Protocol

Study Title: **Effect of exercise on the human skeletal muscle phosphoproteome**

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Rationale

Skeletal muscle is a highly plastic tissue, capable of adapting to changes in nutritional intake and contractile activity. For instance, resistance exercise results in a mild stimulation of rates of muscle protein breakdown (MPB) but a greater stimulation of the rates of muscle protein synthesis (MPS) (1-3). When resistance exercise is performed prior to protein ingestion there is a synergistic combination of the two stimuli such that rates of MPS are stimulated over and above those of MPB (4-6). Thus, repeated bouts of resistance exercise, when coupled with protein ingestion, result in the accretion of skeletal muscle protein referred to as hypertrophy (7). Importantly, by changing the nature of the exercise stimulus, it is possible to redirect the focus of the type of skeletal muscle proteins that are being synthesised. For example, we know that prolonged and repeated lower-load dynamic stimulation of skeletal muscle (*i.e.,* endurance exercise training) results in an increase in the expression of mitochondrial genes (8), proteins (8-10), and ultimately enhanced mitochondrial content (11), leading to a shift towards an oxidative phenotype, and improved fatigue resistance (12). Resistance exercise training also stimulates the transcription of genes and accrual of new muscle proteins (13, 14), but these genes and proteins are largely associated with the myofibrillar protein fraction, and regular resistance exercise leads to muscle hypertrophy and increased force-generating capacity (15, 16). However, during the early stages of exercise training, particularly in training-naïve participants there is a significant increase in the expression of genes common to both modalities of exercise (17, 18). It is only with sustained exercise training that there is a 'fine tuning' of the transcriptome, the protein synthetic response, and then the proteome that gives rise to divergent hypertrophic and oxidative phenotypes (17, 19).

Transcriptional responses to exercise training

Increases in mRNA expression following each exercise session result in enhanced translation of proteins, and ultimately adaptive changes in muscle protein content (12, 19). The temporal pattern of these changes with respect to endurance and resistance exercise training is now becoming clearer. Exercise-induced mitochondrial biogenesis, for example, is a characteristic feature of endurance exercise training (9, 12, 20) and is underpinned by the coordinated up-regulation of both mitochondrial and nuclear transcripts that encode for proteins involved in the electron transport chain (21-23), and lipid metabolism (24). These transcripts include peroxisome proliferator-activated receptor gamma co-activator (PGC)-1α, nuclear respiratory factors (NRFs), and the mitochondrial transcription factor A (TFAM)(25-27). An acute bout of endurance exercise activates sensors of cellular stress (8, 28), decreases methylation of promoter regions (29), stimulates phosphorylation of mediators of translation initiation (16), and increases the expression of the aforementioned transcripts (PGC-1α, NRFs, TFAM) (8, 12). The transient increase in the expression of genes in the hours following an acute endurance exercise session provides the gene template that precedes the respective increases in protein content observed during exercise training (8, 9). Compared to our understanding of transcriptional responses to endurance training, the corresponding picture with

respect to resistance exercise is relatively less complete. Resistance exercise results in the phosphorylation (i.e., activation) of the mechanistic target of rapamycin complex 1 (mTORC1)(30-33). mTORC1 activation serves to enhance MPS by (1) activating downstream protein kinases such as the ribosomal protein of 70 kDa S6 kinase 1 (p70S6K1) and 4E-binding protein-1 (4EBP1), which subsequently promote ribosomal binding to mRNA to initiate protein synthesis (34-37) and (2) up-regulating the transcription of the translational machinery itself (mRNA, ribosomal content) (38). Thus, resistance exercise stimulates an increase in mTORC1 activity and promotes increases in the rates of myofibrillar MPS through both increased translational efficiency (protein synthesized per unit of mRNA) and translational capacity (the abundance of the translational machinery; ribosomes) (31, 33).

Posttranslational modification of proteins

Key to exercise-induced adaptation is post-translational modification of proteins. One important post-translational modification is known as phosphorylation. Phosphorylation is the addition of a phosphate group to a target protein by a protein kinase using ATP and Mg (39). The addition of this phosphate can promote the catalytic activity or binding status of a protein resulting in a biological downstream effect. There are over 500 kinases in the human 'kinome'. We (39, 40) and others (16) often assess the phosphorylation status of single or multiple (~ten) kinases/proteins in response to exercise to gain a mechanistic understanding of how exercise alters the skeletal muscle phenotype. However, current methods are largely semi-quantitative (Western blotting) and are biased in terms of which proteins are studied (*i.e.,* the experimenter can only select a limited number of kinases/proteins to measure). Our understanding of how skeletal muscle adaptation is influenced by phosphorylation is therefore limited. Recent developments in mass spectrometry (41, 42) have enabled a wider interrogation of the phosphorylation status of proteins in response to exercise. Indeed, one study, using advanced liquid chromatography-mass spectrometry, revealed 1,004 unique exercise-regulated phosphorylation sites on 562 proteins in human skeletal muscle biopsy samples obtained before and after a bout of endurance exercise (41). This seminal finding has significantly advanced our knowledge regarding how endurance exercise impacts phosphorylation. Yet, no measurements of end-point MPS were made. Moreover, comparable work has not been performed with resistance exercise. The aim of our investigation is to conduct a similar global phosphoproteomic analysis of skeletal muscle using advanced mass spectrometry following ***both*** endurance and resistance exercise in humans. We will also directly measure rates of MPS for the 3 hours following exercise.

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Specific Objectives

1. Our primary objective is to examine the effect of resistance exercise on the human skeletal muscle phosphoproteome.

2. Our secondary objective is to assess changes in rates of myofibrillar and mitochondrial MPS following resistance and endurance exercise.

Hypotheses

We hypothesize that:

1. Both resistance exercise and endurance exercise will result in the widespread phosphorylation of proteins involved in the regulation of energy metabolism, transcription, translation and muscle protein synthesis.

2. Resistance exercise will increase myofibrillar MPS to a greater extent than endurance exercise.

3. Endurance exercise will increase rates of mitochondrial MPS to a greater extent than resistance exercise.

Participants

Inclusion Criteria

In order to participate in this study, participants must be male between the ages of 18 and 30 years and have performed resistance exercise 3 times per week for at least 1 year.

Exclusion Criteria

Participants cannot participate in, or will be released from, this study if they meet one of more of the following criteria:

- Smoker or user of tobacco products
- High physical activity.
- Have health problems such as: renal or gastrointestinal disorders, metabolic disease, heart disease, vascular disease, rheumatoid arthritis, diabetes, poor lung function, uncontrolled blood pressure, dizziness, thyroid problems, or any other health conditions for which you are being treated that might put you at risk for this study.
- Taking anti-diabetic, anti-inflammatory, platelet inhibitor, or anti-coagulant medications.
- Use of an investigational drug product within the last 30 days.
- Have participated in an infusion protocol in the last year.
- Do not understand English or have a condition the PI believes would interfere with a participants' ability to provide informed consent, comply with

the study protocol, or which might confound the interpretation of the study results or put someone at undue risk.

Recruitment

Twelve participants (6 male: 6 female) participants will be recruited for this study through poster advertisements. Potential participants will be asked if they meet all of the inclusion criteria and none of the exclusion criteria. During an initial screening visit those who qualify and are interested in participating will be invited to the Exercise Metabolism Research Group (EMRG) laboratory, Ivor Wynne Centre (IWC) at McMaster University to begin testing for the study. The potential participants will then be required to read the Information and Consent Form. A written copy of the consent form will be provided for the participants to read by themselves. Written informed consent will then be obtained.

Sample Size

This study is an observational pilot investigation. Previous work has detected changes in our primary outcome (phosphoproteome) following endurance exercise with only 4 male participants (41). For the current study, we wish to recruit both male and female participants. Therefore - accounting for participant attrition - we will aim to recruit 12 participants.

Methodology

A schematic illustration of the experimental design and experimental trial can be seen in Figures 1 and 2, respectively. In a within-subject repeated measures cross-over design, 12 participants (6 male and 6 female) will attend the laboratories at the Dept. of Kinesiology on 6 separate occasions. On the first occasions participants will be assessed for body composition using dual-energy x-ray absorptiometry (DXA) after an overnight fast (~10 hours) followed by familiarization on resistance exercise and endurance exercise equipment. At this point, your two legs will be randomly assigned to the resistance exercise or endurance exercise group.

On the second visit, participants will perform a 1-repetition maximum test on a leg press and leg extension machine. These tests will be used to determine the workload performed on the experimental trials. On the third visit participants will perform a $V0_{2\ max}$ test on a cycle ergometer to determine their aerobic fitness. On the same day, subjects will be familiarized with single-leg cycling. On visit 4, subjects will perform another $V0_{2max}$ test on a cycle ergometer using one leg only. This test will determine the intensity of cycling performed on the experimental trial. To ensure the intensity is appropriate, the subjects will be familiarized with the experimental trial training programs (see “Exercise protocols” below).

Subjects will then embark on a 3-day period of controlled diet and exercise followed by experimental trials (visits 5 and 6) separated by 1 week. The reason for the two independent trails is that the measurement of MPS requires the

infusion of a stable amino acid isotope (L-[*ring*-$^{13}C_6$] phenylalanine), which interferes with the assessment of the phosphoproteome due to the fact they both detect changes in protein mass. Thus the first trial will be conducted to assess exercise-induced changes in the phosphoproteome and the second for MPS, using the L-[*ring*-$^{13}C_6$] phenylalanine isotope.

Acute protocols (Visits 5 + 6)

On visit 5 participants will arrive at the laboratory at approximately 0640 after a 10h overnight fast. After a brief assessment, subjects will rest on a bed for 3 hours. After this period, a skeletal muscle biopsy will be obtained and participants will perform a bout of resistance exercise with a randomised leg, followed by a bout of endurance exercise. Skeletal muscle biopsies will again be obtained immediately following both bouts of exercise and again 3h following exercise. On visit 6, participants will again arrive at the laboratory at approximately 0640 after an overnight fast. This time, a catheter will be placed in the upper forearm. After the placement of the catheter, a primed constant infusion of L-[*ring*-$^{13}C_6$] phenylalanine will be commenced to measure rates of MPS and a catheter placed in the contralateral arm for repeated blood sampling. Exercise and skeletal muscle biopsies will be obtained as in visit 5 (see Figure 2). We have routinely obtained ethical approval and published papers for studies that employ skeletal muscle biopsies and stable amino acid tracers in human volunteers (3, 14, 44-49). Dr. Steven Baker, a liscenced physician, has approved our standard operating procedure and will oversee all muscle biopsy and catheter infusions.

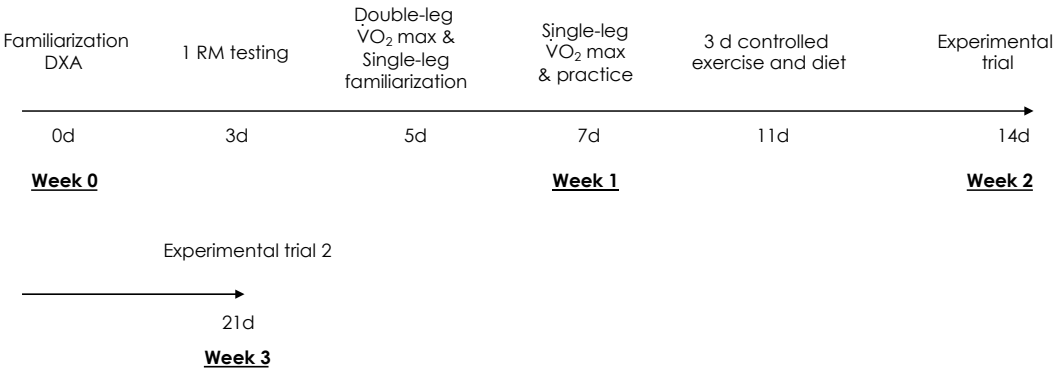
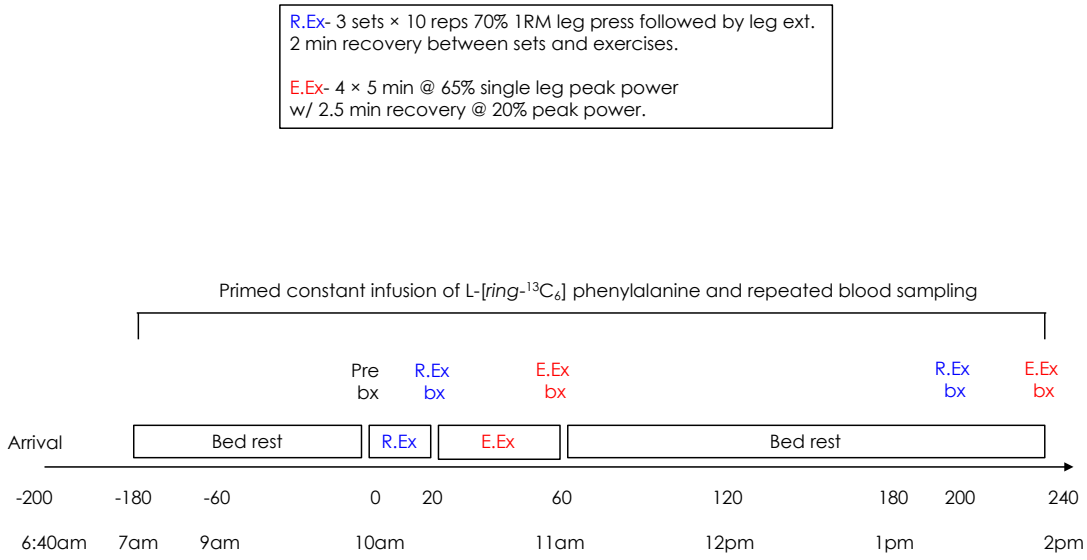


Figure 1 Schematic illustration of experimental design.



* Timing will change depending on exercise order

Figure 2. Schematic illustration of Visit 6.

Measurementsand Measurement Instruments

1 repetition maximum:
The same investigators will administer all strength testing. In short, after a brief general warm-up, a specific warm-up of the given exercise will be performed at approximately 50% of the participant's estimated 1RM based on the 10RM testing. Load will be progressively increased by approximately 10-20% for each repetition until a true 1RM will be reached as previously described (39). Three to 5 min of rest was given between each attempt. A successful attempt required the participant to move the load throughout the full range of motion with correct form.

VO_2peak

Subjects will complete double-leg and single-leg incremental peak oxygen uptake (VO_2peak) tests on a cycle ergometer (Velotron, RacerMate; Seattle, WA). A metabolic cart with an on-line gas collection system (Moxus modular oxygen uptake system, AEI Technologies, Pittsburgh, PA) will acquire oxygen consumption (VO_2) and carbon dioxide production (VCO_2) data and heart rate will be monitored continuously with a heart rate monitor (Polar A3, Lake Success, NY). The test will begin with a 1-min warm up at 50 watts (W), after which the power will be increased by 1 W every two seconds until volitional exhaustion or the point at which pedal cadence falls below 60 rpm. Heart rate will be monitored continuously throughout the test via telemetry with a heart rate monitor (Polar, Kempele, Finland). A similar protocol will be used for single-leg tests, except that power will increase by 1 W every *four* seconds. VO_2peak and will be defined as the highest oxygen consumption achieved over a 30-s period. Maximal workload (Wmax) will be the highest power output achieved during the test. The subject is deemed to have reached VO_2peak if: i) the perceived exertion is >19 (Borg Scale) ii) his HR is within 5bpm of age-predicted maximal HR; iii) his RER is >1.2; and iv) a plateau has been reached in their oxygen consumption.

DXA Scan:
Body composition (i.e. fat (%), appendicular lean mass (kg), and lean body mass (kg)) will be determined by dual-energy x-ray absorptiometry (DXA).

Deuterated Water:
Deuterium is an isotope of hydrogen and is in fact already present in small amounts in the water consumed daily (i.e. 0.02%). It poses no health risk in the quantities consumed during this study, and about half of the water in the body is replaced every week. Once the participant stops drinking this water, body water will return to its normal concentration in 20-30 days. During the study, participants will be required to drink ~1 cup of deuterated water (D2O).

Skeletal Muscle Biopsies and Analyses:
During the study, 7 muscle biopsies will be collected (days -3, 0 [4 biopsies], 1, and 2). This procedure involves the removal of a small piece of muscle tissue using a sterile hollow needle from the quadriceps muscle (*vastus lateralis*) under local aesthetic (lidocaine). A small piece of muscle (~100 mg) will be

collected at each time point. Following the biopsies, the incisions will be closed with instant medical adhesive, and wrapped with a tensor bandage. Phosphoproteomic analysis will be conducted by mass-spectrometry as previously described (41). Myofibrillar MPS will be calculated using the precursor-product equation: myofibrillar MPS =([E_{2b}-E_{1b}]/[E_{ic} x t]) x 100. E_b represents the enrichment of bound myofibrillar protein, E_{ic} is the average intracellular enrichment between two biopsies, and t is the tracer incorporation time in h. As we will employ 'tracer naïve' participants (had not previously participated in a study protocol where L-[*ring*- $^{13}C_6$] phenylalanine was infused), a pre-infusion blood sample will be used for the calculation of resting myofibrillar MPS (50). Myofibrillar and intracellular enrichments of L-[*ring*- $^{13}C_6$] phenylalanine will be measured as previously described (51). Integrated (i.e., days) rates of muscle protein synthesis (MPS) will be determined using the D_2O method. Briefly, ^2H enrichment of saliva (precursor) and muscle (product) pools (relative to ^1H) will be determined using isotope ratio mass spectrometry and myofibrillar fractional synthetic rate determined, as previously described (52, 53).

Diets:

Ad libitum 3-day dietary records for each participant will be collected and analyzed for baseline macronutrient intake. During the study, all food will be provided to the participants as frozen meals (Supplier: Copper County Foods), and pre-weighed and measured snack items.

Physical activity:

Recreationally active young men (assessed through physical activity questionnaire) will be recruited. Participants will be asked to maintain their habitual activity for the duration of the study.

Exercise Protocol:

Resistance exercise: 5 sets × 10 reps 75% 1RM leg press followed by leg extension, with 2 min recovery between sets and exercises (50).

Endurance exercise: 4 × 5 min @ 65% single leg peak power with 2.5 min recovery @ 20% peak power (MacInnis 2016)

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