Protein-carbohydrate supplements improve muscle protein balance in muscular dystrophy patients after endurance exercise: a placebo controlled crossover study

Grete Andersen¹, Mette C. Ørngreen¹, Nicolai Preisler¹, Tina D. Jeppesen¹, Thomas O. Krag¹,
Simon Hauerslev¹, Gerrit van Hall² & John Vissing¹

¹Neuromuscular Research Unit, Section 3342, Department of Neurology, Rigshospitalet, University of Copenhagen, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. ²Department of Biomedical Sciences, University of Copenhagen, Tagensvej 20, DK-2200 Copenhagen, Denmark

Running head: Post-exercise protein supplementation in muscular dystrophy

Correspondence to: Grete Andersen, MD
Neuromuscular Research Unit, Section 3342, Rigshospitalet
Blegdamsvej 9, DK-2100 Copenhagen, Denmark
Phone: + 45 35 45 61 35; Fax: + 45 35 45 61 38
Email: grete.andersen@regionh.dk

Contribution: The authors report no conflicts of interest during this work. Andersen, Ørngreen and Preisler contributed to conception and design of the study, acquisition, analysis and interpretation of data, undertook the statistical analysis and drafted the article. Jeppesen and Krag contributed to conception and design of the study and acquisition of data. Hauerslev contributed to analysis of data. Van Hall contributed to conception and design of the study and interpretation of data. Vissing contributed to conception and design of the study, interpretation of data, and drafted the article. All authors revised and approved the article.
Abstract

In healthy individuals post-exercise protein supplementation increases muscle protein anabolism. In patients with muscular dystrophies, aerobic exercise improves muscle function, but the effect of exercise on muscle protein balance is unknown. Therefore, we investigated 1) muscle protein balance before, during, and after exercise and 2) the effect of post-exercise protein-carbohydrate supplementation on muscle protein balance in patients with muscular dystrophies.

In 17 patients (7 women and 10 men, age 33 ± 11 years (18–52), BMI: 22 ± 3 kg/m² (16–26)) and 8 healthy matched controls (3 women and 5 men, age 33 ± 13 years (19–54), BMI: 23 ± 3 kg/m² (19–27)), muscle protein synthesis, breakdown, and fractional synthesis rates (FSR) were measured across the leg using tracer dilution methodology on two occasions; with and without oral post-exercise protein-carbohydrate supplementation.

In patients, muscle protein breakdown increased in the recovery period (11 ± 1 µmol phenylalanine/min) versus rest (8 ± 1 µmol phenylalanine/min, P = 0.02) enhancing net muscle protein loss. In contrast, post-exercise protein-carbohydrate supplementation reduced protein breakdown, abolished net muscle protein loss and increased the muscle FSR in patients (0.04 to 0.06 % per hour, P = 0.03).

In conclusion, post-exercise protein-carbohydrate supplementation reduces skeletal mixed muscle protein breakdown, enhances FSR, resulting in a reduced net muscle loss in patients with muscular dystrophies. The findings suggest that post-exercise protein-carbohydrate supplementation could be an important add-on to exercise training therapy in muscular dystrophies, and long-term studies of post-exercise protein-carbohydrate supplementation are warranted in these conditions.
Keywords: Endurance exercise; protein-carbohydrate supplementation; muscle protein metabolism; muscular dystrophy

Glossary:

BMD Becker muscular dystrophy
DM1 myotonic dystrophy type 1
FSHD facioscapulohumeral muscular dystrophy
FSR fractional synthesis rate
GC-C-IRMS gas chromatography-combustion-isotope ratio mass spectrometry
GC-MS gas chromatography-mass spectrometry
is internal standard
LBF leg blood flow
LGMD limb girdle muscular dystrophies
NAP n-acetyl-propyl
NB net balance
non-Sup none supplemented trial
PC-Sup protein-carbohydrate supplementation trial
Ra rate of appearance
Rd rate of disappearance
VO$_{2\text{peak}}$ maximal leg oxygen uptake
Introduction

The most common inherited muscular dystrophies in adults are myotonic dystrophy type 1 (DM1), facioscapulohumeral muscular dystrophy (FSHD), Becker muscular dystrophy (BMD), and limb girdle muscular dystrophy type 2 I (LGMD2I), which primarily affect skeletal muscle with clinical manifestations of progressive muscle wasting and weakness. No curative treatment exists, but a number of non-specific, symptomatic treatments are available. Regular cycle exercise at moderate intensity has been shown to be safe and improve fitness and self-reported daily activity levels (18; 19; 27; 28), but muscle protein metabolism during exercise is unknown in these disorders.

In healthy individuals, exercise induces an initial net muscle protein breakdown (22), which increases intramuscular concentration of amino acids. Subsequently, amino acids activate the protein synthesis signaling cascade, resulting in a positive net muscle protein balance after exercise (10; 22). Protein supplementation ingested immediately after both resistance and endurance exercise enhances this exercise-induced muscle protein synthesis (6; 7; 16; 29). Glucose stimulates insulin secretion, and the anabolic effect of insulin in combination with essential amino acids increase muscle protein synthesis more than providing essential amino acids alone (4).

We investigated exercise-induced muscle protein balance before, during and 3 hours after one-legged knee-extensor exercise, with and without post-exercise protein-carbohydrate-supplementation in patients with FSHD, DM1, BMD and LGMD2I, and healthy subjects, 1) to assess whether exercise-induced muscle protein breakdown, synthesis, and fractional synthetic rate (FSR) is enhanced in diseased muscle and 2) to investigate the effect of post-exercise protein-carbohydrate supplementation on these parameters.
Participants and Methods

Subjects: Patients were recruited from our Neuromuscular Clinic, Rigshospitalet, Denmark. We included 7 women and 10 men, age 33 ± 11 years (18–52), BMI of 22 ± 3 kg/m² (16–26), and with genetically verified muscle diseases: 7 DM1 patients carrying 100–300 CTG-repeats in the DMPK gene, 2 BMD patients carrying an exon 26 deletion and an in-frame deletion of exons 45–48 in the dystrophin gene, 2 LGMD2I patients homozygous for the L276I mutation in the FKRP gene, and 6 FSHD patients carrying deletions in subtelomere of chromosome 4q35 with 4–10 remaining D4Z4 units. Exclusion criteria were: 1) other diseases compromising motor function, 2) serious heart or lung conditions, 3) pregnancy and breastfeeding, and 4) inability to perform 40 min of moderate one-legged kicking exercise (severe muscle weakness). All patients were ambulatory. One LGMD2I patient used a walking stick for community ambulation.

Eight healthy subjects matched for gender, age, BMI, and physical activity level were included (3 women and 5 men, age 33 ± 13 (19–54) years, BMI 23 ± 3 (19–27) kg/m²).

Four of the patients (2 DM1, 1 LGMD, 1 BMD) and two of the healthy subjects did not agree to perform a second trial because of the invasive methodology.

Ethics: The study was approved by the Ethical Committees of Copenhagen, Denmark ([KF] 01 320211), and was performed in accordance with the Declaration of Helsinki. Subjects were informed about risks and discomfort involved before giving their written consent to participate.

Pre-experimental testing: To investigate muscle protein balance we chose a one-leg model, where exercise and sampling were isolated to one leg. A maximal incremental exercise-test was performed, by increasing workload every other minute until exhaustion. The test was used to determine a workload that corresponded to 70% of maximal leg oxygen uptake (VO₂peak). Aerobic exercise was performed on a one-leg-kicking cycle (figure 1A), a special ergometer bike.
constructed to investigate aspects during aerobic exercise (1). Subjects exercised with a cadence of 60 kicks per minute. Gas exchanges ($\text{VO}_2$ and $\text{VCO}_2$) were measured with a gas and airflow analyzer (Quark $b^2$, Cosmed, Italy) during the tests. Every other minute the level of perceived exertion was scored on a Borg scale (5; 9). Heart rate was monitored continuously (S&W Medico technique A/S, Denmark). To practice the one-leg kicking technique, and to ensure that subjects could perform the expected exercise program, a 40-min sub-maximal (70% $\text{VO}_2\text{peak}$) test was performed.

**Experimental Design:** After the pre-experimental examination, the subjects came to the laboratory on two other experimental days, separated by at least a month. The first day (non-sup) and the second day (PC-sup) were identical, except that subjects consumed a protein-carbohydrate-supplement (PC-sup) on the second test day (figure 1). Three days before each experiment, subjects were instructed to follow a standardized carbohydrate-rich diet and to refrain from ingesting food items containing corn. All experiments were performed in the morning after an overnight fast. Subjects were served a standardized breakfast containing a whole grain roll with jam approximately three hours before data sampling. Water ad libitum was allowed during both the fasting and the trial periods.

For stable isotope infusion, a catheter (20GA, 26inch BD Venflon$^\text{TM Pro}$) was placed in the cubital fossa vein. For blood sampling, catheters (Pediatric Jugular Catheterization, 20GA, 12cm, 0.02inch diameter, spring wire guide) were inserted in the right femoral artery and vein. Seldinger technique was used, under local anesthesia (Lidocain, 20mg/ml) to place the catheters 1-2cm proximal to the bifurcation and distal to the inguinal ligament. All catheters were kept patent by infusion (0.9% saline, containing heparin).
A priming dose of \([1,2-{^{13}C}]\)-leucine (1mg/kg) and L-[ring-{^2H_5}]\)-phenylalanine (0.5mg/kg) (Cambridge Isotope Laboratories, Andover, US), dissolved in 20ml saline, was delivered and followed by a constant-rate infusion (35.7ml/h L-[ring-{^2H_5}]\)-phenylalanine (0.02mg/kg/min) and \([1,2-{^{13}C}]\)-leucine (0.02mg/kg/min) in 250ml saline). The concentration of the infusate was estimated with a target tracer-to-tracee ratio of 5-10% in arterial blood.

After two hours of isotope infusion, subjects kicked for 40min at a workload of 70% of their \(VO_{2\text{peak}}\).

Blood samples were obtained before, during, and after exercise as shown in figure 1. Simultaneously with blood sampling, blood flow was determined just above the bifurcation of the femoral artery by ultrasound Doppler technique (Vivid I, General Electrics) (23).

Muscle biopsies were taken from the right vastus lateralis muscle (5mm Bergstrøm needle) before tracer infusion, and three hours post-exercise (figure 1). The biopsies were performed under local anesthesia (2ml Lidocain 20mg/ml, norepinephrine 5\(\mu\)g/ml). The tissue was immediately washed free of visible blood, frozen in liquid nitrogen, and stored at -80°C until analysis.

On the second experimental day, subjects consumed a commercially available oral protein-carbohydrate drink within 5 minutes after the 40-min kicking exercise. The drink consisted of 40g 50/50 ProMax powder dissolved in 0.4L skimmed milk, thus containing 32.8g protein, 36.6g carbohydrate, 1.4g fat, and 1238kJ in total.

**Blood sample analyses:** All blood samples were mixed with EDTA (ethylene-diamine-tetraacetic acid) (0.1 ml 0.33 M EDTA/ml blood), immediately centrifuged (5 min, at 4°C, with 3000 rcf, centrifuge 5702R, Eppendorf), and plasma samples were stored at – 80°C until analyzed for lactate and glucose (YSI 2300 STAT PLUS, Incorporated, Yellowsprings, OH), insulin (InsulinELISA96T, DAKO, Denmark), catecholamines (CAT RIA, IBL International GMBH, Hamburg,
Concentration and enrichment of phenylalanine were analyzed in arterial and venous plasma, and in the infusate. To calculate the concentration of total phenylalanine, we analyzed the enrichment of an internal standard with a known concentration of labeled phenylalanine. Internal standards (100 µL) were added to the samples (250 µL) and prepared with 500 µL 50 % acetic acid during the cation exchange under column chromatography (Bio-Rad AG 50Wx8, Hempel Hempstead, UK). The samples were eluted with 2 M NH4OH (ammoniumhydroxid), and dried overnight. To prepare derivates, we added 50 µL acetonitril and 50 µL MTBSTFA (N-methyl-N-(tertbutyldimethylsilyl) trifluoroacetamide) and heated the samples at 70° C for 1h. The enrichment of plasma samples and internal standard was measured by gas chromatography-mass spectrometry (GC-MS, Finnigan Automass II, Paris, France).

Muscle sample analyses: Muscle biopsies were cleaned under a stereomicroscope for blood and visible connective tissue. To measure the dry weight of the muscle tissue, biopsies were freeze-dried under vacuum for four days. 100µl of a ten-times diluted internal standard was added. The samples were homogenized with 500µl sulfosalicylic acid, centrifuged and divided in pellet and supernatant, this process was repeated three times. The supernatant were analyzed with the same method as described for the plasma. The pellet was washed with ethanol and dried with nitrogen. The protein in the pellet was hydrolyzed with 6 M HCl (Hydrogenchlorid), heated and dried, before the cation exchanging and derivation with NAP (n-acetyl-propyl) was undertaken. Then the NAP-leucine derivates were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Finnigan Delta+, Bremen, Germany).
Western blotting was performed as described before (13). In brief, muscle sections were homogenized, equal amounts of extracted muscle proteins were separated and transferred to PVDF membranes. Membranes were blocked, before incubation overnight with the following primary antibodies; PI3K (Tyr458(p85)/Tyr199(p55)), PDK1 (Ser241), mTOR (Ser2448), Akt, phospho-Ser473 (#4060), p70S6K (Thr389), S6 (Ser235/36), 4E-BP1 (Thr37/46) (Cell Signaling Technologies, Danvers, MA), MuRF1 (SAB2105510)/Sigma-Aldrich, St Louis, MO, and alpha-tubulin (12G10) (Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA). Immuno-reactive bands were detected (SuperSignal West Dura kit, Thermo Scientific, Waltham, MA), quantified (Gbox XT16 darkroom), and the intensities of immune-reactive bands were measured (16-bit digital photos, GeneTools software, Syngene, UK).

**Calculations:** Different amino acid pools are involved in muscle protein metabolism. Metabolites were assessed in two plasma pools, the arterial and the venous, and two muscle pools, the intracellular pool (the supernatant of the homogenized muscle biopsy) and the protein pool (pellet of the homogenized muscle biopsy) as described by Robert R.Wolfe (25). In these pools, amino acids flow between the arterial and venous pools through the intracellular pool, and from here to and from the muscle-protein pool. Whole body protein balance was calculated from the arterial pool [2]. Leg protein net balance (NB) was calculated as the difference between the arterial (a) and venous (v) pools [3]. The leg phenylalanine uptake, a measure for leg muscle protein synthesis, was estimated by the rate of labeled phenylalanine disappearance (R_d) from the arterial pool [4] into the leg. The leg phenylalanine release or rate of appearance (R_a) into the circulation, a measure for leg muscle protein breakdown, was assessed from the net-balance and rate of phenylalanine disappearance [5]. The mixed muscle fractional synthesis rate (FSR) was calculated from the measured enrichment of leucine in the two muscle pools, the intracellular pool and the

\[ C_s = \frac{V_a \cdot C_{is}}{V_a \cdot E_{is}} \quad \text{Whole body} \quad Ra = \frac{T}{E_a} \]

\[ NB = (C_a - C_v) \cdot LBF \quad Rd = \frac{(C_a \cdot E_a - C_v \cdot E_v) \cdot LBF}{E_a} \]

\[ Ra = Rd - NB \quad FSR = \frac{E_{\text{Muscle tissue (pellet)}}}{E_{\text{Intracellular muscle water}}} \times 100 \% \]

(V) Volume, (T) phenylalanine infusion rate, (LBF) leg blood flow, (s) sample, (is) internal standard.

**Statistical Analyses:** Descriptive values are presented as mean ± SD (range). Results are presented as means ± SEM. We used a paired Students t-test (two-tailed testing) to compare differences between rest (mean of 3 blood samples) and exercise (mean of 4 samples) and recovery (mean of 6 blood samples), between the first and second muscle biopsy and non-sup and PC-sup. An unpaired Students t-test (two-tailed testing) was used to compare differences between patients and healthy controls in non-sup. A P-value below 0.05 was considered significant.

Patients with four different types of muscular dystrophies were included in the study. The same tendencies were found in results from all subgroups. Thus, all four groups were pooled to increase the statistical power of the analysis. Since DM1, unlike the other muscular dystrophies studied, is a multisystem disorder, we made a sub-group analysis of phenylalanine kinetics in this group (table 2).

**Results**

**Metabolites, hormones and cardiovascular responses:** During the 40-min one-legged kicking exercise, heart rate, perceived exertion, blood flow, plasma lactate, and catecholamine’s were
comparable between non-sup and PC-sup. They increased to the same level in both patients and controls (table 1), indicating that patients and healthy controls performed exercise at the same relative work intensity. Plasma concentrations of leucine, phenylalanine, glucose, and insulin increased and FFA levels decreased 30 minutes after supplementation in all subjects (figure 2).

Without any outliers, insulin increased significantly together with blood-glucose after protein supplementation, indicating a normal insulin secretion. Blood glucose returned to normal (< 6 mmol/L) within an hour after supplementation in all subjects except for two patients (LGMD 7.1 mmol/L) and FSHD (6.4 mmol/L)), indicating a normal glucose-insulin response. Insulin increased similarly in all groups with no single patient being an outlier, indicating that none of the patients were insulin resistant (range: 207–468 pmol/l).

**Muscle phenylalanine balance:** Whole body phenyalalanine rate of appearance was lower in patients than in controls at rest (87 ± 2 versus 100 ± 6µmol/min), during exercise (92 ± 2 versus 104 ± 4µmol/min) and in the recovery period (81 ± 2 versus 94 ± 3µmol/min) (P=0.01).

At rest, muscle phenyalalanine net-balance across the leg was similar among patients and controls, although both the rates of muscle phenyalalanine uptake and release were lower in patients (P=0.005). During exercise, the phenylalanine net-balance and uptake were unchanged, but the phenylalanine release was increased in both patients (P=0.01) and healthy controls (P=0.006), figure 3.

In the 3 hours non-sup recovery period, the phenylalanine release remained increased in patients and caused a significantly exercise-induced net loss of phenylalanine (P=0.01), figure 3.

Post-exercise PC-sup induced an increase in leg phenylalanine net-uptake in patients (P=0.001) and controls (P=0.01). In healthy controls, phenylalanine leg uptake increased (P=0.004), indicating protein synthesis. In patients, the phenylalanine leg uptake was similar at rest and post-
exercise, indicating an unaffected protein synthesis. However, the significant increase in leg phenylalanine release seen in the non-sup recovery period was absent after PC-sup (P=0.003), indicating that PC-sup reduced muscle protein breakdown in the patients with muscular dystrophies (figure 3).

**Muscle protein synthesis rate:** From muscle biopsies we measured the FSRs to compare the effect of PC-sup. The mixed muscle FSR was similar in patients and health individuals without supplementation and increased with PC-sup (patients: P=0.03, n=9; controls: P=0.21, n=3) (figure 4).

**Activation of muscle protein synthesis:** Phosphorylation of proteins involved in the signaling cascade of muscle protein synthesis was analyzed by western blotting (figure 5). Due to lack of muscle specimens, we were unable to perform western blots for all proteins of the cascade to make paired analysis in all other subjects than DM1. The activation appeared to be at different stages of the cascade, therefore, a qualitative presentation of the data is provided in table 3, where exercise activated the muscle protein synthesis cascade in 6/7 patients with DM1.

**Discussion**

Patients with muscular dystrophies suffer from an accelerated loss of muscle proteins resulting in progressive muscle wasting through life. Aerobic exercise training has been shown to effectively improve fitness, and in some cases also muscle strength in these conditions (27). In healthy individuals, exercise induces muscle protein anabolism, can be enhanced by post exercise protein supplementation (6; 7; 16).

In the present study, we found that patients with muscular dystrophies following exercise have an accelerated degradation of muscle proteins, but a similar net muscle protein balance compared to
healthy controls. However, when in recovery from exercise protein-carbohydrates were supplemented the net muscle protein breakdown was abolished, albeit less than in healthy controls via a reduced muscle breakdown and increase muscle FSR in patients. Our findings of an anabolic effect of post-exercise PC-sup in muscular dystrophies are encouraging, and suggest that a long-term aerobic training program with post-exercise protein-carbohydrate ingestion should be undertaken to determine whether this anabolic effect translates into durable improvements in muscle function.

Muscle protein balance: In the recovery period we found that a single bout of exercise significantly increased post-exercise muscle protein breakdown in patients with DM1, FSHD, BMD and LGMD2I during the first three hours of recovery, which contrasted findings in healthy subjects, in whom there was a positive protein balance post-exercise as earlier described (8; 16). This seems to support the old notion that patients with muscular dystrophies should refrain from exercise training, because it can potentially damage muscle fibers and accelerate the disease process. This notion was built on studies in mdx-mice performing eccentric contraction (3; 21; 24), which is known to cause muscle injury, also in healthy humans. However, the time frame of three hours in the current study does not explain what happens later on with muscle protein metabolism. Training without protein supplementation in various muscular dystrophies investigated in the current study, have consistently shown improvements in fitness without evidence of muscle injury (2; 18; 19; 27; 28), which suggests that in the long run, protein balance with training is positive. Furthermore, muscle strength was improved in BMD patients (27), muscle fiber area increased in DM1 patients (19), and capillary density increased in LGMD2I patients (28) after aerobic training, which also indicates a positive net muscle protein balance. In accordance with this, the present
study showed that FSR was similar between patients and healthy controls, and the protein synthesis signaling cascade was activated after exercise in patients in whom it could be assessed (DM1). Based on this, we advise our patients to perform regular endurance training to improve endurance, muscle performance and prevent life-style disorders.

The protein-synthesis signaling-cascade is a “flooding” process where, in growth factor treated normal mice, has a small temporal window of phosphorylated proteins (14), which is essential for the measuring of the cascade. No similar studies have been done in humans, thus the best time to measure each component of the signaling-cascade is unknown. In the current study, exercise induced an active protein-synthesis signaling-cascade in most of patients with DM1, but the cascade was in different phases in the subjects.

Wholebody metabolism and the leg muscle protein balance at rest and during exercise, were qualitatively similar between patients and healthy, but the absolute rates were lower in patients. Since leg muscle mass is considerably lower in patients versus healthy controls, the lower phenylalanine release and uptake in the patients at rest and during exercise does not necessarily imply a lower muscle protein breakdown and synthesis. The influence of muscle mass on the absolute levels of protein turnover could not be ascertained in our study, since the participants’ muscle mass was not measured.

**Post-exercise protein-carbohydrate supplementation**: The effect of different protein- and amino acid supplements in combination with resistant exercise training has been investigated thoroughly in healthy subjects. In contrast, knowledge about the effect of combined protein supplementation and endurance training is much less investigated. In our study, PC-sup, caused net muscle anabolism in healthy controls as expected, whereas in patients PC-sup abolished net catabolism.
Post-exercise protein supplementation in muscular dystrophy

caused by a reduced muscle protein breakdown and increased FSR in the studied 3 hour recovery period. These encouraging findings of an anabolic effect of oral supplementation after endurance exercise are supported by a study using a comparable protocol in which they found an increased whole body protein turnover in elderly, healthy subjects (16). Furthermore, protein supplements after endurance exercise reduce muscle injuries and improve retest results after a recovery period in healthy individuals (8). A higher re-synthesis of muscle glycogen and a greater protein synthesis signaling cascade activation were demonstrated after protein supplementation compared with both an iso-caloric carbohydrate and a placebo supplementation (8).

Whether the positive effect of protein-carbohydrate supplementation after an acute exercise bout on muscle protein balance, as shown in our study, has potential long-term effects is unknown. In healthy, young men, long-term protein supplementation increased the maximal oxygen uptake more than supplementation with carbohydrate or placebo alone (7). Similar results were found in older men (17) and in patients with chronic obstructive pulmonary disease (15), which is encouraging for conducting long-term trials in patients with muscular dystrophies.

In the present study, we focused on the effect of aerobic exercise since it is known to have many beneficial health effects and is safe in muscular dystrophies. The beneficial effect on muscle protein metabolism after supplementation in both patients and in healthy controls in the present study suggests that the effect of protein supplementation after strength training, should also be considered in muscular dystrophies, because of its known anabolic effects in healthy individuals. However, the safety of strength training alone has not been properly investigated in muscular dystrophies.
Protein metabolism in patients with muscular dystrophies has been investigated in patients with DM1 in the post-absorptive resting state. Some older studies suggest that muscle wasting in patients with DM1 is caused by a defect in the muscle protein synthesis rather than in accelerated muscle breakdown (11; 12). In contrast, another study found that muscle wasting is caused by an elevated protein breakdown in DM1 patients, which could be explained by lower levels of plasma IGF-1 and higher levels of TNF-α (20). A direct comparison between these studies and ours is difficult, as we investigated the leg protein balance in relation to exercise and post-exercise PC-sup. However, we found an increased muscle breakdown after exercise and a lack of increase in protein synthesis after post-exercise supplementation. The anabolic effect of insulin is important for protein synthesis, and insulin resistance can abolish this effect. DM1 is a multi-systemic disorder with a high incidence of insulin resistance. In the current study, all DM1 patients had a normal glucose-insulin response, which indicates normal insulin sensitivity.

Investigation of rare diseases is challenging because of difficulties in recruiting large cohorts of patients to reach sufficient power. In the current study, the complicated methodology, need for fasting, invasive procedures, including femoral catheters and muscle biopsies, need for a 40-min exercise bout on three occasions and a total duration of each experiment of eight hours, further complicated the recruiting process and explain why some subjects dropped out. However, such complicated techniques are necessary to provide proof-of-concept inputs to the understanding of muscle protein metabolism during and after exercise, and the effect of protein supplementation after exercise in muscular dystrophies, and the present study is the first of its kind to do this. A distinction among the periods of rest, exercise and recovery would define FSR of muscle proteins in each period better. However, to answer this question, two more muscles biopsies would have
been required in each participant. This was not feasible in this frail group of patients, considering the invasive nature of the study. In our study, we used muscle protein FSR to compare the effect of the supplementation. The only difference in intervention between the two trials from the beginning of the rest period to the end of the recovery period, was the post-exercise supplement. It must therefore be assumed that protein metabolism was similar at rest and during exercise, because the interventions were identical, and that any difference detected in the 3-hr recovery period was caused by the protein supplementation. Results from blood samples during the periods of rest and exercise in the two trials were not different, which corroborates the assumption that the experimental conditions were identical in these parts of the experiment.

Perspectives and significance: In conclusion, we found that post-exercise protein supplementation abolished the rate of acute net muscle protein breakdown following exercise in mildly to moderately affected patients with the muscular dystrophies BMD, DM1, LGMD2I and FSHD. Our findings warrant that long-term studies on post-exercise protein-carbohydrate supplementation should be conducted to verify the time frame of the acute effect on muscle protein synthesis, breakdown, and signalling, there seems different from healthy individuals, and whether these effects are sustained for a longer period of time to result in gain of muscle mass, and should be recommended as an add-on to training in muscular dystrophies.

Acknowledgements

We would like to thank laboratory technicians Christina Andersen, Danuta Olsen, and Thomas Lauridsen, Neuromuscular Research Unit Copenhagen, and laboratory technician Nina Pluszek and engineer Flemming Jessen, Copenhagen Muscle Research Center, for their helpful assistance.
Further we would like to thanks the Danish Council for Independent Research, Medical Sciences (FSS) and The Novo Nordisk Foundation for financial support to this study.

Reference List


Table 1; Metabolites, hormones and cardiovascular responses to exercise.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Non-sup (n = 8)</td>
</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>Rest</td>
<td>0.9 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>End Ex.</td>
<td>2.8 ± 0.35</td>
</tr>
<tr>
<td>Norepinephrine (pmol/l)</td>
<td>Rest</td>
<td>438 ± 42</td>
</tr>
<tr>
<td></td>
<td>End Ex.</td>
<td>849 ± 126</td>
</tr>
<tr>
<td>Epinephrine (pmol/l)</td>
<td>Rest</td>
<td>108 ± 18</td>
</tr>
<tr>
<td></td>
<td>End Ex.</td>
<td>177 ± 22</td>
</tr>
<tr>
<td>Blood flow (l/min)</td>
<td>Rest</td>
<td>0.6 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>End Ex.</td>
<td>6.6 ±0.85</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>Rest</td>
<td>73 ± 5</td>
</tr>
<tr>
<td></td>
<td>Exercise</td>
<td>109 ± 3</td>
</tr>
<tr>
<td>Borg scale</td>
<td>Exercise</td>
<td>14 ± 0.6</td>
</tr>
</tbody>
</table>

End Ex. is the sample at the end of exercise. Exercise represents a mean over the 40 minutes of exercise.

The plasma concentrations are from arterial blood. The Borg scale is a subjective scale of the level of perceived exertion (range 6-20), were 15 is described as hard work. All values are mean ± SEM.
Table 2: Leg phenylalanine balance in patients with dystrophia myotonica.

<table>
<thead>
<tr>
<th></th>
<th>Phenylalanine uptake (synthesis)</th>
<th>Phenylalanine release (breakdown)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DM1</td>
</tr>
<tr>
<td><strong>Non-sup trial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>10.2</td>
<td>7.6</td>
</tr>
<tr>
<td>Exercise</td>
<td>19.3</td>
<td>22.7</td>
</tr>
<tr>
<td>Recovery</td>
<td>10.0</td>
<td>9.3</td>
</tr>
<tr>
<td><strong>PC-sup trial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>10.4</td>
<td>4.4</td>
</tr>
<tr>
<td>Exercise</td>
<td>21.7</td>
<td>19.1</td>
</tr>
<tr>
<td>Recovery</td>
<td>19.2*</td>
<td>6.7</td>
</tr>
</tbody>
</table>

The DM1 (dystrophia myotonica type 1 patients n=5) group was compared with healthy control subjects (n=6), for a comparison between all patients and controls see figure 3. Values are mean (μmol·min⁻¹). * Indicates significant difference (P<0.003) between the none supplementation trial (non-sup) and the protein-carbohydrate supplementation trial (PC-sup).
Table 3: Phosphorylation of proteins involved in the muscle protein-synthesis signaling-cascade in patients with myotonic dystrophy – change from baseline to 3 hour post-exercise.

<table>
<thead>
<tr>
<th>Participants</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-sup trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K (p55)</td>
<td>153</td>
<td>63</td>
<td>75</td>
<td>1337</td>
<td>41</td>
<td>235</td>
<td>297</td>
</tr>
<tr>
<td>PDK1</td>
<td>80</td>
<td>15</td>
<td>349</td>
<td>4</td>
<td>148</td>
<td>14</td>
<td>129</td>
</tr>
<tr>
<td>AKT (S473)</td>
<td>44</td>
<td>16</td>
<td>27</td>
<td>76</td>
<td>25</td>
<td>46</td>
<td>87</td>
</tr>
<tr>
<td>70S6K</td>
<td>503</td>
<td>87</td>
<td>242</td>
<td>5</td>
<td>22</td>
<td>67</td>
<td>1380</td>
</tr>
<tr>
<td>S6</td>
<td>51</td>
<td>15</td>
<td>384</td>
<td>7</td>
<td>115</td>
<td>nd</td>
<td>152</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>276</td>
<td>44</td>
<td>153</td>
<td>6</td>
<td>200</td>
<td>55</td>
<td>58</td>
</tr>
<tr>
<td>MURF1</td>
<td>90</td>
<td>184</td>
<td>81</td>
<td>49</td>
<td>164</td>
<td>88</td>
<td>84</td>
</tr>
<tr>
<td>PC-sup trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K (p55)</td>
<td>114</td>
<td>78</td>
<td>52</td>
<td>57</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDK1</td>
<td>31</td>
<td>114</td>
<td>91</td>
<td>15</td>
<td>19</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>AKT (S473)</td>
<td>36</td>
<td>16</td>
<td>41</td>
<td>19</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70S6K</td>
<td>244</td>
<td>315</td>
<td>751</td>
<td>85</td>
<td>1102</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td>43</td>
<td>207</td>
<td>nd</td>
<td>28</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4E-BP1</td>
<td>112</td>
<td>78</td>
<td>17</td>
<td>62</td>
<td>84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MURF1</td>
<td>92</td>
<td>150</td>
<td>135</td>
<td>113</td>
<td>158</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Phosphorylation of proteins involved in protein-synthesis signalling-cascade 3-hr post-exercise, analyzed by western blotting, expressed as change in pixilation of the band from baseline (100%). An active cascade starts with a dephosphorylation of pPI3K(p55) followed by phosphorylation of PDK1, AKT, 70S6K, S6 and 4E-BP1, respectively. MURF1 is a protein involved in protein degradation by increased phosphorylation. Non-sup: Non supplementation trial (n=7). PC-sup: Protein-carbohydrate supplementation (n=5), two subjects did not performed the PC-sup trial. Nd: not determined.

In the non-sup trial the cascade is active in participants 1, 3, 5, and 7, with phosphorylation of 70S6K, S6 or E4-BP1. In participants 4 and 6, PI3K is overexpressed and the rest of the cascade is de-phosphorylated together with MURF1, thus the cascade has been activated and ended, triggered by a negative feedback mechanism. In participant 2, the cascade is inactive. In the PC-sup trial, the cascade is active in participants 1, 2, 3, and 5, but inactive in participant 4.
Figure 1; Flowchart of the experiments. A) Exercise was performed on a specially constructed one-leg-kicking ergometer cycle to isolate the movement to the quadriceps muscle only. The right leg was fixed to the chair with straps, and the right foot was fixed in a boot connected to the ergometer cycle (Monark 824E Ergometer). The one-leg-kicking ergometer cycle has an “own-load”, and additional loads could be added to increase the workload at the back. A 100 g load added corresponded to increasing the load on the bike by 12 Watts. B) Exercise was performed on the one-leg-kicking ergometer cycle on two occasions. Catheters were inserted in the right femoral artery and vein. Exercise was performed at 70 % of maximal oxygen uptake with a cadence of 60 kicks per minute. We used leucine and phenylalanine to trace muscle protein turnover. A protein-carbohydrate drink (PC-sup) or no supplement (non-sup) was given after exercise.

Figure 2; Arterial concentrations of phenylalanine, leucine, glucose, insulin, and FFA in patients with muscular dystrophies and healthy matched subjects. In one trial, the participants did not consume anything (non-sup), and in the other trial they consumed a protein-carbohydrate containing drink after exercise (PC-sup). Exercise lasted 40 minutes (from 0-40). FFA = free fatty acids. All concentrations changed significantly after PC-sup in patients and controls (mean of the six post-exercise samples). Error bars are SEM.

Figure 3; Leg phenylalanine metabolism. Net phenylalanine uptake represents net muscle protein synthesis (in case of a negative number it signifies a net muscle protein loss). Unidirectional phenylalanine uptake represents muscle protein synthesis, and release represents muscle protein breakdown, assuming that the muscle free phenylalanine pool is unchanged. Rest is the pre-exercise period, exercise covers a 40-min exercise period, and recovery covers a 3-hours period.
following exercise. Significant differences between non-sup and PC-sup in same period and group
(* = P < 0.01). Significant differences from rest in the same group (# = P < 0.04). Error bars are
SEM.

Figure 4; Muscle protein synthesis rate. Muscle protein synthesis rate was calculated from the
amount of leucine incorporated into the muscle tissue over 6 hours. * Significant difference
between the baseline biopsy and the end of recovery biopsy (P = 0.03). Patients n=9. Control n=3.
Error bars are SEM.

Figure 5; Muscle protein synthesis signaling pathway. A) Proteins involved in the cascade of
protein synthesis in muscle cells, which were analyzed in our study. The bold arrows indicate
whether the molecule increases or decreases when the protein synthesis pathway is activated. B)
Western blot analysis of activated enzymes of the protein synthesis-signaling cascade when no
supplements are provided (non-sup) and when a protein-carbohydrate supplementation (PC-sup)
is provided in a representative subject (DM1 patient). Rest represents the first biopsy and recovery
(3h) represents the second biopsy (see figure 1). Alpha-tubulin was used as a load control to
normalize bands. Vertical lines signify discontinuous lanes on the same blot.