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Human muscle response to sprint exercise and nutrient supply with focus on factors related to protein metabolism

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Karolinska Institutet

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Human muscle response to sprint exercise and nutrient supply with focus on factors related to protein metabolism

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The overall aim of the present thesis was to study the muscular response to repeated bouts of 30-s sprint exercise. This type of exercise gives rise to a profound metabolic perturbation that might counteract an exercise-induced stimulation of muscle protein synthesis. Special focus was paid upon nutritional supply and how it can direct the muscular response towards activation of amino acid sensors, cell signalling pathway and muscle protein synthesis. Some questions were analysed by gender.

It was hypothesised that: Amino acids decrease and ammonia increases following sprint exercise and more so in males than females.

Activation of mTOR signalling increases more in females than males in skeletal muscle following sprint exercise. Oral ingestion of essential amino acids and carbohydrates increases the amino acid sensor Vps34 activity, the amino acid transporter and sensor SNAT2, Akt/mTOR signalling and consequently enhances the rate of muscle protein synthesis in skeletal muscle following sprint exercise.

It is concluded that: Plasma leucine decreased and plasma and muscle ammonia increased following sprint exercise and more so in in males than females. Such changes might counteract a possible sprint exercise-induced stimulation of muscle protein synthesis. There was some activation of mTOR signalling in skeletal muscle following sprint exercise and more so in females than males. The larger increase in serum insulin and lower decrease in plasma leucine in females might have contributed to the enhanced signalling response in females. Oral ingestion of essential amino acids and carbohydrates, as compared to placebo, resulted in a markedly higher activation of Akt/mTOR signalling in skeletal muscle following sprint exercise. In contrast to the hypothesis, an oral ingestion of essential amino acids and carbohydrates did not result in an increased activation of hVps34 in skeletal muscle. However, this does not exclude the permissive role of hVps34 in mediating the amino acid-induced activation of Akt/mTOR signalling and skeletal muscle protein synthesis. The interaction between the condition (nutrients or placebo) and correlation between increase in Vps34 activity and increase plasma glucose may reflect the dual role of Vps34, i.e. sensing a nutrient-rich condition and activate mTOR or sensing a nutrient-poor condition and activate autophagy in order to counteract low levels of amino acids. Oral ingestion of essential amino acids and carbohydrates resulted in a higher SNAT2 expression, a higher activation of Akt/mTOR signalling and higher rate of muscle protein synthesis following sprint exercise. Thus, nutrients have a potential to stimulate muscle protein synthesis and related pathways after sprint exercise, in spite of the profound metabolic perturbation.

LIST OF SCIENTIFIC PAPERS

I. Esbjörnsson M, Rooyackers O, Norman B, **Rundqvist H.C**, Nowak J, Bülow J, Simonsen L, Jansson E. Reduction in plasma leucine after sprint exercise is greater in males than in females. *Scand J Med Sci Sports. 2012; 22(3): 399-409*

II. Esbjörnsson M, **Rundqvist H.C**, Mascher H, Österlund T, Rooyackers O, Blomstrand E, Jansson E. Sprint exercise enhances skeletal muscle p70S6k phosphorylation and more so in women than in men. *Acta Physiol (Oxf). 2012 Jul; 205(3): 411-22*

III. **Rundqvist H.C**, Lilja M.R, Rooyackers O, Odrzywol K, Murray J.T, Esbjörnsson M, Jansson E. Nutrient ingestion increased mTOR signaling, but not hVps34 activity in human skeletal muscle after sprint exercise. *Physiol Rep. 2013 Oct; 1(5): e00076*

IV. **Rundqvist H.C**, Esbjörnsson M, Rooyackers O, Österlund T, Moberg M, Apró W, Blomstrand E and Jansson E. Influence of nutrient ingestion and sprint exercise on amino acid transporters and protein synthesis in human skeletal muscle. *Manuscript*

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LIST OF ABBREVIATIONS

4E-BP1	4E binding protein 1
Akt	Protein kinase B
ANOVA	Analysis of variance
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
a.u.	Arbitrary units
BCAA	Branched-chain amino acids
Carb	Carbohydrates
cDNA	Complementary deoxyribonucleic acid
CON	Control
CT	Cycle threshold
c x t	Condition and time
EAA	Essential amino acids
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FFA	Free fatty acids
FSR	Fractional synthetic rate
GC-MS	Gas chromatography-mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG	Immunoglobulin G
LAT1	Large neutral amino acid transporter
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin
NEAA	Non-essential amino acids
p70S6k	Ribosomal protein S6 kinase
PRAS40	Proline-rich Akt substrate of 40 kDa
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
SE	Standard error
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
SNAT2	Sodium-dependent neutral amino acid transporter-2
TLC	Thin-layer chromatography
Tris-HCl	tris(hydroxymethyl)aminomethane - hydrogen chloride
Vps34	Vacuolar protein sorting 34
VO_2max	Maximal oxygen uptake

1 INTRODUCTION

1.1 "Classical" forms of exercise

Physical exercise is often broadly classified as either ”resistance” or ”endurance” exercise. Resistance exercise is characterised by low-volume/high force muscle actions. For one maximal contraction there is no limitation regarding ATP supply and no need for ATP regeneration. Instead the limiting factors are mainly related to muscle cross-sectional area and contractile properties of the muscle fibres. When forceful muscle contractions are repeated the ATP has to be regenerated mainly from creatine phosphate (CP) breakdown and anaerobic glycolysis. Endurance exercise is characterised by high-volume, low- to moderate-intensity work. This type of exercise is associated with ATP regeneration mainly through aerobic pathways. The muscular adaptations to repeated bouts of resistance or endurance exercise is well characterised (1, 2). Resistance exercise usually results in hypertrophy (1), while endurance exercise results in an increased mitochondrial and capillary density (2-4).

1.2 Sprint exercise

In contrast to resistance and endurance exercise, few studies have been carried out to investigate the effects of sprint exercise, even though there is an accelerating interest to explore the effects of sprint exercise due to unexpectedly found health benefits (5). Sprint exercise is similar to resistance exercise with regard to the intense muscular work performed during short-duration bouts (6). Yet one sprint bout is typically built up of a more extensive number of repetitions against lower absolute resistance than during traditional resistance training. Consequently, the limiting factors for power output or speed during sprint exercise are muscle mass, contractile properties and anaerobic energy supplies. A popular form of sprint exercise among various types of athletes and also among individuals who are training to reach health benefits, is sprint or high - intensity interval training. This type of exercise is defined as short bursts of vigorous exercise interspersed by periods of low-intensity exercise or rest (6).

There are a numerous of different ways to design such sprint interval training sessions. The length and intensity of the active bout of sprint and of the "resting" phase can vary as well as the number of "sets". In the present thesis, focus has been on 30-s all-out sprints separated by 20 min of rest, i.e. no exercise was performed during these 20 min. The reasoning behind the relatively long resting period was to guaranty a close-to-full recovery of the power output and the anaerobic ATP regeneration capacity. If the rest period is considerably shorter (i.e. a few minutes), subsequent exercise bouts will show lower power outputs and a considerable switch from anaerobic to aerobic ATP generation (7).
During a 30-s bout of an all-out sprint, ATP has to be regenerated mainly from CP and anaerobic glycolysis and to a lesser extent from aerobic pathways. It has been shown that 30-s of sprint exercise leads to a profound metabolic perturbation and can reduce muscle ATP by 50%, CP by 80%, and glycogen by 35% in type II muscle fibres (8, 9). In type I fibres the reduction of ATP and CP is smaller (8, 9). ATP is broken down to ADP, AMP and finally to

IMP and an equimolar amount of ammonia. However, a small portion of the ATP pool is further broken down to inosine and hypoxanthine (10). The large accumulation of ammonia in plasma and muscle may interfere with amino acid availability and may also interact with muscle protein synthesis (11).

To perform well in sprint exercises there is a need for a large muscle mass and fast contracting muscles are beneficial. Thus, a productive sprint-training program should thus induce an increase in muscle cross-sectional area (12, 13). Interestingly however, longitudinal studies show that such an increase in cross-sectional area is not always the case (12). In fact, there are some observations indicating that sprint exercise may counteract muscle protein accretion, such as a lack of activation of signalling pathways related to the translation machinery (14). Indeed, Coffey et al. reported that resistance exercise induced Akt/mTOR signalling was inhibited when preceded by sprint exercise, indicating that sprint exercise could perhaps interfere with muscle hypertrophic responses if employed over time (15).

1.3 Muscle protein synthesis and muscle hypertrophy

Muscle hypertrophy is known to arise if muscle protein synthesis exceeds degradation of muscle proteins over an extended period of time (16). Important factors for protein synthesis in addition to mechanical stress are amino acid availability and hormones such as insulin, growth hormone, testosterone, IGF-1 (17, 18). Most of these stimuli activate the mTOR-pathway, which is characterized by a cascade of phosphorylation steps leading to the increased expression of factors involved in protein translation, transcription, cell proliferation and ribosome biosynthesis ultimately stimulating skeletal muscle growth (17, 18).
While high mechanical stress and muscular forces activates the mTOR-pathway, high metabolic stress, i.e. when the AMP/ATP ratio increases during high rate of ATP consumption, is associated with AMPK activation. Rodent models have shown that increased AMPK activity may inhibit the mTOR pathway and thereby muscle protein synthesis (19). Past research support the notion that AMPK is heavily activated by sprint exercise. For example, Chen et al 2000 (20) showed a 2-3 fold increase in muscle AMPK activity induced by a single 30-s sprint bout. Furthermore, sprint exercise has been shown to induce skeletal muscle adaptations typically associated with endurance-type training, i.e. increased mitochondrial density and improved oxidative capacity (21). Against this background following research questions were formulated: Is the availability of essential amino acids affected by sprint exercise and is such change related to the accumulation of ammonia? Is the Akt/mTOR pathway activated after sprint exercise?

1.4 Gender

Several gender-related differences in response to single and repeated bouts of sprint exercise have been demonstrated both at the muscular and systemic level (22). Following a period of sprint training, the fibre cross-sectional area is increased in females but not in males (23). Other factors that distinguish males and females in response to sprint exercise are the higher

accumulation of lactate, ammonia and catecholamine in plasma, in males (8, 10, 24). However, serum insulin increases more in females than males after sprint exercise (25). Against this background it is important to study both genders in the experiments and to evaluate gender difference during sprint exercise. Therefore, the first research questions in the present thesis were analysed by gender.

1.5 **Nutrient ingestion**

Nutrient supplementation may have a profound effect on post-exercise muscle proteins synthesis and on the adaptive response to cumulative training (26-30). It has been shown that ingestion of essential amino acids following resistance exercise leads to an even larger increase in the rate of muscle protein synthesis compared with the independent effect of nutrients or muscle contraction (26). In the last years the branched chained amino acids and especially leucine has been studied due of it's potential to activate muscle protein synthesis (31, 32). Interestingly, this seems to be mediated by the activation of the mTOR- pathway and subsequent activation of downstream targets (33).

The effect of nutrient provision on the rate of muscle protein synthesis following 30-s bouts of sprint exercise has not been explored yet, even though an increased myofibrillar protein synthesis was demonstrated, when repeated 6-s bouts of sprint exercise were combined with nutrient provision (15). The metabolic perturbation, however, is much stronger after repeated 30-s bouts than after repeated 6-s bouts (15, 34), arguing for a requirement of a deeper understanding of the consequences of longer 30-s bouts. Thus, the research questions if oral ingestion of nutrients will activate the Akt/mTOR pathway and the rate of muscle protein synthesis following 30-s sprint exercise were formulated in the present thesis.

1.6 **Amino acid sensing**

The underlying mechanisms responsible for the stimulatory effect of essential amino acids on mTOR signalling and muscle protein synthesis are not fully understood, but several have been suggested (35, 36).

Vps34. From in vitro studies it has been suggests that amino acids stimulate mTOR through a class 3 PI3K, the human vacuolar protein sorting 34 (hVps34) (37, 38) and that Vps34 might act as an internal amino acid sensor to mTOR (37, 38). Both fuel depletion (glucose and amino acid) and AMPK activation inhibit hVps34 (33). It has been shown that the activity of Vps34 can be increased by adding glucose or amino acids to cells cultures (37, 38). Also the protein concentration of hVps34 and phosphorylation of p70S6k was increased in human myotubes with leucine supplements (39). These studies show that nutrients affect Vps34 and mTOR signalling. Although Vps34 has been studies in rat skeletal muscle (40, 41) no studies in response to sprint exercise have been executed on Vps34 activity in human skeletal muscle tissue.

SNAT2. The amino acid transporter SNAT2 is known to transport amino acids across the cell membrane and also to act as sensors for changes in extracellular amino acid concentrations and initiator of downstream signalling pathways (42, 43). In rat myotubes, SNAT2 has been shown to be a potential regulator of amino acid-dependent signalling (44). Furthermore, acidosis as well as the lipid metabolite ceramide was shown to inhibit SNAT2 activity (44, 45), which may lead to decreased Akt/mTOR signalling and protein synthesis, and to increased proteolysis through an insulin-mediated mechanism (43, 44). In contrast, insulin has been shown to stimulate SNAT2 in rat myotubes (46).

An increased expression of this transporter has been demonstrated after resistance exercise in human skeletal muscle (47). Upregulation of SNAT2 expression in human skeletal muscle has also been observed during amino acid supplementation at rest (48) and after resistance exercise (49-51). In contrast, recent work suggests that sprint exercise down regulates SNAT2 at the mRNA level (52). However, the influence of nutrient provision on amino acid transporters in relation to repeated bouts of sprint exercise, a form of exercise that is characterized by a profound metabolic perturbation (8), is not known. Therefore, the research questions if oral nutrient ingestion will activate the two amino acid sensors Vps34 and SNAT2 following sprint exercise was formulated in the present thesis.

2 AIM OF THE THESIS

The overall aim of the present thesis was to study the muscular response to repeated bouts of 30-s sprint exercise. This type of exercise gives rise to a profound metabolic perturbation that might counteract an exercise-induced stimulation of muscle protein synthesis. Special focus was paid upon nutritional supply and how it can direct the muscular response towards activation of amino acid sensors, cell signalling pathways and muscle protein synthesis. Some of the questions were analysed by gender.

It was hypothesised that:

- Amino acids decrease and ammonia increases in humans following sprint exercise and more so in males than in females.

- Activation of mTOR signalling increases more in females than in males in human skeletal muscle following sprint exercise.

- Oral ingestion of essential amino acids and carbohydrates increases the amino acid sensor Vps34 activity, as well as mTOR signalling in human skeletal muscle following sprint exercise.

- Oral ingestion of essential amino acids and carbohydrates increases the expression of the amino acid transporter and sensor SNAT2 and Akt/mTOR signalling in and consequently enhances the rate of muscle protein synthesis human skeletal muscle following sprint exercise.

3 MATERIAL AND METHODS

3.1 Subjects

All subjects (n=51) in the studies were recruited from nearby universities and sports/health clubs. The inclusion criteria were: good health, participation in leisure-time sports, but not at an elite level and age 20–35 years. The exclusion criteria were chronic disease, acute infection, severe asthma, or use of products containing nicotine. Health was evaluated by a general health questionnaire and a 12-lead electrocardiogram registration. A questionnaire was used to screen potential subjects for the physical activity level during leisure time (53) with the intention to create two groups with a similar range and average level of physical activity in study I and II. Fat-free body mass (FFM) was estimated from skinfold measurements in triceps, biceps, subscapula and suprailiacal regions (54). In study III and IV the maximal oxygen uptake (VO2 max) was determined using a standardised incremental cycle test to exhaustion on a cycle ergometer (Siemens-Elema, Solna, Sweden). Oxygen uptake was measured continuously utilizing a gas analyser Vmax Encore System (VIASYS Healthcare Inc., Yorba Linda, CA). In study I the females were on day 10-28 of their menstrual cycle (non-bleeding phase), in order to avoid low oestrogen levels. Six females used oral contraceptives. This non-bleeding phase avoids the part of the cycle with the lowest oestrogen levels. In Study II females with varying phases of their menstrual cycle were selected (day 1 to day 28). Four females used oral contraceptive. The physical characteristics of the subjects are presented in Table 1. All subjects were fully informed about the procedures and potential risks of the experiment before giving their written and verbal consent prior to participation. All studies in the thesis were approved by the Ethics Committee of Karolinska Institutet or by the Regional Ethical Review Board in Stockholm, Sweden.

Table 1. The physical characteristics of the subjects in the various studies

	Gender	Number	Age	Height	Body mass	Fat free mass	Body fat	Activity index*
			years	cm	kg	kg	%	
Study I	Female	8	26±7	165±5	64±8	47±7	26±8	17±3
	Male	6	29±6	177±6	75±10	61±9	18±5	15±1
Study II	Female	8	25±2	170±10	66±12	51±7	22±8	16±1
	Male	9	26±4	18±6	84±7	73±7	13±5	16±3
Study III	Female	1	23	167	55	46	16	19
	Male	8	28±5	183±9	80±5	68±5	15±4	15±3
Study IV	Female	3	26±4	171±8	70±4	52±4	26±2	18±2
	Male	9	27±5	182±8	78±10	67±9	15±5	17±2
Mean			26±2	175±8	72±10	58±10	19±5	17±1

* Activity index: range 5 - 20

3.2 Sprint exercise protocol

The experimental design of the different studies is presented in Fig. 1. In all studies the subjects performed the sprint exercise on a mechanically braked cycle ergometer (Cardionics, Bredäng, Sweden). After a brief warm up (1 min, with a braking load of 1 kg) the subjects were instructed to pedal as fast as possible without any resistance. When maximal pedalling speed was reached, an individual's braking load set at 0.075 kp · kg^{-1} body weight was applied to the flywheel, while the subjects continue to pedal "all out" during 30 seconds. The subjects performed in total three 30-s sprint exercise bouts with 20 min rest between the sprints (55). The subjects were in a supine position during the rest periods. Peak power (i.e. the highest 5-s power) and mean power (the average power during the 30-s sprint exercise) were expressed in absolute values (W), relative to body mass (W x kg body $mass^{-1}$) and relative to fat-free body mass (W x kg fat- free body $mass^{-1}$).

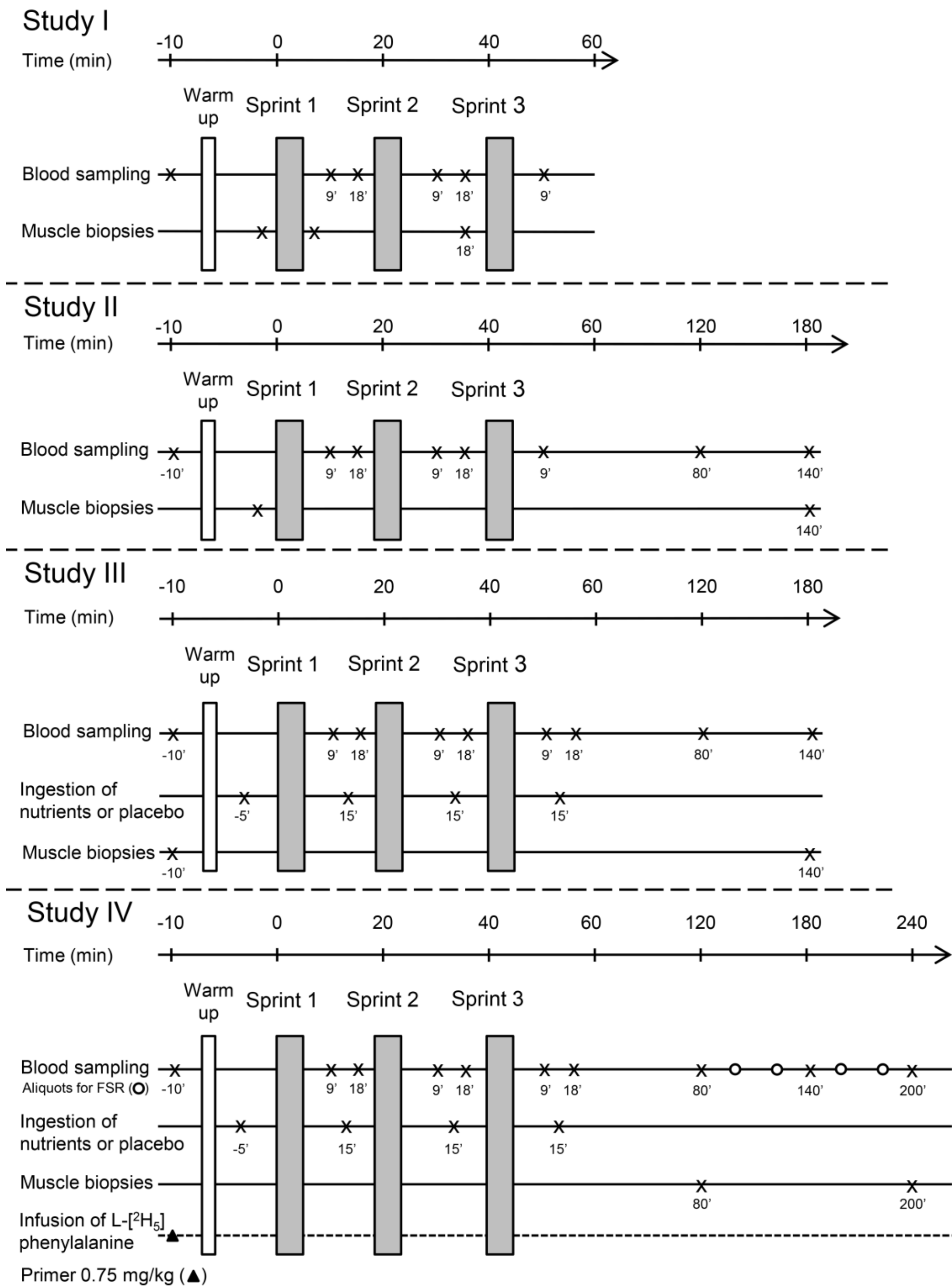

Fig. 1. Experimental protocol for each of the studies in the present thesis, including sprint exercise protocol and point of time for muscle and blood sampling

3.3 Biopsy sampling

The protocol for obtaining biopsy samples in the different studies is presented in Fig. 1. In all studies skeletal muscle biopsy samples were obtained from the lateral portion of the vastus lateralis muscle, ~20 cm above the patella under local anaesthesia without adrenaline by the percutaneous biopsy technique (56). All muscle samples (50-100 mg) were quickly dissected free of any visible fat and connective tissue and frozen by in liquid nitrogen. In study II muscle samples were frozen in isopentane for determination of fibre type composition. Muscle samples were stored at -80 ºC for later analyses.

3.4 Nutritional drink

Studies III and VI were randomised, single-blinded, placebo-controlled, crossover studies (Fig.1). The subjects repeated the sprint exercise protocol with or without nutrient ingestion with a month interval between the experiments. At both occasions the subjects arrived in to the laboratory after an overnight fast from 9 pm the evening before. The subjects were instructed to ingest either an essential amino acid or maltodextrin drink, called "EEA+Carb" drink in study III or "nutrient drink" in study IV or a placebo drink. The intervention drink contained eight essential amino acids, 300 mg x kg body weight^{-1} (Ajinomoto, Kanagawa, Japan) and maltodextrin, 1g x kg body weight^{-1} (Faring AB Sports nutrition, Fast Carbs Natural, Järfälla, Sweden). Both nutrient and the placebo drinks were "Wild Berrie" flavoured and contained salts and artificial sweetener, all to conceal the taste of the amino acids (Funlight, Wild Berries, Procordia Food AB, Eslöv, Sweden, diluted 1:4 in water), see also Table 2. Each subjects ingested a total volume of 12.5 mL · kg^{-1} body weight of the drink delivered at four occasions (5 min before sprint 1 and 15 min after each sprint), see Fig. 1. The reason for adding maltodextrin to the amino acid containing drink was to prevent a possible use of amino acids in gluconeogenesis and oxidative processes that might limit the amino acid availability for muscle protein synthesis.

Table 2. Amino acid composition of the nutritional supplement drink

Essential amino acids	Weight %
Histidine	13.7
Isoleucine	9.4
Leucine	17.3
Lysine	18.0
Methionine	2.9
Phenylalanine	14.3
Threonine	13.7
Valine	10.7
Total	100

300 mg x kg body weight^{-1} of the essential amino acids and 1g x kg body weight^{-1} of maltodextrin were given to the subjects.

3.5 **Blood analysis**

3.5.1 Sample preparation

Blood samples in the studies were divided into two equal portions. One portion was transferred into sodium- heparinised tubes, and immediately centrifuged at 2000 x g (4°C) for 10 min. One-millilitre aliquots of this plasma portion was frozen in liquid nitrogen and stored at -80°C. The other portion of blood was transferred to a serum tube stored at room temperature for 20 min and then treated the same way as the heparinised blood. In study IV also small aliquots (~200 µL) were taken 100 - 200 min after the last sprint for fractional synthetic rate analysis. These small aliquots were treated the same way as the heparinised blood.

3.5.2 Glucose, Lactate, Insulin, Ammonia, Growth hormones, FFA

Glucose concentration was determined, using a Beckman Coulter Oxygen Electrode (LX-20 Brea, CA, USA). The plasma was pipetted into a reaction cup containing glucose oxidase solution. The amount of consumed oxygen is proportional to the concentration of glucose. Concentration of plasma lactate was established by applying a Radiometer ABL 800 Flex blood gas analyser (Berman & Beving Lab, Triolab, Göteborg, Sweden). An electro-chemiluminescence immunoassay was used to analyse serum insulin Modular (E170, Roche, Pharma, Stockholm, Sweden). Plasma ammonia was analysed using an enzymatic method according to van Anken and Shiphorts (57) with the following modifications. We used an optical 96-well reaction plate (MicroAmp) and an absorbance microplate reader (Molecular device Spectra max plus 384, Sunnyvale, CA, USA) to measure the decrease in absorbance Unlike van Anken and Schiphorts, we allowed the reaction to go to completion at a so-called end point. Serum growth hormone (GH) was determined using the Immulite 2000 Growth Hormone (hGH) chemiluminescent enzyme immunoassay (Diagnostic Products Corporation, Germany). Serum concentration of free fatty acids (FFA) was analysed by an enzymatic colorimetric assay (Wako Chemicals GmbH, Neuss, Germany) according to manufacturer's instructions.

3.5.3 Amino acids

Amino acid concentrations were determined by HPLC, using precolumn derivatisation with orthophthaldialdehyde/3-mercaptopropionic acid (Alliance, Waters 2690, fluorescence detector Waters 474: Waters, Stockholm, Sweden) as described by Vesali et al (58).

3.6 **Muscle analysis**

3.6.1 Amino acids

Specimens of muscle tissue for the analysis of amino acids were homogenised using a minibead beater (Biospec Products, Bartlesville, Oklahoma, USA) and deproteinised in 4% sulfosalicylic acid (SSA) with 0.2mM norvaline as an internal standard in a volume to weight

ratio of 20 (mL/mg wet muscle). Amino acids were analysed by an HPLC as described under 3.5.3. Briefly, amino acids were analysed using precolumn derivatisation with orthophthaldialdehyde/3-mercaptopropionic acid on an HPLC system (Alliance, Waters 2690, fluorescence detector Waters 474: Waters, Stockholm, Sweden).

3.6.2 Immunoblotting

Muscle samples (~20 mg) were homogenised on ice using glass homogenisers in ice-cold homogenisation buffer (20 μL x mg^{-1} wet weight). The protein concentration of the homogenate was determined spectrophotometrically. The samples were diluted with homogenisation buffer and Laemmli buffer to a final protein concentration of 2 μg x μL^{-1}. The diluted protein was denatured (95ºC for 5 min) and stored at −20 °C until further analysis.

Samples containing total protein were loaded and separated by SDS-PAGE on precast Criterion TGX gradient gels (BioRad). Gels were subsequently equilibrated in transfer buffer (30 min at 4º) and then transferred to a polyvinylidine fluoride membrane. In study IV, the transfer of proteins was confirmed through staining of the membranes with MemcodeTM Reversible Protein Stain Kit. After blocking for 1h at room temperature in Tris-buffered saline containing 5 % nonfat dry milk, the membranes were incubated overnight with primary phospho- or total protein specific antibodies diluted in TBS supplemented with 0.1 % Tween-20 and 2.5 % non-fat dry milk. Next, the membranes were washed serially and incubated for 1h at room temperature with HRP-conjugated secondary antibodies. Then, the membranes were washed serially again and finally the target proteins were visualised by application of Super SignalTM West Femto Chemiluminescent Substrate (Thermo Scientific) to the membrane. The results were detected using a Molecular Imager ChemiDocTM XRS system. In study II and IV, following visualisation, the membranes were stripped of the phosphor specific antibodies, using Restore Western Blot Stripping Buffer after which the membranes were washed and re-probed with primary antibodies for each respective total protein as described above. In study III, the membranes were re-probed with monoclonal anti-α-tubulin as a loading control. In study IV, the memcodeTMstaining using memcodeTM was used as loading control for SNAT2.

3.6.3 Lipid kinase assay (Study III)

To produce protein extracts, muscle samples (~30 mg) were powdered under liquid nitrogen using a mortar and pestle. Powdered frozen extracts were lysed using analysis buffer containing 50 mmol L^{-1} Tris-base pH 7.5, 1 mmol L^{-1} EGTA, 1 $mmol^{-1}$ EDTA, 1 mmol L^{-1} sodium orthovanadate, 10 mmol L^{-1} b-glycerophosphate, 50 mmol L^{-1} sodium fluoride, 5 mmol L^{-1} sodium pyrophosphate, 0.27 mol L^{-1} saccharose, 0.1% (v/v)b-mercaptoethanol and 0.3% (w/v) 3[(3-Cholamidopropyl) dimethylammonio]-propanesulfonic acid, and onetablet (per 10 mL) of Complete protease inhibitor (Roche, Diagnostics, Indianapolis, IN) to a final protein extract concentration of 2–5 mg muscle powder mL^{-1}. The protein extract was agitated (Vibrax orbital shaker, Sigma-Aldrich, Belfast, Ireland) at 1000 rpm, for 10 min

at 4°C and centrifuged at 4°C for 15 min at 16, 000 x g, to remove insoluble material. The protein concentration was determined using the DC protein assay (Bio-Rad) utilizing a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany). Details of the hVps34 activity assay procedures have been previously published (41) with slight modifications for this study. Samples were immunoprecipitated overnight at 4°C with 2 µg of sheep anti-hVps34 antibody (hVps34 antibodies, for activity measurements and Western blot analysis, were obtained from Dr. James Murray's laboratory at Trinity Biomedical Science Institute, Trinity Collage Dublin, Ireland) using a protein extract volume corresponding to 0.7–1.5 mg of total protein extract. Vps34 protein complexes were then immobilised on protein-G sepharose. Immunocomplexes were washed in Lysis buffer and in 60 µL TNE (10 mmol L^{-1} Tris-base, pH 7.5, 150 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} Na3VO4), resuspended in TNE+ (TNE, 0.5 mmol L^{-1} EGTA, pH 8.0, 1: 1000 2-mercaptoethanol), and incubated with 20 µg hVps34 antigen peptide. Substrates for the assay were prepared by adding 10 µL of 100 mmol L^{-1} MnCl2 and 10 µL of 2 mg mL^{-1} phosphoinositol (PtdIns) (bovine liver, Avanti Polar Lipids, Alabaster, AL) to each sample. PtdIns was sonicated for 5 min prior to addition to the assay to generate micelles. Reactions were performed at 30°C with shaking throughout the assay and initiated with the addition of ATP mix (1 mmol L^{-1} unlabelled ATP, [γ-^{32}P] ATP, H_2O). After 10 min, reactions were terminated by the addition of 20 µL of 8 mol L^{-1} HCl and phase separated with 160 µL 1:1 chloroform and methanol and centrifuged for 1 min at 16,000 x g. The lower organic phase was spotted on an aluminium-backed silica TLC gel 60 F254 plate (Merck, Damstadt, Germany) and analysed in a TLC chamber solvent system (60-mL chloroform, 47-mL methanol, 11.2-mL water, and 2-mL ammonium hydroxide). After the plates had developed sufficiently, they were air dried, wrapped in protective film, and exposed to a phosphorimager screen for 24 h before imaging with a phosphorimager (FLA-7000, Fujifilm, Tokyo, Japan). Incorporation of [γ-^{32}P] ATP was quantified using Multi-Gauge software (Fujifilm).

3.6.4 mRNA expression (Study II)

Total RNA was isolated by homogenising 10-20 mg frozen tissue with a homogenising dispenser (Polytron, Kinematica) and a standard TRIzol® protocol (InvitrogenTM Life Technologies, Carlsbad, CA, USA). The concentration and purity of the RNA was determined using nanodrop (Spectrometer ND-1000: Nanodrop®; Wender Aveen). One microgram of total RNA was reverse transcribed using transcription kits from Applied Biosystems, Foster City, CA, USA (High-capacity cDNA reverse Superscript) and random hexamer primers (Roche Diagnostics GmbH, Mannheim, Germany) in a total volume of 20 µL reaction. Real-time PCR was used to measure mRNA expression on an ABI-PRISMA 7700 Sequence Detector (Applied Biosystems Inc., Foster City, CA, USA). Primers and probes were supplied as a TaqMan® Reagents kit (Applied Biosystems). RPS18 (Hs01375212_g1) was used as an endogenous control to correct for potential variation in RNA loading errors. All reactions were performed in 96-well MicroAmp Optical plates (Applied Biosystems). Amplification reagents (25 µL) contained the sample 5 µl cDNA

diluted 1:100 and TaqMan Universal PCR Mastermix. For each individual, all samples were simultaneously analysed in duplicate in one assay run. Measurements of the relative expression were performed for each individual; a CT value was obtained by subtracting RPS18 mRNA CT values from respective target CT values. The expression of each target was then evaluated by $2^{-\Delta\Delta CT}$ and fold changes were calculated for each gene (59).

3.7 Fraction synthetic rate analysis (study IV)

Approximately 40 mg of wet muscle was freeze dried and dissected free of visual blood, fat and connective tissue. Approxemately10 mg of the freeze-dried muscle tissue was homogenised in 500 µL 4 % sulfosalicylic acid (SSA) and left on ice for 1h before centrifugation (10 min, 16,600 x g, 4°C). The supernatant was saved for amino acid analysis. The pellet was washed in 4 % SSA and dissolved in 1 mL 0.3 M NaOH and left for 1 h in a 37°C. Proteins were precipitated with 40 % SSA, incubated for 10 min on ice followed by centrifugation (10 min, 16600 x g). Protein pellets were washed with 4 % SSA and then hydrolysed for 24h in 6 M HCL at 110°C. The hydrolysed samples were dissolved in 0.5 M trisodium citrate, pelleted (5 min, 10,600 x g), and decarboxylated into phenyl-ethylamine using a suspension containing 2 mg tyrosine-decarboxylase and 0.25 mg pyridoxal phosphate. Samples were incubated over night at 50°C, before adding 6 M NaOH centrifuged (10 min, 16,600 x g) the following day. To extract the phenyl-ethylamine, the supernatant was mixed with diethyl ether. The mix was shaken and placed in an ethanol bath with dry ice. When the bottom layer had frozen, the liquid ether phase was transferred to a new tube containing 0.1 M HCL and the phenyl-ethylamine was back-extracted to an aqueous phase from the ether phase. The tubes were shaken and placed in ethanol bath and the bottom layer containing phenyl-ethylamine was frozen and the ether phase was discarded. Sample were transferred to GC-MS vials, dried and then derivatised by N-metyl-N-(tert- butyldimetysilyl) trifluouroacetamide and ethyl acetate (ratio 1:1) incubated at 60°C for 1h. The ratio of labelled to unlabelled phenylalanine was measured by monitoring ion 180 (m+2) and 183 (m+5) of the derivatised phenyl-ethylamine. The analysis of protein-bound tracer abundances was carried out by analysing the ratio of labelled and unlabelled phenylethylamine to a standard curve containing 0-0.189 atomic percent excess (APE) of L-[2H5] phenylalanine and treated the same way as the samples.

Muscle precursor enrichment: To determined muscle tissue fluid enrichment of free phenylalanine, the supernatant from the homogenised muscle was purified on resin columns (AG 50 W-X8 resin, Bio-Rad Laboratories, Hercules, CA, USA). After being washed, eluted using 4 M NH4OH and dried in a rotary evaporator (Speedvac) (24 h), the purified amino acids were derivatised by N-metyl-N-(tert- butyldimetysilyl) trifluouroacetamide and ethyl acetate (ratio 1:1) incubated at 60°C for 1h. Muscle tissue fluid enrichment of phenylalanine as well as enrichment of the standard curve was measured using GC-MS by selective ion monitoring for 336 and 341 m/z.

Plasma precursor enrichment: Plasma samples were deproteinised using 40 % sulfosalicylic acid (SSA) and left on ice for 10 min before centrifugation (15 min, 20, 800 x

g, 4°C). The supernatant was purified, derivatised and analysed as described above for the muscle free fraction.

Fractional synthesis rate calculation: Mixed muscle protein fractional synthetic rate was calculated both with plasma and muscle tissue fluid enrichment as precursors pool, as follows:

- FSRplasma = (ΔE / (Eplasma x t)) x 100
- FSRmuscle = (ΔE / (Emuscle x t)) x 100

ΔE is the difference in protein bound L-[2H5] phenylalanine enrichment between biopsies taken 200 min and 80 min after sprint 3. Eplasma is the AUC for L-[2H5] phenylalanine enrichment in plasma between the two biopsies. Emuscle is the muscle tissue free L-[2H5] phenylalanine enrichment in the biopsies. The time period for tracer incorporation in hours is denoted by t. To express FSR in percent per hour (% h) values were multiplied by 100. FSR was also calculated using the ratio of the muscle tissue free phenylalanine enrichment in the biopsies and the corresponding phenylalanine enrichment in plasma as a correction factor to weighting the average of the Eplasma, titled FSRadjusted (60).

3.8 **Statistical analysis**

Generally, a two-way ANOVA was used to study the effect of gender and time or between conditions (nutrient or placebo) and time. In the case of significant interaction, a one-way ANOVA of repeated measures design or Student's t-test for paired observations or independent groups were used to evaluate specific changes by time or gender. Values in the text are means ± standard error (SE) or standard deviation (SD) unless otherwise stated. The P values were accepted as statistically significant at $P \leq 0.05$. Correlations were calculated by Pearson's correlation coefficient. No corrections for multiple comparisons were performed due to the limited use of multiple post hoc comparisons.

4 SUMMARY OF STUDY I-IV

For experimental protocols and subject characteristics for study I-IV, see Fig. 1 and Table 1.

4.1 Study I

Previous studies have shown a pronounced difference between males and females in the accumulation of ammonia in plasma after sprint exercise. There are no studies on gender differences on exercise-induced changes in amino acid levels, although there are studies exploring the effects of exercise, regardless of gender, on amino acid levels (61-66)

It was hypothesised that amino acid levels would decrease while ammonia levels would increase in humans following repeated 30-s of bouts of sprint exercise and more so in males than females.

The major findings were that sprint exercise induced a decrease in plasma amino acids, especially leucine (Fig. 2A). This decrease was 2-fold higher in males than in females. The accumulation of ammonia both in plasma (Fig. 2B) and in muscle (data in paper I is show in table 3) was smaller in females compared to males. A correlation was shown between the decrease in plasma leucine and the accumulation of muscle ammonia (Fig. 3).

It was concluded that plasma leucine decreased while plasma ammonia increased following sprint exercise and more so in in males than in females. Such changes might counteract a possible sprint exercise-induced stimulation of muscle protein synthesis.

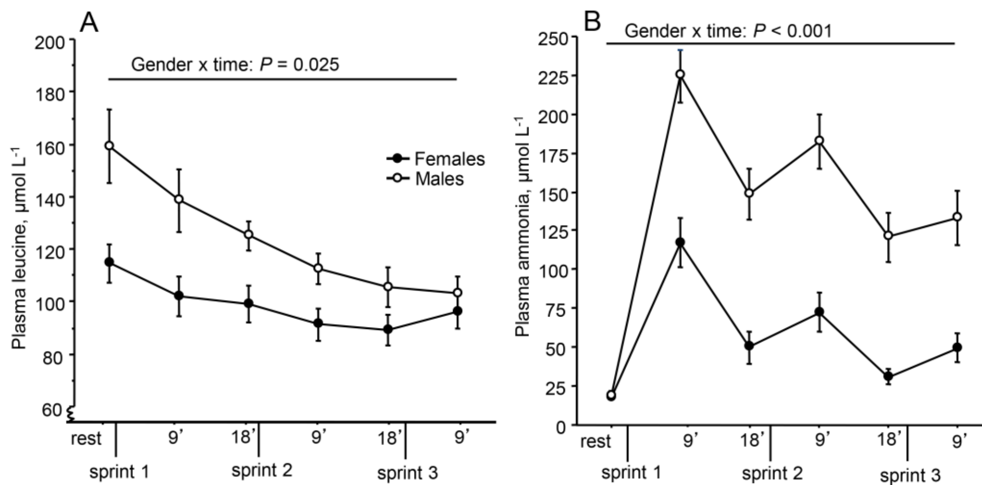

Fig. 2. (A) Plasma leucine and (B) Plasma ammonia concentration at rest and during the course of three bouts of sprint exercise with 20 min rest in between in eight females (closed symbols) and six males (open symbols). Values are the mean ± SE.

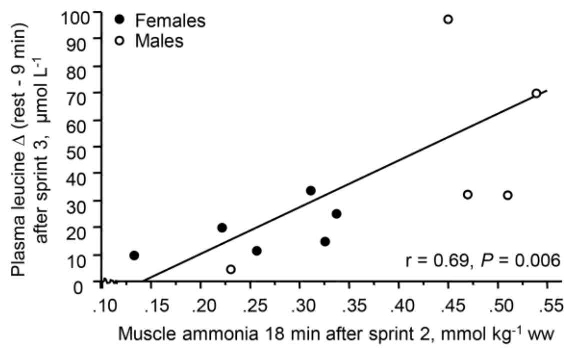

Fig. 3. Correlation between the exercise-induced decrease in arterial plasma leucine (rest -9 min after sprint 3) and the muscle ammonia content 18 min after sprint 2 in six females (closed symbols) and six males (open symbols).

4.2 **Study II**

As shown in study I, males showed a more pronounced decline in plasma leucine following sprint exercise. An earlier sprint training study showed a greater increase in muscle typ II fibre cross-sectional area in females than males (23). However, the effect on Akt/mTOR signalling pathway, an important link in the regulation of muscle protein synthesis, is not so well studied in relation to gender.

It was hypothesised that the activation of the mTOR signalling pathway is more pronounced in females than in males in skeletal muscle, following repeated 30-s bouts of sprint exercise.

The major findings were that plasma lactate (Fig. 4A) increased and plasma leucine decreased (data shown in paper II) during the sprints and more so in males. Serum insulin increased by sprint exercise and more so in females than in males (Fig. 4B). Phosphorylation of Akt in skeletal muscle increased by 50 % (Fig. 5A) and mTOR by 120 % (Fig. 5B) independent of gender. The elevation in p70S6k phosphorylation was greater in females (Fig. 5C) and averaged 230 % as compared to 60 % in males. The increase in the phosphorylation of p70S6k was directly related to the increase in serum insulin (Fig. 5D).

It was concluded that some activation of mTOR signalling occurred in skeletal muscle following sprint exercise, and that this activation was greater in females than in males. The larger increase in serum insulin and lower decrease in plasma leucine might have contributed to the enhanced signalling response in females.

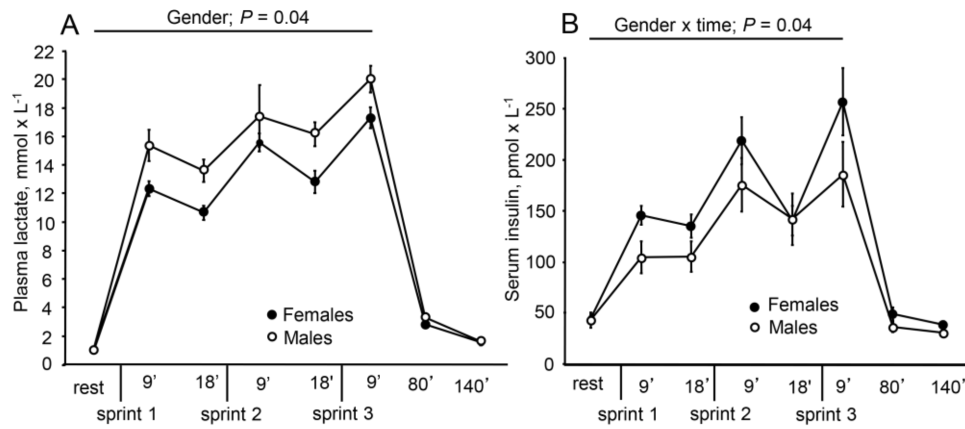

Fig. 4. (A) Plasma lactate and (B) serum insulin concentration at rest and during the course of three bouts of sprint exercise with 20-min rest in between in eight females (closed symbols) and nine males (open symbols). Values are the mean ± SE.

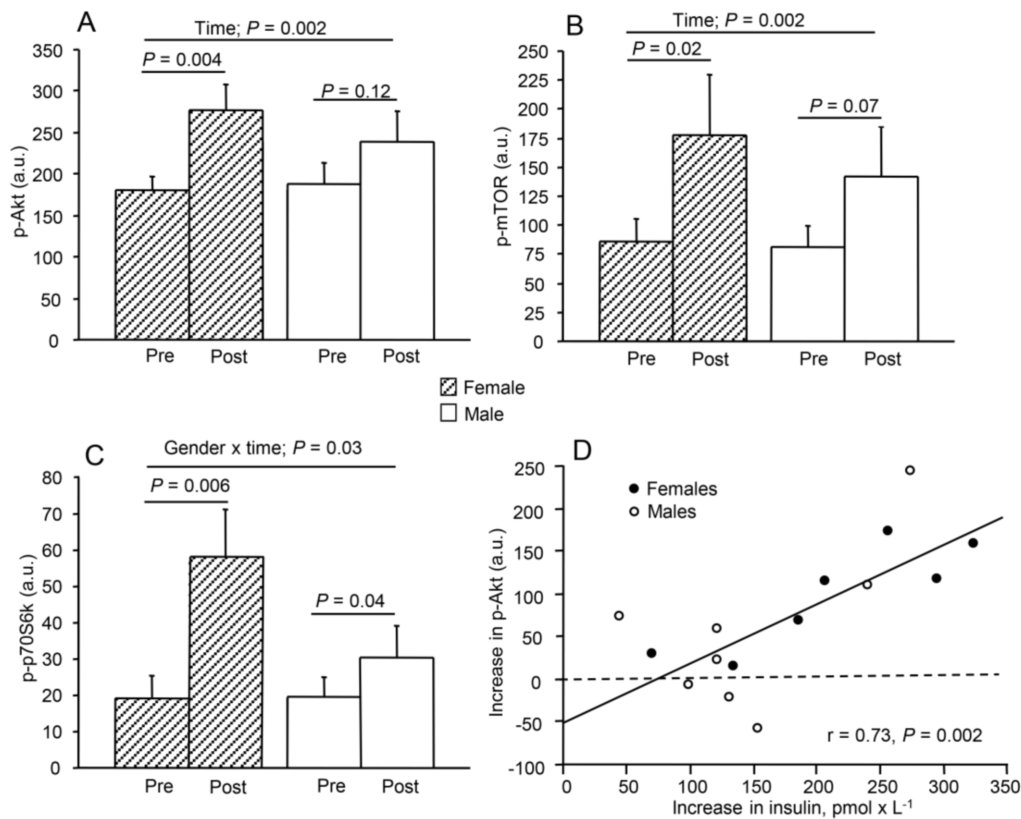

Fig. 5. (A) Phosphorylation of Akt^{Ser473}, (B) $mTOR^{Ser2448}$, (C) $p70S6k^{Thr389}$ pre-exercise and 140 min post-exercise in eight women (hatched bars) and nine men (white bars). (D) Correlation between exercise-induced increase in (d) phosphorylation of Akt^{Ser473} (140 min post-exercise – rest) on the one hand and exercise induced increase in insulin concentration (peak-rest) on the other in seven women (closed symbols) and nine men (open symbols). Values are arbitrary units (a.u.) and mean ± SE.

4.3 **Study III**

In study II it was shown that the increase in Akt/mTOR-signalling in skeletal muscle following sprint exercise was relatively low, especially in males. This could have been the result of decreases in amino acids and especially concentration of leucine, which was also higher in the males. Therefore, the question was raised if the activation of Akt/mTOR signalling could be increased by ingestion of amino acids. Further, the mechanisms how amino acids are sensed in the cell are not fully known. One suggested nutrient sensor is the class III phosphatidylinositol 3-kinase, vacuolar protein sorting 34 (Vps34), not previously studied in human skeletal muscle.

It was hypothesised that oral ingestion of essential amino acids and carbohydrates increase the activity of the amino acid sensor Vps34 activity and Akt/mTOR signalling in human skeletal muscle following repeated 30-s bouts of sprint exercise.

The major findings were that oral ingestion of nutrients increased the accumulation of plasma leucine and serum insulin markedly between 18 to 140 min after sprint 3 compared to placebo (Fig 6A, 6B). Plasma glucose accumulation increased somewhat more in in the nutrition condition (Fig. 6C). After the last sprint, glucose decreased by 25 % independent of condition. The exercise-induced increase in plasma lactate was somewhat smaller in nutrient condition (data shown in paper III). A marked increase was shown in phosphorylation of both mTOR and p70S6k in the nutrient condition (Fig. 7B; 7A) 140 min after the last sprint, compared to placebo. The activity of Vps34 was not significantly changed from the resting state by nutrients 140 min after the last sprint (Fig.7C). However, hVps34 activity tended to increase in the placebo condition. A negative correlation was seen between the increase in hVps34 activity and plasma glucose levels expressed as AUC in placebo condition, whereas a positive correlation was seen in the nutrient condition (Fig. 7D).

It was concluded that oral ingestion of essential amino acids and carbohydrates, as compared to placebo, resulted in a markedly higher activation of Akt/mTOR signalling in human skeletal muscle following sprint exercise. On contrast to the hypothesis, an oral ingestion of essential amino acids and carbohydrates did not result in an increased activation of hVps34 in human skeletal muscle. However, this does not exclude the permissive role of hVps34 in mediating the amino acid-induced activation of Akt/mTOR signalling and skeletal muscle protein synthesis. The interaction between the condition (nutrient or placebo) and correlation between increase in Vps34 activity and increase plasma glucose may reflect the dual role of Vps34, i.e. sensing a nutrient-rich condition and activating mTOR or sensing a nutrient-poor condition and activating autophagy in order to counteract low levels of amino acids.

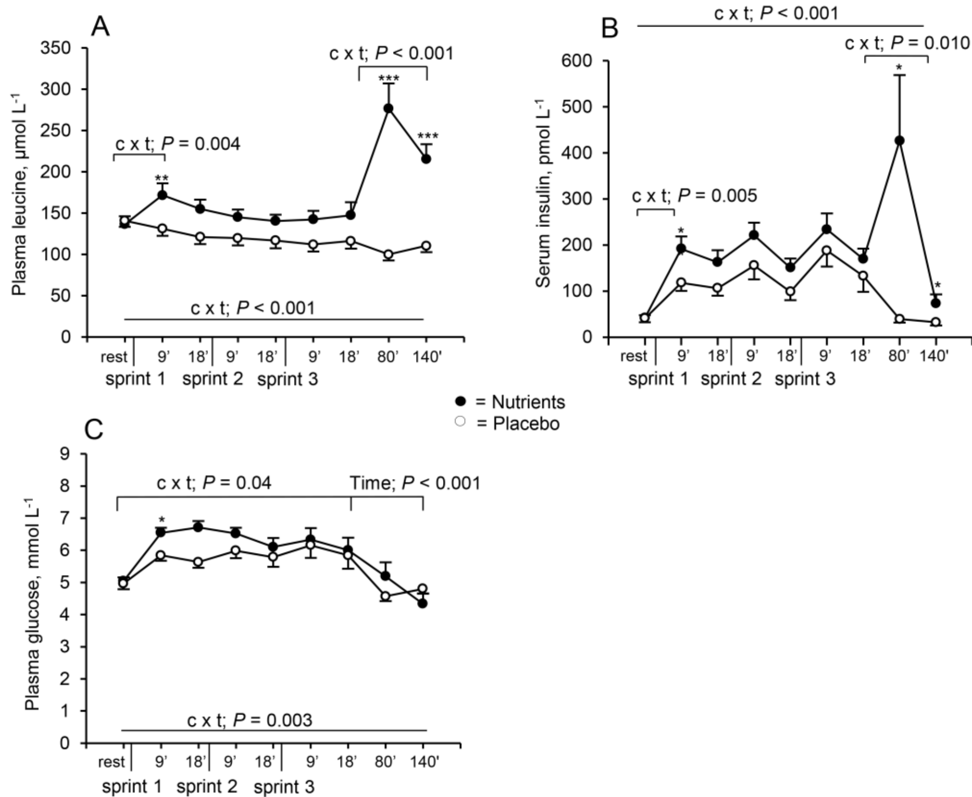

Fig. 6. (A) plasma leucine (B) serum insulin and (C) plasma glucose concentration at rest and during the course of three bouts of sprint exercise with 20-min rest in nutrient (hatched bars) or placebo (white bars) condition in seven subjects. Values are the mean ± SE; c = condition; t = time. Significant difference between the conditions is denoted by *P < 0.05, **P < 0.01, ***P < 0.001.

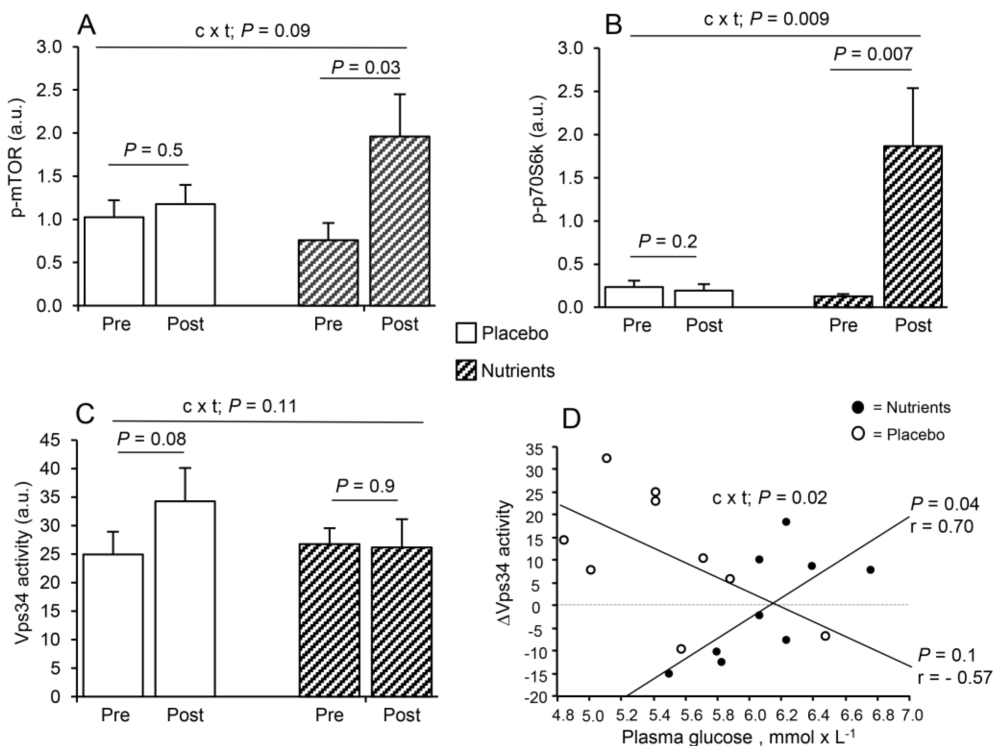

Fig. 7. (A) Phosphorylation of mTOR^{Ser2448} and (B) p70S6k^{Thr389} relative to alfa-tubulin at rest pre-exercise and 140 min post exercise during nutrient (hatched bars) or placebo (white bars) condition in nine subjects. Values are arbitrary units (a.u.) and mean±SE; c = condition; t = time. (C) Activity of hVps34 and (D) correlation between the sprint exercise-induced increase in Vps34 activity and plasma glucose concentration (before sprint 1–9 min after sprint 2. Note the opposite slopes when comparing the two conditions. Values are arbitrary units (a.u.) and mean ± SE; c = condition; t = time.

4.4 **Study IV**

The increase in Akt/mTOR signalling with oral amino acid supplement combined with sprint exercise in study III indicates that the muscle protein synthesis is increased. Further, the mechanisms how amino acids are sensed in the cell are not fully known. One suggested nutrient sensor is the amino acid transporter and sensor SNAT2.

It was hypothesised that oral ingestion of essential amino acids and carbohydrates increases the expression of the amino acid transporter and sensor SNAT2 and Akt/mTOR signalling in human skeletal muscle following repeated 30-s bouts of sprint exercise and consequently enhances the rate of muscle protein synthesis.

The major findings were that in the nutrient condition both plasma leucine and serum insulin accumulation peaked at 80 min after the last sprint and were 2.6-fold and 4-fold higher compared to placebo (Fig. 8A, 8B). Four subjects vomited after exercise during the nutrient condition and showed only a minor increase in plasma leucine. They were not included in the group comparisons, but in the correlation analyses (Fig. 8A). Post exercise increase in serum FFA was lower in nutrient condition (for data see paper IV). The phosphorylation of Akt, mTOR and p70S6k were all higher in the nutrient compared to the placebo condition 80 min and 200 min following sprint exercise (Fig. 9A, 9B, 9C). The rate of muscle protein synthesis was measured as the fractional synthesis rate (FSR). The post-sprint FSRplasma (enrichment based on plasma phenylalanine) in the nutrient compared to the placebo condition was 44 % higher. The FSRmuscle (enrichment based on intramuscular phenylalanine) or FSRadjusted (enrichment based on intramuscular phenylalanine) did not reach a significant difference between the nutrient and the placebo condition (Fig 10A, 10B, 10C) but showed a similar trend to increase in the nutrient condition. A positive correlation was found between post-exercise FSRplasma and the increase in plasma leucine in nutrient conditions ($r = 0.70$; $P = 0.02$). Positive correlations were also found in the nutrient condition between both post-exercise FSRplasma and FSRmuscle on the one hand and serum insulin levels as estimated by area under the curve ($r = 0.84$; $P < 0.001$ and $r = 0.68$; $P = 0.02$). These correlations could be considered as a kind of validation of the FSR measurements and justifies the conclusion that FSR was increased in the post sprint exercise condition by nutrients. SNAT2 expression was higher at both mRNA (1.4-fold) and protein level (40 % higher) after the nutrient than after the placebo 200 min post-exercise (Fig 11A, 11B).

It was concluded that nutrient ingestion increased the expression of the amino acid transporter SNAT2 and the Akt/mTOR signalling after acute sprint exercise, and that this was accompanied by enhanced rate of muscle protein synthesis following sprint exercise. Thus, nutrients have a potential to stimulate protein synthesis and related pathways after sprint exercise, in spite of the profound metabolic perturbation.

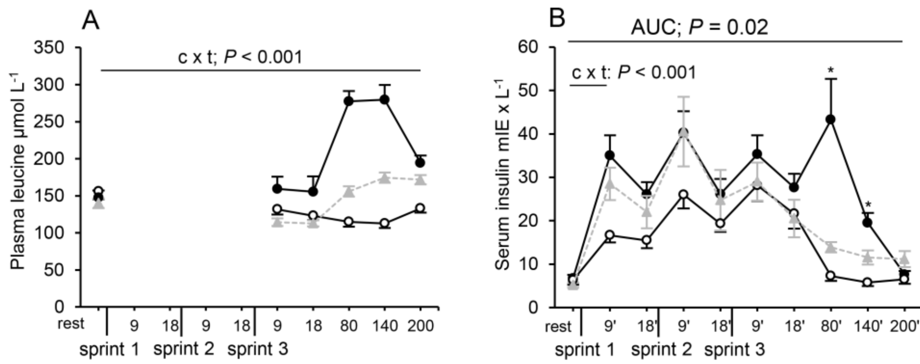

Fig. 8. (A) plasma leucine, (B) serum insulin concentration at rest, during the course of three bouts of sprint exercise with 20 min rest in between and during recovery after the last sprint in nutrient (closed symbols) and placebo condition (open symbols) in eight subjects. Values are mean ± SE. Grey lines represent the four subjects that vomited and thereby retained a lower nutrient dose. *denotes a significant difference at the level of least p<0.05.

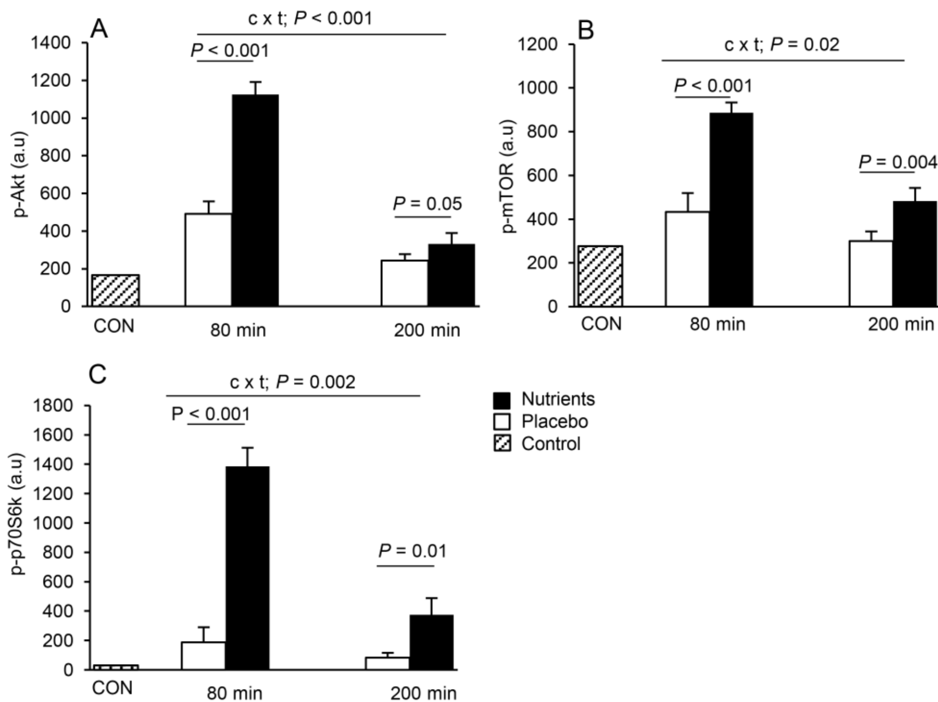

Fig. 9. (A) Phosphorylated Akt^{Ser47}/totAkt, (B) mTOR Ser^{2448}/tot mTOR, (C) $p70S6k^{Thr389}$/tot p70S6k 80 min and 200 min post exercise in nutrient (black bars) and placebo condition (white bars). Values are arbitrary units (a.u.) and mean ± SE in eight subjects. CON (hatched bars) are resting values from a matched control group of four subjects. The interactions between condition and time are denoted c x t.

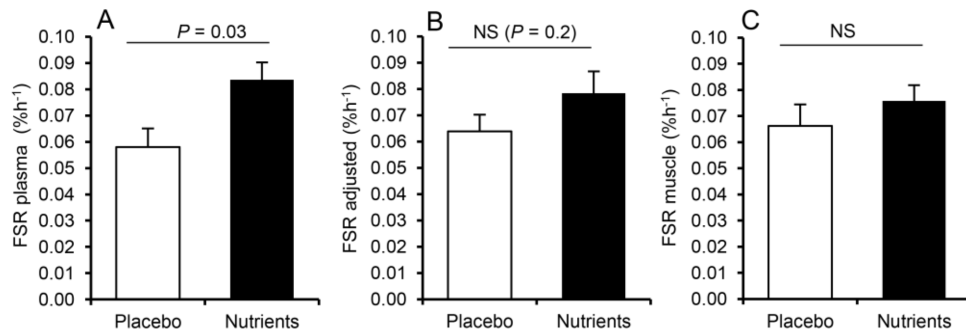

Fig. 10. (A) FSRplasma, (B) FSRadjusted, (C) FSRmuscle in nutrient (black bars) and placebo (white bars) condition. Values are mean ± SE in 8 subjects. FSRadjusted denotes an adjusted calculation of FSRmuscle according to Mikkelsen et al (60), see methods.

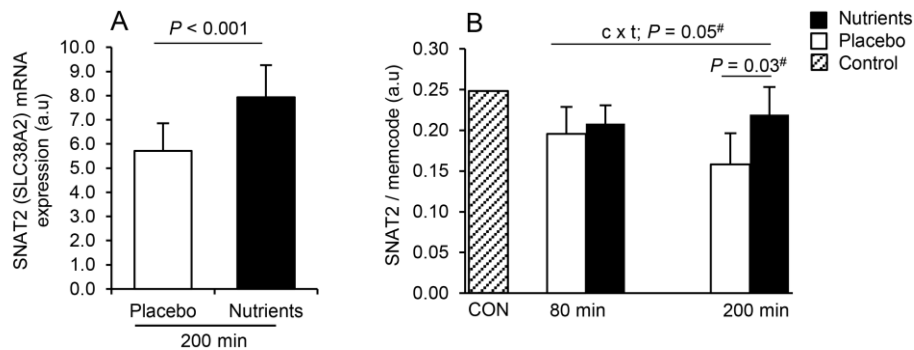

Fig. 11. (A) Gene expression of SNAT2/SLC38A2 and (B) protein expression of SNAT2 at 80 and 200 min after the last third sprint in nutrient (black bars) and placebo (white bars) condition in eight subjects. Values are arbitrary units (a.u.) mean ± SE. CONS (hatched bars) are resting values from a matched control group of four subjects The interactions between condition and time are denoted c x t. # The statistics is based on relative changes, because these were closer to a normal distribution curve than the absolute changes.

5 METHODOLOGICAL CONSIDERATIONS

5.1 Nutrient ingestion and plasma amino acids

There was no increase in plasma essential amino acids (EEA), when comparing basal rest with 9 min after the last sprint in the nutrient condition. Between basal rest and 9 min after the last sprint, no samples were collected for amino acid analysis in study IV. The reason for this was that in study III, with the same study protocol, only minor or no increases were found between basal rest and 9 min after the last sprint. In both studies III and IV a marked increase occurred first between 18 and 80 min post-exercise. The reason for this delayed increase in plasma EEA might be related to the large sympathoadrenergic activation during this type of sprint exercise (8) and thereby a vasoconstriction in the gastrointestinal tract and a delayed gastric emptying. In four of the subjects the combined effect of sprint exercise and oral ingestion of the drink resulted in an involuntary vomiting. The plasma amino acids in these subjects were more similar to the corresponding levels in the subjects in the placebo condition (Fig. 8A).

5.2 Fractional synthetic rate

A stable isotope tracer technique was applied in the present study to measure FSR. One prerequisite is an accurate assessment of a labelled precursor for protein synthesis in order to measure the precursor enrichment. There are strong supports for that t-aminoacyl-tRNA is a true precursor (67, 68). However, the very low concentration of aminoacyl-tRNA in tissues limits the use of this precursor, especially in in vivo experiments in humans (69, 70). Therefore, surrogate precursor pools have been used in the majority of studies. Commonly studied surrogate pools are the plasma and the skeletal muscle intracellular pool (tissue fluid pool) of labelled amino acids for estimation of the precursor enrichment. In the literature an agreement between various surrogate precursor pools and aminoacyl-tRNA varies between e.g. species, tissues, tracers and experimental set up can be found (71). Therefore, it is difficult to find support for the choice of a specific precursor that is validated for each experimental set up and study question. A single study on human skeletal muscle addressed the various precursor pools in comparison to aminoacyl-tRNA (70) and found that the plasma precursor-based measurements of FSR underestimated the "true" FSR (based on labelled aminoacyl-tRNA) and that the precursor pool based on the tissue fluid pool (skeletal muscle) overestimated the "true" FSR. Thus, based on studies of human skeletal muscle there is no consensus on which precursor pool gives the most accurate estimate of the true precursor. This is despite reports from other species and experimental models indicating that the tissue fluid-based pools represents the most accurate values of the true precursor (72). In contrast, additional methodology has been proposed to give even more accurate estimations of by the plasma pool precursor (73). However, it has been shown even if the absolute values for FSR as calculated by using the plasma precursor pool or the tissue fluid pool significantly differed, the changes in FSR in skeletal muscle after metabolic perturbations like feeding or insulin

infusion were in the same direction, independently of precursor pool used, in the calculations of FSR (74, 75). In the current study the absolute values are of less importance, rather the study question was related to changes induced by the nutrient ingestion.

In the present study both the plasma and the skeletal muscle tissue pool of labelled amino acids were used for estimation of the precursor enrichment. The enrichment of precursor in the plasma pool showed a close to steady state level during the measurement period, even though there were some changes in enrichment at the end of the experiment. However due to the frequent sampling of plasma this change is controlled for in the AUC calculation of the precursor enrichment. The enrichment of precursor in the muscle tissue pool was based on only two biopsy samples and there was a decrease in enrichment under placebo conditions. Due to the limited number of samples available for this analysis, the average enrichment is rather uncertain. Combined with the fact that only two points of measurements were available, comparison to the calculation of FSRmuscle is less accurate than the calculation of the FSRplasma. The FSRplasma was 44 % higher under nutrient conditions than in the placebo condition, but the FSRadjusted and FSRmuscle values did not reach significance, despite the fact that the average values were 23 % and 15 % higher, respectively in the nutrient compared to the placebo condition. Moreover, there were high correlations between the FSRplasma and the FSRmuscle in each of the conditions and correlations between FSR and AUC for plasma insulin were demonstrated for both precursors, see Figs. 8C, 8D shown in paper IV.

Collectively, the mean value comparisons (nutrients vs placebo), the correlations between FSRplasma and FSRmuscle and the correlations between both FSRplasma and FSRmuscle and plasma insulin strengthen the validity of the measurements of MPS in the present study. It is therefore justified to conclude that nutrient supplementation stimulated the MPS after sprint exercise.

Finally, an interesting finding in the present study was that the difference between FSRplasma and FSRmuscle was much smaller than previously reported (71). An important difference between the current study and those reported by Smith et al (71), is that the FSR in the current study was measured in a post-exercise phase with muscle hyperaemia (76), while previous reports measured FSR at rest without a preceding exercise session.

5.3 Repeated biopsies

One limitation in study IV was that MPS only was exclusively measured in the post-exercise period, i.e. no data on MPS at rest are available. Therefore, it is not possible to draw any conclusion about the effect of sprint exercise per see on MPS from the data in the present study. One reason for this choice of study protocol, with the current limitations, was to avoid very long experimental time periods with increasing interference of fasting and circadian rhythms. Another reason was to avoid a potential damaging effect of repeated muscle biopsies. Several studies in the literature show such effect of repeated biopsies (77-80) but not all (81). To get an estimation of the basal resting levels for the specific proteins analysed

by Western blot, these proteins were also analysed in a separate control group of four age- and physical activity-matched subjects, from which biopsies were obtained only at rest.

Also in study II, there was a limitation that only one biopsy was taken during the post-exercise period. As in study IV a residual effect of a preceding biopsy on protein activation was to be avoided. However, it cannot be excluded that an earlier transient increase in hVps34 activity following the last bout of exercise, related to the period of with increasing plasma EAA level, had occurred. However, the plasma EAA levels were still clearly elevated at the time of the post-exercise biopsy obtained 140 min after the last sprint, suggesting activation conditions for hVps34. Further studies are necessary to explore the possibility of transient hVps34 activation.

6 GENERAL DISCUSSION

6.1 Major aims and findings

The overall aim of the present thesis was to study the muscular response to sprint exercise including recovery and especially how nutritional supply can direct this response towards activation of amino acid sensors, cell signalling pathways and muscle protein synthesis. Muscle hypertrophy is known to occur when muscle protein synthesis exceeds degradation of muscle proteins. Mechanical stress, amino acid availability and hormone exposure are known to stimulate the protein synthesis (17, 18).

To perform sprint exercise and delivering the highest possible power, there is a need for a large muscle mass and fast contracting muscles are required. However, longitudinal studies show that sprint training not always increases muscle cross-sectional area (12). For example, following a period of sprint training, the fibre cross-sectional area increased in females but not in males (23).

There are numerous of different ways to design sprint exercise or sprint interval training protocols. The length and intensity of the active bout of sprint and the number of "sets" can vary vastly. Another critical issue is the length of the "resting" phase. The present thesis focused on 30-s all-out sprints separated by 20 min of rest periods, i.e. no exercise was performed during these 20 min. The reason for the relatively long resting period was to get as close to full recovery as possible of the anaerobic ATP storage. Longer resting periods may also allow for more favourable prerequisites for muscle hypertrophy. If the resting period is too short the exercise will switch from anaerobic to aerobic ATP generation and the power output becomes depressed (7), which may favour aerobic adaptations.

Sprint exercise is characterised by a large anaerobic ATP regeneration during the active phase. Repeated 30-s bouts of exercise influence both central cardiovascular and peripheral skeletal muscle functions. The focus in the current thesis has been on factors related to regulation of muscle mass. One 30-s bout of sprint exercise give rise to a profound metabolic perturbation and can markedly reduce muscle ATP, creatine phosphate and glycogen levels, especially in type II muscle fibres (9), with a large accumulation of e.g. ammonia and lactate, catecholamines, growth hormone and also an increase in insulin. Most of these sprint exercise effects are related to gender, with smaller changes occurring in females with the exception of insulin and growth hormone levels (8, 25).

In the first part of this thesis (study I and II) focus was placed on factors related to gender-specific changes in muscle mass, such as amino acid availability, insulin exposure and muscle Akt/mTOR signalling (Fig.11).

Major findings included the observation that sprint exercise decreased arterial plasma leucine levels more profoundly in males than in females. This reduction was directly related to the increase in muscle ammonia, i.e. the larger the increase in muscle ammonia the larger the reduction in plasma leucine. In addition, sprint exercise lead to a greater increase of Akt/mTOR signalling in skeletal muscle of females in comparison to males. Moreover, serum insulin increased more significantly in females during the sprint exercise period in

comparison to males, and that an increase in Akt and p-70S6k phosphorylation respectively was correlated to the exercise-induced increase in serum insulin (AUC).

The second part of this thesis (study III and IV) focused on the effects of oral ingestion of essential amino acids and carbohydrates on factors related to muscle hypertrophy such as Akt/mTOR signalling, amino acid sensing and rate of MPS (Fig.12)

The major findings in this second part of the thesis were that oral ingestion of essential amino acids and carbohydrates, following sprint exercise, resulted in a markedly higher activation of Akt/mTOR signalling as compared to placebo, in skeletal muscle. In contrast to the initial hypothesis, oral ingestion of essential amino acids and carbohydrates did not result in increased activation of the amino acid sensor hVps34 in skeletal muscle. However, this does not exclude the permissive role of hVps34 in mediating the amino acid induced activation of the mTOR-pathway and skeletal muscle protein synthesis. Furthermore, oral ingestion of essential amino acids and carbohydrates, after sprint exercise, resulted in a higher expression of the amino acid transporter SNAT2 and a higher rate of muscle protein synthesis. Thus, nutrients have the potential to stimulate muscle protein synthesis and related pathways, despite of the profound metabolic perturbation, observed after sprint exercise.

6.2 Sprint exercise and cell signaling

In an earlier study on repeated 30-s sprint exercise no activation of the Akt/mTOR pathway was found (14). Instead, the authors reported increased AMPK and p38 MAPK signalling, suggesting the induction of mitochondrial biogenesis in skeletal muscle (82). However, the study by Gibala et al. included only male participants (14), which might have contributed to the different results between these two studies. The divergent results could also be explained by different lengths of the rest periods, which were 20 min in the current as compared to only 4 min in the Gibala et al. study. The length of the rest period may be critical for the muscle perfusion and consecutive activation of the Akt/mTOR pathway and muscle protein synthesis. The muscle blood flow and perfusion is most likely of importance for the activation of mTOR signalling and muscle protein synthesis (83). In fact, a marked post-exercise hyperaemia has been demonstrated following a 30-s bout of sprint exercise (76), which allows for an enhanced inflow of important nutrients and stimulators of protein synthesis, such as amino acids and glucose (83, 84).

6.3 Sprint exercise and gender

The exercise-induced increase in the phosphorylation of p70S6k has been shown to be a good marker for increased muscle protein synthesis (15, 85-87) and hypertrophy (88, 89). However, dissociation between changes in Akt/mTOR signalling and muscle protein synthesis has been demonstrated in a non-exercise situation (90). Therefore, findings from signalling events could not directly be translated into conclusions regarding muscle protein synthesis. However, the larger increase in the phosphorylation of p70S6k in females than

males, as found in study II, is in line with the previous finding of an increased in the type II fibre cross-sectional area in females but not in males, in response to a four-week session of sprint training (23).

There are, to the best of our knowledge, very few studies on cell signalling following sprint exercise analysed by gender. As mentioned above, Gibala et al. (14) found no increase mTOR signalling following 30-s bouts of sprint in males, but they did not compare with females. Fuentes et al (91) compared the response to one 30-s bout of sprint exercise in males and females with regard to cell signalling related to an activation of mitochondrial pathways. They found that ACC beta phosphorylation, a downstream target for AMPK activation, tended to increase more in the males than in the females that is in accordance with a lower mTOR signalling in males.

Possible explanations for the presented gender difference in sprint exercise-induced activation of p70S6k signalling might be (Fig.12):

1) The different ammonia and plasma leucine levels observed in males and females might contribute to the reduced mTOR signalling in males. Ammonia has been shown to inhibit mTOR (92), and is less activated when leucine availability is reduced (84).

2) The greater breakdown of ATP level in type II fibres was faster in males than in females, during repeated bouts of sprint exercise, and this may have potentiated an activation of AMPK, a known mTOR inhibitor (93).

3) The lower increase in serum insulin levels in males in comparison to females, in response to sprint exercise, may reduce the Akt/mTOR activation. Lower plasma insulin levels in males may also contribute to a lower muscle protein accretion due to less inhibition of MPB (90) and to a lower SNAT2 activation (46). Thus, there are several potential mechanisms behind the gender-related difference in cell signalling response to sprint exercise.

Support for a sprint exercise-induced inhibition of Akt/mTOR signalling of MPS was found by Coffey et al, who demonstrated that resistance-exercise induced activation of Akt/mTOR Akt/mTOR signalling was inhibited when preceded by sprint exercise (15).

The explanations for the, in the current thesis, presented metabolic, hormonal and cell signalling differences between males and females are not fully known. Possibly gender-related differences in muscle fibre composition and body composition may contribute to the explored gender-related differences. Females generally have a larger relative type I fibre area (94, 95). ATP depletion and IMP accumulation are much lower in type I than in type II fibres. In mixed muscle ATP depletion and IMP, ammonia and lactate accumulation is lower after 30-s bouts of sprint exercise as demonstrated in study I. There are also typically gender differences in body composition, where females have a smaller relative muscle mass and a larger relative fat mass. Females also have a smaller muscle mass relative to blood or plasma volume (10). Therefore, certain obtained concentration of e.g. muscle lactate or ammonia is diluted in a, relative to muscle mass, larger blood or plasma volume. Moreover, the relatively larger fat mass may constitute a sink or buffer for ammonia (24). Finally, a more favourable condition for tissue perfusion after sprint exercise in females than males, i.e. lower plasma catecholamines and higher serum insulin, may add to the gender-related differences in muscle

fibre composition and body composition to explain some of the presented differences between males and females the current thesis.

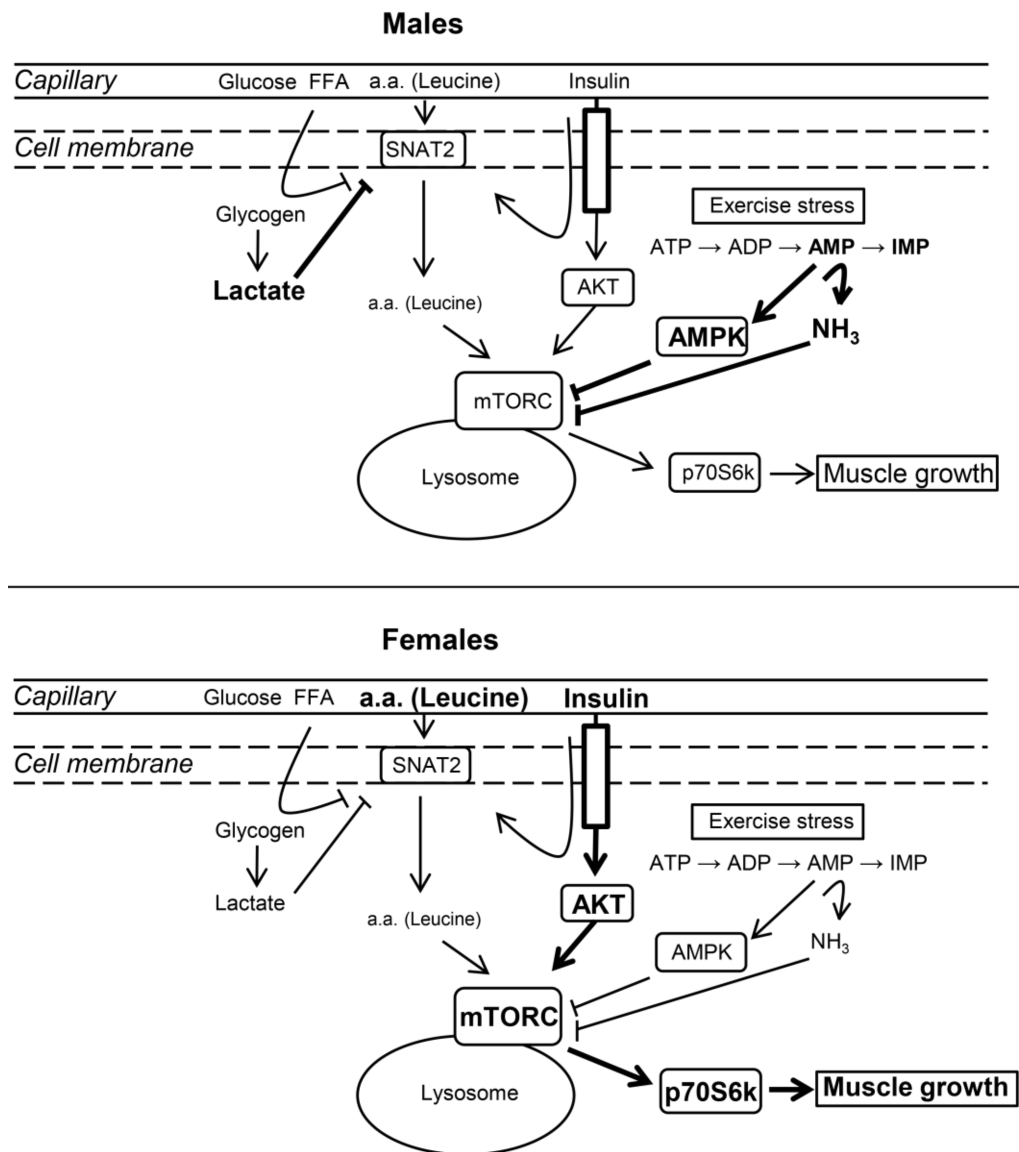

Fig. 12. Schematic presentation of differences between males and females in acute and chronic response to sprint exercise. Indicated blood-borne substances, intramuscular anaerobic metabolism and relevant signalling pathways are especially based on results from the current thesis and from earlier sprint exercise studies (8, 20, 23). Pathways, transport and metabolic reactions are presented in bold in the gender with the most profound activations or inhibitions. a.a = amino acids

6.4 Sprint exercise and nutrients

One question is if acute sprint exercise-induced changes in MPS can predict changes in muscle protein accretion. A prerequisite, however, for an increase in muscle protein accretion, is a positive net balance between muscle protein synthesis and breakdown (MPB) and ideal this balance would be measured to predict such changes. In the present study, MPB was not measured and thereby net protein balance could not be calculated. However, the potentiated response in serum insulin, after nutrient ingestion during the sprint exercise session and especially after the last sprint, could have led to a reduced MPB. In a human clamp experiment it was demonstrated, that insulin reduced MPB up to a systemic level of 30 mU x L^{-1} (90). Thus, the nutrient- increased MPS in the current thesis together with a potential decreased in MPB will increase the protein net balance. In fact, a reduced net protein balance was supported by the divergent changes in intramuscular tyrosine between the two conditions in the current study suggesting that, the net protein balance was enhanced by nutrients during the post-sprint exercise period.

Another question is if the nutrient-induced increase in serum insulin, as presented in paper IV, could have contributed to the increased MPS or to an increased net protein balance in the nutrient condition. The finding of a large increase in Akt and PRAS40 phosphorylation indicates that insulin signalling was activated (96) and therefore might have potentiated the mTOR signalling and rate of MPS (97). Also the positive correlation between MPS and AUC for serum insulin in the nutrient condition supports this idea, even though this relationship might simply reflect a direct relationship between MPS and plasma EEA. In fact, literature data strongly support that insulin above basal levels do not further stimulate MPS under the prerequisite of an optimal amino acid availability in experiments performed at rest (90, 98) or after resistance exercise (51, 99-103), even though there are some conflicting results (104-106). Thus, no conclusion can be drawn from the present study regarding the influence of insulin on MPS.

6.5 Amino acid sensing

The underlying mechanisms responsible for the stimulatory effect of essential amino acids on mTOR signalling and muscle protein synthesis are not fully known (35, 36).

Vps34. (Fig.13). In vitro work suggested that amino acids could stimulate mTOR signalling through the class 3 PI3K, the human vacuolar protein sorting 34 (hVps34) (38). Additionally, it has been proposed that the hVps34 might act as an internal amino acid sensor to mTOR after resistance exercise, thus forming the basis of the current experimental hypothesis. The hypothesis in this study was based on in vitro experiments, showing that Vps34 is activated by amino acids or glucose (37, 38, 41).

Study III presents, to our knowledge, for the first time data on hVps34 activity in human skeletal muscle, the first ones reported in the literature. However, contrary to our hypothesis, the hVps34 activity tended to increase following sprint exercise in the placebo condition, i.e. a nutrient-poor condition. The interaction reported between increased hVps34

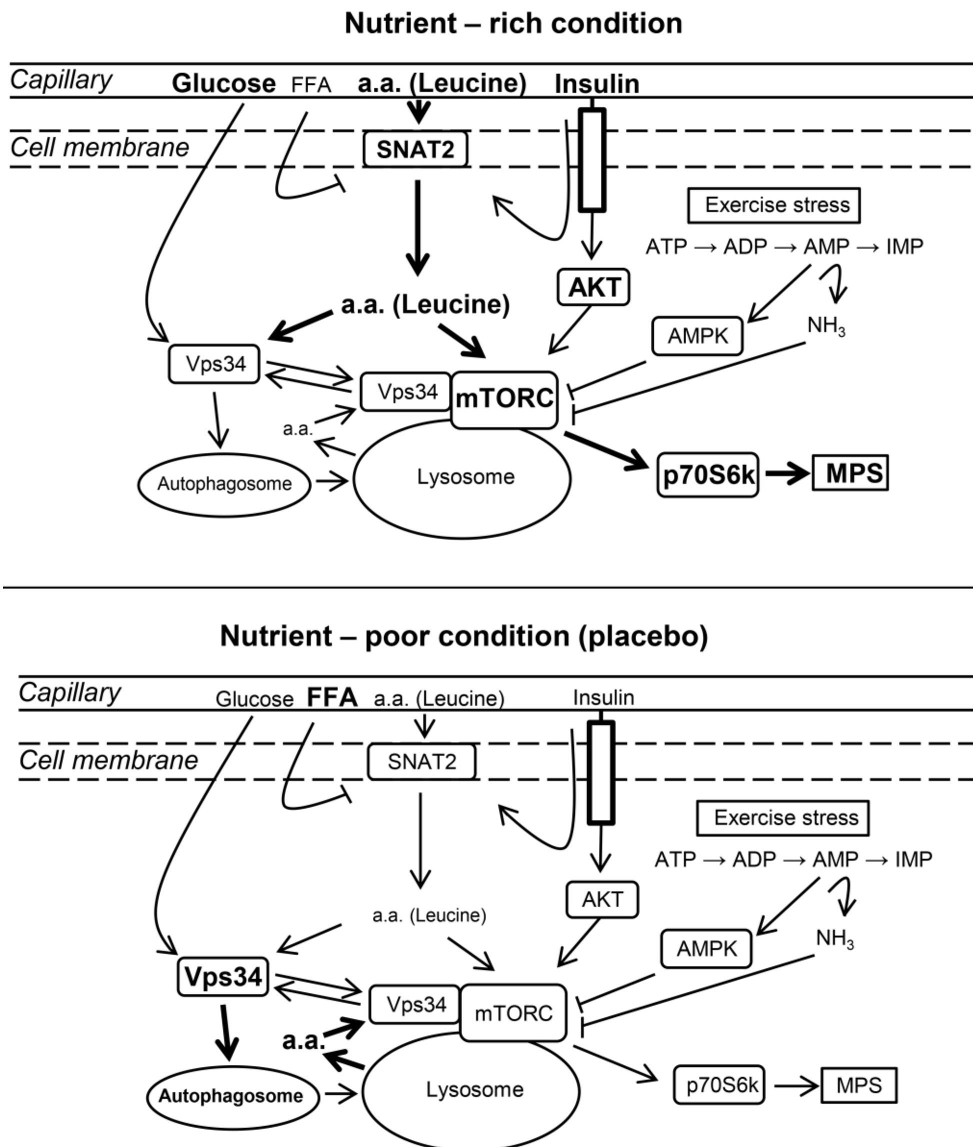

Fig. 13. Schematic presentation of differences between nutrient-rich and nutrient-poor (placebo) condition in acute response to sprint exercise. Indicated blood-borne substances, intramuscular anaerobic metabolism and relevant signalling pathways are especially based on results from the current thesis. Pathways, transport and metabolic reactions are presented in bold in the condition with the most profound activations or inhibitions. a.a. = amino acids

activity and plasma glucose levels in the nutrient and in placebo condition (Fig. 7D), highlight the complexity of hVps34 activation and regulation. It supports the model of the dual functions of this enzyme (107). One of these functions is to control growth by activating mTOR in nutrient-rich conditions. The other one is to induce autophagy during nutrient-poor conditions. These opposing roles of Vps34 are thought to be possible by the formation of spatially distinct Vps34 containing complexes mediating either mTOR activation and cell growth or autophagy (107, 108).

SNAT2. (Fig.13). Amino acid transporters are required for the transport of amino acids across the cell membrane, but can also act as sensors for changes in extracellular amino acid concentrations and initiate downstream signalling (42, 109). Findings reported in paper IV show increased gene and protein expression of the amino acid transporter and sensor SNAT2, as a result of nutrient ingestion during the early post-exercise period. In addition to the increased EEA availability, increased serum insulin and lowered serum FFA may contribute to the increased expression of SNAT2 in the nutrient condition (45, 46). In earlier studies of human skeletal muscle, oral ingestion of amino acids at rest (48) and after exercise (48-50) has been shown to increase expression of SNAT2 at both the mRNA and protein levels. The work reported here, for the first time, observe nutrient-induced changes in SNAT2 protein content after sprint exercise, possibly induced by the found increase in SNAT2 gene expression.

7 CONCLUSIONS

It is concluded that:

- Plasma leucine decreased and plasma ammonia increased, following sprint exercise and more so in males than in females. Such changes might counteract a possible sprint exercise-induced stimulation of muscle protein synthesis.

- There was some activation of mTOR signalling in human skeletal muscle following sprint exercise. This activation was more pronounced in females than in males. The larger increase in serum insulin and lower decrease in plasma leucine might have contributed to the enhanced signalling response in females.

- Oral ingestion of essential amino acids and carbohydrates, as compared to placebo, resulted in a markedly higher activation of Akt/mTOR signalling in human skeletal muscle following sprint exercise. In contrast to our initial hypothesis, an oral ingestion of essential amino acids and carbohydrates did not result in an increased activation of hVps34 in human skeletal muscle, instead it tended to increase in the placebo condition. However, this does not exclude the permissive role of hVps34 in mediating the amino acid-induced activation of Akt/mTOR signalling and skeletal muscle protein synthesis. The interaction between the condition (nutrients and placebo) and the correlation between the increase in Vps34 activity and the increase in plasma glucose may reflect the dual role of Vps34, i.e. sensing a nutrient-rich condition and activate mTOR or sensing a nutrient-poor condition and activate autophagy in order to counteract low levels of amino acids.

- Oral ingestion of essential amino acids and carbohydrates resulted in a higher SNAT2 expression, a higher activation of Akt/mTOR signalling and a higher rate of muscle protein synthesis following sprint exercise in humans. Thus, nutrients have a potential to stimulate protein synthesis and related pathways after sprint exercise, in spite of the profound metabolic perturbation.

8 FUTURE PERSPECTIVES

The results in the present thesis may have implications for designing performance-related training programmes for athletes as well as health-related training programmes in health and disease. The results have increased the understanding of muscular response to severe metabolic stress in males and females, and how to direct the response by oral ingestion of nutrients towards increased muscle protein synthesis. For example, a sprinter needs both a large muscle mass and fast contracting muscles to develop high power. If sprint training leads to a decreased muscle protein synthesis, this may counteract the gains obtained by strength training sessions. Therefore, it is important to optimise the sprint training sessions with regard to post-exercise muscle (myofibrillar) protein synthesis. Another future perspective is treatment of anabolic resistance in e.g., ageing and various chronic diseases with sprint exercise combined with nutrient supply. This kind of exercise activates type II fibres that commonly are atrophied in muscle wasting and may stimulate muscle protein synthesis in these fibres. Moreover, the sprint exercise combined with nutrient supply may induce an increased inflow of substrates and hormones during the pronounced post sprint exercise hyperaemia. There is, however, a need to follow up the results in the current thesis by performing longitudinal studies on the effect of nutritional supply on changes in muscle mass or muscle cross-sectional area following sprint training in various populations.

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