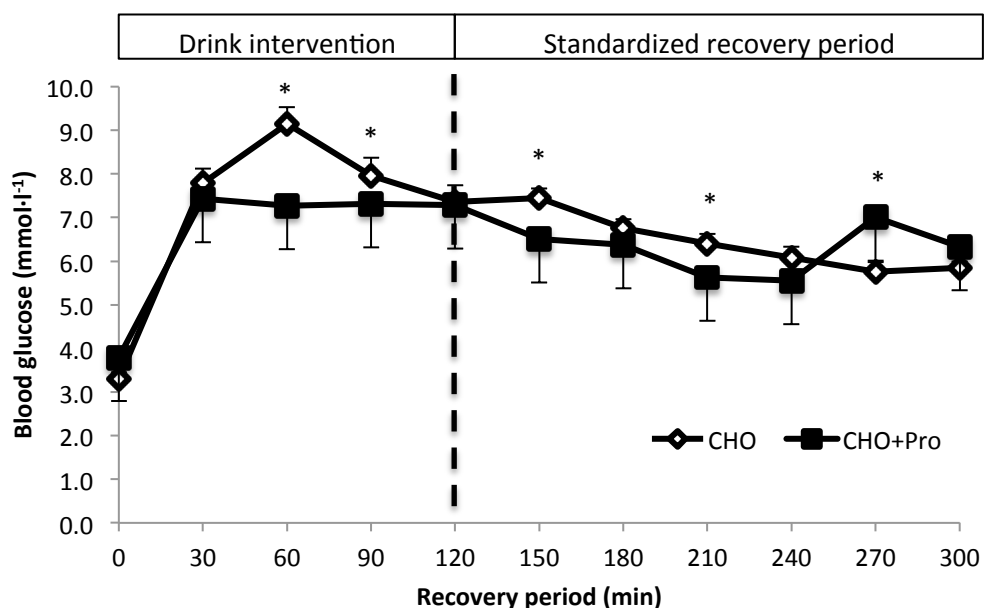


Figure 4.1: Blood glucose during the 5 h recovery following CHO (open) or CHO+Pro (filled) ingestion. *Significant difference between CHO and CHO+Pro ($p < 0.05$). $N = 9$. Data are mean \pm SEM.

Plasma insulin increased rapidly during the drink intervention period (fig. 4.2). A tendency to higher area under curve during the first 120 min was observed in CHO+Pro compared to CHO ($p = 0.062$). At 120 min, plasma insulin concentration was significantly higher in CHO+Pro ($265 \pm 38 \text{ pmol} \cdot \text{l}^{-1}$) compared to CHO ($213 \pm 33 \text{ pmol} \cdot \text{l}^{-1}$) ($p < 0.05$). In both CHO and CHO+Pro, a peak in plasma insulin concentration was seen after 150 min of recovery. Plasma insulin declined after 150 min of recovery until a standard recovery drink was ingested at 240 min. Further, a tendency to significantly lower plasma insulin concentration in CHO+Pro was seen at 150 and 240 min ($p = 0.051$ and 0.058 , respectively).

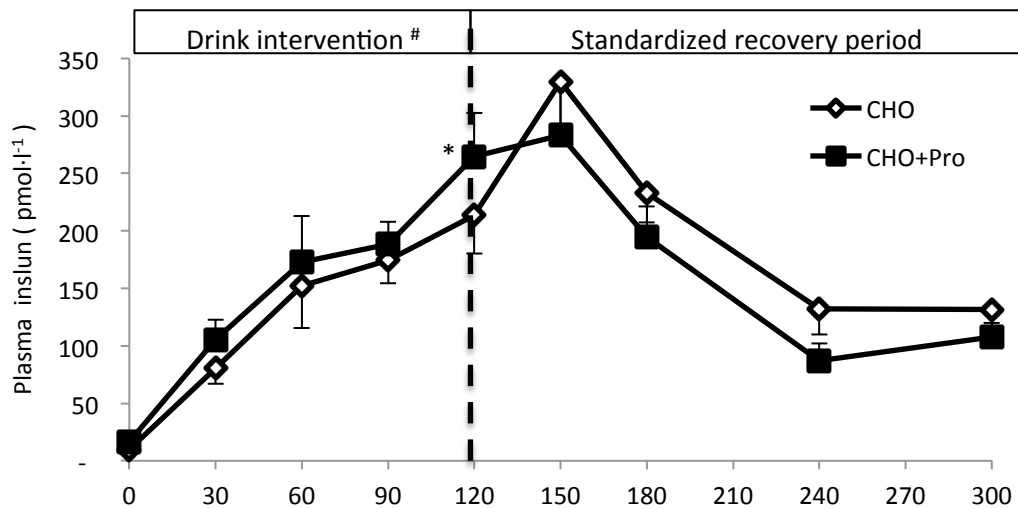


Figure 4.2: Plasma insulin ($\text{pmol}\cdot\text{l}^{-1}$) during the 5 h recovery period when CHO (open) or CHO+Pro (filled) was ingested. * CHO+Pro significantly higher than CHO ($p<0.05$). # Tendency of between trial difference expressed as area under curve ($p=0.062$). $N=9$. Data are mean \pm SEM.

Following exercise, blood lactate fell to resting levels after 30 min (figure 4.3). A difference between trials was seen immediately after exercise and after 60 min of recovery.

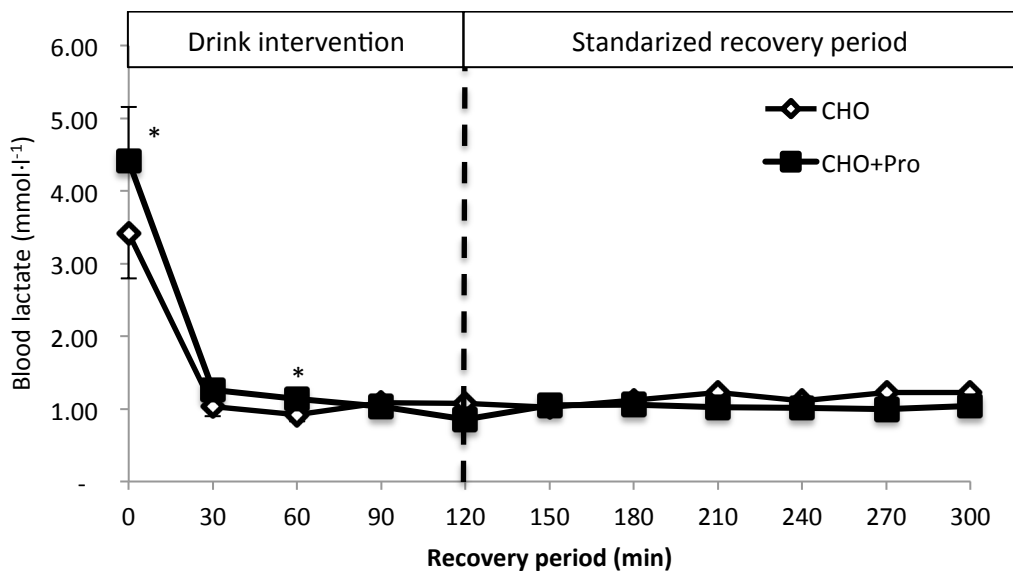


Figure 4.3: Blood lactate ($\text{mmol}\cdot\text{l}^{-1}$) during 5 h recovery when CHO (open) or CHO+Pro (filled) was ingested. * Significant difference between CHO and CHO+Pro ($p<0.05$). $N=9$. Data are mean \pm SEM.

HR was similar between CHO and CHO+Pro during the recovery period. During the first 120 min of recovery, HR was 79.9 ± 3.7 and 82.1 ± 4.0 beats·min⁻¹ in CHO and CHO+Pro, respectively. The following 180 min, HR was 77.9 ± 3.4 and 77.3 ± 2.7 beats·min⁻¹ in CHO and CHO+Pro, respectively. During the 10 min rest sampling after 270 min recovery, HR was 65.1 ± 3.3 and 65.2 ± 2.1 beats·min⁻¹ in CHO and CHO+Pro, respectively. Compared to resting samples in the morning, HR was significantly higher during the resting after 4.5 h of recovery ($p<0.01$).

Resting VO₂ and RER after 4.5 h recovery was similar between CHO and CHO+Pro. In CHO, VO₂ was 306.8 ± 18.8 ml·min⁻¹. In CHO+Pro VO₂ was 310.9 ± 18.2 ml·min⁻¹. RER was 0.91 ± 0.03 in CHO and 0.90 ± 0.02 in CHO+Pro. Compared to the resting samples in the morning, VO₂ was significantly higher in both trials ($p<0.01$). RER was not changed between the resting samples in the morning compared to after 4.5 h of recovery.

Net nitrogen balance was higher in CHO+Pro than in CHO (fig 4.4, $p<0.05$). In CHO+Pro, net nitrogen balance was 19.6 ± 7.6 mg·kg⁻¹ and significantly positive ($p<0.05$). In CHO, net nitrogen balance was at -10.7 ± 6.3 mg·kg⁻¹, but not significantly negative.

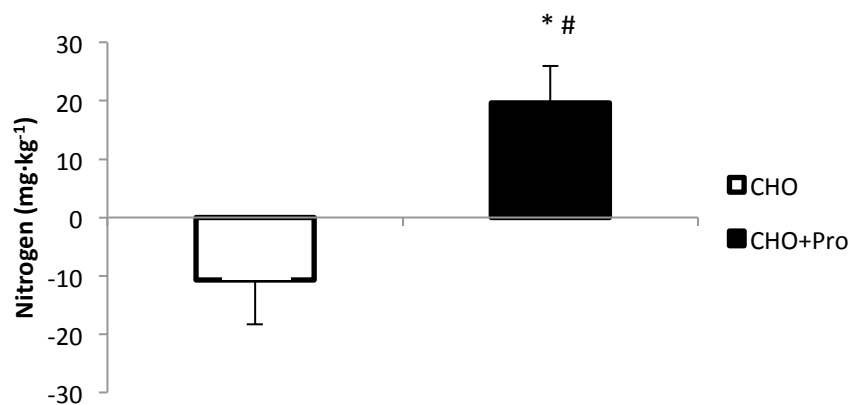


Figure 4.4: Net nitrogen balance during the 5 h recovery when CHO (open) or CHO+Pro (filled) was ingested. * CHO+Pro significantly higher than CHO ($p<0.05$). # CHO+Pro significantly positive ($p<0.05$). N=9. Data are mean±SEM.

The exhaustive exercise reduced the muscle glycogen stores to a similar degree before both interventions (figure 4.5). During recovery, muscle glycogen increased similarly in both trials. Muscle glycogen was reduced after performance test. Glycogen utilization

rate, based on the baseline concentration, during the initial glycogen depleting exercise was 3.7 ± 0.3 and 4.4 ± 0.3 $\text{mmol} \cdot \text{kg dw}^{-1} \cdot \text{min}^{-1}$ in CHO and CHO+Pro, respectively. Thus, before CHO and CHO+Pro dietary interventions, muscle glycogen was 152.1 ± 30.7 and 109.3 ± 31.9 $\text{mmol} \cdot \text{kg dw}^{-1}$, respectively ($p > 0.05$). After the recovery period, muscle glycogen was resynthesized to 357.5 ± 46.1 and 342.3 ± 41.2 $\text{mmol} \cdot \text{kg dw}^{-1}$ in CHO and CHO+Pro, respectively. Thus, the resynthesis rate was 41.3 ± 5.8 and 46.8 ± 8.0 $\text{mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ in CHO and CHO+Pro, respectively. Following the performance test protocol, muscle glycogen was reduced to 145.5 ± 34.2 and 158.2 ± 40.2 $\text{mmol} \cdot \text{kg dw}^{-1}$ in CHO and CHO+Pro, respectively. Utilization rate of muscle glycogen was 4.7 ± 0.8 and 3.4 ± 0.9 $\text{mmol} \cdot \text{kg dw}^{-1} \cdot \text{min}^{-1}$ during the performance test protocol in CHO and CHO+Pro, respectively ($p > 0.05$).

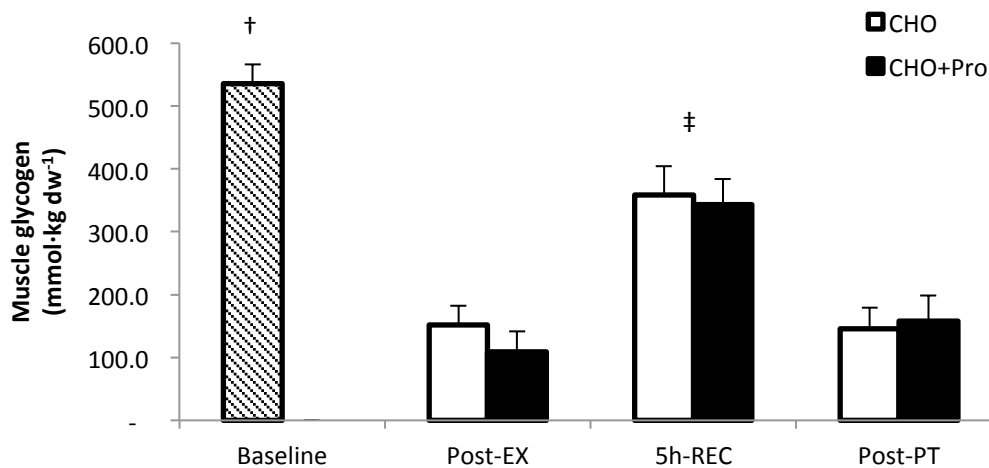


Figure 4.5: Muscle glycogen ($\text{mmol} \cdot \text{kg dw}^{-1}$) following overnight fasting (baseline), after the glycogen depleting exercise (Post-EX), after the 5 h recovery period (5h-REC) and after the performance test protocol (Post-PT). † = Basal measurements significantly higher than Post-EX, 5h-REC and Post-PT ($p < 0.01$). ‡ = 5h-REC significantly higher than Post-EX and Post-PT in both trials ($p < 0.01$). $N = 9$, except Post-PT, $N = 8$. Data are mean \pm SEM.

$\text{GS}^{\text{ser641}}$, $\text{AS160}^{\text{ser588}}$ and $\text{PKBser}^{\text{473}}$ phosphorylation and respective blots are shown in figure 4.6.A-D. Phosphorylation of $\text{GS}^{\text{ser641}}$ was down regulated following the exhaustive exercise protocol and kept below basal levels throughout both experimental days (fig. 4.6.A). No difference was seen between trials in $\text{GS}^{\text{ser641}}$ phosphorylation. However, variation in phosphorylation of $\text{GS}^{\text{ser641}}$ was seen after recovery in CHO. This was a result of two subjects having large phosphorylation at this time point. In

CHO+Pro, variation in phosphorylation of GS^{ser641} was seen Post-PT compared. This was also a result of large phosphorylation for two subjects at this time point.

Phosphorylation of AS160^{ser588} was up regulated after 5h of recovery compared to baseline, post-EX and post-PT (fig.4.6.B). No differences were seen between trials.

No significant differences between groups were seen in PKB^{ser473} at any time points or between treatments. In CHO, a variation was seen on phosphorylation of PKBser⁴⁷³ 5h-REC. This was because of two subjects having large phosphorylation at this time point. Variability was also seen on phosphorylation of PKB^{ser473} post PT (fig 4.6.A). This was also because of two subjects having large phosphorylation at this time point.

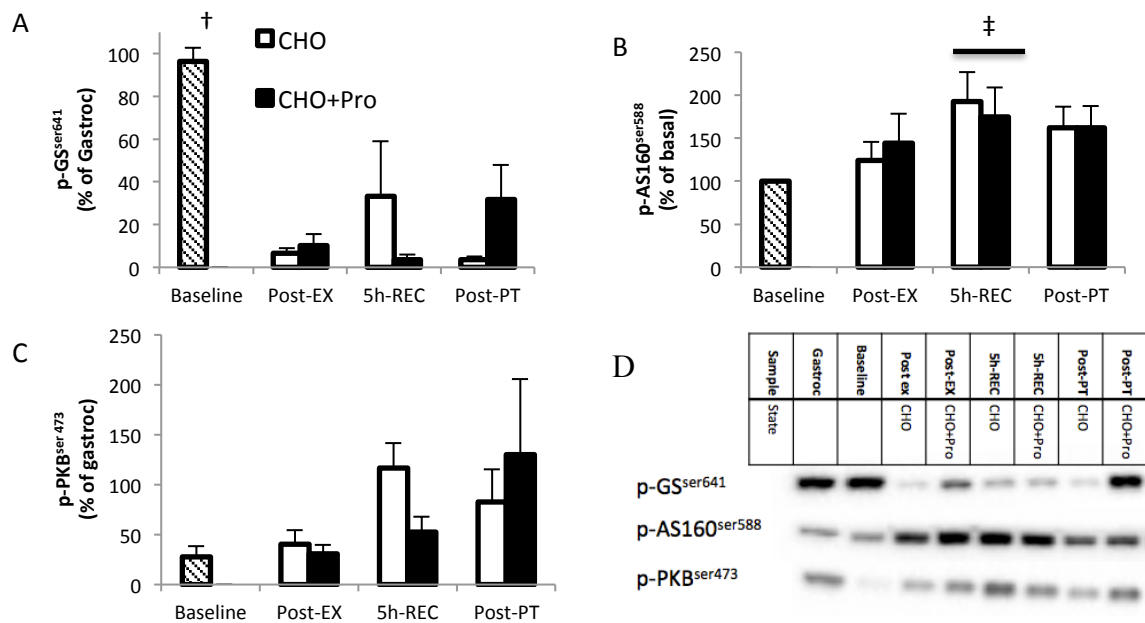


Figure 4.6: *A: GS^{ser641} phosphorylation relative to rat gastrocnemius muscle. B: AS160^{ser588} phosphorylation relative to basal measurements. C: PKB^{ser473} phosphorylation relative to rat gastrocnemius muscle. D: Blots for a representative subject. †: Basal significantly higher than Post-EX, 5h-REC and Post-PT ($p < 0.05$). ‡: 5h-REC significantly higher than basal measurements ($p < 0.05$). $N = 9$, except Post-PT: $N = 8$. Data are mean \pm SEM.*

4.4 Performance test (n=5)

The time to exhaustion performance test for the five subjects lasted on average 8.4 ± 1.8 min longer in CHO+Pro compared to CHO. In CHO, subjects cycled 46.1 ± 9.8 min before exhaustion. During CHO+Pro, subjects cycled 54.6 ± 11.0 min before exhaustion, significantly longer than in CHO ($p < 0.01$, fig. 4.7).

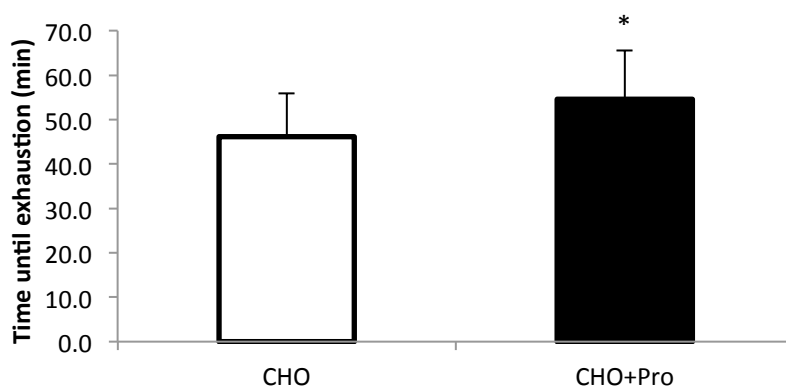


Figure 4.7: Time until exhaustion (min) during the performance test. *Significant difference between CHO+Pro and CHO ($p < 0.01$). $N=5$. Data are mean \pm SEM.

Data of VO_2 , RER, HR and RPE from the performance test are summarized in table 4.4. VO_2 was higher after 15 min and at exhaustion compared to 5 min in CHO, however not significant. In CHO+Pro, less increase in VO_2 was observed. No difference between trials was seen on VO_2 . RER was not different after 15 min or at exhaustion compared to after 5 min of biking. Also, no difference between trials was seen. HR was higher after 15 min and at exhaustion compared to after 5 min ($p < 0.05$). No difference was seen between trials. No increase in RPE was seen after 15 min compared to 5 min. At exhaustion RPE was higher than both 5 and 15 min of biking in both trials.

Data of blood glucose and blood lactate are presented in table 4.5. Blood glucose was lower during the performance test protocol compared to concentrations following recovery in CHO and CHO+Pro ($p < 0.05$). In both trials, blood glucose was higher at exhaustion compared to 5 and 15 min ($p < 0.05$), and no difference was seen between groups. Blood lactate increased from after the recovery period to 5, 15 min and at exhaustion during the performance test protocol ($p < 0.05$). In CHO+Pro, blood lactate was lower at 15 min and at exhaustion compared with the 5 min sampling ($p < 0.05$).

Blood lactate was not different between CHO and CHO+Pro during the performance test.

Table 4.4 *VO₂, RER, HR and RPE measured at 5, 15 min and at exhaustion during the performance test.*

	5 min	15 min	Exhaustion
VO₂ (ml·min⁻¹)			
<i>CHO</i>	3061.0±130.1	3301.6±167.7	3462.6±146.5
<i>CHO+Pro</i>	3128.4±145.8	3155.3±126.0	3209.9±146.7
RER (VCO₂·VO₂⁻¹)			
<i>CHO</i>	0.98±0.03	0.99±0.02	0.99±0.03
<i>CHO+Pro</i>	0.98±0.02	1.00±0.02	0.99±0.02
HR (beats·min⁻¹)			
<i>CHO</i>	154.0±2.9	158.4±3.1†	166.6±3.3†
<i>CHO+Pro</i>	154.0±3.8	158.8±3.7†	162.4±5.0†
RPE			
<i>CHO</i>	14.4±1.2	16.5±0.7	19.0±0.3‡
<i>CHO+Pro</i>	14.8±0.5	15.6±1.0	19.0±0.6‡

† =significant different from 5 min (p<0.05). ‡=significant different from 5 and 15 min (p<0.05). N=5. Data are mean±SEM.

Table 4.5 *Blood glucose and lactate following 5 h recovery (Post-Rec), at 5, 15 min and exhaustion during the performance test.*

	Post-Rec	5 min	15 min	Exhaustion
Blood glucose (mmol·l⁻¹)				
<i>CHO</i>	5.7±0.2†	3.8±0.2	3.4±0.2	4.8±0.1†
<i>CHO+Pro</i>	6.1±0.1†	4.1±0.2	3.2±0.1	4.6±0.4†
Blood lactate (mmo·l⁻¹)				
<i>CHO</i>	1.19±0.22†	2.39±0.31	1.90±0.42	2.03±0.39
<i>CHO+Pro</i>	1.03±0.16†	2.60±0.25	1.78±0.23†	1.75±0.25†

†=significant different from 5 min and 15 min (p<0.05). †=Post-Rec significantly different from 5, 15 min and exhaustion (p<0.05). N=5. Data are mean±SEM.

5. Discussion

The main finding in the present study was improved performance 5 h after exhaustion when CHO+Pro was supplemented, as compared to CHO. Muscle glycogen synthesis was similar during recovery when either CHO+Pro or CHO was ingested. After the performance test, muscle glycogen was reduced to similar levels in CHO+Pro and CHO. These data suggest that there are other mechanisms than glycogen resynthesis that explain the better recovery of performance after intake of CHO+Pro compared to CHO.

5.1 Initial glycogen depleting exercise

On the dietary intervention days, an exhaustive exercise was performed to deplete muscle glycogen and cause fatigue. Cycling to exhaustion was chosen to reduce the variability in muscle glycogen and fatigue before the dietary intervention period started, and minimum variability in duration between the test days was a goal for the study. Duration during the initial glycogen depleting exercise was not different before CHO+Pro and CHO (101 and 107 min, respectively), and we succeeded with a low day-to-day variability on duration. Using an identical glycogen depleting exercise, Rustad (2011) and Sollie (2013) found similar results to the present study. Cycling time to exhaustion was 82.4, 89.5 and 87.3 min on three occasions in Rustad (2011). Sollie (2013) reported the duration to be 115.1 and 108.9 min when a glycogen depleting protocol was completed two times. Training level and perhaps initial glycogen level of the subjects, could be a reason for the different duration on the glycogen depleting exercise between the present study, Rustad (2011) and Sollie (2013). Supporting this is a study by Hermansen et al. (1967) who found longer duration when trained compared to untrained subjects cycled to exhaustion at 77 % of VO_{2max} .

Training at intensities above 65 % of VO_{2max} utilizes muscle glycogen as the main energy substrate (Romijn et al., 1993; van Loon et al., 2001). In the current study, exercise at a workload corresponding to 72 % of VO_{2peak} significantly reduced muscle glycogen concentration from baseline (fig. 4.5). The muscle glycogen concentration was 152.1 ± 30.3 and 109.3 ± 31.3 mmol·kg dw⁻¹ after exhaustion before subjects ingested CHO or CHO+Pro, respectively. This was not different between trials ($p=0.22$), and

similar muscle glycogen concentrations following exhaustive exercise have been reported in many studies (Hickner et al., 1997; van Loon et al., 2000; Wallis et al., 2008; Betts & Williams, 2010). Wallis et al. (2008) reported muscle glycogen concentration to be 128 and 112 mmol·kg dw⁻¹ following an exhaustive glycogen depleting exercise. However, large variation in muscle glycogen content following prolonged endurance exercise has been reported (Betts & Williams, 2010). Hermansen et al. (1967) reported muscle glycogen concentration to be much lower (14.2 mmol·kg dw⁻¹) after exhaustive exercise at 77 % of VO_{2max}, compared to what Hill et al. (2013) reported after cycling to exhaustion at 90 % of VO_{2max} (390 mmol·kg⁻¹). This could be a result of the intensity used to exhaust the subjects as 75 % of VO_{2max} seems to be an optimal intensity for a large decrease in glycogen content (Betts & Williams, 2010). The current study exhausted subjects at a workload corresponding to 72 % of VO_{2peak}, and succeeded in partially depleting the muscle glycogen before the diet intervention.

Variability in muscle glycogen after the initial glycogen depleting exercise was moderate in the current study, and similar variation has been shown in other studies (Bergström et al., 1967; Zawadzki et al., 1992; van Loon et al., 2001; Betts & Williams, 2010; Hill et al., 2013). The variability in post exercise glycogen concentrations was higher than desired, and could be caused by the slightly longer duration of the initial glycogen depleting exercise during CHO+Pro compared to CHO. CHO was combusted during the whole exercise (table 4.4), and glycogen was likely to be the main energy substrate. Another reason for the moderate variability in post exercise muscle glycogen could be initial glycogen concentrations (Bergström et al., 1967). Bergström et al. (1967) reported higher muscle glycogen content after biking until exhaustion at 77 % of VO_{2max} when the initial muscle glycogen content was high, compared to normal. We did not take biopsies before the dietary intervention days to reduce the number of biopsies with one. This also implicates that we did not measure muscle glycogen concentration before the intervention days. Training and diet were standardised the last 24 h before the baseline biopsy and dietary intervention days to reduce variability in glycogen before the test days. The moderate variability in muscle glycogen after the initial glycogen depleting exercise could be a result of the preparations before the dietary intervention days. It could also be caused by the low variation in cycling time during the initial exercise. Regardless of the variability, muscle glycogen concentration was reduced to levels comparable with other groups. We therefore succeeded with the initial glycogen

depleting exercise, as variability was low in TtE and moderate in muscle glycogen after exercise.

Depletion of liver glycogen can cause a decrease in blood glucose during exercise (Suh, Paik, & Jacobs, 2007). Blood glucose declined during the initial glycogen depleting exercise. This is in agreement with several studies (Ivy et al., 1988a; Zawadzki et al., 1992; Nybo et al., 2003; Rustad, 2011). We did not measure liver glycogen content, but we might speculate that the liver glycogen concentration was reduced after the initial glycogen depleting exercise.

Blood lactate was stable during the glycogen depleting exercise (table 4.3). This indicates that subjects were exhausted by other mechanisms than an increase in muscle lactate. After exhaustion at the workload corresponding to 72 % of VO_{2peak} , 1 min intervals repeated to exhaustion at a workload corresponding to 90 % of VO_{2peak} . After the intervals, blood lactate was higher in CHO+Pro ($4.4 \text{ mmol}\cdot\text{l}^{-1}$) than CHO ($3.4 \text{ mmol}\cdot\text{l}^{-1}$) ($p < 0.05$). This could be a result of daily variation, as blood lactate concentration tended to be higher the whole initial glycogen depleting exercise before CHO+Pro compared to CHO ($p > 0.05$). However, these values are comparable to what Rustad (2011) and Ferguson-Stegall et al. (2011b) reported after 1 min intervals at 90 % of VO_{2max} .

Power output from the SRM ergometer was not correct for the last four subjects in this study. As a result, one subject had shorter duration of the exercise on the second test, whereas the remaining three had longer duration compared to day one. This led to 15 min longer TtE on the second test compared to the first (table 4.1). This was detected when the subject cycled shorter the second test day, and we decided to change the workload corresponding to VO_2 and HF for the remaining three subjects. By doing this, we managed to reduce muscle glycogen to similar levels (appendix 3), and exhaust the subjects as described in the method. However, duration of the exercise would be influenced by even a small variation in VO_2 and HR, and the subjects were therefore excluded for analysis from the TtE performance test. In addition, as a result of the failing ergometer, one of the four subjects was not able to partly perform the performance test successfully. He was therefore excluded for the analysis of the muscle biopsies taken after the performance test.

5.2 The recovery period

The diet during the recovery was designed to optimize the glycogen resynthesis. Therefore, an ingestion rate of $1.2 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ was chosen to serve as control for the experimental diet. This ingestion rate is recommended to saturate the glycogen resynthesis (Jentjens & Jeukendrup, 2003; Betts & Williams, 2010). The CHO+Pro diet was isocaloric with the CHO control diet, and the intervention diets were only ingested during the first 2 h of recovery. The subsequent 3 h of recovery, the subjects ingested a standardised lunch and drank a standardised CHO recovery drink. Protein ingestion during the first 2 h of recovery was therefore the only variable changed between the dietary intervention days. This enabled us to compare glycogen resynthesis and performance outcome as result of protein intake during the first 2 h of recovery.

An important strength to the present study was the standardised set up during recovery. Subjects rested all 5 h on a bed, only disturbed by assistants involved in the project. Toileting next door was allowed whenever needed, and subjects watched series on a computer or read a book while resting. This was reflected by very small variability between CHO and CHO+Pro in HR throughout the whole recovery period, as presented in section 4.3.

Glycogen resynthesis after exercise is dependent on amount CHO ingested, frequency of supplementation, glycogen concentration and training level of the subjects (Betts & Williams, 2010). Comparison of different studies is therefore challenging, and this can also be one reason for the large variability in reported muscle glycogen resynthesis during recovery (Betts & Williams, 2010). Glycogen resynthesis in the present study was not different between trials at $41.3 \pm 5.8 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ when CHO was ingested, and $46.8 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ when CHO+Pro was ingested. The glycogen resynthesis reported in the present study is comparable to the highest reported glycogen resynthesis rates after exercise, indicating a successful diet intervention (Betts & Williams, 2010), and well-trained subjects (Hickner et al., 1997).

After exercise, glycogen resynthesis remains low if no CHO is ingested (Ivy et al., 1988a), typically at $2 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$ (Ivy et al., 1988b). By increasing the amount of CHO ingested to $0.75 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, Ivy et al. (1988b) reported an increase in muscle glycogen resynthesis ($18.4 \text{ mmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Though, no further increase in glycogen

resynthesis was seen after $1.5 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ intake. In the present study, subjects ingested CHO immediately after exercise to activate glycogen synthesis rapidly. We provided CHO more frequent than Ivy et al. (1988b) because we wanted to optimize the glycogen resynthesis during recovery. The consensus is that high frequency of supplementation diets leads to higher glycogen resynthesis compared to less frequent feeding (Betts & Williams, 2010). The higher glycogen resynthesis reported in the present study compared to Ivy et al. (1988b) is therefore likely a result of frequency of the feeding.

Protein intake in combination with carbohydrate after exercise may increase glycogen resynthesis. In a study of Zawadzki et al. (1992), subjects were provided either 0.8 g CHO or $0.8 \text{ g CHO} + 0.4 \text{ g Pro} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ after a glycogen depleting exercise. The study found muscle glycogen resynthesis to be improved by CHO+Pro compared to CHO after 4 h recovery. However, Zawadzki et al. (1992) did not compare isocaloric trials, and the effect may have been a result of the extra available energy.

Intake of CHO+Pro compared to isocaloric CHO intake has in some studies been reported to improve glycogen resynthesis during recovery (Ivy et al., 2002; Berardi et al., 2006). Berardi et al. (2006) reported glycogen resynthesis after exercise when ingesting $0.8 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ and $0.4 \text{ g Pro} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ to be higher ($19.8 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$) compared to $1.2 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ingestion ($14.8 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$). Supplementation was given immediately, 1 and 2 h after exercise. We reported rate of muscle glycogen resynthesis to be similar after CHO and CHO+Pro intake, and several studies support this (van Loon et al., 2000; Howarth et al., 2009; Ferguson-Stegall et al., 2011b; Rowlands et al., 2011). van Loon et al. (2000) compared identical diets as the present study the first 2 h during recovery, and also reported the glycogen resynthesis after CHO+Pro ($35.4 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$) to be similar compared to CHO intake ($44.8 \text{ mmol} \cdot \text{kg dw}^{-1} \cdot \text{h}^{-1}$). van Loon et al. (2000) and the present study reported higher rate of glycogen resynthesis compared to Berardi et al. (2006), and this could be one reason for the different findings. Higher glycogen resynthesis is likely caused by feeding intervals (Betts & Williams, 2010), which was more frequent in van Loon et al. (2000) and the present study compared to Berardi et al. (2006).

Subjects were hypoglycaemic after exercise until the first supplementation of CHO or CHO+Pro caused a rapid increase in the blood glucose concentration (fig.4.1). In CHO+Pro, blood glucose peaked after 30 min. In CHO, blood glucose continued to increase until it peaked after 60 min of recovery. After 60 min, blood glucose was lower in CHO+Pro than CHO. This has also been supported by other groups (Ivy et al., 2002; Betts et al., 2007; Rustad, 2011). The reason for lower blood glucose during CHO+Pro compared to CHO could be improved glucose uptake from the blood or reduced glucose availability.

Plasma insulin concentration in the present study was low after the initial glycogen depleting exercise. After ingestion of CHO or CHO+Pro, plasma insulin increased rapidly until 150 min after exercise. After 120 min, plasma insulin was higher in CHO+Pro than CHO, and a tendency to higher plasma insulin concentration during the 2 h was seen in CHO+Pro compared to CHO ($p = 0.062$, fig. 4.2). Rustad (2011) reported a similar tendency on plasma insulin after CHO+Pro intake compared to CHO. Other groups also support this (Ivy et al., 2002; Hill et al., 2013), though this is not consistent (van Loon et al., 2000; Ferguson-Stegall et al., 2011b). Glucose as well as some amino acids are signal molecules for insulin secretion, and this could be one reason for the higher insulin concentration following CHO+Pro supplementation.

GS is activated by exercise and regulated by glycogen concentration (Jensen et al., 2006; Lai et al., 2010; Jensen et al., 2011). Insulin stimulates muscle glycogen synthesis (Lai et al., 2010; Jensen et al., 2011), and the mechanism behind this involves the proteins PKB, GSK3 and GS (fig 2.1). Compared to baseline in the present study, GS^{ser641} phosphorylation was reduced after exercise in both CHO and CHO+Pro (fig 4.5). Thus, GS was active before any recovery supplementation was ingested. Active GS could be a result of the exercise and/or low muscle glycogen content (Lai et al., 2010). GS was also active after the 5 h recovery, as GS^{ser641} phosphorylation was still reduced in CHO and CHO+Pro. One reason for the active GS could be muscle glycogen content, which was below resting levels (fig. 4.5). GS was also in its active form after the performance test, as GS^{ser641} phosphorylation was reduced in CHO and CHO+Pro. Again, this could be because of the preceding exercise and glycogen content. No differences were seen between CHO and CHO+Pro and protein ingestion did not affect GS activity.

Two upstream targets for GS are PKB and GSK3 (fig 2.1). PKB is activated by insulin binding to the muscle cell membrane (Lai et al., 2010). In its active form, PKB phosphorylates GSK3 (Jensen et al., 2011). Activity of GSK3 leads to inhibition of GS, and GSK3 activity is down regulated when the enzyme is phosphorylated. In the present study, PKB^{ser473} phosphorylation was unchanged after exercise compared to baseline (fig 4.6.C). Plasma insulin concentration was low after exercise (fig. 4.2), and this supports that the active form of GS after the initial glycogen depleting exercise was a result of the preceding exercise and/or low muscle glycogen concentrations. After 5 h recovery, PKB^{ser473} was similar between trials, reflecting the similar plasma insulin concentrations (fig 4.2). After the TtE performance test, PKB^{ser473} was still not significantly affected. We did not measure insulin at this time point. However, insulin secretion after exercise remains low until CHO or protein intake (Ivy et al., 2008), and the PKB^{ser473} phosphorylation in the present study was likely down regulated as a result of reduced insulin binding.

Insulin binding and exercise will also lead to translocation of GLUT-4 to the muscle membrane. The mechanism behind insulin stimulated GLUT-4 translocation is via PKB and AS160 (fig. 2.1). PKB phosphorylates AS160 and TBC1D1, whose activity leads to GLUT-4 translocation by inhibition of Rab GTPase (Jensen et al., 2011). AS160^{ser588} phosphorylation was unchanged from baseline after the initial glycogen depleting exercise (fig 4.6.B). Thus, AS160 was active after exercise. This can be a result of the exercise, as contraction activates AS160 (Lai et al., 2010). AS160 was equally more phosphorylated after recovery than at baseline in both trials, which is likely a result of similar insulin concentrations (fig.4.2).

5.3 Recovery of performance

The present study reported improved recovery of performance when ingesting CHO+Pro compared to CHO during the first 2 h of recovery. The five subjects with an adequate performance test had a positive performance effect from protein intake during the first 2 h following exercise. A positive effect of protein on performance has previously been reported from our laboratory (Rustad, 2011; Sollie, 2013). This is supported also by several studies from other research groups (Williams et al., 2003; Saunders et al., 2004; Betts et al., 2007; Berardi et al., 2008; Ferguson-Stegall et al., 2011b; Lunn et al., 2012).

In the study of Rustad (2011), and Sollie (2013), the initial exercise and diet during the first 2 h after exhaustion were identical to the current study. Rustad (2011) used a TtE performance test, and found a positive effect of protein ingestion on performance approximately 18 h following an exhaustive exercise. TtE in Rustad's study was 14 min longer when CHO+Pro was supplemented, as compared to an isocaloric CHO diet. This corresponds well with the present study, where TtE was 8 min longer in CHO+Pro compared to CHO after 5 h of recovery. Sollie (2013) also found improved performance on a TT 18 h after an exhaustive exercise when CHO+Pro was ingested compared to CHO. Rustad (2011) showed CHO+Pro intake during the first 2 h after an exhaustive exercise caused improved endurance capacity compared to CHO. Sollie (2013) showed that the subjects were able to cycle at a higher workload when subjects ingested CHO+Pro compared to CHO the first 2 h after an exhaustive exercise. Protein intake immediately after exercise may therefore improve subsequent performance in terms of both capacity and workload.

Data from other groups also supports the finding of improved performance when protein has been ingested during recovery. Williams et al. (2003) provided the subjects with 0.15 g CHO or 0.40 g CHO + 0.10 g Pro·kg⁻¹·h⁻¹ after a 2 h exercise at 65-75 % of VO_{2max}. After 4 h of recovery, TtE at 85 % of VO_{2max} was improved with 55 % when CHO+Pro was ingested. CHO+Pro was higher in energy content than CHO, and the enhanced performance was likely because of more energy provided and 128 % greater muscle glycogen resynthesis. A study by Saunders et al. (2004) found 29 % enhanced performance when providing the subjects 0.48 g CHO + 0.12 g Pro compared to 0.48 g CHO·kg⁻¹·h⁻¹ during exercise. A second TtE at 85 % of VO_{2max} was performed the day after, and CHO+Pro improved performance by 40 % compared to CHO. Saunders et al. (2004) also provided more energy during CHO+Pro, which may be the reason for the enhanced performance.

There are several studies comparing isocaloric diets of CHO+Pro and CHO that do not find a positive effect of protein ingestion on performance recovery (Table 2.2). Different exercise protocols, diets, performance tests and subjects make a comparison of studies challenging (Betts & Williams, 2010). However, according to table 2.2, studies using running activity or repeated sprint performance tests, are also usually the studies where no effect of protein intake has been reported (Betts et al., 2005; Millard-Stafford

et al., 2005; Berardi et al., 2006; Betts et al., 2007; Rowlands et al., 2007; Rowlands et al., 2008; Rowlands & Wadsworth, 2011; Nelson et al., 2012). Tsintzas, Williams, Boobis, and Greenhaff (1996) reported muscle glycogen to be $285.4 \text{ mmol} \cdot \text{kg dw}^{-1}$ after running to exhaustion at 70 % of $\text{VO}_{2\text{max}}$. This is higher than what most others, and the present study, reported after cycling to exhaustion (Betts & Williams, 2010). This could indicate that running performance at 70 % of $\text{VO}_{2\text{max}}$ is determined by other mechanisms than muscle glycogen.

Protein ingestion during recovery has been investigated on repeated sprint ability in several studies (table 2.2). The majority of these studies have been conducted on the laboratory of Rowlands et al. (Rowlands et al., 2007; Rowlands et al., 2008; Rowlands & Wadsworth, 2011; Thomson et al., 2011; Nelson et al., 2012), and two studies reported a positive outcome (Rowlands et al., 2008; Thomson et al., 2011). When performing repeated sprints 15-18 h after an initial exercise, intake of CHO with a high proportion of protein was reported not to affect performance, compared to both a diet with CHO and a low protein proportion (Rowlands et al., 2007), and only CHO (Rowlands et al., 2008). One reason could be that 18 h recovery period was too short for sprint performance to be influenced by protein intake, which was confirmed when the recovery period was increased to 39 and 60 h (Rowlands et al., 2008; Thomson et al., 2011). Both Rowlands et al. (2008) and Thomson et al. (2011) argued that less disruption to skeletal muscle integrity caused improved performance when CHO+Pro compared to a CHO diet (Rowlands et al., 2008), or a CHO diet with a small proportion of protein (Thomson et al., 2011). Supporting this was a study by Nelson et al. (2012) where reduced plasma creatine kinase was reported after CHO+Pro intake compared to CHO after exercise. Rustad (2011), Sollie (2013) and the present study found improved performance in an earlier phase of recovery than any of the studies conducted at the laboratory of Rowlands. We tested performance as TtE, as did Rustad (2011), and Sollie (2013) tested performance as a prolonged TT. Workload and duration of the performance test could be reasons for the different outcome in the present study compared to the studies conducted on the laboratory of Rowlands.

Berardi et al. (2006) compared 1.2 g CHO ingestion after exercise with 0.8 g CHO+0.4 g Pro $\cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ on recovery of performance. Surprisingly, no effect on performance during a 1 h TT was seen even though muscle glycogen was 15 % higher after 6 h of

recovery in CHO+Pro compared to CHO. Muscle glycogen utilization and heart rate during the TT were similar between CHO and CHO+Pro, and they argue that the subjects did not motivate themselves enough to utilize the extra amount of muscle glycogen.

Two years later, Berardi et al. (2008) performed another study investigating the effect of CHO+Pro on recovery of performance. Subjects received the same recovery diet and performance was tested with a 1 h TT as the previous study, however more feedback was given during the TT. They reported improved recovery of performance after CHO+Pro compared to CHO. FA oxidation rate was higher during CHO+Pro compared to CHO, and they argue that a glycogen sparing effect of protein intake could be one reason for the improved performance. They further compared the study with the previous findings from 2006, and speculated that muscle glycogen content could have been higher after recovery in CHO+Pro compared to CHO.

The findings in the study of Berardi et al. (2008) have also been supported by other groups (Thomas et al., 2009; Ferguson-Stegall et al., 2011b; Lunn et al., 2012). In the study of Ferguson-Stegall et al. (2011b), subjects cycled 90 min at 70 % of VO_{2max} before five 1 min intervals at 90 % of VO_{2max} were performed. After this, either 1.34 g CHO or 1.02 g CHO + 0.32 g Pro \cdot kg⁻¹ \cdot h⁻¹ was ingested for 2 h. Cycling time during a 40 km TT was in CHO+Pro (79 min) significantly faster than CHO (86 min). Muscle glycogen resynthesis during 4 h recovery was not different between CHO and CHO+Pro. This is in agreement with our data, where improved performance was not caused by glycogen resynthesis during recovery. Ferguson-Stegall et al. (2011b) reported increased phosphorylation of mTOR and rpS6 in CHO+Pro intake compared to CHO after 45 min of recovery. This indicates that CHO+Pro activated the protein synthesis, which could be one reason for the improved recovery of performance compared to CHO.

Type of performance test could be of importance when assessing the effect of protein intake on recovery of performance. This has already been discussed earlier on repeated sprint ability, where more than 18 h of recovery is needed for protein intake to be effective. We used a TtE at a workload corresponding to 72 % of VO_{2peak} because it has been shown to be valid in assessing performance after different initial glycogen

concentrations (Bergström et al., 1967), and as effect of protein ingestion (Rustad, 2011; Thomas et al., 2009). The workload in the present study was chosen out of experience. Because of the biopsies, we did not expect to recruit elite cyclists for the study and less trained subjects were included. Thus, higher workload would be very tough, and exercise could terminate as a result of increasing levels of muscle metabolites (Mann, Lamberts, & Lambert, 2013). If the workload was too low, fat would be the primary energy source and the exercise would not deplete the muscle glycogen. It should however be recognized that TtE performance tests may have a 10 % coefficient of variety (Currell & Jeukendrup, 2008), which should be acknowledged when interpreting the results.

5.4 Peripheral or central fatigue?

Exhaustion has been reported to occur when muscle glycogen content was depleted after cycling at 77 % of VO_{2max} (Hermansen et al., 1967). An interesting finding of the present study was that muscle glycogen content was similar between CHO+Pro and CHO after the performance test (fig.4.5). Even though CHO+Pro improved TtE, no significant difference in muscle glycogen combustion during the performance test was found ($p=0.26$), and muscle glycogen was reduced to $145 \text{ mmol}\cdot\text{kg dw}^{-1}$ in CHO, and $158 \text{ mmol}\cdot\text{kg dw}^{-1}$ in CHO+Pro. Much lower concentrations have been reported in the same musculature following cycling to exhaustion (Hermansen et al., 1967). Exhaustion was therefore not a result of limited muscle glycogen availability or reduced CHO combustion. This is supported by the RER values in the present study, indicating that a high proportion of CHO was combusted throughout the performance test (table 4.4).

Several theories have tried to link decrease in muscle glycogen to exhaustion (Abbiss & Laursen, 2005). Supporting a peripheral model of fatigue is depletion of muscle glycogen and impaired sarcoplasmic reticulum (SR) function (Ørtenblad et al., 2013). This was shown in a study by Ørtenblad et al. (2011), who reported reduced SR Ca^{2+} release when muscle glycogen concentration was depleted from $540 \text{ mmol}\cdot\text{kg dw}^{-1}$ at rest to $167 \text{ mmol}\cdot\text{kg dw}^{-1}$ after a TT. Subjects in the present study also depleted their muscle glycogen during exercise, and it is tempting to speculate that SR function was reduced at exhaustion following both the initial glycogen depleting exercise and the performance test.

Reduction in muscle glycogen content could also lead to central fatigue. According to the central governor model of fatigue, performance is regulated by feedback mechanisms between the muscle and the brain to keep the body in homeostasis (Noakes, 2012). Muscle glycogen may be part of an efferent feedback mechanism (Rauch et al., 2005). In the study of Rauch et al. (2005), subjects performed two TT where initial muscle glycogen was manipulated to obtain high or normal glycogen concentrations. Performance was greater when muscle glycogen was high, and muscle glycogen concentration was almost equal after exercise at 100 and 72 mmol·kg dw⁻¹. They argue that the subjects selected a pace according to available muscle glycogen, supporting the central governor model. An analogue could be the gauge for remaining fuel on a car. The driver (governor) would drive more conservatively to save fuel if the tank initially was half-empty compared to a full tank. The present study used a TtE test, and thus, the subjects could not select a conservative pace. The decrease in muscle glycogen concentration could have initiated afferent signals, and subjects would have to increase the central drive to postpone exhaustion. RPE increased towards the end of both the initial glycogen depleting exercise (table 4.2) and performance test (table 4.4), indicating enhanced central drive to withstand the workload. Central fatigue could therefore have led to exhaustion as result of impaired ability to adequately activate the motor neurons.

Hypoglycaemia during prolonged exercise may also be involved in fatigue and increased RPE. In the present study, blood glucose decreased during the initial glycogen depleting exercise (table.4.3), while RPE increased (table 4.2). In a study by Nybo et al. (2003), subjects performed a 3 h exercise at 60 % of VO_{2max} while supplemented with CHO (0.7 g CHO·kg⁻¹·h⁻¹) or no energy. During exercise when CHO was provided, blood glucose remained at 5 mmol·l⁻¹ and RPE was not significantly changed. When no energy was provided during exercise, blood glucose decreased from 5.2 to 2.9 mmol·l⁻¹ and RPE increased from 12 to 17 on Borg scale. The decreased blood glucose caused reduced cerebral metabolism (Nybo et al., 2003). This could in the present study explain the increased RPE during the initial glycogen depleting exercise. However, our data during the performance test are not consistent with this. We reported higher RPE at exhaustion compared to 5 and 15 min into the performance test (table 4.4). Interestingly, blood glucose also increased towards the end of the performance test (table 4.5). The increased RPE during the performance test in the present study was

therefore not a result of insufficient blood glucose, and other mechanisms than blood glucose availability to the brain must explain the increased RPE during the performance test.

Indeed, both the initial glycogen depleting exercise and the TtE performance test reduced muscle glycogen in the present study. However, a considerable amount of muscle glycogen was still available after both the initial glycogen depleting exercise and TtE after CHO+Pro and CHO. CHO was still combusted at exhaustion, and other mechanisms than insufficient availability of glucose or glycogen must be the explaining factor for the exhaustion. A reduction in central drive as a result of afferent signalling to prevent further muscle glycogen depletion could be the cause of exhaustion, as exercise terminated at similar levels after CHO and CHO+Pro. However, peripheral fatigue could also cause exhaustion, as low muscle glycogen concentrations may decrease SR function. We cannot conclude if exhaustion was a result of reduced central activation of the motor neurons, as we did not test this specifically. The mechanism behind fatigue following prolonged exercise is still unclear, and more research is needed (Abbiss & Laursen, 2005).

5.5 Protein synthesis

CHO intake has been shown to reduce muscle protein degradation (Børsheim et al., 2004a), but amino acids are required for activation of muscle protein synthesis (Blomstrand et al., 2006; Howarth et al., 2009). After 2 h of cycling exercise, Howarth et al. (2009) provided 1.2 g CHO + 0.4 g Pro·kg⁻¹·h⁻¹ in a recovery diet for 3 h. A positive nitrogen balance was found after 4 h recovery when protein was ingested, and was negative in both CHO content and caloric matched controls. The positive nitrogen balance was related to positive muscle amino acid balance. Additionally, Howarth et al. (2009) reported the fractional muscle protein synthesis to increase after CHO+Pro intake, compared to CHO intake. This is supported by studies investigating the effect of CHO+Pro intake on activity of proteins involved in muscle protein synthesis (Ferguson-Stegall et al., 2011b; Rowlands et al., 2011). In the present study, nitrogen balance was measured based on urine and whole body basis. Thus, we cannot specify the whereabouts of any remaining nitrogen; it could be in the muscle cell or other tissues. However, Howarth et al. (2009) found protein intake to cause positive protein nitrogen balance, positive muscle amino acid balance and increased fractional muscle protein

synthesis rate after an exercise and recovery period comparable to the present study. We could therefore speculate that the nitrogen was located in the muscle cell when the nitrogen balance was positive.

Positive whole body protein balance is reflected by positive nitrogen balance. The present study found a positive nitrogen balance when CHO+Pro was supplemented during the first 2 h of recovery (fig 4.4). During CHO, average nitrogen balance was negative, however not significantly. This was calculated based on a total protein ingestion of 1.2 g Pro·kg⁻¹ in CHO+Pro, and 0.5 g Pro·kg⁻¹ in CHO. Supporting the positive nitrogen balance in CHO+Pro is a study of Tarnopolsky et al. (1988). Tarnopolsky et al. (1988) reported a positive nitrogen balance based on urine, feces and sweat when endurance athletes ingested 1.6 g Pro·kg⁻¹ for 24 h. In the present study, positive urine nitrogen balance was achieved when protein was supplemented during the first 2 h of recovery. Nelson et al. (2012) also found nitrogen balance to be positive when providing protein in the immediate recovery period. Protein ingestion immediately after exercise may therefore be important for achieving a positive nitrogen balance.

Muscle protein synthesis may be activated by PKB activity via the mTOR pathway (Egan & Zierath, 2013). PKB can also inhibit protein degradation via the FOXO pathway. Ivy et al. (2008) reported PKB activity to be upregulated after 45 min of recovery when CHO+Pro intake was compared to no energy after cycling exercise. Supporting this is a study by Ferguson-Stegall et al. (2011b). They reported reduced FOXO activity after CHO+Pro and CHO intake, likely as a result of phosphorylation by PKB. mTOR activity was increased by CHO+Pro compared to CHO after 45 min. After 4 h of recovery, both FOXO and mTOR activity was unaffected by CHO+Pro compared to CHO. The present study found no effect of CHO+Pro or CHO feeding on PKB 5 h after exercise, and this was likely a result of the time course of for this enzyme.

We did not measure muscle protein balance. However, it is tempting to speculate that the improved recovery of performance in CHO+Pro partly was a result of enhanced amino acid uptake and protein synthesis in the muscle cell.

5.6 Limitations of the study

Valid performance data was only obtained on five subjects. A total of nine subjects completed the study, and were included to increase the statistical power. However, technical issues led to exclusion of the latter four subjects, which increases the risk of a statistical type 1 error. However, the possibility of the performance outcome from the present study to be a result of chance alone was less than 1 %.

A biopsy taken after 2 h of recovery would provide evidence for the state in glycogen and protein synthesis caused by CHO+Pro or CHO. We did not take this, and may therefore only speculate on the muscle state at this time point. We could in addition have done more analysis on the muscle tissue. Further analysis on the biopsies on gene expression and phosphorylation of proteins involved in muscle protein synthesis could explain the reason for improved recovery of performance after CHO+Pro.

The present study did not measure muscle protein balance. This could have been done by infusion of stable isotopes and mass spectrometry analysis on the muscle biopsies. For protein balance analysis, we measured urine nitrogen concentrations. This underestimates nitrogen content, as nitrogen also is adherent in hair, sweat and feces.

We did not control the origin of fatigue. This could have been measured by electrically stimulating the muscle during a maximal voluntary contraction. If the force generation increased during electrically stimulation, central fatigue would be a possible cause of exhaustion. We did not measure this, and can therefore not differentiate between central or peripheral fatigue.

5.7 Importance of the study and practical implications

This study shows that composition of the diet after exercise is important for recovery of same day performance. Inclusion of protein in the recovery diet improved time until exhaustion, compared to a CHO diet. Further, we compared energy-matched diets, and found that the beneficial effect on performance was a result of protein ingestion *per se*. We also found that other mechanisms than muscle glycogen resynthesis enhanced recovery of performance. The study further emphasizes the importance of composition of recovery diet when an athlete is to perform multiple times on the same day.

The mechanism behind the improved performance is still unclear. It would have been interesting to include tracer methodology on a similar study design to elucidate the importance of protein intake on muscle protein synthesis. This has only been done on studies where performance has not been tested (Howarth et al., 2009), and application of the methodology during a performance test could provide evidence of the faith of the amino acid.

We only investigated our hypothesis on male volunteers. Future research should investigate the effect of protein inclusion in a recovery diet on women, as they may respond differently to protein intake.

5.8 Summary and conclusions

This study found a positive effect of protein ingestion on recovery of performance. When protein was ingested in a combination of carbohydrates during the first 2 h of recovery, time to exhaustion was significantly improved as compared to an energy matched carbohydrate diet.

The effect of carbohydrate and protein ingestion on glycogen resynthesis is equivocal. Compared to isocaloric diets containing only CHO, some have found improved resynthesis, while others has found unaffected resynthesis. We found muscle glycogen resynthesis during recovery to be similar between CHO and CHO+Pro. Further, muscle glycogen utilization during the performance test was also similar. We therefore conclude that other mechanisms than glycogen resynthesis is the reason for the improved performance.

Nitrogen balance was positive during CHO+Pro. Positive nitrogen balance may reflect and underlying positive muscle protein balance, which is associated with improved recovery of the muscle cell. The present study found that protein ingestion immediately after exercise might turn whole body nitrogen balance to be positive. This could be one mechanism that cause improved performance after CHO+Pro compared to CHO.

The conclusions for the hypotheses investigated in this thesis is thus:

1. Recovery of performance was improved 5 h after an exhaustive exercise when ingestion of protein in combination of carbohydrates was compared to an isocaloric carbohydrate diet ingested during the first 2 h of recovery.
2. Muscle glycogen resynthesis was not affected by protein ingestion in combination with carbohydrates compared to ingesting an isocaloric carbohydrate diet during the first 2 h of recovery.

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Appendix

Appendix 1. Solutions used in western blot analysis

Homogenizing buffer

Solution	Conc. in solution	15 ml
H ₂ O		9953 μ l
0.926 M HEPES ph 7.4	50 mM	810 μ l
3 M NaCl	150 mM	750 μ l
100 mM Na ₄ P ₂ O ₇	10 mM	1500 μ l
500 mM NaF	30 mM	900 μ l
100 mM Na ₃ VO ₄	1 mM	150 μ l
200 mM EDTA	10 mM	750 μ l
250 Benzamidin	2.5 mM	150 μ l
Sigma cocktail (P-8340)	0.5 μ l · 2 mg dw ⁻¹	37.5 μ l

PBS tween 20 washingbuffer

Salt	Manufacturer	Amount
80 mM Na ₂ HPO ₄	Merck, 6580	56.92 g
20 mM NaH ₂ PO ₄	Merck 6343	11.04 g
100 mM NaCl	BDH, 27810	23.36 g
0.1 % Tween-20	Sigma, P-1379	4 ml
H ₂ O		ad 4 litres

pH adjusted to 7.4

Transfer buffer

Solution	Amount
10 X running buffer	200 ml
Etanol	200 ml
H ₂ O	Ad 2 litre

Running buffer

Solution	Amount
10 X running buffer	100 ml
20 % SDS	5 ml
H ₂ O	Ad 1 litre

10 X running buffer

Solution	Manufacturer	Amount
Tris	Sigma, T-1503	121.1 g
Glycin	Bio-Rad. Cat. 161-0724	577 g
H ₂ O		Ad 4 litre

SDS

Solution	Manufacturer	Amount
	Bio-Rad, cat. 161-0301	9 g
H ₂ O		ad 45 ml

H₂O was dH₂O in all buffers.

Appendix 2. Reagents used in glycogen analysis.

Analysis buffer (on ice): 25 ml (1 plate)

H ₂ O _{millipore}	22.4ml
1M Tris pH 8.1	2.5 ml
1M MgCl ₂	25 µl
0.5M DTT	25 µl
100mM ATP	75 µl
50mM NADP+	15 µl
1750U/ml G-6-PDH	1.1 µl
1% BSA	6.3 µl

20 mM Tris / 0.02% BSA pH 8.1: (thaw)

H ₂ O _{millipore} :	4.8 ml
1 M Tris pH 8.1:	100 µl
1% BSA:	100 µl

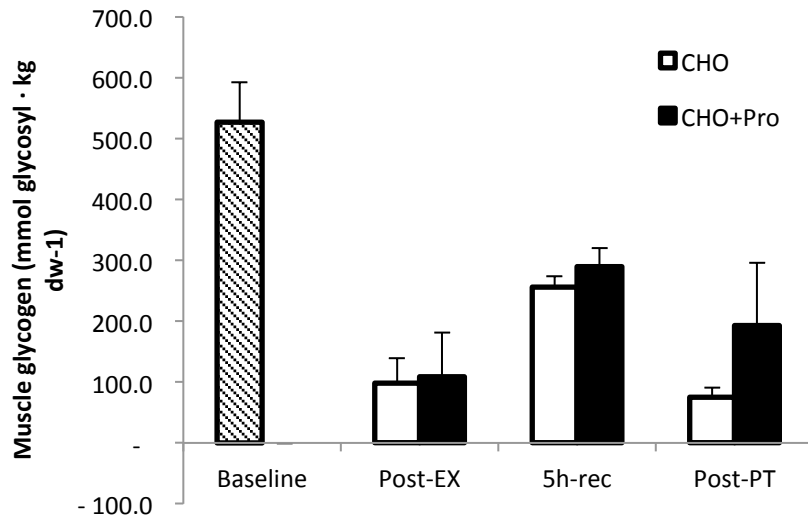
Hexokinase : (on ice)

HK 1500 U/ml:	15 µl
20mM Tris w/0.02% BSA pH 8.1:	1.80 ml
Will be 0.30 U/ml HK in sample	

Hydrolysis buffer:

1 N HCl:	2.5 ml
0.2 N NaOH:	12.5 ml

Appendix 3. Statistical data of muscle glycogen for subjects 6-9.



Muscle glycogen concentrations for subjects 6-9. Subject number eight is excluded from Post-PT.

Appendix 4 Study information provided the subjects

Deltagerinformation

Forsøgets titel: **Effekt af protein+kulhydrat sammenlignet med kulhydrat indtag på præstationen 5 timer efter et intensivt cykellarbejde**

Forespørgsel om deltagelse i videnskabeligt forsøg

Før du beslutter, om du vil deltage i forsøget, skal du fuldt ud forstå, hvad forsøget går ud på, og hvorfor vi gennemfører forsøget. Vi vil derfor bede dig om at læse følgende deltagerinformation grundigt.

Hvad ved vi?

Under aktivitet med høj intensitet er kulhydrat (glykogen) den vigtigste energikilde og udmattelse/træthed opstår når muskelen har tømt glykogenlageret. Hård fysisk aktivitet fører også til ødelæggelse af muskelproteiner, og det er vigtigt at kroppen tilføres protein for at genopbygge musklerne. For at udnytte pauserne mellem træninger og konkurrencer optimalt er kostindtagelsen vigtig. Indtagelse af protein og kulhydrat i pausen mellem træningerne er gavnligt for restitutionen af udholdenheden. Årsagerne til den positive effekt af protein på restitutionsprocessen er imidlertid ikke kendt

Formålet med forsøget

Målet med studiet er at undersøge hvorfor man præsterer bedre efter at have drukket en drik bestående af kulhydrat sammen med protein, sammenlignet med en drik bestående udelukkende af kulhydrat.

Deltagerne

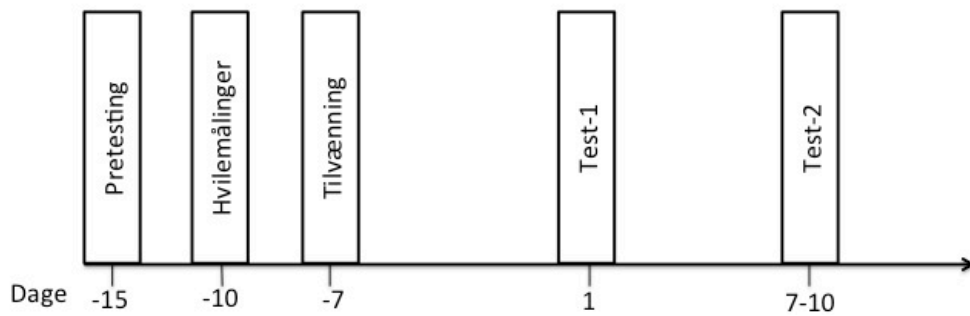
I alt 12 raske, veltrænede mandlige frivillige forsøgspersoner i alderen 18-40 år, skal gennemføre forsøget i løbet af foråret 2013.

- Inklusionskriterier: Veltrænede raske mænd med minimum 2 træninger om ugen på cykel.
- Eksklusionskriterier:
 1. Personer, der indtager medicin eller kosttilskud, der kan have indflydelse på glykogensyntesen.
 2. Personer der ikke er i stand til at gennemføre forsøgsprotokollen.

Plan for studiet

Forsøgspersonere skal gennemføre to testdage, hvor de først gennemfører en hård cykeltræning for at tømme glykogenlagrene og derefter indtager konrollerede diæter med forskellig sammensætning. På den ene testdag får forsøgspersonerne kulhydrat sammen med protein og på den anden testdag får de ren kulhydrat. De to diæter gives i randomiseret rækkefølge

Forsøgspersonerne skal i tillæg møde 3 gange før de to testdage til forskellige forundersøgelser (se under). Fremmøder på laboratoriet er vist i figur 1.



Figur 1. Oversigt over studiets tidsforløb.

Under prætestingen måles den maksimale iltoptagelseshastighed og vi beregner watt som skal bruges i de to test. Hvilemålinger indebærer at en muskeltvævsprøve og blodprøve tages fra det ene ben på en morgen efter en natts faste. Nogle dage efter gennemføres en tilvænningstest, hvor der cykles i 40 minutter ved moderat belastning (70 % af maksimal iltoptagelseshastighed).

På Test-1 og Test-2 møder forsøgspersonen på laboratoriet klokken 8.00. Aktiviteterne er identiske på de to dage og indeholder to arbejds-sessioner. Dagene adskiller sig kun ved diætten som serveres de første to timer efter den første arbejds-session. Totalt tages der 16 blodprøver ved hjælp af et kateter placeret i en underarmsvene (Total 110 ml). Det samlede blodtab er væsentligt mindre end hvad der eksempelvis gives ved bloddonation. Det tages en muskeltvævsprøve umiddelbart efter den første arbejds-session, så igen efter 2 og 5 timer samt en efter dagens anden arbejds-session. I løbet af studiet tages der 9 muskeltvævsprøver (ca. 200 mg pr vævsprøve). Dagen før hviledagmålinger registreres kostindtagelse og træningaktivitet. Kostindtagelsen repeteres før de to testdage. Træning dagen før skal undgås eller begrænses til moderat konditionstræning.

Nytte ved forsøget

Vi håber at resultaterne fra dette studie kan give os viden om årsagerne til at proteinindtagelse sammen med kulhydrat umiddelbart efter en hård træning forbedrer præstationen i timerne herefter.

Risiko forbundet med forsøget

Anlæggelsen af katetre er forbundet med minimale gener på niveau med oplevelsen af at få taget en blodprøve, og kan således være forbundet med lette smerter, og der er en beskedent risiko for infektion og blodansamling ved indstiksstedet. Blodtabet i løbet af en testdag forventes ikke at medføre symptomer.

I forbindelse med udtagningen af muskeltvævsprøver (ca. 200 mg per prøve) lægges der lokal bedøvelse i området omkring selve prøvestedet, hvorved selve indgrebet oftest ikke er forbundet med nævneværdig smerte, men snarere en ubehagelig trykkende fornemmelse, som forsvinder så snart prøven er taget. Efter indgrebet kan der forekomme lokal ømhed i muskulaturen, ligesom der kan forekomme en let blodansamling i området (ses som et blåt mærke). De gentagne biopsier taget over 6 timer ifm. gennemførelsen af arbejdsprotokollen vil forstærke denne ømhed. Eventuelt kan der være ømhed i dagene efter ligesom et ”trælår”. Ømheden vil stadig kun strække sig over et par dage og en minimale risiko for infektion. Der er i sjældne tilfælde set påvirkning af nerve ved indstiksstedet i form af nedsat følesans i huden. For at fremme sårhelingen anbefaler vi at undgå kraftig fysisk aktivitet det følgende døgn. Personalet

på Institut for Folkesundhed – Idræt har mange års erfaring med disse procedurer.

Håndtering af biologisk materiale.

Det indsamlede biologiske materiale (blodprøver og muskelprøver) vil blive opbevaret anonymiseret i fryser på de involverede forskningsinstitutioner. Loven om behandling af personoplysninger vil blive overholdt. Efter at alle analyser er afsluttet eller senest 5 år efter afslutning af sidste forsøgsgang, vil evt. resterende biologisk materiale blive destrueret.

I forbindelse med studiet vil der blive etableret en forskningsbiobank bestående af plasma- og vævsprøver. Deltagerne vil blive informeret om og give skriftlig tilladelse til at prøverne gemmes i en forskningsbiobank. Prøvene mærkes med et IDnummer som ikke kan kobles til forsøkspersonens personalia. Mappen med tilhørende identifikation, vil blive opbevaret et separat aflåst sted, således at det kun er de af forskergruppen autoriserede, der har adgang dertil. Patienten vil i samtykkeerklæringen blive informeret om deres rettigheder vedr. indsamling til forskningsbiobanken.

Forventninger til dig i forbindelse med studiet

Foruden stabile træningsvaner i studieperioden forventer vi, at du møder op til de planlagte forsøg, og at du følger forsøgsansvarliges anvisninger.

Udelukkelse fra studiet

Hvis du på baggrund af resultaterne fra screeningsbesøget (dag -15) ikke opfylder inklusionskriterierne, kan du ikke deltage i studiet. Den forsøgsansvarlige kan desuden vælge at trække dig ud af studiet, såfremt du ikke følger de anviste retningslinjer for kosten, motionsvaner osv.

Frivillighed

Det er frivilligt at deltage i studiet, og du kan når som helst og uden at give en grund trække dit samtykke tilbage, uden at det vil få nogen konsekvenser.

Finansiering

Projektet er et samarbejde mellem Aarhus Universitet og Norges Idrættshøgskole, Oslo. Projektet er en delprojekt som indgår i studiet «**Udholdenhedstræning og restitutionsevne: implikationer for optimal ernæring og præstationsoptimering**» som støttes af Team Danmark (1.200.000 kr) og Arla (1.000.000 kr). Udgifter i forbindelse med analyser i Oslo finansieres af Norges Idrættshøgskole.

Udover gennem sit ansættelsesforhold på Aarhus Universitet har den projekt/forsøgsansvarlige ingen økonomisk tilknytning (ansættelsesforhold, mulighed for økonomisk vinding ect.) ift. de nævnte bidragsydere.

Ved gennemførelse af hele forsøget udbetales en økonomisk skattepligtig kompensation på 4000 kr. til dig som forsøgsperson. Dersom du vælger at trække dig fra studiet vil en forholdsmæssig kompensation udbetales.

Hvis du, efter at have haft mulighed for at læse deltagerinformationen hjemme, stadig er interesseret i studiet, inviteres du ind til en mundtlig samtale, hvor projektansvarlig vil kunne besvare eventuelle spørgsmål. Du har mulighed for at medbringe en bisidder til samtalen. Hvis du beslutter dig for at deltage i forsøget, vil vi bede dig underskrive en

Samtykke- og fuldmagtserklæring. Samtykke- og fuldmagtserklæring omfatter at de involverede forskningsinstitutter kan udveksle og behandle nødvendige oplysninger, der er nødvendige for forsøget.

Husk du har ret til mindst en uges betænkningstid, før du beslutter om du vil underskrive samtykke- og fuldmagtserklæring.
Deltagelse i studiet er frivilligt, og du kan på ethvert tidspunkt trække dig fra undersøgelsen.

Har du yderligere spørgsmål vedrørende undersøgelsen, må du gerne kontakte projektansvarlig: Lektor, PhD, Kristian Overgaard.

Vi beder dig også læse vedlagte materiale ”Forsøgspersons rettigheder i et sundhedsvidenskabeligt forskningsprojekt”.

Projektansvarlig:

Lektor, PhD, Kristian Overgaard
Institut for Folkesundhed, Sektion for Idræt, Aarhus Universitet,
Dalgas Avenue 4, 8000 Århus C
Mail: ko@sport.au.dk

Appendix 5 Written consent

SAMTYKKEERKLÆRING

Forskningsprojektets titel:

Effekt af protein+kulhydrat sammenlignet med kulhydrat indtag på præstationen 5 timer efter et intensivt cykelarbejde

Erklæring fra den forsøgsansvarlige:

Jeg erklærer, at nedenstående forsøgsperson har modtaget mundtlig og skriftlig information om forsøget. Efter min overbevisning er der givet tilstrækkelig information, herunder om fordele og ulemper til at træffe et informeret valg.

Navn: _____ Dato: _____

Underskrift: _____
(forsøgsansvarlig)

Erklæring fra forsøgsperson:

Jeg har læst den skriftlige information og fået mundtlig information i et sprog som jeg forstår. Jeg ved nok om formålet, metoderne, fordele og ulemper til at sige ja til deltage. Jeg er informeret om, at det er frivilligt at deltage, og at jeg når som helst uden begrundelse kan trække mit samtykke tilbage uden at dette påvirker min ret til behandling eller andre rettigheder. Jeg samtykker, at biologisk materiale udtaget i projektet må opbevares i en forskningsbiobank.

Navn: _____ Dato: _____

Underskrift: _____
(forsøgsperson)

Forsøgsansvarlig: Kristian Overgaard, lektor, Ph.D.

Institut for folkesundhed, sektion for idræt, Aarhus Universitet, Dalgas Avenue 4, 8000 Aarhus C.

Tlf. 87 16 81 74 /51 66 65 48

Kopi af ovenstående underskrevne erklæring udleveres til forsøgspersonen