DISSERTATION

PROTEIN SYNTHESIS RATES IN RESPONSE TO EXERCISE AND β-ADRENERGIC SIGNALING IN HUMAN SKELETAL MUSCLE

Submitted by

Matthew McHutcheson Robinson

Department of Health and Exercise Science

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Doctoral Committee:

Advisor: Benjamin F Miller

Michael J. Pagliassotti
Karyn L Hamilton
Adam J Chicco
Matthew S Hickey

ABSTRACT

PROTEIN SYNTHESIS RATES IN RESPONSE TO EXERCISE AND

β-ADRENERGIC SIGNALING IN HUMAN SKELETAL MUSCLE

Skeletal muscle protein turnover is determined by the synthesis and degradation of skeletal muscle proteins and is the mechanism that determines skeletal muscle protein content. A loss of skeletal muscle mass and function occurs during aging (sarcopenia) due to a net imbalance between synthesis and degradation pathways. Mitochondrial protein turnover, a component of skeletal muscle protein turnover, is decreased with aging. A decline in mitochondrial protein turnover and subsequent decline in mitochondrial function is associated with the progression of chronic diseases associated with aging. Aging populations are commonly prescribed medications to combat age-related chronic diseases. Among commonly prescribed medications are β-adrenergic receptor blockers as anti-hypertensive therapy. Decreased β-adrenergic signaling may impair skeletal muscle adaptations to exercise, particular the mitochondrial fraction, and potentially diminish the benefits of exercise training on skeletal muscle protein synthesis. The regulation of post-exercise mitochondrial protein synthesis by β-adrenergic receptor signaling is not well known in humans.

Protein consumption following exercise induces net synthesis of skeletal muscle proteins in younger populations, however the effect appears to be blunted with aging and likely lead to sarcopenia. The net positive protein synthesis following exercise with

protein feeding occurs for several hours and may be effective therapy for age-related declines in skeletal muscle mass, yet it is not known whether these short increases will persist over longer periods.

The overall objective of our three projects was to investigate the regulation of skeletal muscle protein synthesis in response to exercise, protein consumption, and β-adrenergic signaling in humans. We tested the hypothesis that β-adrenergic signals can regulate mitochondrial biogenesis by examining non-selective β-adrenergic stimulation during resting conditions (Experiment #1) and non-selective β-adrenergic blockade during aerobic exercise (Experiment #2). Furthermore, we tested the hypothesis that protein consumption following exercise can promote skeletal muscle protein synthesis over several weeks of aerobic training (Experiment #3). We used stable isotopic methods to determine rates of skeletal muscle protein synthesis including analysis of the mitochondrial fraction as a measure of mitochondrial biogenesis. Additional measures of mitochondrial biogenesis included mitochondrial DNA content and mRNA content of signaling pathways for mitochondrial adaptations. Deuterium labeling over several weeks was used to measure the synthetic rates of skeletal muscle proteins and DNA during aerobic training.

Experiment #1 involved examining the short-term response of skeletal muscle protein synthesis and mitochondrial biogenesis following infusion of a non-selective β-adrenergic agonist. We found that non-selective β-adrenergic activation did not increase skeletal muscle synthesis, whole body protein turnover, or measures of mitochondrial biogenesis. Experiment #2 included investigation of the short-term response of skeletal muscle protein synthesis following infusion of a non-selective β-adrenergic antagonist

during a one-hour bout of cycling. Mitochondrial protein fractional synthesis rates were decreased following cycling with non-selective β-adrenergic blockade, yet signals for mitochondrial biogenesis were not different compared to a saline control infusion. Experiment #3 included evaluating the ability for post-exercise protein consumption during aerobic training to stimulate long-term measures of multiple skeletal muscle synthetic processes. We determined that consuming protein compared to carbohydrates after exercise did not lead to differences in protein synthesis or mitochondrial DNA content over several weeks. Interestingly, we measured the amount of newly synthesized DNA in skeletal muscle to be ~5%. Skeletal muscle does not undergo regular cell division, therefore the DNA synthesis was higher than expected. It is likely that the DNA synthesis is due to satellite cell activation.

We conclude that β-adrenergic signaling during exercise is a signal for mitochondrial protein synthesis in skeletal muscle. Additionally, the ability for protein consumption following exercise to increase protein synthesis over several hours does not lead to long-term increases in protein synthesis. Collectively, these results provide insight into the regulation of skeletal muscle protein turnover with exercise and β-adrenergic signaling. Understanding potential negative drug and exercise interactions can help improve future therapeutic recommendations for healthy aging.

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CHAPTER I - INTRODUCTION/EXPERIMENTAL AIMS

It is important to understand the regulation of skeletal muscle protein turnover because of its direct and indirect effects on a variety of health disorders. For example, the loss of muscle mass with age leads to decreased physical function and alterations in metabolic rate and substrate metabolism that indirectly promote obesity and diabetes. Diet and exercise can promote health during aging through their impact on skeletal muscle protein turnover. In addition, various drug classes can alter skeletal muscle protein turnover and potentially impair beneficial adaptations to exercise. Further investigations should consider the effects of drugs on skeletal muscle protein synthesis.

Skeletal muscle protein turnover is the combination of the synthesis and breakdown of proteins and is a mechanism for skeletal muscle remodeling. Acute fluctuations in protein turnover occur following exercise or feeding and accumulate over time to change the protein composition of skeletal muscle. A net gain in skeletal muscle protein content occurs when synthesis exceeds breakdown, and net loss occurs when breakdown exceeds synthesis. Extended periods of net loss, such as what occurs with aging, can lead to significant decreases in skeletal muscle protein mass and impaired physical function. Although there is an obvious imbalance of synthesis and breakdown, the etiology of sarcopenia is not known. An initial hypothesis was that protein degradation exceeds protein synthesis with aging under resting conditions, however many

reports indicate that younger and older people have similar rates of protein turnover, both synthesis and breakdown, at rest. Instead, evidence now supports that aging leads to an impaired ability to respond to anabolic stimuli, such as exercise or protein consumption. Thus, a bout of exercise or protein consumption leads to smaller increases in protein synthesis and smaller decreases in protein breakdown, and contributes to an overall negative net protein balance with age.

The rates of skeletal muscle protein synthesis and breakdown can be measured using isotopic tracer methods. The most common method for human research is an intravenous infusion of stable isotopes of amino acids that are incorporated in newly synthesized skeletal muscle proteins. Muscle biopsies samples are collected and analyzed for the change of tracer incorporation into proteins over time. Such studies have provided much insight into how exercise, nutrition, and aging alter the regulation of skeletal muscle protein turnover. Using isotopic tracers, it is possible to accurately quantify subtle differences over several hours in skeletal muscle protein kinetics using tightly controlled in-patient study designs. However, in-patient studies designs are limited to several hours and are not representative of free-living conditions. Long-term studies that allow extended kinetic measures throughout periods of exercise, feeding, and fasting are also necessary to understand how protein kinetics change with aging and contribute to sarcopenia. Long-term protein synthesis rates of multiple skeletal muscle pathways can be measured using oral consumption of deuterium oxide. The use of deuterium oxide is not novel, yet few studies have used the method to evaluate long-term synthetic processes in humans.

Mitochondrial protein turnover is a component of skeletal muscle protein turnover and is recognized as an important contributor to health or disease. There is a loss of mitochondrial content and function with aging and a variety of diseases, and various therapeutic interventions appear to mediate their health benefits through improved mitochondrial function. Conversely, negative health effects can arise from drugs or conditions that impair mitochondrial function. In particular, consumption of β-adrenergic antagonists is common during aging and can potentially impact mitochondrial adaptations to exercise. Drug treatments are often prescribed with dietary and exercise recommendations, however there is a potential for negative interactions between therapies. It is necessary to understand the signals that regulate mitochondrial protein turnover in order to avoid negative drug interactions between therapies.

The overall objective of our projects was to investigate skeletal muscle protein kinetics in response to exercise and nutritional interventions. We performed a literature review and discussed the potential for commonly consumed drugs to impair skeletal muscle adaptations to exercise, in particular discussing the mitochondrial sub-fraction. Next, we performed a series of studies using short and long-term isotopic tracer methods to evaluate the regulation of synthetic pathways in response to β-adrenergic signaling, exercise, and feeding. Our experimental designs included measurements of key regulatory points (e.g. mRNA content or activation state of regulatory proteins) in combination with pathway kinetics (e.g. isotopic tracers). The combined approach provided insight into how exercise or drug interventions altered key regulatory points within a metabolic pathway and whether the overall pathway flux was altered.

Our first project was designed to evaluate if non-selective stimulation of β-adrenergic receptors could increase skeletal muscle protein synthesis and mitochondrial biogenesis. Second, we investigated if non-selective blockade of β-adrenergic receptors during aerobic exercise could decrease skeletal muscle protein synthesis and mitochondrial biogenesis. Third, we used a long-term isotopic labeling method during several weeks of aerobic training with nutritional intervention to simultaneously assess multiple skeletal muscle synthesis pathways in an aging population.

Overall hypothesis: We tested the overall hypothesis that stimulation of skeletal muscle protein synthesis with exercise can be modulated with β-adrenergic signaling and protein consumption.

Specific aim of Experiment #1: To determine if non-selective β-adrenergic stimulation can acutely stimulate skeletal muscle protein synthesis and mitochondrial biogenesis.

Specific aim of Experiment #2: To determine if non-selective β-adrenergic blockade during aerobic exercise can decrease the post-exercise stimulation of skeletal muscle protein synthesis and mitochondrial biogenesis.

Specific aim of Experiment #3: To determine if post-exercise protein consumption can increase skeletal muscle synthetic processes over several weeks of aerobic training.

Specific aim of Experiment #4 (appendix): To determine if a localized sampling method can be used to assess skeletal muscle collagen breakdown following an acute bout of resistance exercise.

Main conclusions

These projects represented novel investigations into short and long-term regulations of skeletal muscle protein synthesis. Our review of literature identified a variety of mechanisms by which drug treatments potentially have negative interactions with mitochondrial adaptations to exercise. Experiment #1 showed that non-selective β-adrenergic stimulation that evoked a systemic cardiovascular response was not sufficient to stimulate whole body protein turnover, skeletal muscle protein synthesis or mitochondrial biogenesis during resting conditions. Experiment #2 showed that non-selective β-adrenergic blockade during cycling blunted post-exercise mitochondrial protein synthesis in skeletal muscle. The increased sympathetic activity during exercise appears to stimulate post-exercise mitochondrial protein synthesis, without changes to mRNA signals for mitochondrial biogenesis. Experiment #3 showed that consuming protein following exercise during aerobic training does not improve long-term skeletal muscle protein synthesis measurements, contrary to current paradigms based on short-term studies. Our use of deuterium labeling in humans provided important insight into aerobic training adaptations and showed that short-term increases in protein synthesis shown by acute studies may not persist over several weeks. Additionally, the study demonstrated that aerobic exercise stimulated skeletal muscle DNA synthesis in adults. Skeletal muscle does not undergo regular cell division; therefore it is likely that the DNA synthesis is due to satellite cell activation. Satellite cell activation is known to occur during periods of skeletal muscle growth such as during childhood development or resistance training, yet our results indicated that satellite cell activation may occur in

older people during aerobic training. Experiment #4 demonstrated that, using a localized sampling method, no change in skeletal muscle collagen breakdown was detected following an acute bout of resistance exercise.

Collective, these projects provide insight into the regulation of protein turnover following exercise, nutrition, and drug interventions. Understanding how such interventions influence protein turnover will benefit future recommendations for improving health.

CHAPTER II-MANUSCRIPT I

The interactions of some commonly consumed drugs with mitochondrial adaptations to exercise

By: Matthew M Robinson, Karyn L Hamilton, and Benjamin F Miller

From: Department of Health and Exercise Science
Colorado State University
200 Moby B Complex
Fort Collins, CO 80523-1582

Running Title: Drug and exercise interactions

Please address correspondence to:

Benjamin F Miller PhD
Department of Health and Exercise Science
Colorado State University
200 Moby B Complex
Fort Collins, CO 80523-1582
Fax: 970-491-0445
Office: 970-491-3291
Email: bfmiller@cahs.colostate.edu

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Abstract

The importance of mitochondrial dysfunctions in the progression of chronic disease has been well established. Patients with chronic diseases are often prescribed a variety of medications, many of which have been shown to induce mitochondrial dysfunction. Exercise is a known stimulus for mitochondrial biogenesis and also recommended to patients as a lifestyle modification to supplement drug therapy. However, the potential interference of those drugs with mitochondrial adaptations to exercise has not been thoroughly investigated. This review provides a summary and discussion of known and potential interactions of commonly consumed drugs with exercise induced mitochondrial adaptations.

Introduction

Chronic diseases are the leading killers in the United States. Current health recommendations encourage physical activity and prevention and treatment for chronic disease (Haskell *et al.*, 2007). In addition, patients with chronic disease are often prescribed a variety of drugs in an attempt to reduce the progression of disease or to alleviate symptoms of the disease. While these patients are frequently encouraged to engage in routine physical activity, the interaction between exercise and drugs, both prescription and non-prescription, is not well known. Thus, patients may be inadvertently attenuating potential adaptations to exercise that could otherwise be beneficial.

It is now apparent that mitochondrial dysfunction is causal in many disease states (Ritz & Berrut, 2005; Wallace, 2005) and that improvement in mitochondrial function could be an important therapeutic target. It has been demonstrated that mitochondrial dysfunction is associated with obesity (Kim *et al.*, 2000), insulin resistance (Petersen *et al.*, 2004), heart disease (Chicco & Sparagna, 2007), and aging (Trifunovic *et al.*, 2004). Endurance exercise is associated with increased mitochondrial size, number, function, and this improvement is thought to contribute to the observed decreased incidence of chronic disease (see (Kim *et al.*, 2008) for review) and improvement in aging associated declines in function (Menshikova *et al.*, 2006) in those that are physically active. It is also thought that improvements in metabolic and cardiovascular disease progression after treatment with pharmaceuticals such as thiazolidinediones (Bogacka *et al.*, 2005), or non-pharmaceutical treatments including caloric restriction (Lopez-Lluch *et al.*, 2006), are at least partially mediated by increases in mitochondrial biogenesis and function.

The focus of this review is to examine current research regarding common prescription and non-prescription drug interactions on mitochondrial turnover and function, specifically in skeletal muscle. We identified a need for a review of the area because of problems we have encountered when determining inclusion/exclusion criteria for studies of exercise, mitochondria, and aging. Sympathetic nervous system β-adrenergic blockers, non-steroidal anti-inflammatory drugs (NSAIDS) and 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors (statins) will be considered as these are the three classes of compounds that we see most commonly when screening older subjects for our studies. The present review includes discussion of measures of mitochondrial size and function and focuses on *in vivo* studies performed in animal models and humans. The focus on whole organisms is not meant to take away from the importance of cell culture systems for studying drug induced mitochondrial toxicity as these studies have provided important insight into pharmacotoxicity and mitochondria (Krause *et al.*, 2003; Redlak *et al.*, 2005; Dikshit *et al.*, 2006). Mitochondrial protein turnover is a sub-fraction of muscle protein turnover and studies that include alterations to mixed protein turnover will be included due to possible effects on the mitochondrial fraction. As much as possible, the methods used to measure mitochondrial biogenesis (e.g. changes in mtDNA, respiratory activity, protein content etc) are included in this text, however the reader is encouraged to obtain the original source for further clarification of specific procedures. PubMed Central was used to search the National Center for Biotechnology Information databases using keywords including "mitochondria", "mitochondrial biogenesis", "protein turnover", "drug", "interactions", "exercise", "adaptations" and the specific classes and names of drugs. A recently published book

titled “Drug Induced Mitochondrial Dysfunction” provides additional information on drug interactions and mitochondrial toxicity including guidance for laboratory methodology (Dykens & Will, 2008).

Mitochondrial biogenesis

Mitochondrial biogenesis refers to changes in the volume, number of mitochondria per mass unit of muscle, or protein and lipid composition of mitochondrial membranes. Mitochondrial biogenesis is well known to occur following exercise training and results in an increased capacity for ATP production (Holloszy, 1967). However, viewing mitochondria in reference to cellular energy status overlooks important roles of mitochondria in managing oxidative stress and apoptosis. Indeed, the generation of apoptotic signals and reactive oxygen species is central to theories of aging and may mediate progression of disease and disability (Balaban *et al.*, 2005; Short *et al.*, 2005; Hiona & Leeuwenburgh, 2008). Other authors have presented detailed reviews of mitochondrial biogenesis (Hood, 2001; Joseph *et al.*, 2006; Scarpulla, 2008), but key components of the process are briefly highlighted here to provide the background for the discussion of drug interactions with exercise adaptations that will follow.

Mitochondrial biogenesis is the result of a highly complex, coordinated effort that results in expression of proteins encoded by both the nuclear (nDNA) and mitochondrial genomes (mtDNA). Although smaller than the nuclear genome, mtDNA (~16.5 kb) encodes for 13 subunits within complexes I, II, IV, and V that are required for electron transport chain respiration (Scarpulla, 2008). The coordination of the two genomes to make functional mitochondria appears to involve the transcription factor peroxisome-proliferator-activated receptor γ coactivator-1 α (PGC-1α) (Lin *et al.*, 2005). When

PGC-1α is overexpressed in myotubes there is an increase in mitochondrial biogenesis (Wu *et al.*, 1999), while PGC-1α knockout mice display decreased mRNA for mitochondrial proteins (Lin *et al.*, 2004). PGC-1α is also increased following exercise (Pilegaard *et al.*, 2003) and impairing the response of PGC-1α hinders mitochondrial adaptations (Leick *et al.*, 2008). It is important to note that although responses to exercise are decreased compared to wild-type littermates, PGC-1α knockout mice show mitochondrial adaptations indicating other factors influence mitochondrial biogenesis (Leick *et al.*, 2008).

Although the signaling pathways regulating mitochondrial biogenesis are not fully elucidated, it is well established that exercise exerts at least some of its beneficial health effects by inducing mitochondrial biogenesis. It is also clear that some of these mitochondrial biogenic pathways are primary or secondary drug targets. The present review will explore where drug and exercise interactions occur in order to better optimize the combination of drugs and exercise for therapeutic treatment.

β-blockers

An estimated 50 million people in the United States and one billion people worldwide (Chobanian *et al.*, 2003; Kearney *et al.*, 2005) are hypertensive (blood pressure > 140/90 mmHg). Furthermore, data from the Framingham Heart Study indicate people who are normotensive at 55 years are at a 90% residual lifetime risk for developing hypertension (Vasan *et al.*, 2002). Lifestyle modification is the primary recommendation to reduce blood pressure, however modification is routinely used in combination with pharmaceutical treatments such as diuretics, angiotensinogen converting enzyme (ACE) inhibitors, and β-adrenergic receptor antagonists. Of these

drug classes, β-adrenergic antagonists have a strong potential to affect mitochondrial biogenesis by impairing aerobic exercise capacity as well as inhibiting the post-exercise PCG-1α response.

β-adrenergic receptor blockers (β-blockers) effectively treat hypertension by antagonizing the effects of β-adrenergic receptor stimulation by the sympathetic nervous system and circulating catecholamines. β-blockers function by inducing vasodilation of vascular smooth muscle cells (predominately via β_2 receptors) or reducing cardiac rate and contractility (predominately β_1). Nonselective β-blockers antagonize all classes of β-adrenergic receptors ($\beta_{1\text{-}3}$ and putatively β_4) with various affinities (Reiter, 2004; Baker, 2005). Common β-blockers are β_1-selective (metoprolol, atenolol), β_2-selective (ICI 118551) or nonselective (carvedilol, propranolol) with the latter class often having a greater affinity for β_1 receptors (Reiter, 2004; Baker, 2005). Importantly, skeletal muscle predominately expresses β_2-receptors. Therefore, use of a nonselective β-blocker can limit aerobic exercise performance by antagonizing both cardiovascular (β_1) and skeletal muscle (β_2) responses to exercise.

The effects of β-blockade on adaptations to aerobic exercise have been studied in both healthy and hypertensive subjects. Maximum oxygen consumption (VO_{2max}), cardiac output, and stroke volume are common primary outcomes, however a few studies have evaluated mitochondrial function from skeletal muscle biopsies. We focus on VO_{2max} and mitochondrial function results since VO_{2max} is a strong indicator for health and is affected by mitochondrial adaptations. β-blockers decrease endurance exercise capacity, which may be mediated by interactions with mitochondrial adaptations (**Figure 1**).

It is known that β-blockers impair the expected adaptations to aerobic training. For example, Ades et al (Ades *et al.*, 1990) evaluated 10 weeks of endurance training (50 min, 4x/week, 75%-85% VO_{2max}) with β_1-selective (metoprolol) and a nonselective β-blockers (propranolol) on cardiac and skeletal muscle adaptations in hypertensive adults (46.5±7 yrs). The placebo group showed an increase (+24%, $p<0.05$) in VO_{2max} that was attenuated with β_1-blockade (+8%, $p<0.05$) and completely absent in the nonselective β-blocker group (Ades *et al.*, 1990). These results provide evidence that aerobic training adaptations may be attenuated with β-blockade and suggest that the nonselective inhibitors, which also antagonize β_2-receptors, can restrict aerobic training effects. However, from these results it is not possible to distinguish whether the inhibition was a mitochondrial effect.

Mitochondrial adaptations to endurance exercise may be attenuated concomitantly with aerobic adaptations while on β-blockade. Six weeks of aerobic training (50 min 3x/week, 85% $VO_{2\ max}$) increased VO_{2max} in healthy young men (21-35 yrs) on a placebo (+16%, $p<0.05$) to a greater extent than a β_1-selective (+6%, $p<0.05$) or nonselective β-blocker (no change) (Wolfel *et al.*, 1986). Mitochondrial function (succinate dehydrogenase and citrate synthase activity) also showed the greatest increase while on placebo, with attenuated responses in both β-blocker groups. Similar results were shown by Svedenhag et al. (Svedenhag *et al.*, 1984) following 8 weeks of cycling training (40 min 4x/week, 60-75% $VO_{2\ max}$) in young men (20-31 yrs). Maximal oxygen consumption increased for placebo and nonselective β-blocker groups, but cytochrome c oxidase and 3-hydroxyacyl-Coa deydrogenase increased to a greater extent in placebo (+87% and +63%, respectively) than β-blocker (+38%, +22%, respectively).

The results discussed above reveal differences in aerobic training adaptations between β_1-selective and non-selective β-blockers in humans. Data from rodent studies confirm that β_2-blockade may impair the adaptation to endurance exercise to a greater extent that β_1-blockade. Ji et al. reported 10 weeks of treadmill training (26.8 m/min 15% grade for 60 min, 5x/week) in rats increased skeletal muscle activity of enzymes in the tricarboxylic acid cycle (citrate synthase and malate dehydrogenase), electron transport change (cytochrome c oxidase), and fatty acid oxidation (hydroxyacyl coenzyme A dehydrogenase) activity in placebo and β_1-selective treatment, but there was no change in any of these with non-selective β-blockade (Ji *et al.*, 1986). However, a rate-limiting enzyme of fatty acid oxidation (carnitine palmitoyl transferase) was increased in all three groups indicating that mitochondrial enzyme adaptations can occur during β-blockade, possibly dependent on substrate availability (Ji *et al.*, 1986). Collectively, these results suggest β-blockade can impair training induced activities of mitochondrial enzymes in skeletal muscle, and the additional non-selective blockade of β_2-receptors having a more restrictive effect.

Recent evidence provides insight into a potential mechanism of β_2-receptor activation and mitochondrial adaptations through PGC-1α. Data collected from mice demonstrate that β_2-selective activation (clenbuterol) increases PGC-1α mRNA under resting conditions, but this effect was not evident during selective stimulation of α, β_1, or β_3 receptors, or in transgenic mice lacking β-adrenergic receptors (Miura *et al.*, 2007). Furthermore, β_2-blockade inhibited PGC-1α mRNA transcription following a 45-minute bout of treadmill running, an effect that was reversed with β_2-stimulation (Miura *et al.*, 2007). Interestingly, the same authors followed their initial results with a novel finding of

multiple isoforms of PGC-1α and demonstrated that both β_2-agonists and endurance exercise induced PGC-1α-b and PGC-1α-c, but not PGC-1α-a (Miura *et al.*, 2008). Thus, it appears that PGC-1α can be regulated by β_2-adrengeric stimulation or blockade.

In summary, β-adrenergic blockade can impair VO_{2max} and mitochondrial adaptations to endurance exercise (**Figure 1**). However, the effects are variable and may, at least in part, depend on the receptor specificity of the β-blocker. The impaired mitochondrial response to exercise training with non-selective β-blockers may be due to a β_2-receptor-mediated decrease in PGC-1α. Therefore, the potential for impaired exercise capacity associated with β-blockade, and the potential for attenuated mitochondrial adaptations mediated by β_2-blockade should be considered when prescribing β-adrenergic antagonist therapy.

HMG-CoA reductase inhibitors

HMG-CoA reductase inhibitors (statins) are commonly prescribed for patients with hypercholesterolemia (total cholesterol >200 mg/dl), with 24 million prescriptions filled in the United States during 2003-2004 (Mann *et al.*, 2008). The primary mechanism of statins is to inhibit the rate-limiting step for cholesterol synthesis, the conversion of HMG-CoA to mevalonate by the enzyme HMG-CoA reductase. The inhibition of HMG-CoA reductase by statins can effectively reduce circulating cholesterol concentrations and may also have beneficial effects on other cardiovascular disease risks, further reinforcing the use of statins in treatment of cardiovascular disease (Davidson *et al.*, 1997). Although statin treatments are highly effective and generally free of clinically relevant incidents, the most common side effect of statin treatments are skeletal muscle myopathies ranging from mild pain, weakness, and decreased function to

very rare but fatal cases of rhabdomyolysis (0.15 deaths in 1 million) (Thompson *et al.*, 2003).

While statin treatments are considered free of serious side effects (death, severe functionally impairment), epidemiological data have shown ~10% of patients on statins (40-80 mg/day) report at least mild muscular symptoms after one month of beginning therapy (Bruckert *et al.*, 2005). Additionally, ~38% of those with symptoms reported pain with moderate exertion during activities of daily living (Bruckert *et al.*, 2005). Biopsy samples from four patients with statin-associated myopathy revealed histological evidence for mitochondrial dysfunction including ragged red fibers, negative staining for cytochrome c oxidase, and increased lipid accumulation. These effects were reversed following administration of a placebo (Phillips *et al.*, 2002). Interestingly, all four patients were able to correctly identify placebo versus return to statin treatment. It is possible that statin induced myopathies are mediated through mitochondria (**Figure 2**). Initial research evaluated the possibility of ubiquinone depletion as mediating statin induced skeletal muscle myopathies.

The product of HMG-CoA reductase, mevalonate, is not only used as a precursor for cholesterol but also for multiple downstream products such as ubiquinone, dolichol, and intermediates of the cholesterol synthesis pathway that are used for isoprenylation of proteins (Olson & Rudney, 1983; Appelkvist *et al.*, 1994). The decreased activity of HMG-CoA reductase by statin treatment decreases flux through the cholesterol synthetic pathway and potentially has further reaching effects than simply lowering circulating cholesterol concentrations. For example, ubiquinol, the reduced form of ubiquinone, functions as an electron shuttle between the flavoproteins and cytochromes in the electron

transport chain (ETC). A branch point of cholesterol synthesis is the formation of farnesyl-pyrophosphate (FPP) that can be used to synthesize ubiquinone for the ETC or squalene for eventual cholesterol formation. Inhibition of HMG-CoA reductase can decrease the formation of isopentenyl diphosphate units needed for ubiquinone formation. In addition to sharing a common pathway, ubiquinone is transported by low-density lipoprotein cholesterol (LDL-C). A combination of impaired synthesis and a decrease in LDL-C most likely contributes to the decreased concentration of circulating ubiquinone observed following statin treatment (Elmberger *et al.*, 1991; Laaksonen *et al.*, 1996; Davidson *et al.*, 1997; Rundek *et al.*, 2004).

Intramuscular ubiquinone concentrations are less well characterized and may not respond to statin treatment in a similar fashion to circulating levels. Indeed, statin treatment in humans has shown inconsistent effects with reports of decreases (Paiva *et al.*, 2005), increases (Laaksonen *et al.*, 1995), and no change (Laaksonen *et al.*, 1996) on skeletal muscle concentrations of ubiquinone. The difference in effects on ubiquinone concentration may be related to differences in treatment doses and pharmacokinetics of the drugs. For example, the liver metabolizes 90-95% of an oral simvastatin dose which leaves 5-10% to enter general circulation as a bioactive metabolite whereas cerivastatin has about 60% active bioavailability (Williams & Feely, 2002). The variable and limited peripheral exposure to active metabolites of statins may contribute to the varied response of skeletal muscle to statins.

The variability in peripheral exposure to bioactive statin metabolites may contribute to varied responses of skeletal muscle concentrations of ubiquinone and mitochondria respiratory activity. For example, Lamperti et al. evaluated skeletal muscle

concentrations of ubiquinone and cytochrome c oxidase in hypercholesterolemic patients receiving low doses of statins (5-20 mg/day of a variety of statins) and did not show differences as compared to controls subjects in either variable (Lamperti *et al.*, 2005). In contrast, higher doses of simvastatin (80 mg/day) for eight weeks decreased intramuscular ubiquinone and decreased respiratory activity (-24% to -74%) of complexes II-IV and citrate synthase with no changes shown in groups receiving atorvastatin (40 mg/day) or placebo (Paiva *et al.*, 2005). It is important to note that these later findings by Päivä et al. were not significant after normalizing ETC activity for changes in citrate synthase activity, suggesting that the changes in complex activity are explained by decreased mitochondrial content (Paiva *et al.*, 2005). In a follow up report, the simvastatin treatment resulted in a decrease in the ratio of mtDNA to nDNA, providing further support that changes in mitochondrial respiratory capacity were due to decreased mitochondrial content (Schick *et al.*, 2007). Recent data from embryonic zebra fish showed lovastatin treatment decreased mitochondrial function (determined via MitoTracker and fluorescence activated cell sorting) in a dose dependent manner (Hanai *et al.*, 2007). The effect was abolished with concomitant transfection with PGC-1α cDNA suggesting that stimulation of mitochondrial biogenesis may protect against statin induced impairments in mitochondria function (Hanai *et al.*, 2007). Thus, although high-dose statin therapy could impair mitochondrial function through depletion of muscle ubiquinone, the primary mechanism for statins to impair mitochondria function may be mediated through apoptosis and decreased mitochondrial content.

In addition to respiratory chain activity effects, evidence exists suggesting that apoptosis pathways may contribute to statin associated myopathies. Mitochondrial

initiated apoptosis is thought to be influenced by the net contribution of pro- versus anti-apoptotic members in the B-cell CLL/lymphoma 2 (Bcl-2) family of proteins. The anti-apoptotic Bcl-2 subfamily includes Bcl-2, Bcl2-like 1 (Bcl-XL), and myeloid cell leukemia sequence-1 (Mcl-1), while pro apoptotic subfamilies include the Bcl-2 associated-X (Bax) proteins. Bax proteins are induced by BH3 interacting domain death agonist (BID) proteins to form channels in the outer membrane that release apoptosis promoting factors (including cytochrome c and apoptosis inducing factor). Bcl-2 and Bcl-XL proteins inhibit channel formation. The detrimental effects of statins are suggested to be induction of a pro-apoptotic state by down regulation of the anti-apoptotic Bcl-2 protein (Blanco-Colio *et al.*, 2002) and Mcl-1, a Bcl-2 homologue (Blanco-Colio *et al.*, 2002; Demyanets *et al.*, 2006). In cell cultures, the decrease in Bcl-2 with statins can be reversed upon treatment with farnesyl pryophosphate and geranyl-geranyl pyrophosphate suggesting that isoprenoid intermediates have a role in statin induced apoptosis (Blanco-Colio *et al.*, 2002).

Statins may also promote muscle fiber atrophy, specifically through atrogin-1, a muscle specific ubiquitin ligase. Recent evidence from human muscle samples showed atrogin-1 mRNA is increased with statin therapy (Hanai *et al.*, 2007). Atrogin-1 null myotubes and zebra fish embryos appear to be resistant to statin induced damage suggesting atrogin-1 plays an important role in statin myopathies (Hanai *et al.*, 2007). Thus, mitochondrial induced apoptosis or skeletal muscle atrophy may contribute to statin induced skeletal muscle myopathies.

The alterations to mitochondrial content and function discussed above likely play a role in contributing to skeletal myopathies that can occur during statin therapy. The

known side effects of muscle pain and weakness can impair mobility or exercise capacity and may hinder functional capacity of patients. Indeed, leg strength was reduced in four older patients while on statin therapy but improved within two weeks of stopping treatment (Phillips *et al.*, 2002). Upon return to treatment, each patient reported muscle pain and weakness again. The effect of four different statin treatments on muscle pain and weakness has also been evaluated in professional athletes with familial hypercholesterolaemia (Sinzinger & O'Grady, 2004). Out of 22 athletes, 16 were not able to tolerate statin treatment of any form due to adverse muscle symptoms. These were relieved in one to three weeks following removal of treatment and indicate statins can induce muscle myopathy in athletes.

In summary, statins have the potential to adversely affect aerobic exercise tolerance possibly through impaired mitochondrial function, decreased mitochondrial content, and apoptotic pathways. Although clinically serious myopathies are rare side effects of statin therapy, minor impairments to mitochondrial function that do not elicit severe symptoms could hinder mitochondrial adaptations to aerobic exercise. Thus, mitochondrial adaptations, which are a primary response to aerobic exercise, could be limited in patients who are consuming statins.

Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are a generic class of common nonprescription medications with analgesic, antipyretic, and antithrombotic capabilities. Ibuprofen, acetaminophen, and aspirin are regularly consumed NSAIDS and have an estimated 25 million users everyday (Wilcox *et al.*, 2005). The primary mechanism of NSAIDs is to impair the activity of cyclooxygenase (COX), which produces pro-

inflammatory prostaglandins from arachidonic acid. NSAIDs competitively bind to the arachidonate-binding site of COX to decrease formation of prostaglandins. The COX family has constitutive (COX-1) and inducible (COX-2) isoforms with additional variants of each. A putative COX-3 isoform has been identified as an intron-retaining version of COX-1 and is also sensitive to COX inhibitors thus providing a mechanism of action for drugs that do not target COX-1 or COX-2 (Chandrasekharan *et al.*, 2002). NSAIDs can induce negative gastrointestinal side effects due to inhibition of COX-1 located within the endothelium of the stomach and kidneys (Warner *et al.*, 1999). In an effort to avoid gastrointestinal side effects, selective COX-2 inhibitors (e.g. Celebrex and Vioxx) were developed to decrease COX-2 activity, which is locally induced at sites of inflammation (Warner *et al.*, 1999).

NSAIDs have the potential to impair mitochondrial function through a variety of mechanisms (**Figure 3**). *In vitro* models have shown NSAIDs can inhibit β-oxidation and promote uncoupling of oxidative phosphorylation (Nulton-Persson *et al.*, 2004), while *in vivo* human studies have shown decreased post-exercise skeletal muscle protein synthesis compared to placebo (Trappe *et al.*, 2002). The effects of NSAIDs on *in vitro* oxidative phosphorylation and *in vivo* protein synthesis will be considered separately.

NSAIDs and in vitro mitochondrial function

The carboxyl group and lipid soluble nature of NSAIDs allow the drug to pass through the mitochondrial membranes and act as a protonophore to disrupt the membrane potential across the inner membrane. NSAID exposure to isolated mitochondria from rat liver showed increased proton leak and decreased rates of ATP synthesis (Krause *et al.*, 2003). In agreement, mitochondria isolated from rat kidneys also showed uncoupled

oxidative phosphorylation following NSAID treatment (Mingatto *et al.*, 1996). The extent of uncoupling may be due to the pKa of the acid group. Nulton-Persson et al. showed that salicylic acid (the primary metabolite of acetylsalicylic acid or aspirin) exerted a larger inhibition of state-3 respiration than acetylsalicylic acid in mitochondria isolated from rat hearts (Nulton-Persson *et al.*, 2004). It is important to note that the doses of salicylic acid and acetylsalicylic acid used by Nulton-Persson et al. were in the range of estimated plasma concentrations during therapeutic use (low millimolar concentrations) suggesting that mitochondrial inhibition may occur with commonly consumed doses. The study further reported that salicylic acid and acetylsalicylic acid were both able to inhibit a rate controlling Kreb's cycle enzyme, α-ketoglutarate dehydrogenase. However, the inhibitory effects of salicylic acid, but not acetylsalicylic acid, could be reversed with increased concentrations of the substrate α-ketoglutarate (Nulton-Persson *et al.*, 2004). Collectively, these *in vitro* results indicate that aspirin can impair mitochondrial function, but the inhibitory characteristics can vary between metabolites of the drug.

In addition to uncoupling oxidative phosphorylation, NSAIDs can also impair mitochondrial function by inducing oxidative stress. Mice injected with a high dose (300 mg/kg body weight) acetaminophen showed increased liver mtDNA damage with concomitant increases in the highly reactive species peroxynitrite compared to control mice (Cover *et al.*, 2005). The mitochondria also showed much greater protein nitration than other cellular locations (nucleus, cytosol, microsomes) suggesting that mitochondria are a site for oxidative damage presumably due to accelerated peroxynitrite production (Cover *et al.*, 2005). Injection with glutathione either immediately or 1.5 hours after

acetaminophen injection blunted the effects of the drug treatment suggesting that oxidative stress may be mediating the adverse drug effects (Cover *et al.*, 2005). Although the dose of acetaminophen represents a hepatotoxic dose and was much higher than commonly consumed, the results indicate mitochondrial induced oxidative stress and mtDNA damage can occur with NSAID treatment.

Mitochondrial mediated apoptosis may also be stimulated by NSAIDs. Aspirin is being investigated for its ability to stimulate apoptosis through both extrinsic and intrinsic pathways. The intrinsic mechanism is suggested to be through generation of a pro-apoptotic environment by up regulating Bax and down regulating Bcl-2 proteins (Dikshit *et al.*, 2006). Neuronal cell cultures had concentration dependent decreases in cell survival with aspirin treatment (2.5-50 mM aspirin), and parallel increases in cytochrome c release and caspase 3 and 9 activities (Dikshit *et al.*, 2006). Similar increases were also observed in the pro-apoptotic Bax and proteins involved in cell cycle arrest (p53, and $p27^{kip1}$) suggesting aspirin promotes cell cycle arrest and apoptosis in concentrations of 2.5 to 50 mM. Although the upper range (greater than 10 mM) of these aspirin concentrations are higher than circulating concentrations (~1-10 mM), the lower concentrations of aspirin (2.5-10 mM) still exerted negative effects suggesting physiological concentrations of aspirin may promote apoptosis and cell cycle arrest (Nulton-Persson *et al.*, 2004; Dikshit *et al.*, 2006).

NSAIDs and in vivo protein synthesis

It is clear that NSAIDs can impair cell survival and mitochondrial function in cell cultures and animal models. The negative effects of NSAIDs on mitochondrial function may also occur through impairments to skeletal muscle protein synthesis. Mitochondrial

protein synthesis is a fractional component to mixed muscle protein synthesis; therefore any decreases in mixed muscle protein synthesis could also include decreased mitochondrial protein synthesis. Recent evidence in humans identify that NSAIDs can impair mixed muscle protein synthesis following resistance exercise through a COX mediated mechanism (Weinheimer *et al.*, 2007). Although mitochondrial adaptations are commonly considered adaptations to aerobic exercise, resistance exercise has been shown to increase mitochondrial protein synthesis (Wilkinson *et al.*, 2008) and should be included when evaluating the potential of drugs to impair exercise induced mitochondrial biogenesis.

COX-1 and COX-2, but not COX-3, mRNA are increased following resistance exercise in humans (Weinheimer *et al.*, 2007). All isoforms of COX act on arachidonic acid liberated from lipid membranes to produce prostaglandin-H_2 (PGH_2), which is further modified to active forms including prostaglandin-$F_{2\alpha}$ ($PGF\text{-}_{2\alpha}$) (Breyer *et al.*, 2001). $PGF_{2\alpha}$ mediates skeletal muscle growth by participating in myoblast fusion and multinucleation of fibers (Horsley & Pavlath, 2003). In human skeletal muscle, $PGF_{2\alpha}$ is increased (~60%) following eccentric resistance exercise, an effect that is abolished with acetaminophen or ibuprofen (Trappe *et al.*, 2001). Additionally, these drugs also attenuate the post-exercise increase in fractional synthetic rate of mixed skeletal muscle protein 24-hours following high intensity resistance exercise (Trappe *et al.*, 2002). Thus, COX inhibition can impair mixed skeletal muscle protein synthetic response to resistance exercise. Since mitochondrial protein synthesis is a subfraction of mixed muscle protein synthesis and these studies did not separate the mitochondrial fraction, the effects of NSAIDs on mitochondrial protein synthesis are not known. Early results from an

isolated mitochondrial fraction from rat liver showed decreased basal rates of incorporation of a radioactive leucine tracer with increasing concentrations of salicylate (0.6-20 mM) and suggest that aspirin may impair mitochondrial protein synthesis (Dawkins *et al.*, 1966). The inhibitory effects on leucine incorporation were concentration dependent beginning at low concentrations (~66% inhibition at 0.6 mM), which is within the estimated circulating concentration following aspirin consumption (Dawkins *et al.*, 1966; Nulton-Persson *et al.*, 2004). Thus, NSAIDs can impair mixed protein synthesis following resistance exercise, which may include negative effects on the mitochondrial fractional synthesis rate.

In summary, data from *in vitro* and animal models demonstrate that NSAIDs can impair mitochondrial function by uncoupling oxidative phosphorylation and can induce mitochondrial-mediated apoptosis. Further, NSAIDs can impair protein synthesis following resistance exercise and may subsequently impair mitochondrial protein synthesis.

Conclusions, future directions, and recommendations

As research continues to evaluate mitochondrial function and potential countermeasures for dysfunction, the effects of drugs on mitochondria content and activity will need to be considered. This review has presented a brief background and evidence for drugs that are commonly consumed and subsequently encountered by researchers and clinicians. Increasing physical activity is a common lifestyle recommendation for hypertension and hypercholesterolemia, however drugs such as β-blockers and statins that are commonly prescribed for their treatment, have the potential to limit the beneficial effects of exercise on mitochondria. Similarly, NSAIDs used to

ameliorate post-exercise pain or decrease cardiovascular disease risk may also impact skeletal muscle recovery from exercise.

In this brief review only a small subset of common medications were discussed. Many of the findings discussed herein were limited in their interpretation to skeletal muscle effects rather than mitochondria per se. Although evidence was presented that these medications may affect mitochondria function, there is need for mitochondrial specific study designs. In addition, experiments should be designed to evaluate the impact of pharmaceutical compounds during rest, exercise, and post-exercise recovery, with emphasis on the post-exercise period since this in when most exercise-induced adaptations occur.

Population specific designs (e.g. young, aged, obese) should also be considered when examining potential exercise and drug interactions. It is realistic to expect that treatment in one population where mitochondrial or exercise function is optimal may differ from other populations where mitochondrial or exercise function is compromised. Careful attention must also be paid to the drugs used by study participants with clear indication of the medications in the resulting publication. For those who prescribe exercise to patients, careful attention must be paid to the drugs patients are using and what the intended goal of drug/exercise treatment is. Finally, researchers, especially those investigating exercise and chronic disease, should continue to explore the interaction between drugs, exercise, and mitochondrial adaptation. If it can be determined where detrimental effects occur, it may be possible to eliminate negative interactions to facilitate the transition to a state of health based on exercise rather than medication.

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Figures

Figure 1: Interactions of β-blockers and mitochondrial biogenesis. Aerobic exercise activates β_2-adrenergic receptors (β-AR) on skeletal muscle and induces PGC-1α transcription, a regulator of mitochondrial biogenesis. Selective and non-selective β-blockers can blunt β_2-AR signaling, which restricts the expected PGC-1α response following exercise and can impair adaptations to mitochondria and aerobic capacity.

Figure 2: Statin induced skeletal myopathies may originate with mitochondrial dysfunction. The decreased flux through the cholesterol biosynthesis pathway with statin therapy can impair production of necessary components of the electron transport chain including isoprenylated proteins (for membrane attachment), heme a (necessary for complex-IV), and ubiquinone (electron shuttle). Statins may also negatively affect mitochondria by increasing apoptosis (via Bcl-2) and protein degradation (via atrogin-1). A combination of diminished functional respiratory chain components and pro-apoptotic environment can lead to statin-induced mitochondrial dysfunction.

Figure 3: NSAIDs can impair mixed muscle protein synthesis and may decrease the fractional synthetic rate (FSR) of mitochondrial proteins. Exercise promotes muscle protein synthesis including the mitochondrial fractional synthesis rate. $PGF_{2\alpha}$ production by cyclooxygenase appears to be a necessary stimulus for protein synthesis, however NSAIDs inhibit cyclooxygenase activity and block $PGF_{2\alpha}$ production. NSAIDs can also induce cell cycle arrest and apoptosis leading to decreased cell proliferation and

subsequently decreased mixed muscle FSR, which includes the mitochondrial fraction. NSAIDs can impair mitochondrial adaptations to exercise by attenuating protein synthesis and promoting protein breakdown.

Figure 1

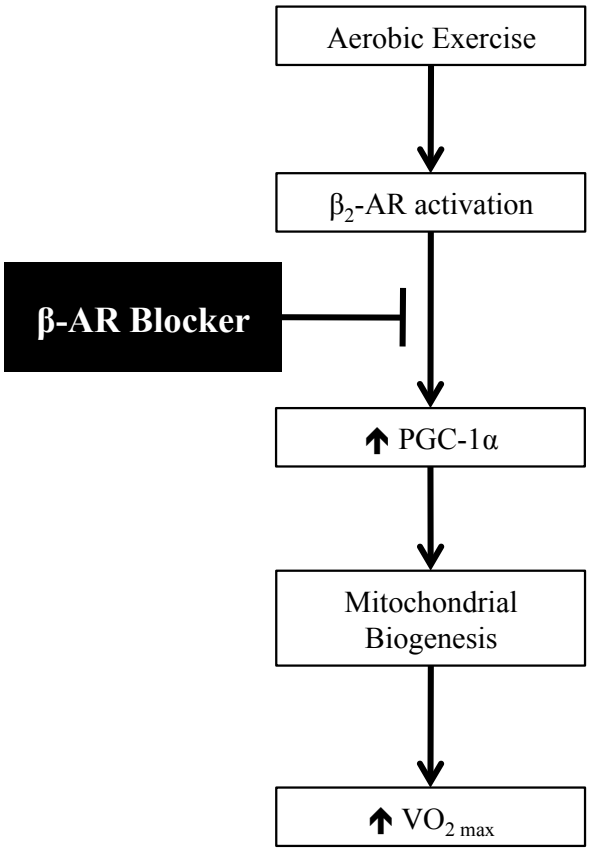

Aerobic Exercise
β_2-AR activation
β-AR Blocker
↑ PGC-1α
Mitochondrial Biogenesis
↑ $VO_{2\ max}$

Figure 2

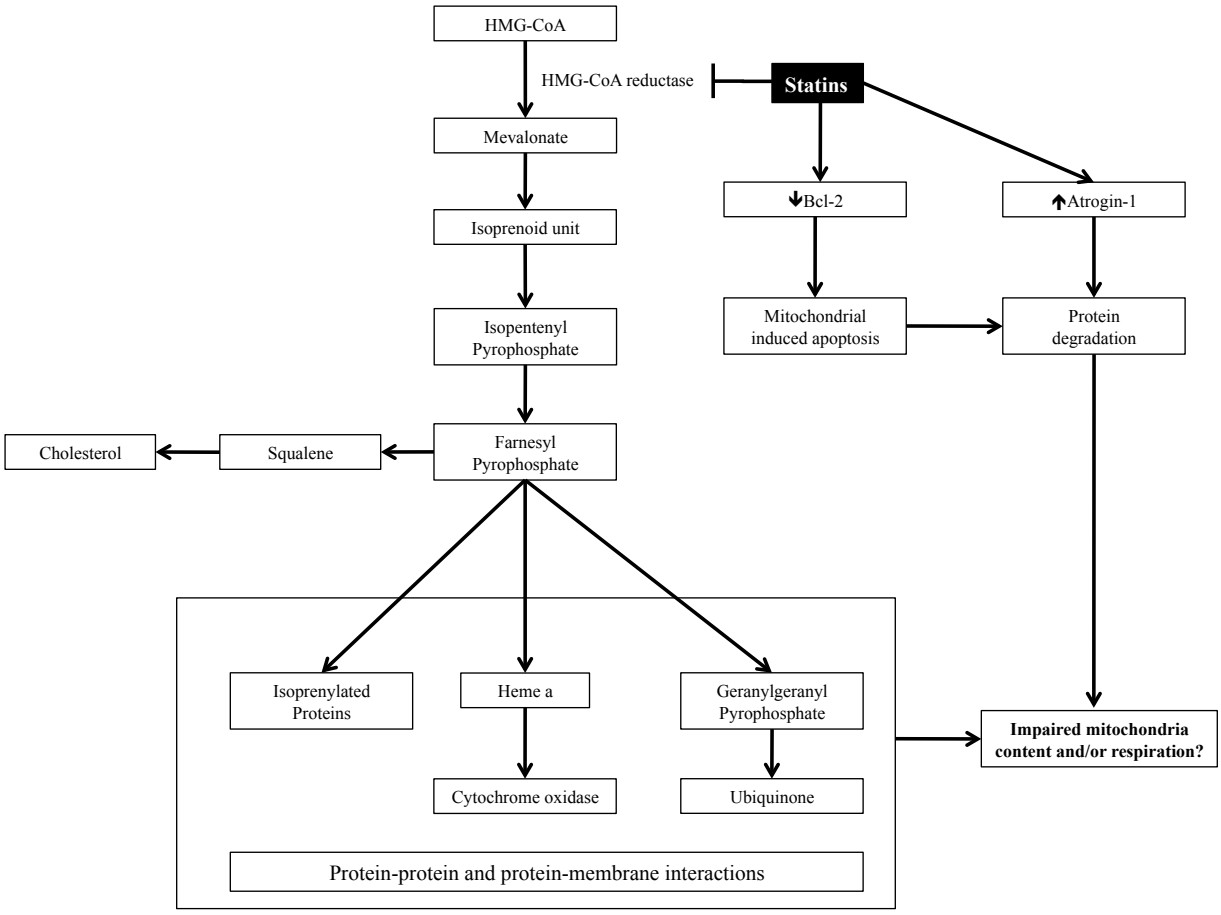
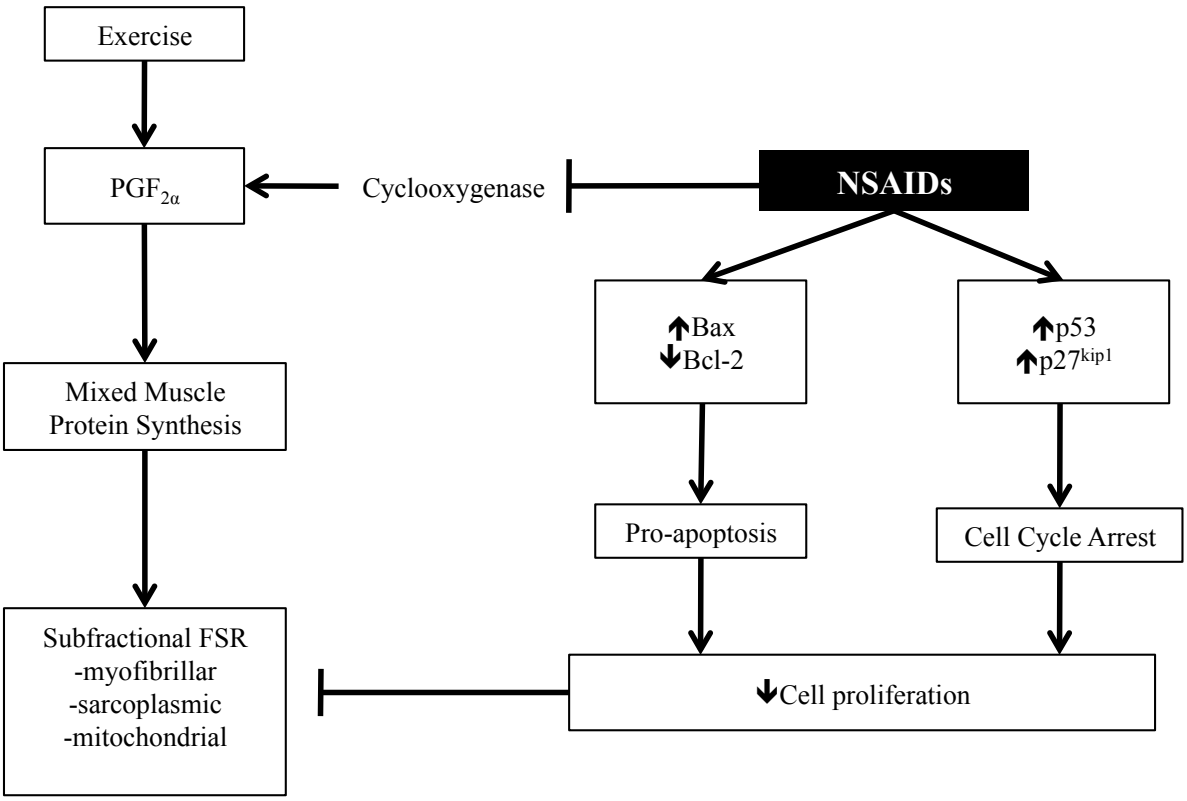

HMG-CoA
HMG-CoA reductase
Statins
Mevalonate
↓Bcl-2
↑Atrogin-1
Isoprenoid unit
Isopentenyl Pyrophosphate
Mitochondrial induced apoptosis
Protein degradation
Cholesterol
Squalene
Farnesyl Pyrophosphate
Isoprenylated Proteins
Heme a
Geranylgeranyl Pyrophosphate
Impaired mitochondria content and/or respiration?
Cytochrome oxidase
Ubiquinone
Protein-protein and protein-membrane interactions

Figure 3


Exercise
$PGF_{2\alpha}$
Cyclooxygenase
NSAIDs
↑Bax
↓Bcl-2
↑p53
↑$p27^{kip1}$
Mixed Muscle
Protein Synthesis
Pro-apoptosis
Cell Cycle Arrest
Subfractional FSR
-myofibrillar
-sarcoplasmic
-mitochondrial
↓Cell proliferation

CHAPTER III-MANUSCRIPT II

Acute β-adrenergic stimulation does not alter mitochondrial protein synthesis or markers of mitochondrial biogenesis in adult men

Matthew M. Robinson[1], Jennifer C. Richards[1], Matthew S. Hickey[1], Daniel R. Moore[2], Stuart M. Phillips[2], Christopher Bell[1], and Benjamin F. Miller[1]

[1]Department of Health and Exercise Science, Colorado State University, 200 Moby B Complex, Fort Collins, CO 80523-1582, and [2]Exercise Metabolism Research Group, Department of Kinesiology, McMaster University, Hamilton, Canada.

Correspondence to:

BF Miller
Department of Health and Exercise Science
Colorado State University
200 Moby B Complex
Fort Collins, CO 80523-1582

Fax:(970) 491-0445
Office: (970) 491-3291
Email: bfmiller@cahs.colostate.edu

Running Title: Beta-Adrenergic Stimulation and Mitochondria

Key Words: protein synthesis, stable isotopes, sympathetic nervous system, isoproterenol, skeletal muscle

Abstract

Exercise induced expression of peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) is dramatically inhibited in mice pre-treated with a beta-adrenergic receptor (β-AR) antagonist, suggesting that β-ARs play an important role in

the regulation of skeletal muscle PGC-1α expression, and potentially, mitochondrial biogenesis. Accordingly, we hypothesized that acute β-AR stimulation would induce transcriptional pathways involved in skeletal muscle mitochondrial biogenesis in humans. Whole body protein turnover (WBPT), myofibrillar protein synthesis (MyPS), skeletal muscle mitochondrial protein synthesis (MiPS) and mitochondrial biogenic signaling were determined in samples of vastus lateralis obtained on two separate occasions in 10 young adult males following 1-hour continuous intravenous administration of saline (CON) or a non-specific β-AR agonist (isoproterenol (ISO): 12 ng•kg fat free mass^{-1}•min^{-1}), combined with co-infusion of [1,2]^{13}C-leucine. β-AR stimulation induced appreciable increases in heart rate and systolic blood pressure (both $P<0.001$) but did not affect mitochondrial biogenic signalling (no change in PGC-1α, TFAM, NRF-1, NRF-2, COX, or NADHox expression via RT-PCR; $P>0.05$). Additionally, MiPS (CON: 0.099±0.028, ISO: 0.074±0.046 (mean±SD); $P>0.05$) and MyPS (CON: 0.059±0.008, ISO: 0.055±0.009; $P>0.05$) and measures of whole body protein turnover (WBPT) were unaffected. Based on this investigation we conclude that acute intravenous β-AR stimulation does not increase mitochondrial protein synthesis or biogenesis signals in skeletal muscle.

Introduction

It has been demonstrated that some degree of mitochondrial dysfunction occurs with obesity (Kim *et al.*, 2000), insulin resistance (Petersen *et al.*, 2004), heart disease (Chicco & Sparagna, 2007), and aging (Trifunovic *et al.*, 2004). It is now apparent that mitochondria dysfunction may be causally related to many disease states and that improvement in mitochondria function could be an important therapeutic target (Ritz & Berrut, 2005; Wallace, 2005). It is thought that increases in mitochondrial biogenesis and/or function are partially responsible for reductions in disease progression following pharmaceutical treatments such as thiazolidinediones (Bogacka *et al.*, 2005), or non-pharmaceutical treatments including caloric restriction (Lopez-Lluch *et al.*, 2006) and endurance exercise ((Menshikova *et al.*, 2006) and see (Kim *et al.*, 2008) for review). Therefore, understanding the factors that regulate mitochondrial biogenesis is important for the therapeutic treatment of chronic disease.

A recent report in mice demonstrated that the exercise-induced increase in peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 alpha (PGC-1α) is at least partially due to β-adrenergic receptor (β-AR) activation (Miura *et al.*, 2007). In this study, a series of experiments were performed on wild type and transgenic mice lacking β-ARs using exercise and selective β-AR agonist/-antagonist treatments to examine PGC-1α expression. Similarly, through a combination of exercise, epinephrine treatment, and β-AR blockade it was determined that β-AR stimulation increases PGC-1α mRNA in rat adipose tissue extracts (Sutherland *et al.*, 2009). Since PGC-1α is a major regulator of genes related to mitochondrial biogenesis (Joseph *et al.*, 2006), the ability for β-AR stimulation to increase, or β-AR blockade to decrease PGC-1α may provide a

mechanism for blunted mitochondrial enzyme adaptations following training with β-AR blockade in rats and humans (Ji *et al.*, 1986; Ades *et al.*, 1990).

Data from studies in humans are conflicting as some authors have concluded that β-AR blockers do not influence exercise conditioning (Wilmore *et al.*, 1985) while others suggest the contrary (Sable *et al.*, 1982; Marsh *et al.*, 1983). Specific to skeletal muscle adaptations, β-AR blockade during endurance training showed a blunted increase on maximal activity of selected oxidative enzymes (Svedenhag *et al.*, 1984; Wolfel *et al.*, 1986). Additionally, hypertensive adults receiving β_1-AR selective or non-selective blockers had blunted training effects in maximal exercise capacity ($VO_{2\ max}$) as compared to placebo (Ades *et al.*, 1990). We propose that the regulation of mitochondrial biogenesis by β-AR stimulation has important medical and practical implications for the enhancement of health and quality of life with chronic disease (Robinson *et al.*, 2009).

The current study investigated the potential role of β-AR in mitochondrial biogenesis. We chose to avoid the complications associated with β-AR blockade during exercise, such as incomplete β-AR blockade and β-AR hypersensitivity after stopping treatment (Nattel *et al.*, 1979; Frishman, 1987; Gilligan *et al.*, 1991), and instead used β-AR stimulation on skeletal muscle mitochondrial biogenesis. Further, although changes in mRNA expression of PGC-1α were noted in the study on mice (Miura *et al.*, 2007), the report did not directly measure mitochondrial biogenesis. In many experimental models, changes in transcript levels do not always mirror changes in the proteome (Mootha *et al.*, 2003) and it is important to directly measure changes in mitochondrial biogenesis. We therefore took an integrative approach to examine the effect of β-AR stimulation by isoproterenol, a non-specific β-AR agonist, on protein synthesis and the

signals specific for increased mitochondrial biogenesis. Our measures of protein synthesis included skeletal muscle sub-fractions and whole body protein turnover (WBPT). WBPT can be altered with β-AR stimulation and was included to provide a more complete evaluation of protein turnover (Kraenzlin *et al.*, 1989). Based on the previous report in mice (Miura *et al.*, 2007), we hypothesized that: 1) β-AR stimulation would increase mRNA content of PGC-1α and downstream gene targets, and 2) during the recovery period from β-AR stimulation, there would be an increase in whole body protein turnover (WBPT), myofibrillar protein synthesis (MyPS), and mitochondrial protein synthesis (MiPS).

Methods

Subjects

The Institutional Review Board at Colorado State University approved this study design. Young healthy adult males were recruited for participation. Compared with sedentary, obese and/or older adults the young healthy subject population recruited for participation in the current study would be expected to have good β-AR function (Kerckhoffs *et al.*, 1998; Schiffelers *et al.*, 2001; Bell *et al.*, 2006b). After providing oral and written informed consent, participants underwent screening and preliminary testing. A standard health and medical screening determined those able to participate in a graded exercise exam, as well as screened for potential contraindications and medications that affect β-AR signalling or mitochondrial biogenesis. In the end, only those subjects who were non-smokers, weight stable (± 2 kg) for one year, and demonstrated no signs or symptoms of disease, or no contraindications to isoproterenol were invited to participate in the study. Six healthy men (Age: 19 ± 1 yr, Height: 181 ± 6 cm, body mass: 80 ± 11 kg, body fat %: 14.6 ± 8.8%, and $VO_{2\ max}$: 51 ± 5 $mL \cdot kg^{-1} \cdot min^{-1}$ (mean ± SE)) were enrolled for the full study, while an additional five (Age: 21 ± 1 yr, Height: 176 ± 8 cm, body mass: 69 ± 13 kg, body fat %: 13.0 ± 3.5%, and VO_{2max}: 54 ± 10 $mL \cdot kg^{-1} \cdot min^{-1}$) were enrolled for the determination of cellular signalling only.

Experimental design

The study examined whether acute intravenous β-AR stimulation increased mitochondrial biogenesis, as measured by stable isotope infusion, in resting human subjects (**Figure 1**). We used a randomized design consisting of intravenous

administration of isoproterenol (ISO; a non-specific β-AR agonist) or saline (CON) infusion. Subjects reported to the laboratory on two separate occasions following a 12-hr fast, 24-hr abstention from exercise, and 48-hr abstention from caffeine (a sympathetic nervous system stimulant). Procedures were identical on both days except for β-AR stimulation. Upon arrival, a baseline breath sample was obtained for background enrichment of $^{13}CO_2$. After the breath sample, a catheter was inserted in the antecubital vein of one arm for infusion of isotope and drug/saline. In the contralateral hand, a catheter was inserted into a dorsal hand vein for heated, arterialized-venous samples. After catheter placement and baseline blood draw, a primed-continuous infusion of L-[1,2-^{13}C]leucine (see below) began followed immediately by initiation of infusion (60 min duration) of either isoproterenol or saline. Blood and breath were sampled hourly for the determination of isotopic enrichment. At 2-hr and 6-hr skeletal muscle samples (vastus lateralis) were acquired to determine the change in protein enrichment over time and for analysis of gene transcripts related to mitochondrial biogenesis using real-time polymerase chain reaction (RT-PCR). After our initial investigation it was determined that results from our signalling analysis were approaching significant differences. In order to avoid a Type II error, five additional subjects were recruited. These five subjects went through the identical protocols as described except that no stable isotope was infused.

Procedures: β-AR stimulation

Subjects received a continuous intravenous infusion of isoproterenol (ISO, 12 ng·kg fat free mass (ffm)$^{-1}$·min^{-1}) or an isovolumetric infusion of saline (CON) over 60 minutes using a Harvard syringe pump (Harvard Apparatus, Holliston, MA) as performed

previously by our group (Bell *et al.*, 2006a, c; Stob *et al.*, 2007a; Stob *et al.*, 2007b). During the infusion period, beat-by-beat heart rate was monitored continuously via electrocardiogram, while blood pressure was determined every 10 minutes using an automated sphygmomanometer.

Percutaneous muscle biopsies

Incision sites of the vastus lateralis were prepared with local anaesthetic (lidocaine 1%) and samples (≈100mg) were obtained using a Bergstrom needle with suction. To minimize the potential for interference, serial biopsy samples were collected in contralateral legs on a given testing day (Bickel *et al.*, 2005). Muscle biopsies were immediately frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

PGC-1α and its downstream effectors

Expression of PGC-1α, its downstream effectors mitochondrial transcription factor A (TFAM), nuclear respiratory factors 1 and 2 (NRF-1, and NRF-2), and respiratory chain complex subunits cytochrome-c oxidase VIIa (COX7a, nuclear coded), and NADH dehydrogenase IV (mtND4, mitochondrial coded) were analyzed by real-time-PCR (RT-PCR). Total RNA was extracted from whole muscle samples (~30 mg) using standard chloroform-phenol extraction methods (Trizol, Invitrogen Inc). RNA concentration and protein contamination were determined using spectrophotometry, and RNA degradation was determined by agarose gel separation. RNA was reverse transcribed to cDNA and target sequences (**Table 1**) amplified using standard primer-probe reagents (Taqman, Applied Biosystems Inc) and RT-PCR procedures (7300 RT-PCR System, Applied Biosystems Inc). The relative quantification of each target gene was normalized to an

endogenous control (β2-microglobulin) and compared against the baseline sample using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Stable isotope methodology

Stable isotopically-determined protein synthesis rates were performed according to our previously published methods (Miller *et al.*, 2005; Miller *et al.*, 2006) with further isolation of mitochondrial proteins. Certified sterile isotopes (Cambridge Isotope, Andover, MA) were mixed and sterility and pyrogenicity tested at the Department of Pharmacy, Medical Center of the Rockies. A primed, continuous infusion (0.8 $mg \cdot kg^{-1}$, 1.0 $mg \cdot kg^{-1} \cdot min^{-1}$) of L-[1,2-$^{13}C_2$]leucine was used to increase label incorporation over time. Blood and breath samples were taken at 1 hr intervals for determination of tracer/tracee ratios.

Plasma ketoisocaproic acid (KIC) was derivatized as previously described (Miller *et al.*, 2005) and analyzed as its t-BDMS derivatives by gas chromatography mass spectrometry (GC-MS Agilent 5975 GC and 7890 MS) (Schwenk *et al.*, 1984; Miller *et al.*, 2005).

Subsarcolemmal mitochondria isolation

Subsarcolemmal mitochondria (SM) proteins were isolated by mechanical procedures used in our laboratory as modified by the method of Butz *et al*. (Butz *et al.*, 2004). Briefly, muscle tissues (~60 mg) were powdered in liquid nitrogen and homogenized for 15 seconds at 50% power with a handheld polytron homogenizer in 1ml ice-cold solution 1 (100 mM KCl, 30 mM Tris-HCl, 10 mM Tris base, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP, pH 7.5) and centrifuged at low speed (800g) for 10 min at 4°C (Eppendorf, model 5415R). The supernatant from the low-speed spin was carefully removed and

centrifuged at high speed (9000g) for 10 min to pellet a crude mitochondrial fraction. The crude SM pellet was washed and suspended in 200 µl solution 2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4), then centrifuged (8000g, 10 min, 4°C). The pellet was washed a second time, suspended in 100 µl solution 2 and centrifuged (6000g, 10 min, 4°C). The final SM pellet and myofibrillar pellet from the initial 800g spin were washed with 500 µl of 100% ethanol, centrifuged (1000g, 30 sec, 4°C), and rinsed with water (repeated twice). Protein pellets were solubilised in 1N NaOH (50°C, 15 min) and hydrolyzed into free amino acids (6 M HCl, 120°C, 24 hrs). Hydrolyzed amino acids were analyzed as their NAP derivatives and analyzed by GC-C-IRMS as previously described (Moore *et al.*, 2009).

A series of western blots with positive and negative controls were run to confirm that our isolation procedures obtained relatively pure mitochondrial samples (**Supplemental Figure 1**). Briefly, SM (~100 µg) were isolated from rat quadriceps muscle using the above protocol with the final mitochondrial pellet suspended in 100 µl of RIPA buffer. Mitochondrial proteins were diluted using Laemmli sample buffer (Biorad) and 14 µg of protein was separated using standard SDS-PAGE methods (4-15% Tris-HCl gels, Biorad). Proteins were transferred to nitrocellulose membranes (50V per gel, 75 min in 25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol, pH 8.3), blocked for 90 minutes (5% BSA in TBST: 20 mM Tris, 150 mM NaCl, 0.1% Tween 20, ph 7.5), washed in TBST (3x10min), and incubated for 90 minutes with primary antibodies in 0.05% BSA in TBST (Santa Cruz Biotechnology, Inc). Membranes were then washed in TBST (3x10 min) and incubated for 90 minutes with HRP conjugated secondary

antibodies (Pierce, 1:2000 in TBST with 0.05% BSA,) and used with chemiluminescent (West-Dura, Pierce) to obtain digital images (Biospectrum, UVP inc.). Our mitochondrial isolation protocol yielded SM that showed a strong signal for cytochrome-c oxidase IV (1:200 primary) and little signal of contamination by contractile proteins (myosin heavy chain, 1:500 primary), cytoskeletal proteins (desmin, 1:500 primary) and plasma membrane (Na/K ATPase, 1:500 primary). Thus, the calculated MiPS was primarily SM protein synthesis and the MyPS was primarily myofibrillar protein synthesis, however other contaminating proteins may also influence the calculated fractional synthesis rates.

Rates of MiPS and MyPS were calculated using standard equations (Rennie *et al.*, 1982); thus for the constant infusion approach, fractional protein synthesis (FSR, %/h) = $\Delta Em/Ep \times 1/t \times 100$, where ΔEm is the change in enrichment of leucine in each specific protein fraction between the final and initial muscle biopsy, Ep is the mean enrichment over time of the precursor for protein synthesis (taken as venous KIC enrichment) and t is the time (h) between the venous isotopic steady state and the muscle biopsy. Venous KIC represents the immediate precursor for muscle protein synthesis i.e. leucyl-t-RNA (Watt *et al.*, 1991). Finally, whole body protein turnover was calculated by the single pool model of leucine kinetics (Wolfe & Chinkes, 2005), where rates of oxidation (Rox) determined from breath $^{13}CO_2$ enrichment (Metabolic Solutions, Nashua, NH) is subtracted from plasma derived leucine rate of appearance (Ra) to obtain non-oxidative leucine disposal (NOLD).

Statistics

Values are presented as mean ± standard deviation (SD). Changes in heart rate, blood pressure, and whole body protein turnover over time were determined by a two-way analysis of variance (ANOVA) as treatment by time with a Bonferroni correction applied when differences were noted. RT-PCR was compared by a one-way ANOVA with all comparisons made to 2 hr CON time-point. Other variables were compared by a paired two-tailed t-test to compare saline and drug infusion trials. Statistical significance was set at $P<0.05$.

Results

Cardiovascular Responses to β-AR Stimulation: Intravenous isoproterenol administration elicited appreciable increases in heart rate and systolic blood pressure (**Figure 2**) reflecting significant systemic β-AR stimulation.

Protein Synthesis: There were no differences in leucine Ra in the period following ISO or CON infusions (**Figure 3A,** time by treatment: P = 0.138, treatment: P = 0.621). Similarly, there was no difference in time by treatment interaction of ISO and CON for Rox (**Figure 3B**, P = 0.099) and NOLD (**Figure 3C**, P = 0.092), nor an effect of treatment on NOLD (P = 0.074). There was no difference between ISO and CON for either MiPS (CON: 0.099 ± 0.028, ISO: 0.074 ± 0.046 %/hr) or MyPS (CON: 0.059 ± 0.008, ISO: 0.055 ± 0.009 %/hr, **Figure 4**).

RT-PCR: Values for RT-PCR were compared to the 2 hr post infusion time point of CON. CON was normalized to 1.0 and fold changes above the normalized values were reported (**Figure 5**). There were no significant differences between 2 hr CON or 2 or 4 hr ISO for signals of mitochondrial biogenesis (PGC-1α, NRF-1, NRF-2, and TFAM). Similarly, mRNA of nuclear (COX7a) and mitochondrial genes (mtND4) were not different at any time point.

Discussion

In mice, β-ARs appear to play an important role in the regulation of skeletal muscle mitochondrial biogenesis (Ji *et al.*, 1986; Miura *et al.*, 2007), whereas data from humans are less clear (Sable *et al.*, 1982; Marsh *et al.*, 1983; Svedenhag *et al.*, 1984; Wilmore *et al.*, 1985; Wolfel *et al.*, 1986). Accordingly, we have investigated the hypothesis that acute intravenous β-AR stimulation would increase skeletal muscle mitochondrial biogenesis in adult humans. The main finding from this study was that mitochondrial protein synthesis and the increase in the mRNA of mitochondrial biogenesis signalling pathways and respiratory chain subunits were unaffected by 60-minutes continuous intravenous administration of the non-specific β-AR agonist, isoproterenol.

β-AR stimulation

In order to demonstrate that β-AR stimulation increased expression of PGC-1α in skeletal muscle of mice, Miura *et al*. (Miura *et al.*, 2007) subcutaneously administered a large (1 $mg \cdot kg^{-1}$) bolus of the β_2-AR specific agonist, clenbuterol. During periods of increased activation of the sympathetic nervous system (e.g. during exercise) β-ARs are stimulated in a non-specific manner, that is, all three β-AR subtypes are stimulated. In humans, Ades *et al.* demonstrated that ten-weeks of endurance training with β-AR blockade impaired VO_{2max} and mitochondrial enzyme adaptations, an effect that was more pronounced in the group receiving a non-selective β-AR antagonist compared to β_2-AR or β_1-AR selective antagonists (Ades *et al.*, 1990).

In the current study we chose to use a non-selective β-AR agonist. The dose of isoproterenol administered in the current study has been shown previously to increase

energy expenditure by 10-15% in humans (Bell *et al.*, 2006a, c; Stob *et al.*, 2007a; Stob *et al.*, 2007b), thus it represents a significant systemic β-AR stimulus. This stimulus is further illustrated by the observation that our intravenous isoproterenol administration elicited appreciable increases in heart rate and systolic blood pressure. For these reasons we believe that our selection of a non-specific β-AR agonist, such as isoproterenol, was the appropriate study design. Our study design did not consider the role of α-AR because the study of Miura *et al.* demonstrated that phenylephrine, an α-agonist, had no effect on PGC-1α expression (Miura *et al.*, 2007).

Timing of Tissue Sampling

In the current study, skeletal muscle was sampled at 1-hr and 5-hr after the termination (2- or 6-hr after the initiation) of the experimental infusion. By using an β-AR agonist, rather than an β-AR antagonist, and by avoiding chronic stimulation, we did not have the complications associated with hypersensitivity of the β-AR upon withdrawal of an antagonist (Nattel *et al.*, 1979; Frishman, 1987; Gilligan *et al.*, 1991). Further, by using a combination of skeletal muscle biopsies in both control and β-AR stimulation conditions, we were able to capture label incorporation into mitochondria for synthetic measurements, and two time points likely for increases in PGC-1α mRNA and its downstream effectors. Miura *et al.* sampled tissue at 4-hr or 24-hr after injection (Miura *et al.*, 2007). Depending on the experimental condition, significant increases in PGC-1α mRNA were reported at both time points. β-AR stimulation is one of the many stimuli induced by exercise, and could be affecting mitochondrial biogenesis following exercise. Following an acute bout of exercise in human subjects, increases in PGC-1α mRNA have been reported immediately (Mathai *et al.*, 2008), 2-hr (Pilegaard *et al.*, 2003; Norrbom *et*

al., 2004; Mortensen *et al.*, 2007; Mathai *et al.*, 2008), 4-hr (Harber *et al.*, 2009) and 6-hr (Pilegaard *et al.*, 2003) after the completion of the exercise bout. Exercise activates many pathways in addition to β-AR that are known to regulate PGC-1α. We avoided these confounding variables by using short-term β-AR activation and sampling time points that were previously shown to have increased PGC-1α mRNA (Harber *et al.*, 2009) and protein synthesis (Wilkinson *et al.*, 2008a). Therefore, we are confident that our sampling times would have captured acute changes in PGC-1α mRNA or protein synthesis. It is possible that the short-term study design could have missed longer-term changes in mRNA content or mitochondrial biogenesis (e.g protein content or respiratory activity), however there were no acute indications that our β-AR agonist promoted mitochondrial biogenesis. Furthermore, although it has been demonstrated that increases in PGC-1α activity may regulate initial increases in mitochondrial biogenesis (Wright *et al.*, 2007), we failed to see an increase in mRNA for mitochondrial proteins (mtND4 and COX7a) or overall MiPS.

Methods of Analysis

We took an integrative approach to analyzing whether β-AR stimulation increased mitochondrial biogenesis. In a previous investigation, activation of pathways of mitochondrial biogenesis were examined without a true measure of changes in mitochondrial synthesis rates or mitochondrial mass (Miura *et al.*, 2007). We are of the opinion that because of energetic constraints and the integration of multiple pathways that an increase in PGC-1α does not always equate to an increase in mitochondrial biogenesis and that some measure of mitochondrial turnover is warranted. Although we agree that PGC-1α is very important for the transcriptional regulation of mitochondrial biogenesis,

we feel that post-transcriptional events, including translation initiation, are important for mitochondrial biogenesis. Since mitochondrial biogenesis is an ambiguous term and can refer to alterations in mitochondrial size and protein:lipid ratio of the reticulum (Hood *et al.*, 2006), we chose to evaluate both transcriptional and post-transcription indicators of mitochondrial biogenesis.

We determined mitochondrial protein synthesis by isotope incorporation into mitochondrial proteins. Western blotting confirmed the relatively pure mitochondrial fraction obtained by our procedures (**Supplemental Figure 1**). In addition, our mean values (0.07 – 0.10 %/hr) are similar to previously published values using similar techniques (Rooyackers *et al.*, 1996; Wilkinson *et al.*, 2008b). In the current study, we were interested in an acute effect of a β-AR agonist on MiPS, thus we did not feel that a measure of mitochondrial protein breakdown, a measurement not technically feasible at this point, was necessary. In addition, if mitochondria remodelling in skeletal muscle follows that of mixed muscle, it is thought that synthesis, not breakdown, is the driving force behind changes in protein content (Rennie *et al.*, 2009). We must therefore conclude that in the five hours following β-AR stimulation, there is no change in the rate of mitochondrial protein synthesis in human skeletal muscle.

Our study design assessed mitochondrial biogenesis using mRNA content and rates of protein synthesis to capture both transcriptional and translational effects of β-AR stimulation. Although we did not detect changes in the methods we used, there are additional regulatory processes that could be affected, but were not assessed. For example, a limitation of measuring mRNA content of transcription factors is that the protein content or activity of the transcription factors is not known. Additionally, the

protein content of downstream targets was not assessed. Although we did not detect changes in protein synthesis, we cannot rule out the possibility of changes to regulation steps such as protein content or activity of the transcription factors. Future studies could assess additional regulation steps such as transcription factor activation and ribosomal binding to mRNA transcripts.

Drug administration

We used a study design that allowed us to avoid complications in relation to administration and withdrawal of medication. The conflicting data of Wolfel *et al.* (Wolfel *et al.*, 1986) and Svedenhag *et al. (Svedenhag et al., 1984)* could be explained by the fact that the former measured VO_{2max} at four time-points, before and after training while participants were receiving or had stopped the drug treatment, while the latter measured before and four days after the secession of drug treatment and exercise training. As previously discussed, increased β-AR sensitivity can occur following treatment with β-AR blockers (Nattel *et al.*, 1979; Frishman, 1987; Gilligan *et al.*, 1991), thus differences in reported training effects may be influenced by the timing of measurements around drug treatments and whether the participants are consuming the drugs while measurements are made.

Species Differences

Previous studies have examined isoproterenol induction of PGC-1α in brown fat cells (Puigserver *et al.*, 1998), effects of β-AR stimulation or blockade on PGC-1α mRNA in the adipose tissue of exercising rats (Sutherland *et al.*, 2009), and a series of receptor-specific blockades and stimulations in skeletal muscle of mice (Miura *et al.*, 2007) and suggest that β-AR stimulation is important for mitochondrial adaptations. The

data in humans concerning β-AR and mitochondrial adaptations to exercise are conflicting with some reports of attenuated rise in selected mitochondrial enzymes and $VO_{2\ max}$, while others show no impairments (Svedenhag *et al.*, 1984; Wolfel *et al.*, 1986; Ades *et al.*, 1990). Possible reasons for these conflicting results on β-AR and exercise adaptations in humans include: 1) the inability of adults receiving β-AR blockers to exercise at the same intensity as their placebo-controlled counterparts; 2) typical therapeutic doses of β-AR blockers that do not actually achieve complete β-AR blockade; and 3) the observation that there is a hypersensitivity of β-AR two to four days after β-AR blocker withdrawal (Nattel *et al.*, 1979; Frishman, 1987; Gilligan *et al.*, 1991), making the timing of β-AR blocker withdrawal and post-training data collection crucial.

From the data presented in the current study, there is no indication that stimulation of β-AR can induce mitochondrial adaptations at rest. It must be acknowledged that long-term use of β-AR blockade could affect substrate use and selection (Mora-Rodriguez *et al.*, 2001), and potentially influence mitochondrial biogenesis in response to energetic signals. Additionally, exercise includes a variety of signals including β-AR stimulation, thus acute β-AR stimulation would not include other signals that may be required to stimulate mitochondrial protein synthesis.

Whole body and muscle protein synthesis

Although our primary interest was mitochondrial protein synthesis, we are also interested in the factors that control WBPT and skeletal muscle protein synthesis. It has been recorded that long-term intake of β-AR agonists has anabolic effects on skeletal muscle (Carter *et al.*, 1991). Also, anabolic effects of β-AR stimulation (180 minutes of 50 $ng \cdot kg^{-1} \cdot min^{-1}$ epinephrine) on WBPT and isolated limbs were reported in healthy

males (Straumann *et al.*, 1988; Kraenzlin *et al.*, 1989). In contrast, β-AR stimulation is part of the stress response and contributes to increased protein breakdown, as suggested by improved protein retention following β-AR blockade in hyper-catabolic burn victims (Herndon *et al.*, 2001). It is possible that the overall catabolic or anabolic effects of β-AR stimulation depend on additional metabolic signals. During the present study, we did not find a difference in WBPT in young-healthy subjects although at one time point NOLD was greater with β-AR stimulation than CON. Although one time point may indicate that, at least acutely, β-AR stimulation might be anabolic, our data from mixed muscle protein synthesis did not indicate that skeletal muscle was the site of this increase. More studies are needed to confirm these findings.

Mitochondria population

Studies using stable isotopes to determine protein synthesis rates cannot use protease treatment of muscle tissue because of the potential for liberating bound tracer and artificially decreasing the calculated synthesis rates (Rooyackers *et al.*, 1996). Mitochondria are a reticulum that extends throughout the cell (Plecita-Hlavata *et al.*, 2008), and mechanical homogenization of muscle tissue samples yields a loosely bound population (subsarcolemmal, SM) and a tightly bound population (intermyofibrillar) (Palmer *et al.*, 1977). Previous studies have shown that both mitochondria populations can adapt to stimuli, however the SM fraction appears to have a relatively greater response (Bizeau *et al.*, 1998; Menshikova *et al.*, 2006). Since our mitochondria isolation yields primarily SM mitochondria, the calculated MiPS is for SM mitochondria and any different effects on the intermyofibrillar fraction are not known.

Fiber type

Specific β-AR actions in skeletal muscle vary depending on the muscle fiber type. For instance, the influence of adrenergic agonists on the contractile properties of skeletal muscle differs dramatically between slow- and fast-twitch fibers, mediated in part by differences in calcium handling in the sarcoplasmic reticulum (Ha *et al.*, 1999). Further, chronic β-AR stimulation leads to greater hypertrophy in fast-twitch fibers compared with slow-twitch (Oishi *et al.*, 2002), possibly due to greater extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) signalling in fast-twitch muscle (Shi *et al.*, 2007). (Shi, Zeng et al. 2007). In the current study we likely avoided fiber type specific effects by repeatedly sampling the vastus lateralis, a mixed muscle in humans. It is also important to note that the potential influence of this variability should have been minimized with our repeated measures, within subjects design.

Perspectives and Significance

If, as demonstrated previously in animal models, β_2-AR stimulation is an important mechanism to increase mitochondrial turnover, the clinical use of β-AR blockers may have important consequences for those that use endurance type exercise as a preventative or therapeutic treatment for chronic diseases. Further investigation should evaluate β-AR signalling and mitochondrial adaptations to exercise in humans. In the current study, acute whole body β-AR stimulation had no effect on myofibrillar or mitochondrial protein synthesis, or on the expression of transcription factors involved in mitochondrial biogenesis. Therefore, at this point we conclude that acute β-AR

stimulation is not an important mechanism for increased skeletal muscle mitochondrial biogenesis in resting humans.

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Table 1. RT-PCR target gene information and probe sequence (Applied Biosystems).

Target	*Gene Symbol*	*Assay ID*	*Chromosome*	*Target Exons*	*Probe Sequence*
PGC1-α	PPARGC1A	Hs00173304_m1	4	7	TGGAACTGCAGGCCTAACTCCACCC
TFAM	TFAM	Hs01082775_m1	10	5	GATTCACCGCAGGAAAAGCTGAAGA
NRF1	NRF1	Hs00602161_m1	7	8	TGATGGAGAGGTGGAACAAAATTGG
NRF2	GABPA	Hs01022023_m1	21	9	ACTCAGTCGTGCATTAAGATATTAT
COX7a	COX7A1	Hs00156989_m1	19	1	GGCCCTTCGGGTGTCCCAGGCGCTG
mtND4	MT-ND4	Hs02596876_g1	MT	n/a	CAAACTCCTGAGCCAACAACTTAAT

Figures

Figure 1. Study design. Subjects were investigated twice in a randomized, crossover design.

Figure 2. Heart rate (HR) and systolic blood pressure (BP) during intravenous administration of saline (CON) and β-AR receptor stimulation (Isoproterenol: ISO). For HR there was a significant effect of treatment (P=0.0079) and time by treatment interaction (P<0.0001). For BP there was a significant effect of treatment (P=0.0464) and time by treatment interaction (P<0.0001). CON values were compiled from the second group of subjects only (n=5) while ISO values represent all subjects (n=11).

Figure 3. Whole body protein turnover (WBPT) in the period after ISO or CON infusions. Leucine rate of appearance (Ra, P = 0.621) (**panel A**), leucine rate of oxidation (Rox, P = 0.099, **panel B**) and non-oxidative leucine disposal (NOLD, P = 0.092, **panel C**) were not different between treatments (n = 5).

Figure 4. Skeletal muscle mitochondrial (MiPS) and myofibrillar (MyPS) protein synthesis in the period after ISO or CON infusions. Neither MiPS (**panel A**, P = 0.344) nor MyPS (**panel B**, P = 0.666) were significantly different between CON and ISO trials. (n = 5).

Figure 5. RT-PCR on pathways of mitochondrial biogenesis. Values for RT-PCR were compared to the 2-hr time point of CON. CON was normalized to 1.0 and fold changes above the normalized values were reported. When compared to 2-hr CON, there were no significant differences at 2-hr or 4-hr for PGC-1α (**panel A**), TFAM (**panel B**), NRF-1 (**panel C**), NRF-2 (**panel D**), COX7a (**panel E**), and mtND4 (**panel F**) (n = 11).

Supplemental Figure 1. The mitochondrial isolation yielded subsarcolemmal mitochondria (SM) that are relatively free of major contaminating proteins as compared to whole muscle (WM), myofibrillar (MYO) and sarcolemmal (SARC) preparations. SARC was prepared from the supernatant of the first 9000g centrifugation for representative blot and not used for determination of FSR.

Figure 1

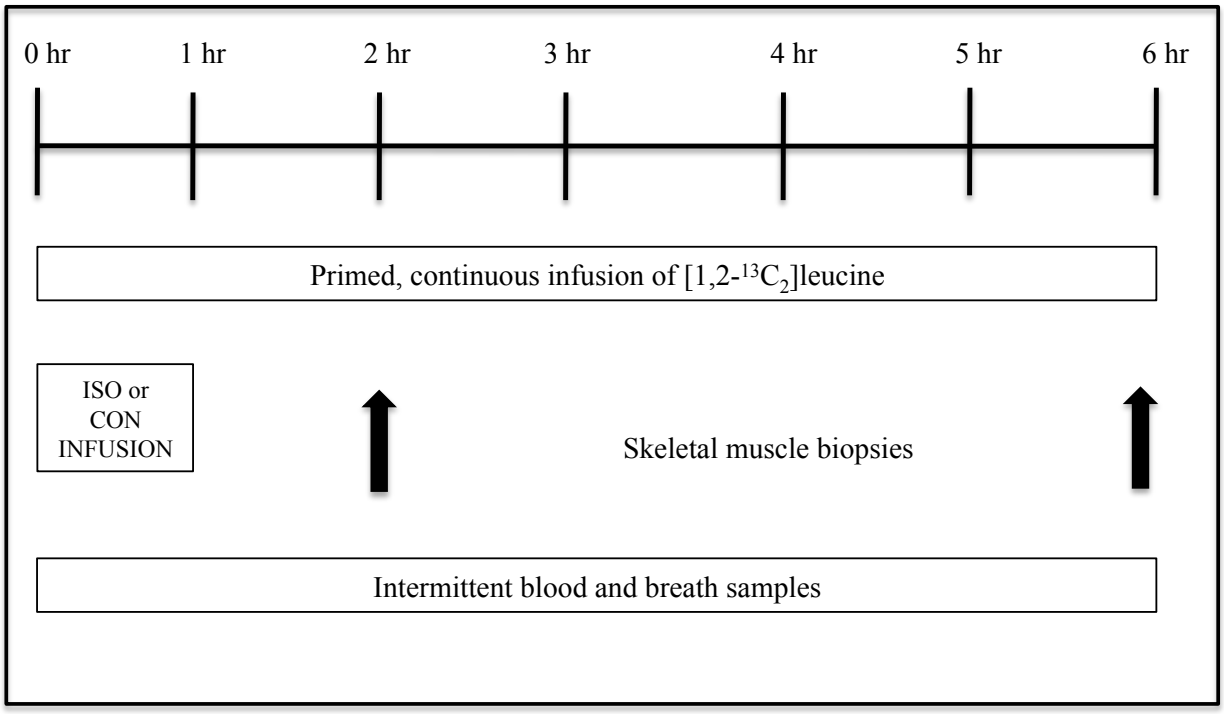

0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
Primed, continuous infusion of [1,2-13C2]leucine
ISO or CON INFUSION
Skeletal muscle biopsies
Intermittent blood and breath samples

Figure 2

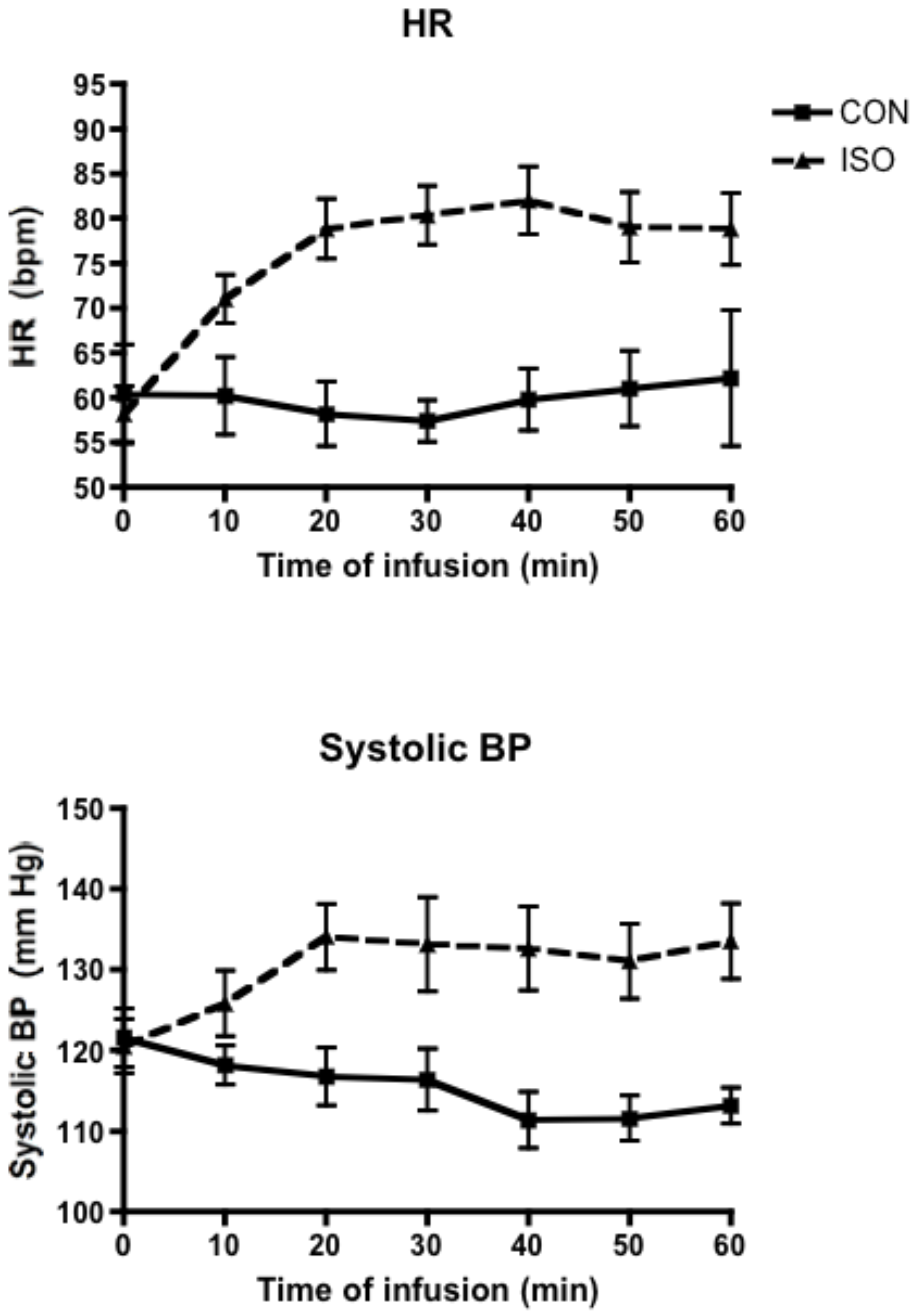

HR
95
90
85
80
75
70
65
60
55
50
HR (bpm)
0
10
20
30
40
50
60
Time of infusion (min)
CON
ISO
Systolic BP
150
140
130
120
110
100
Systolic BP (mm Hg)
0
10
20
30
40
50
60
Time of infusion (min)

Figure 3

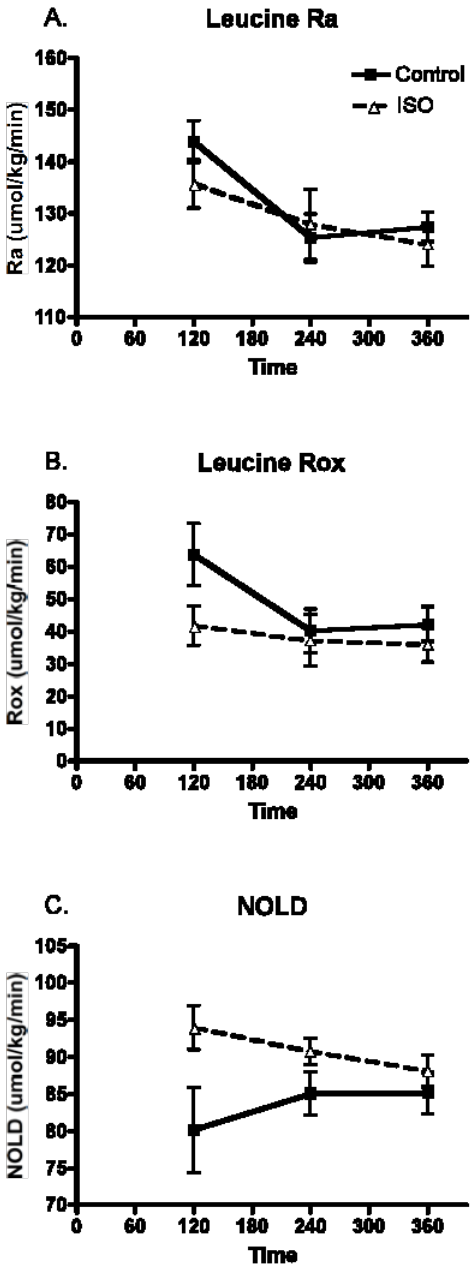

A.
Leucine Ra
Control
ISO
Ra (umol/kg/min)
160
150
140
130
120
110
0
60
120
180
240
300
360
Time
B.
Leucine Rox
Rox (umol/kg/min)
80
70
60
50
40
30
20
10
0
0
60
120
180
240
300
360
Time
C.
NOLD
NOLD (umol/kg/min)
105
100
95
90
85
80
75
70
0
60
120
180
240
300
360
Time

Figure 4

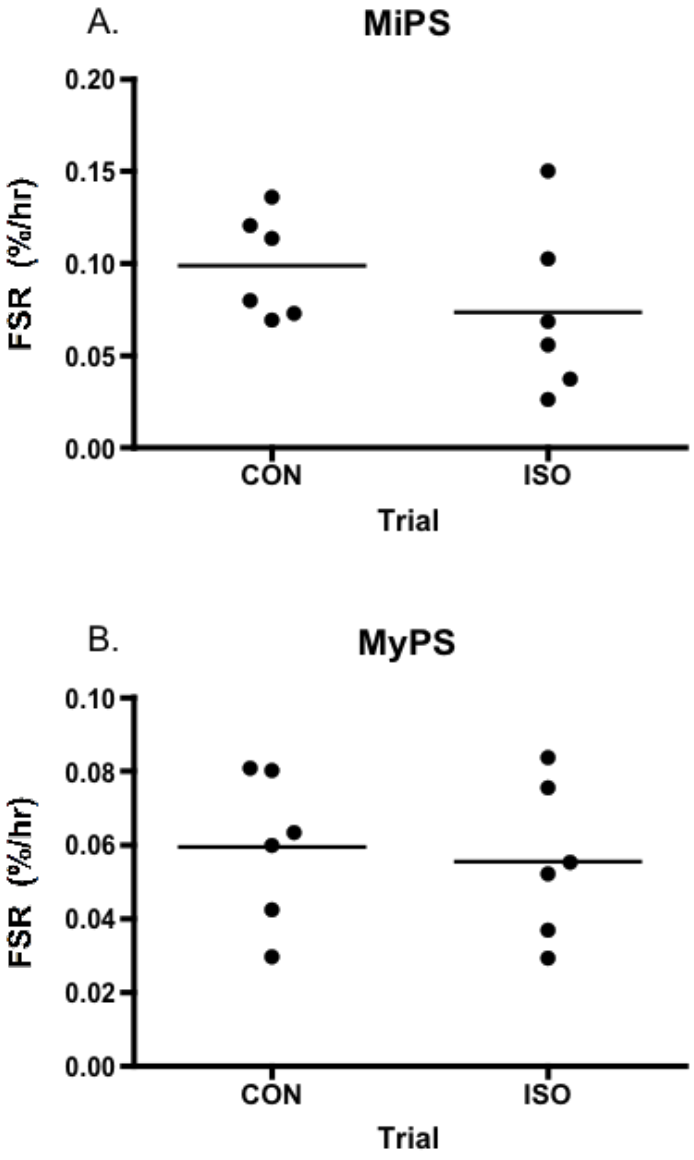
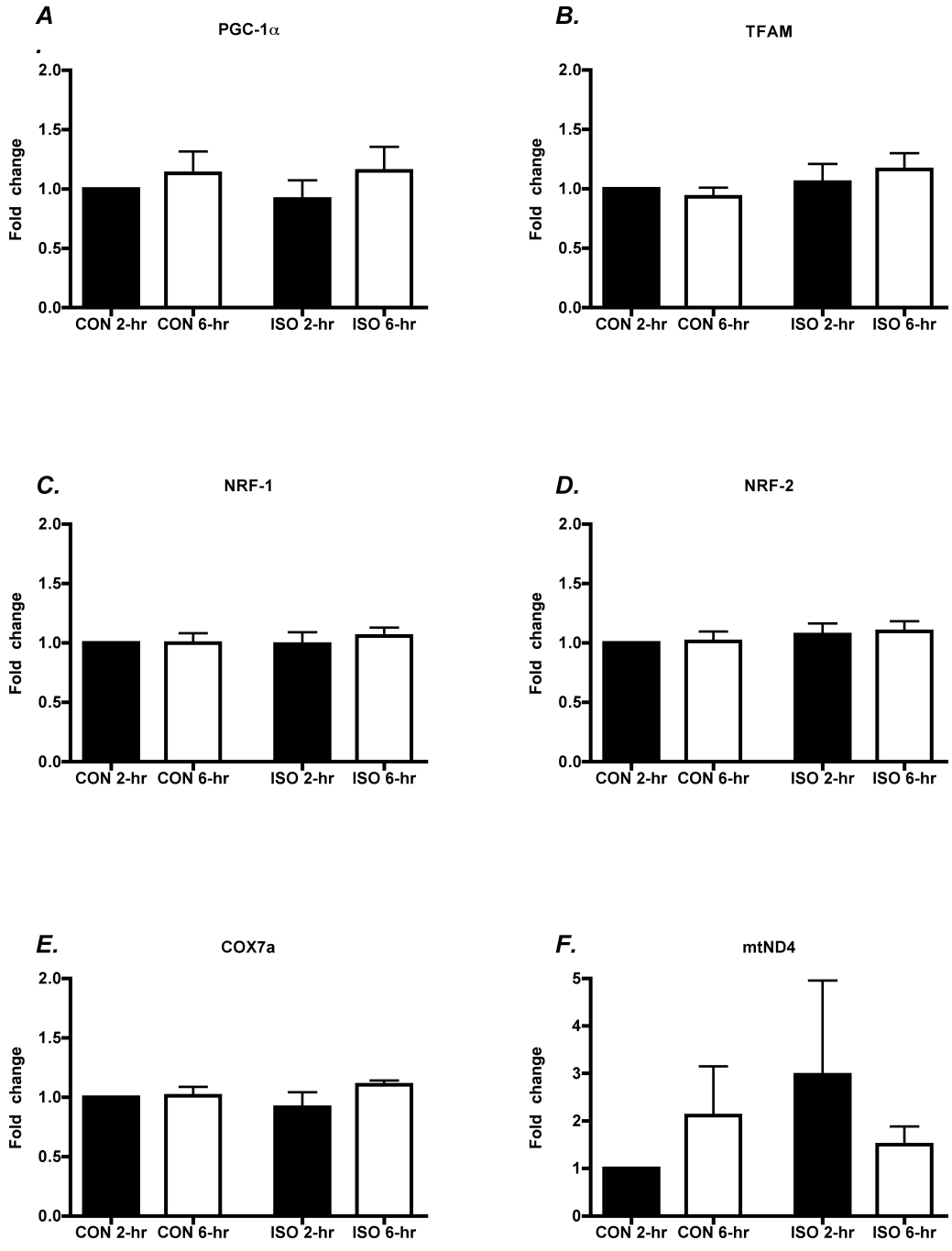
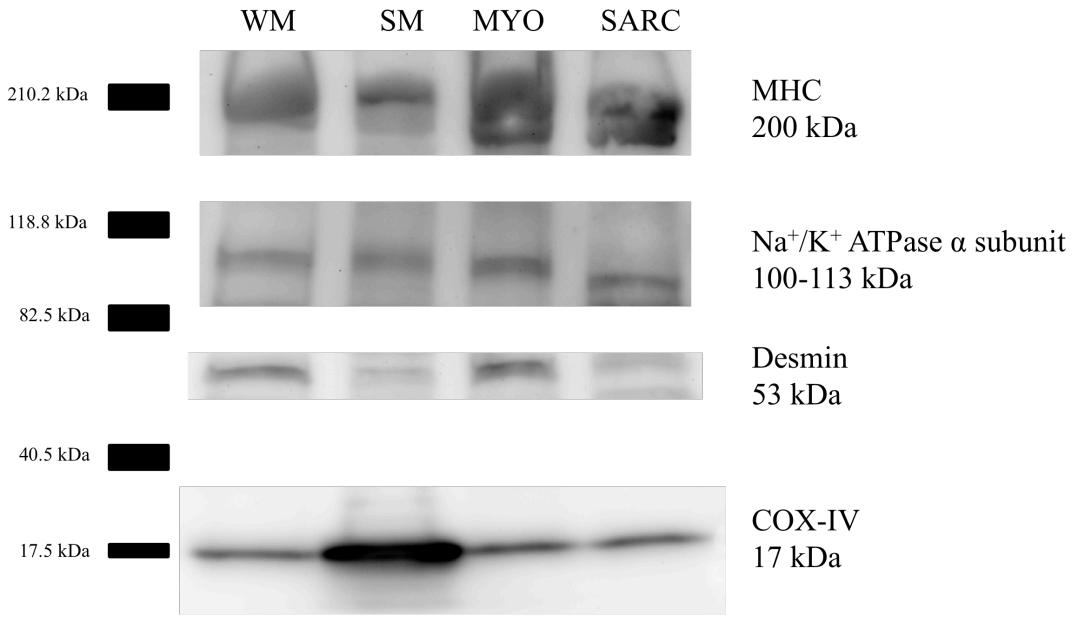

A.
MiPS
FSR (%/hr)
0.20
0.15
0.10
0.05
0.00
CON
ISO
Trial
B.
MyPS
FSR (%/hr)
0.10
0.08
0.06
0.04
0.02
0.00
CON
ISO
Trial

Figure 5


A.
PGC-1α
B.
TFAM
C.
NRF-1
D.
NRF-2
E.
COX7a
F.
mtND4
Fold change
CON 2-hr
CON 6-hr
ISO 2-hr
ISO 6-hr

Supplemental Figure 1



CHAPTER IV-MANUSCRIPT III

Propranolol blunts post-exercise skeletal muscle mitochondrial protein synthesis rates in human subjects

Matthew M Robinson, Christopher Bell, Frederick F Peelor III, and Benjamin F Miller*

Author affiliations
Department of Health and Exercise Science
Colorado State University
Fort Collins CO 80523

Corresponding author
*benjamin.f.miller@colostate.edu
970-491-3291

Running head
β-adrenergic signaling and mitochondrial biogenesis

Keywords
mitochondrial biogenesis, β-adrenergic antagonist, stable isotope tracer, aerobic exercise

Abstract

Recent evidence from mouse models indicates that β-adrenergic signaling regulates skeletal muscle protein synthesis and mitochondrial biogenesis. If so, then β-adrenergic antagonists may impair mitochondrial adaptations to exercise. We hypothesized that decreased β-adrenergic signaling during a bout of cycling would impair mitochondrial biogenesis following exercise. Six healthy males (age: 26±6 y, $VO_{2\ max}$: 39.9±4.9 $ml*kg^{-1}*min^{-1}$, BMI 26.7±2 $kg*m^{-2}$) performed two trials days of a one-hour bout of cycling (60% $VO_{2\ max}$) with either intravenous infusion of propranolol (PROP, non-selective β-adrenergic antagonist) or isovolumetric saline. Skeletal muscle mitochondrial, myofibrillar and sarcoplasmic protein synthesis rates were assessed using $^{2}H_5$-phenylalanine incorporation into skeletal muscle proteins post-exercise. The mRNA content of signals for mitochondrial biogenesis was determined using real-time polymerase chain reaction (rtPCR). Propranolol infusion decreased mitochondrial protein synthesis rates (Saline: 0.217 ±0.076 vs. PROP: 0.135±0.031 $\%*hr^{-1}$, $p<0.05$), but not myofibrillar or sarcoplasmic protein synthesis rates. Peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) mRNA was increased ~2.5 fold ($p<0.05$) at five hours compared to one hour post-exercise, but not different between propranolol or saline. We conclude that decreased β-adrenergic signaling during cycling can blunt the post-exercise increase in mitochondrial protein synthesis rates without changes to mRNA content of transcripts for mitochondrial biogenesis.

Introduction

Decreased mitochondrial content and function have been reported with aging and may contribute to chronic disease (Petersen *et al.*, 2003) and muscle wasting (Trifunovic *et al.*, 2004; Romanello *et al.*, 2010). Changes to mitochondrial function occur during lifestyle and pharmacological therapies and may influence the pathogenesis of disease. For example, aerobic exercise stimulates mitochondria proliferation (Holloszy, 1967) and may contribute to the health benefits of exercise training (Menshikova *et al.*, 2006). Conversely, certain drug classes cause mitochondrial dysfunction (Dykens & Will, 2007) and may contribute to skeletal muscle myopathies (Gambelli *et al.*, 2004). Given the high prevalence of chronic disease and the widespread use of drug therapies and lifestyle recommendations for treatment of chronic diseases, it is necessary to understand how concurrent exercise and drug therapy affect mitochondrial biogenesis.

Recent evidence suggests that mitochondrial biogenesis following aerobic exercise is partly mediated through β-adrenergic signaling (Miura *et al.*, 2007). Selective β_2-adrenergic stimulation under resting conditions increased the mRNA content of a regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor-gamma coactivator 1α [PGC1-α, (Miura *et al.*, 2008)]. Additionally, mice that were treated with the non-selective β-adrenergic antagonist propranolol had a blunted increase of PGC1-α mRNA following aerobic exercise (Miura *et al.*, 2007). Such findings are consistent with human aerobic training studies reporting blunted adaptations of select mitochondrial enzymes and maximal aerobic capacity during non-selective β-adrenergic antagonist treatment (Svedenhag *et al.*, 1984; Wolfel *et al.*, 1986; Ades *et al.*, 1990). β-adrenergic antagonists (β-blockers) are antihypertensive drugs that are commonly prescribed along

with exercise recommendations. It is possible that β-blockers may impair mitochondrial adaptations and limit the beneficial effects of aerobic exercise (Robinson *et al.*, 2009).

We previously reported that a one hour infusion of the non-selective β-adrenergic agonist isoproterenol did not increase mitochondrial biogenesis, as measured by mRNA signaling and incorporation of stable isotope into mitochondrial proteins (Robinson *et al.*, 2010). Our dose of isoproterenol increased heart rate, increased blood pressure and was equivalent to previous reports that increased resting metabolic rate (Bell *et al.*, 2006), but did not stimulate whole body or skeletal muscle protein synthesis. A recent report using selective β_2-adrenergic agonist treatment for several weeks in mice showed skeletal muscle protein synthesis rates were not elevated acutely, but were increased following seven days of treatment (Koopman *et al.*, 2010).

The aforementioned report of Miura *et al.* concluded an increase in mitochondrial biogenesis using changes in mRNA content (Miura *et al.*, 2007). Changes in mRNA content may not lead to changes in mitochondrial protein content (Mootha *et al.*, 2003), therefore it is necessary to determine mitochondrial biogenesis using measures of mRNA content and protein synthesis rates. It is also possible that there are inter-species differences in β-adrenergic signaling between mice and humans. If β-adrenergic signaling regulates mitochondrial biogenesis in humans, then drugs that block β-adrenergic receptors may impair mitochondrial biogenesis. Such a negative relationship between β-blockers and exercise adaptations implies that drug prescription may be counter-productive to exercise recommendations.

Our previous study was a *gain of function* design to evaluate if isoproterenol could stimulate mitochondrial biogenesis at rest (Robinson *et al.*, 2010). Here we

conduct the *loss of function* experiment in which β-adrenergic signaling is diminished during exercise. We hypothesized that blocking β_1- and β_2-adrenergic receptors using a propranolol infusion during a one-hour bout of cycling would decrease mitochondrial protein synthesis and mRNA content of genes related to mitochondrial biogenesis.

Methods

Ethics approval

The Institutional Review Board of Colorado State University approved the protocol (#09-1124H). Each volunteer was informed of the potential risks and written consent was obtained prior to enrollment. The study followed the guidelines set forth by the Declaration of Helsinki.

Study overview

Six healthy adult males were studied during two or three days separated by at least three weeks. First, a subset of three subjects was used to confirm that our propranolol infusion would achieve a β-adrenergic blockade (see details below). All subjects then completed an experiment in which the non-selective β-adrenergic antagonist propranolol was infused intravenously during a one-hour bout of cycling at ~60% maximal aerobic capacity ($VO_{2\ max}$). On the final trial, an isovolumetric dose of saline was infused and the participants cycled at the same absolute workload as during the propranolol trial. The propranolol trial was always performed before the saline trial to make any adjustments in workload necessary for the subject to complete one-hour of exercise. The same absolute workload was used on the saline control day. Following exercise on both days, the participants rested for five hours during infusion of a stable isotope and subsequent muscle biopsy sampling to determine protein synthesis rates and mRNA content of skeletal muscle.

Subject characteristics

The participants (age 26±6 y, BMI 26.7±2 $kg*m^{-2}$, $VO_{2\ max}$ 39.9±4.9 $ml*kg^{-1}*min^{-1}$) were healthy based on a medical history questionnaire and free of any cardiac

abnormalities as determined by an exercising 12 lead electrocardiogram. The three participants (age 28±5 y, BMI 27.4±0.3 $kg*m^{-2}$, $VO_{2\ max}$ 39.9±2.4 $ml*kg^{-1}*min^{-1}$) who volunteered for verification of β-adrenergic blockade was a subset of the entire group. People with bradycardia (heart rate at rest <40 $beats*min^{-1}$) were not eligible due to the risk of hypotension during propranolol treatment. $VO_{2\ max}$ was determined on a cycle ergometer (Velotron, RacerMate Inc., Seattle WA) using indirect calorimetry (ParvoMedics, Sandy UT). Body composition was determined using dual energy x-ray absorptiometry (DEXA, Lunar Discovery W, GE Medical Systems, Madison WI).

Verification of β-adrenergic blockade

To verify β-adrenergic receptor blockade, participants arrived to the laboratory after an overnight fast and graded doses (9, 12, 15, 18, 21 and 24 $ng*kg\ fat\ free\ mass^{-1}*min^{-1}$) of the non-selective β-adrenergic agonist isoproterenol were infused to raise heart rate ~25 $beats*min^{-1}$ above resting values (Bell *et al.*, 2006). Next, propranolol was intravenously infused as a priming dose (0.25 $mg*kg^{-1}$ at 1 $mg*min^{-1}$) followed by a maintenance dose (0.006 $mg*kg^{-1}*min^{-1}$) through the one-hour cycling at 60% $VO_{2\ max}$. Following exercise, the infusion was stopped and the participant rested in bed. At one hour post-exercise, the isoproterenol dose that previously raised heart rate to 25 $beats*min^{-1}$ above resting was repeated to determine if heart rate and blood pressure increased. β-adrenergic blockade was demonstrated by a lack of stimulation of heart rate or blood pressure following the dose of isoproterenol.

Study protocol

Participants were instructed to refrain from physical activity on the day before each trial and were provided an evening snack to consume the night before each trial.

The participants reported to the laboratory following an overnight fast and intravenous catheters were inserted into a dorsal hand vein for arterialized blood sampling and antecubital vein for infusions. Beat-by-beat electrocardiograms were continuously recorded throughout the study day and blood pressure was monitored with an automated cuff. On the first trial, propranolol was administered as a priming dose (0.25 $mg*kg^{-1}$ at 1 $mg*min^{-1}$) followed by a maintenance dose (0.006 $mg*kg^{-1}*min^{-1}$) using a precision pump (Harvard Apparatus, Holliston MA). Immediately following the priming dose, the participants began cycling for one hour at 60% $VO_{2\ max}$ during infusion of the maintenance dose. Expired air was collected every 15 min to maintain steady state oxygen consumption as determined by indirect calorimetry. On the second trial day, the participants received an isovolumetric dose of saline and the same absolute workload was performed. Immediately following exercise on both days, the participants consumed a recovery beverage (Ensure, Abbott Laboratories, Abbott Park IL) to replace the calories burned during exercise as determined by average oxygen consumption and respiratory exchange ratio. The beverage also contained sufficient protein (>20 grams) to maximally stimulate protein synthesis following exercise (Moore *et al.*, 2009). A primed continuous infusion of $^{2}H_5$-phenylalanine (2 $\mu mol*kg^{-1}$ prime, 0.05 $\mu mol*kg^{-1}*min^{-1}$ continuous infusion) was performed for five hours following exercise. Skeletal muscle biopsy samples (~100-150 mg) were collected with a Bergstrom needle under local anesthesia (1% lidocaine) at one hour and five hours following exercise. Intermittent arterialized blood samples were collected from a heated dorsal hand vein. $^{2}H_5$-phenylalanine (Cambridge Isotope, Cambridge MA) was prepared in sterile isotonic saline by a

pharmacy (Medical Center of the Rockies, Loveland CO) and tested for pyrogenicity. All solutions were passed through a 0.2 μm filter before being infused intravenously.

Muscle protein synthesis

Skeletal muscle protein synthesis rates were determined of subsarcolemmal mitochondria (SM), myofibrillar (MYO), and sarcoplasmic (SARC) fractions as previously described (Robinson *et al.*, 2010). Briefly, ~70 mg of muscle tissue was homogenized (Bullet Blender, Next Advance Inc., Averill Park NY) in one milliliter ice-cold solution 1 (100 mM KCl, 30 mM Tris-HCl, 10 mM Tris base, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP, pH 7.5) and centrifuged at low speed (800g) for 10 min at 4°C (Eppendorf, Hauppauge NY). The supernatant from the low-speed spin was carefully removed and centrifuged at high speed (9000g) for 10 min to pellet a crude mitochondrial fraction and a sarcoplasmic supernatant. The crude SM pellet was washed and suspended in 200 μl solution 2 (100 mM KCl, 10 mM Tris-HCl, 10 mM Tris base, 1 mM $MgSO_4$, 0.1 mM EDTA, 0.02 mM ATP, and 1.5% BSA, pH 7.4), then centrifuged (8000g, 10 min, 4°C). The pellet was washed a second time, suspended in 100 μl solution 2 and centrifuged (6000g, 10 min, 4°C). The sarcoplasmic fraction of the first 9000g spin was deproteinated with equal volume of 14% sulfosulfuric acid and centrifuged at 17000g for 10 min at 4°C. The final SM pellet, myofibrillar pellet from the initial 800g spin, and sarcoplasmic pellet were washed with 500 μl of 100% ethanol, centrifuged (1000g, 30 sec, 4°C), and rinsed with water (repeated twice). Protein pellets were solubilized in 1N NaOH (50°C, 15 min) and hydrolyzed into free amino acids (6 M HCl, 120°C, 24 hrs). Free amino acids were added to cation exchange columns then derivatized to their TBDMs derivatives as previously described (Harber *et al.*, 2010).

Samples were analyzed using gas-chromatography mass spectrometry (GC-MS 7890A GC with 5975C MS, Agilent Technologies Inc, Santa Clara CA) in electron impact mode with selected ion monitoring at m/z at 234 (m+0), 237 (m+3) and 239 (m+5), with m+0 representing the parent ion. Tracer enrichments of muscle samples were determined as tracer to tracee ratio from a standard curve using the m+5/m+3 ion ratios (Harber *et al.*, 2010). Plasma enrichments were determined from m+5/m+0 ion ratios and phenylalanine concentration was determined using the internal standard technique using 50 µl of 50 µM $^{13}C_6$-phenylalanine added to each plasma sample (Biolo *et al.*, 1995). The fractional synthesis rate (FSR) was determined using the standard precursor product relationship $FSR=\Delta E_m * E_p^{-1}$ with ΔE_m as the change in enrichment of muscle proteins and E_P as the enrichment of the precursor determined from the integral of the plasma phenylalanine enrichment over time. The low natural abundance of the infused tracer and washout period between separate trials allowed the assumption that background tracer enrichment is zero (Smith *et al.*, 2010).

Real-time PCR

Real-time PCR was used to determine changes in mRNA content for PGC-1α and downstream targets (Table 1). Total RNA was extracted from ~10 mg of skeletal muscle using standard Trizol methods (Invitrogen, Carlsbad CA) and reverse transcribed to cDNA as previously described (Robinson *et al.*, 2010). Approximately 10 ng of cDNA were amplified using 20 µl reactions with a manufactured master mix (Thermo Fisher, Rockford IL) in triplicate in clear 96 well plates using Taqman® probes and 7300 Real-time PCR system (Applied Biosystems, Carlsbad CA). PCR conditions were a hot start (2 minutes at 50°C, 15 minutes at 95°C) followed by 40 cycles of denaturing and

annealing (15 sec 95°C, 1 min 60°C). The relative quantity of each gene target was normalized to a reference gene (β2-microglobulin) and fold changes determined using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Statistics

Heart rate, blood pressure, and VO_2, were compared between trials using two-way (Trial x Time) analysis of variance (ANOVA) with repeated measures. Skeletal muscle FSR was compared using a one-way ANOVA. Changes in mRNA content were determined using two-way (Trial x Time) ANOVA with repeated measures on the ΔCt values (Yuan *et al.*, 2006) and are expressed as fold increase compared to the one hour biopsy on the saline control day. Multiple comparisons were performed using the Bonferroni correction. Statistical significance was set at p=0.05.

Results

Verification of β-adrenergic blockade

Infusion of isoproterenol increased heart rate and blood pressure above resting values. Repeating the isoproterenol infusion following propranolol infusion and one-hour cycling showed no stimulation of heart rate or blood pressure indicating that the propranolol protocol achieved sufficient β-adrenergic blockade (**Figure 2**).

Exercise response during exercise

Heart and blood pressure were decreased during cycling with propranolol infusion (**Figure 3A and B**). Absolute workload was the same between propranolol and saline infusions resulting in a VO_2 that was not different between trials (**Figure 3C**). Respiratory exchange ratio was not different between trials (data not shown).

Fractional synthesis rates (FSR)

Skeletal muscle fractional synthesis rate (FSR, **Figure 4**) of subsarcolemmal mitochondrial was decreased with propranolol infusion [SM Saline: 0.217±0.076 vs. SM PROP: 0.135±0.031 ($p<0.05$)]. The FSR was not different between trials for myofibrillar proteins [MYO Saline: 0.154±0.061 vs. MYO PROP: 0.102±0.038 ($p>0.05$)] or sarcoplasmic proteins [SARC Saline: 0.122±0.034 vs. SARC PROP: 0.0898±0.025 ($p>0.05$)].

Real-time PCR

Real-time PCR revealed PGC-1α mRNA was increased at five hours compared to one hour after cycling, with no differences between propranolol or saline infusions ($p<0.01$ for time, **Figure 5A**). Downstream mRNA signals for mitochondrial biogenesis

were not different between time points or between propranolol or saline infusions (**Figure 5B-F**).

Discussion

Propranolol was infused during a one-hour bout of cycling to determine if non-selective β-adrenergic blockade could alter skeletal muscle protein synthesis and mitochondrial biogenesis. Post-exercise mitochondrial protein synthesis was greater during the saline than propranolol trial. PGC-1α mRNA was increased at five hours post-exercise compared to one hour, but was not different between trials. Other downstream mRNA signals for mitochondrial biogenesis were not different between one and five hours after exercise or between trials. We conclude that mitochondrial biogenesis following aerobic exercise can be impaired with non-selective β-adrenergic blockade.

Verification of β-adrenergic blockade

Non-selective β-adrenergic blockade was confirmed in three participants. The ability for isoproterenol to stimulate heart rate and blood pressure was determined before and after cycling with propranolol. The lack of cardiovascular stimulation at one hour following cycling with propranolol indicates non-selective β-adrenergic blockade during the acute period following cycling, which is when skeletal muscle protein synthesis rates and signals for mitochondrial biogenesis are elevated (Pilegaard *et al.*, 2003; Haus *et al.*, 2007; Wilkinson *et al.*, 2008). Our biopsies were collected at one and five hours following propranolol infusion, which is during the three to six hour half-life of propranolol (Reiter, 2004). We cannot determine if there was complete blockade of all β-adrenergic receptors on skeletal muscle. However, the demonstration of non-selective β-adrenergic blockade combined with the half-life of propranolol indicates that β-adrenergic receptor signaling was decreased during our measures of skeletal muscle protein synthesis and mRNA. As expected, heart rate and blood pressure were decreased

during cycling with propranolol infusion and indicates that decreased β-adrenergic receptor signaling was achieved in all participants.

Our study was designed as an integrative approach to assess mitochondrial biogenesis during recovery from aerobic exercise. PGC-1α and downstream mRNA content are increased by two hours and have remained elevated for eight hours following exercise (Pilegaard *et al.*, 2003; Pilegaard *et al.*, 2005). Additionally, skeletal muscle protein synthesis is increased in the several hours following exercise and can remain elevated for 72 hours (Miller *et al.*, 2005; Wilkinson *et al.*, 2008). We are confident that our the timing of muscle sampling was adequate to capture any changes in mRNA content for proteins related to mitochondrial biogenesis or changes in protein synthesis rates. Our measurements of mitochondrial adaptations included mRNA transcript content for proteins regulating mitochondrial biogenesis and mitochondrial protein synthesis. Previous studies of β-adrenergic signaling and mitochondrial biogenesis have reported changes in mRNA content (Miura *et al.*, 2007; Miura *et al.*, 2008), however such measurements do not take into account post-transcription or translational regulation. Our kinetic measurement of mitochondrial protein synthesis represents the cumulative regulation of signals for mitochondrial biogenesis.

Fractional synthesis rate (FSR)

The synthesis rate of skeletal muscle subsarcolemmal mitochondrial proteins was blunted in the several hours following cycling with propranolol infusion. Our results provide short-term evidence that non-selective β-adrenergic blockade during cycling can impair mitochondrial adaptations, and supports long-term studies that showed decreased mitochondrial enzyme activity during training with β-blocker drugs (Ades *et al.*, 1990).

A blunted response of mitochondrial protein synthesis to individual training sessions may lead to decreased adaptations over long-term.

The regulation of β-adrenergic signaling on mitochondrial biogenesis and skeletal muscle protein synthesis appears to be mediated through β_2-adrenergic receptors. Previous reports showed that selective β_2-adrenergic receptor agonists, but not β_1 or α agonists, increased PGC-1α mRNA at rest and selective β_2-adrenergic receptor blockers abolished the post-exercise increase of PGC-1α mRNA (Miura *et al.*, 2007). Additionally, the blunted increase of mitochondrial enzymes during aerobic training is reported during treatment with non-selective β-adrenergic blockers (e.g. atenolol or propranolol), but not selective β_1-adrenergic blockers (e.g. metoprolol or atenolol) (Svedenhag *et al.*, 1984; Ji *et al.*, 1986; Ades *et al.*, 1990). Others have reported no difference in $VO_{2\ max}$ adaptations between selective or non-selective β-blocker treatments (Savin *et al.*, 1985; Wolfel *et al.*, 1986). It is possible that different degrees of β-adrenergic blockade due to various drug doses or binding preferences within selective or non-selective classes can explain variable training adaptations. It is important to note that studies with specific mitochondrial measures (e.g. mitochondrial enzyme activity) can detect impairments with non-selective β-blockers that are not detected with whole body measures (e.g. $VO_{2\ max}$). Our approach was to measure acute changes in protein synthesis that may explain long-term differences in mitochondrial adaptations with non-selective β-adrenergic blockade.

Myofibrillar protein synthesis was not different between the propranolol and saline trials. While β-adrenergic antagonists are used to treat cardiovascular disease, β-adrenergic agonists have been used for the treatment of muscle loss, such as with aging or

disease. In particular, β_2-selective agonists stimulate skeletal muscle protein gains over several weeks in aging rats (Ryall *et al.*, 2004). We have shown that acute infusion of isoproterenol (non-selective β-adrenergic agonist) did not acutely stimulate skeletal muscle protein synthesis in humans (Robinson *et al.*, 2010). Recent data showed that chronic treatment with a selective β_2-adrenergic agonist increased skeletal muscle protein synthesis of myofibrillar, mitochondrial, and sarcoplasmic fractions at seven days, but not acutely or at 28 days of treatment (Koopman *et al.*, 2010). Koopman *et al*. also showed that Ca^{++} dependent pathways of protein breakdown were decreased within 24 hours and throughout β_2-adrenergic agonist treatment (Koopman *et al.*, 2010). Thus, β-stimulation may acutely decrease protein breakdown, but requires repeated treatments to increase protein synthesis. Skeletal muscle protein breakdown rates are increased following exercise (Phillips *et al.*, 1997), but the effects of β-blockade on post-exercise breakdown are not well known. Our current results indicate that post-exercise myofibrillar protein synthesis is not altered with propranolol, but future studies should consider β-adrenergic signaling on post-exercise skeletal muscle protein breakdown.

Our values of skeletal muscle fractional synthesis rates were determined following exercise in the fed state. The fractional synthesis of skeletal muscle mitochondria is higher than myofibrillar proteins at rest (Rooyackers *et al.*, 1996; Robinson *et al.*, 2010), following exercise (Hartman *et al.*, 2007) and is increased with intravenous amino acid infusion (Bohe *et al.*, 2003). Our measured mitochondrial protein synthesis rates are slightly higher than those reported by others due to the experiment conditions (i.e. post-exercise and feeding) and calculating rates using a zero background labeling (Smith *et al.*, 2010). The high rate of mitochondrial protein synthesis reported

here and by others indicates a high rate mitochondrial protein turnover that can be stimulated by exercise (Wilkinson *et al.*, 2008) and nutrition (Bohe *et al.*, 2003).

Exercise indirect calorimetry

The one-hour cycling workload was adjusted to maintain 60% $VO_{2\ max}$ during the first trial (propranolol) and the same absolute workload was repeated for the second trial (saline). The respiratory exchange ratios were not different between trials indicating that substrate utilization was the same during the trials. Others have reported similar indirect calorimetry results during cycling exercise following propranolol treatment (van Baak *et al.*, 1995). Post-exercise signals for mitochondrial biogenesis can be influenced by dietary habits that alter glycogen content (Pilegaard *et al.*, 2005) but not related to circulating glucose or free fatty acid availability (Russell *et al.*, 2005). Thus, the potential influence of β-adrenergic signaling on mitochondrial biogenesis is likely to be within skeletal muscle and not due to changes in circulating substrate metabolism during exercise.

PCR

Contrary to our hypothesis and previous studies with mice (Miura *et al.*, 2007), mRNA content of markers of mitochondrial biogenesis were not different between exercise with propranolol compared to saline. PGC-1α mRNA was increased in both trials at five hours post exercise compared to one hour, which is similar to previous reports in human exercise trials (Pilegaard *et al.*, 2003). To minimize subject burden, we did not collect a biopsy sample before exercise and cannot determine the change in mRNA content from resting values. However, our biopsy time points at one hour and five hours after exercise provide two times points to compare between propranolol and

saline trials and are within the timeframe that PGC-1α and downstream mRNA are elevated (Pilegaard *et al.*, 2005).

Exercise-induced changes to PGC-1α mRNA content are dependent on exercise intensity (Tadaishi *et al.*, 2010) and appear to be regulated by the relative intensity rather than the absolute intensity (Nordsborg *et al.*, 2010). Despite an acute blunting of heart rate response and increased perceived effort with propranolol, our exercise bouts were likely performed at the same relative intensities. Support for the same relative exercise intensity was that subjects were tested within four weeks with no changes in exercise patterns in the intervening period, and equal RER, which scales to relative exercise intensity (Brooks & Mercier, 1994), between trials.

Our current results indicate that β-adrenergic blockade can decrease mitochondrial protein synthesis without changes to mRNA content of select respiratory subunits or signaling proteins for mitochondrial biogenesis. β-adrenergic signaling may alter mitochondrial protein content through post-transcriptional mechanisms. For example, β-adrenergic signaling can decrease initiation of protein translation (Gelinas *et al.*, 2007), which will result in decreased protein synthesis. Our data indicate that the mitochondrial subfraction was decreased with β-adrenergic blockade, but there was a trend for the other fractions to be decreased as well. We cannot exclude the possibility that a global decrease in protein synthesis occurred during β-adrenergic blockade. Additionally, β-adrenergic stimulation can decrease Ca^{++} dependent protein degradation (Koopman *et al.*, 2010) and contribute to maintaining skeletal muscle protein content, including mitochondria. The regulation of β-adrenergic signaling on post-transcriptional regulation of mitochondrial protein content should be considered in future studies.

Perspectives

We previously reported that acute non-selective β-adrenergic stimulation during resting did not increase mitochondrial protein synthesis or markers of mitochondrial biogenesis. Our current investigation indicates that β-adrenergic receptor signaling can modulate exercise induced mitochondrial protein synthesis. The post-exercise recovery period is a critical time for skeletal muscle remodeling and decreasing sympathetic signals to skeletal muscle may attenuate adaptations. Additional work should consider if chronic intake of β-adrenergic blockers would decrease mitochondrial protein synthesis and contribute to impaired mitochondrial adaptations during training.

Conclusion

We conclude that non-selective β-adrenergic blockade can blunt the post-exercise increase in mitochondrial protein synthesis rates. Non-selective β-adrenergic blockade did not alter the mRNA content for signals of mitochondrial biogenesis, suggesting that the impaired protein synthesis response is independent of changes to mRNA transcripts. Our results indicate that non-selective β-adrenergic receptor antagonists can impair mitochondrial adaptations to acute aerobic exercise and may lead to decreased training adaptations.

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Tables

Table 1: Subject characteristics of subjects who volunteered for the verification of β-blockade using isoproterenol and propranolol (ISO+PROP) and the propranolol study (PROP). The three subjects for ISO+PROP were from final PROP group.

Table 2: PCR Sequences

Table 1

	ISO+PROP	PROP
	n=3 males	n=6 males
Age (yr)	28±5	26±6
Height (m)	1.8±0.1	1.78±0.1
Weight (kg)	86.5±4.0	84.4±6
BMI ($kg*m^{-2}$)	27.4±0.3	26.7±2
Fat (%)	26.3±5.1	25.1±6
$VO_{2\ max}$ ($ml*kg^{-1}*min^{-1}$)	39.9±2.4	39.9±4.9
$VO_{2\ max}$ ($l*min^{-1}$)	3.5±0.3	3.35±0.3

Table 2

Target	*Gene Symbol*	*Assay ID*	*Chromosome*	*Target Exons*	*Probe Sequence*
PGC1-α	PPARGC1A	Hs00173304_m1	4	7	TGGAACTGCAGGCCTAACTCCACCC
TFAM	TFAM	Hs01082775_m1	10	5	GATTCACCGCAGGAAAAGCTGAAGA
NRF1	NRF1	Hs00602161_m1	7	8	TGATGGAGAGGTGGAACAAAATTGG
NRF2	GABPA	Hs01022023_m1	21	9	ACTCAGTCGTGCATTAAGATATTAT
COX7a	COX7A1	Hs00156989_m1	19	1	GGCCCTTCGGGTGTCCCAGGCGCTG
mtND4	MT-ND4	Hs02596876_g1	MT	n/a	CAAACTCCTGAGCCAACAACTTAAT

Figures

Figure 1: Study schematic. β-adrenergic receptor blockade was verified in three participants using a graded isoproternol (ISO) infusion to raise HR and BP, then infused propranolol (PROP) during cycling and repeated the ISO infusion at one hour post exercise (A). Six participants complete two trials with either propranolol (PROP) or saline infused during cycling followed by stable isotope infusion and muscle biopsies (B).

Figure 2: β-adrenergic receptor blockade was demonstrated in three subjects by a lack of β-adrenergic stimulation by isoproterenol of heart rate (HR, A), systolic (B) or diastolic blood pressure (C). Data are expressed as change from resting following isoproterenol (ISO) infusion or propranolol followed by isoproterenol (PROP+ISO).

Figure 3: Heart rate (HR, A), systolic (SYS), and diastolic (DIA) blood pressure (BP, B) were all lower during exercise with propranolol (PROP) infusion. Absolute workloads were identical resulting in similar steady state oxygen consumption (C). *$p<0.01$

Figure 4: Propranolol infusion (PROP) during cycling decreased skeletal muscle fractional synthesis (FSR) for subsarcollemal mitochondrial (SM) but not myofibrillar (MYO) or sarcoplasmic (SARC) proteins. *$p<0.05$

Figure 5: Real-time PCR revealed PGC-1α mRNA was increased at five hours following cycling (*$p<0.01$ for time, A) but not different between saline (open) or propranolol

(PROP, shaded) trials. Downstream mRNA signals for mitochondrial biogenesis were not different at one hour and five hours following cycling between saline or propranolol trials (B-F). Data are expressed as fold change compared to one-hour saline

Figure 1

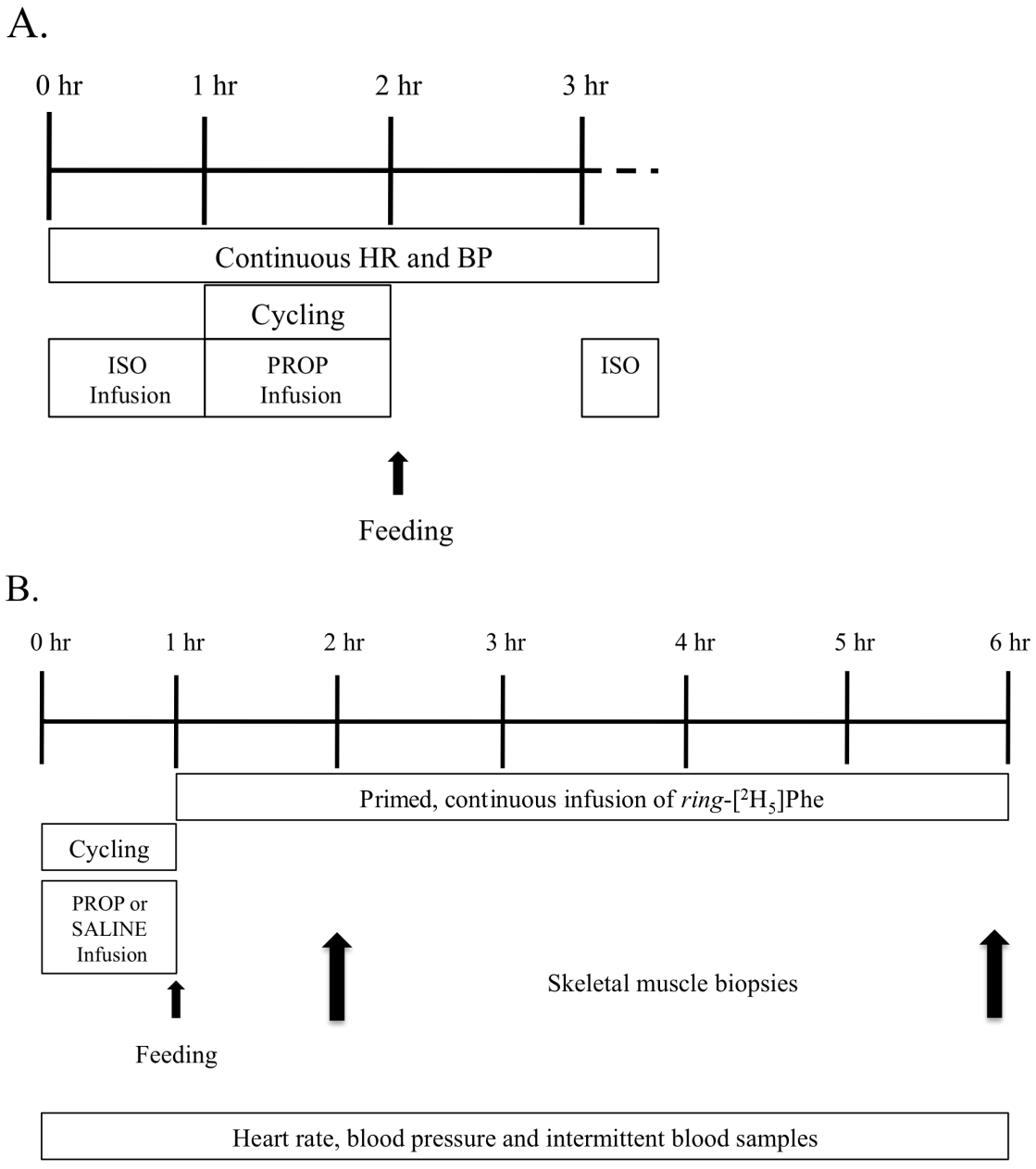

A.
0 hr
1 hr
2 hr
3 hr
Continuous HR and BP
Cycling
ISO Infusion
PROP Infusion
ISO
Feeding
B.
0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
6 hr
Primed, continuous infusion of ring-[2H5]Phe
Cycling
PROP or SALINE Infusion
Skeletal muscle biopsies
Feeding
Heart rate, blood pressure and intermittent blood samples

Figure 2

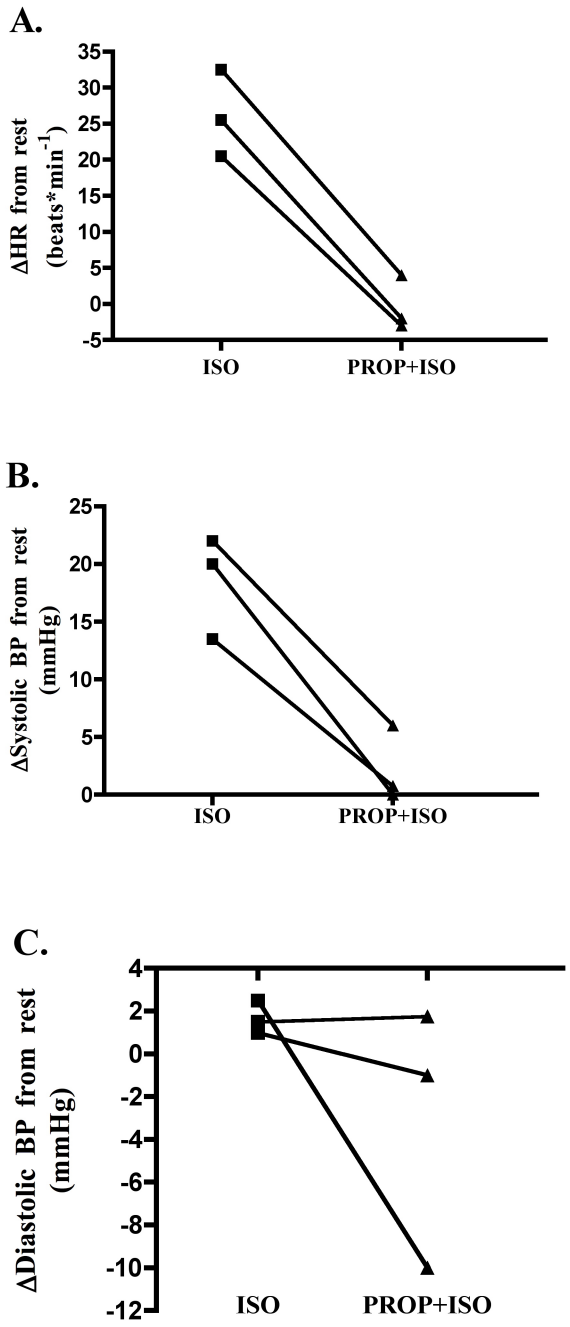

A.
ΔHR from rest (beats*min-1)
35
30
25
20
15
10
5
0
-5
ISO
PROP+ISO
B.
ΔSystolic BP from rest (mmHg)
25
20
15
10
5
0
ISO
PROP+ISO
C.
ΔDiastolic BP from rest (mmHg)
4
2
0
-2
-4
-6
-8
-10
-12
ISO
PROP+ISO

Figure 3

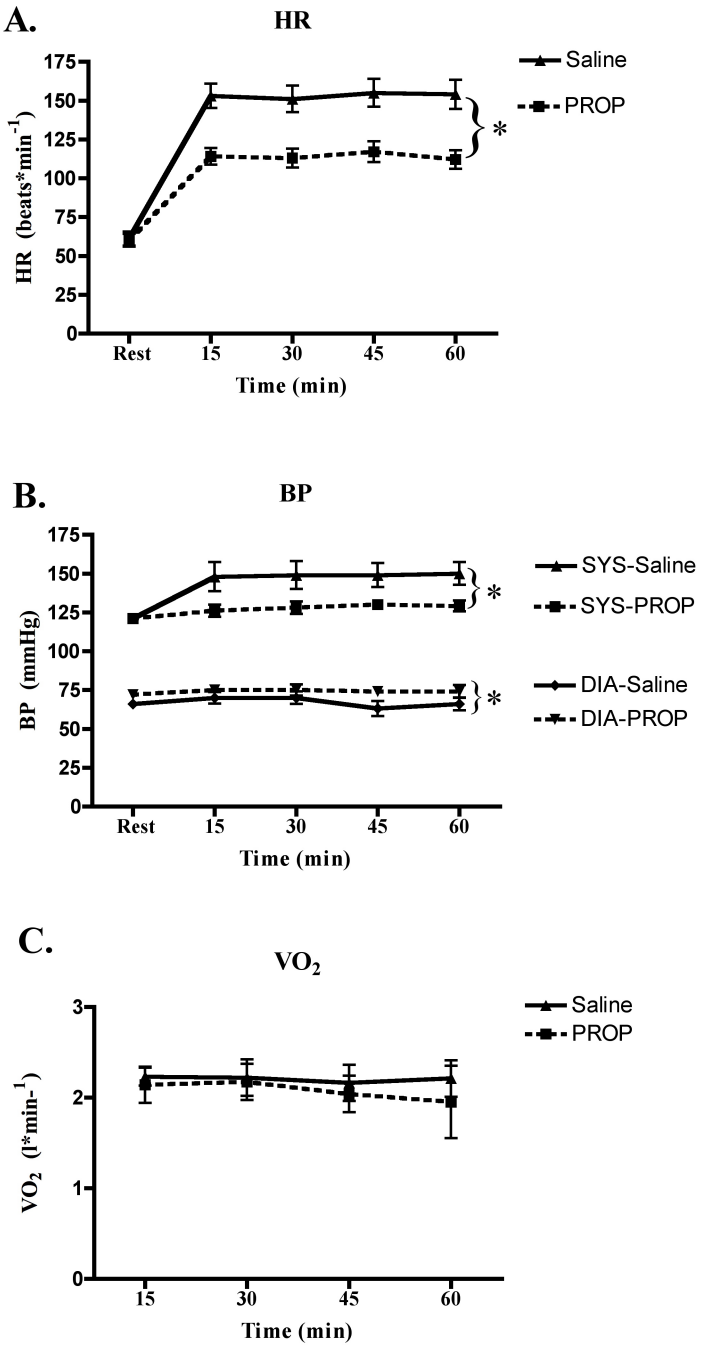

A.
HR
HR (beats*min-1)
175
150
125
100
75
50
25
0
Rest
15
30
45
60
Time (min)
Saline
PROP
*
B.
BP
BP (mmHg)
175
150
125
100
75
50
25
0
Rest
15
30
45
60
Time (min)
SYS-Saline
SYS-PROP
DIA-Saline
DIA-PROP
*
*
C.
VO2
VO2 (l*min-1)
3
2
1
0
15
30
45
60
Time (min)
Saline
PROP

Figure 4

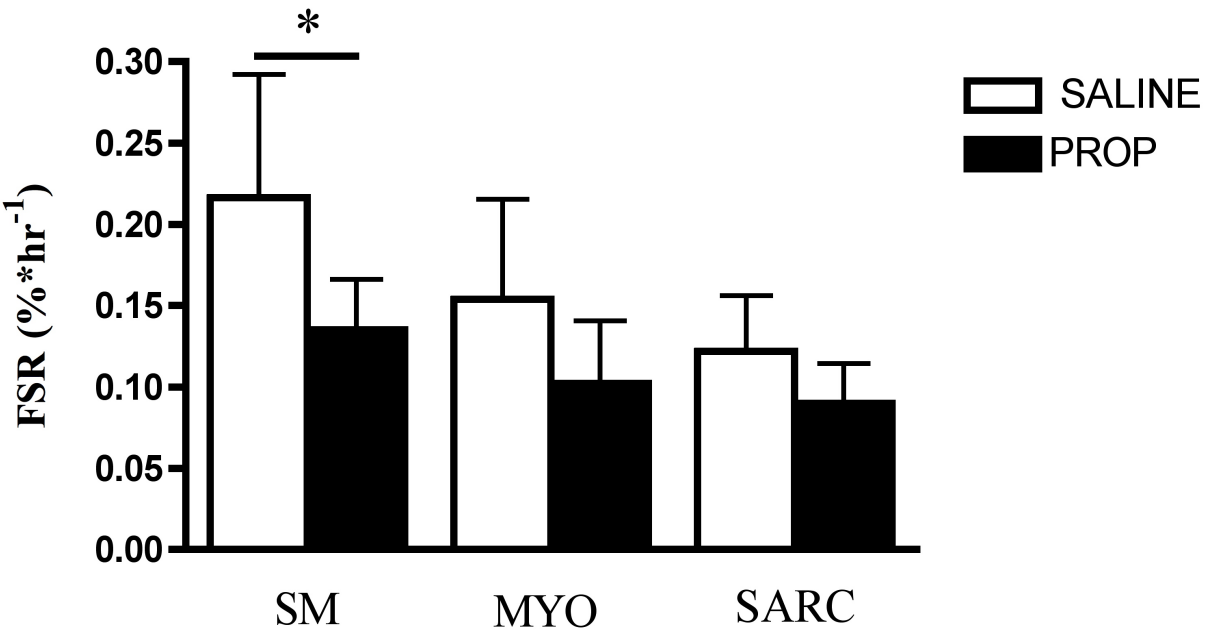
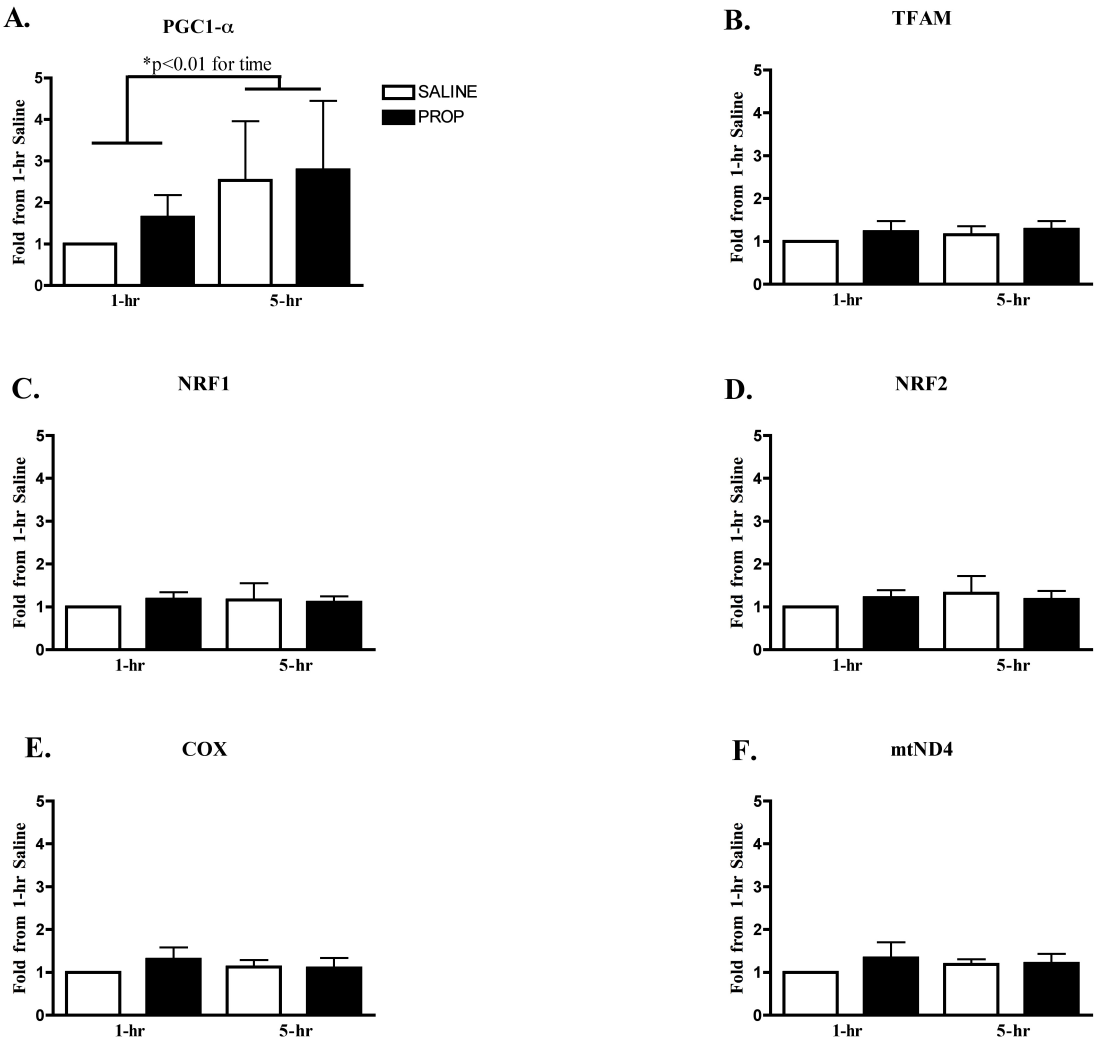


Figure 5


A.
PGC1-α
*p<0.01 for time
SALINE
PROP
Fold from 1-hr Saline
1-hr
5-hr
B.
TFAM
C.
NRF1
D.
NRF2
E.
COX
F.
mtND4

CHAPTER V-MANUSCRIPT IV

Myocellular remodeling during aerobic exercise training in aging human subjects goes beyond just protein synthesis

Matthew M Robinson[1], Scott Turner[2], Marc K Hellerstein[2,3], Karyn L Hamilton[1], and Benjamin F Miller[1*]

[1]Department of Health and Exercise Science
Colorado State University, Fort Collins CO

[2]KineMed Inc
Emeryville CA

[3]Department of Nutritional Sciences
University of California, Berkeley CA

[*]Corresponding Author
benjamin.f.miller@colostate.edu
200 Moby B-Complex
Colorado State University, Fort Collins CO 80523-1582

Keywords
stable isotope tracer, kinetics, deuterium oxide, heavy water, endurance, satellite cell

Abbreviations
D_2O: Deuterium oxide
DNA %F: DNA synthesis rate
MPS: Mixed muscle protein synthesis rate
GLY %F: Membrane phospholipid bound glycerol synthesis rate

Abstract

Consuming protein following exercise stimulates protein synthesis within skeletal muscle and is recommended to prevent sarcopenia; however it is not known if acute stimulations will persist long-term or include other macromolecules. We determined if consuming protein following exercise during aerobic training could increase long-term protein and DNA synthesis rates in skeletal muscle of adult humans. Sixteen previously untrained participants (50±8 years) consumed either a carbohydrate or carbohydrate and protein drink following each session during six weeks of treadmill training. A younger untrained group provided a non-exercising comparison. Participants consumed deuterium oxide to isotopically label newly synthesized skeletal muscle proteins and DNA. Contrary to acute studies and our hypothesis, consuming protein after exercise did not increase skeletal muscle protein synthesis rates. We also found no difference in DNA synthesis between nutrition groups, however the average of the exercise groups (~4%) was higher than the younger sedentary group (not different than zero) and may be attributed to satellite cell activation. We conclude that post-exercise protein consumption does not increase average rates of protein synthesis over six weeks and that aerobic exercise stimulates DNA synthesis in skeletal muscle. Long-term synthesis rates provide important insight into aging and exercise adaptations.

Introduction

Aging is a major public health concern due to high social and economic costs associated with impaired mobility and physical function (Janssen *et al.*, 2004). These costs will continue to rise as the world's population over 60 years old increases to an estimated two billion by 2050 (Koopman & van Loon, 2009). The loss of skeletal muscle mass and function with age (sarcopenia) contributes to increased morbidity and understanding the progression of sarcopenia may help with developing lifestyle recommendations to promote healthy aging.

There is a ~1-2% per year loss of skeletal muscle mass in people over 50 years (Hughes *et al.*, 2002; Goodpaster *et al.*, 2006). The mass of skeletal muscle tissue is determined by the net contribution of the synthesis of new proteins and degradation of existing proteins, collectively called protein turnover. Decreased basal rates of skeletal muscle protein synthesis with aging have been reported (Balagopal *et al.*, 1997; Henderson *et al.*, 2009) and could contribute to loss of mass with age if not countered by decreased protein breakdown. However, other reports showed no difference at rest in either muscle protein synthesis or breakdown between younger and older people (Volpi *et al.*, 2000; Volpi *et al.*, 2001; Cuthbertson *et al.*, 2004) suggesting that sarcopenia is not due to basal differences in protein turnover. Instead, it is possible that aging induces resistance to anabolic stimuli. In support, exercise and protein consumption are repeatedly shown to increase muscle protein synthesis in younger people (Phillips *et al.*, 1997; Moore *et al.*, 2009) while older people showed blunted responses to feeding (Cuthbertson *et al.*, 2004), insulin (Wilkes *et al.*, 2009) and exercise (Kumar *et al.*, 2009).

The blunted response to anabolic signals may lead to lower average rates of protein synthesis over prolonged time frames and contribute to sarcopenia.

Providing protein nutrition following exercise has been used to overcome the impaired anabolic response. A recent report studying older humans showed that even when consuming identical diets over a period of three days, protein consumption after daily endurance exercise increased nitrogen retention compared to consuming the same beverage earlier in the day (Jordan *et al.*, 2010). Nitrogen balance provides an index of protein accretion over extended periods, however a direct measure of skeletal muscle protein synthesis would allow greater insight into whether exercise and nutrition could attenuate sarcopenia. Aerobic exercise is a common activity for aging populations (Simpson *et al.*, 2003), but little is known about skeletal muscle protein kinetics following aerobic exercise. Recent studies show that aerobic exercise can stimulate skeletal muscle protein synthesis (Wilkinson *et al.*, 2008) and protein consumption after cycling can increase whole body protein turnover in older people (Murphy & Miller, 2010). Consuming protein after aerobic exercise is a simple lifestyle modification for aging populations, but it is not known if any short-term increases in skeletal muscle protein kinetics will persist over longer duration.

Much of our understanding of amino acid kinetics is based on in-patient settings using intravenous infusion of stable isotope over several hours. Such studies are limited to acute time frames due to practical (e.g. avoiding prolonged intravenous infusions) and theoretical complications (e.g. reappearance of tracer from protein breakdown). Furthermore, tracer studies commonly analyze only a single pathway, or possibly separate pathways if multiple tracers are infused (Zhang *et al.*, 1996; Volpi *et al.*, 1999;

Levenhagen *et al.*, 2001). In addition to protein turnover other pathways that are affected by aging should be considered, such as depletion of satellite cells (Renault *et al.*, 2002) and decay of mitochondria (Short *et al.*, 2005). Satellite cells are resident myogenic precursors that promote muscle growth and repair following exercise and a decrease in the content (Renault *et al.*, 2002; Shefer *et al.*, 2010) or activation (Beccafico *et al.*, 2010) of satellite cells with age is suggested to contribute to sarcopenia. Activation of satellite cells to grow, divide and fuse to an existing myofibril requires DNA synthesis, protein synthesis, and membrane remodeling; however simultaneously analyzing these pathways raises technical challenges with short-term intravenous tracer infusions. For example, several days of intravenous infusion may be necessary for sufficient incorporation of labeled precursors into newly synthesized DNA (Macallan *et al.*, 1998). Long-term kinetic studies that include multiple pathways could further our understanding of how exercise and nutrition may help maintain muscle mass with age.

Deuterium oxide (^2H_2O, or D_2O) can be used to determine kinetics of multiple synthetic processes over several weeks to months. The methodology is based on standard precursor-product relationships with the true precursor enrichment being determined from a fully turned over pool and a maintained isotopic steady state through oral consumption of D_2O. D_2O equilibrates with the body water pool and achieves steady isotopic enrichment within one week that can be maintained for several weeks with continued D_2O consumption (Neese *et al.*, 2002). Deuterium from the body water pool is incorporated into a wide variety of metabolic precursors that produce deuterium labeled products including protein (Previs *et al.*, 2004), DNA (Busch *et al.*, 2007) and lipids (Turner *et al.*, 2003). D_2O has been validated for multiple synthetic processes in different

tissues (Hellerstein, 2003). To our knowledge, there are no reports characterizing multiple long-term synthetic processes in skeletal muscle during exercise training in humans.

The purpose of our study was to assess long-term synthetic processes in skeletal muscle in older individuals undergoing an aerobic exercise-training program with post-exercise nutrient ingestion. We hypothesized that protein consumption following exercise would increase synthesis rates of skeletal muscle protein and DNA. Contrary to our hypothesis, post-exercise protein nutrition did not enhance anabolic responses to a greater extent than post-exercise carbohydrate ingestion. A novel finding was that aerobic exercise training increased DNA turnover in skeletal muscle of older individuals, a finding we attribute to increased satellite cell recruitment.

Methods

Ethical approval

The Institutional Review Board at Colorado State University approved this study (07-199H and 09-1363H). Each volunteer was informed of the potential risks and benefits and provided written consent before participating. The study followed the guidelines set forth by the Declaration of Helsinki.

Subject characteristics

Sixteen sedentary males and females (mean±SD age: 50±8, range 37-64 yrs) volunteered for the study (**Table 1**). We initially planned to study people over 55 years old but later expanded to a wider age range because it has been demonstrated that protein synthesis rates are decreased by 50 years of age (Rooyackers *et al.*, 1996). Participants were matched for age, sex, body mass index (BMI), and maximal aerobic capacity ($VO_{2\ max}$) then randomized to consume either a carbohydrate (CHO) or protein+carbohydrate (PRO) drink following each exercise session. There were no baseline differences ($p>0.05$) between PRO and CHO in age, BMI, or $VO_{2\ max}$ (**Table 1**). The participants were healthy based on medical history questionnaire and results from a Bruce protocol graded exercise test with 12-lead electrocardiogram. Body composition was determined using dual-energy x-ray absorptiometery (DEXA, Lunar Discovery W, GE Medical Systems, Madison WI). All subjects were free of any medications that could impair mitochondrial adaptations (Robinson *et al.*, 2009). Because of novel findings regarding DNA turnover, a second group of participants (3F, 1M; age: 21±2, range 19-23 yrs) that did not perform exercise training was added to provide a young sedentary comparison group (SED) (**Table 1**).

Study overview

Participants completed a progressive aerobic exercise protocol (three sessions per week for six weeks) and consumed a post-exercise drink of either CHO or PRO. The drinks were isocaloric (300 kcal) and PRO included 20 grams of protein (drinks were kindly provided by the Gatorade Sports Science Institute, Barrington IL). Deuterium oxide (D_2O) was consumed daily to isotopically label newly synthesized skeletal muscle proteins, DNA, and membrane phospholipid glycerol. $VO_{2\ max}$, DEXA, muscle biopsy and blood sampling were performed before and after the six week training protocol (**Figure 1**).

$VO_{2\ max}$

$VO_{2\ max}$ was measured using a cycle ergometer (Lode Excalibur, Medical Graphics Corporation, St. Paul MN) and indirect calorimeter (True One, ParvoMedics, Sandy UT) as previously described (Jordan *et al.*, 2010). Heart rate was continuously recorded during the test and used to determine the intensity of exercise sessions. The heart rates recorded at various percentages of $VO_{2\ max}$ (60%, 65%, 75%, and 85%) were used to determine exercise intensity during the six-week exercise protocol.

Exercise protocol

Exercise was performed on a treadmill and included warm-up (15 minutes at 60%) and workout stages (30 minutes, progressing from 65% to 85% by wk-5). The participants wore heart rate monitors (Polar Electro Inc, Lake Success NY) and the speed or grade of the treadmill was changed to maintain heart rate at the specified percentage of $VO_{2\ max}$. A student investigator supervised each exercise session and monitored all exercise intensities and times. The drinks were consumed immediately after exercise

under supervision. We had 100% compliance with all exercise sessions and drink consumption.

Deuterium labeling

Deuterium labeling of newly synthesized products was achieved using oral consumption of D_2O (70%, Cambridge Isotope Laboratories Inc, Andover MA) throughout the entire six-week exercise protocol (or 4-weeks for SED). A target of 1-2% enrichment was achieved during a one-week priming stage (50 ml three times daily=150 ml*day^{-1}) and maintained for five weeks (50 ml two times daily=100 ml*day^{-1}). Body water enrichment was determined from saliva swabs collected at weeks two, four and six as previously described (Neese *et al.*, 2001; Turner *et al.*, 2003). Participants were instructed to not eat or drink anything for 30 minutes prior to saliva sampling. Saliva swabs were stored at −80°C until analysis.

Tissue sampling

Participants arrived at the laboratory following an overnight fast for blood and muscle sampling before and after the six-week exercise protocol. Venous blood was collected and one milliliter of whole blood was removed and stored at −80°C for peripheral blood mononuclear cell (PMBC) isolation as described below. The remaining whole blood was centrifuged (1200g, 4°C, 15 minutes) to separate plasma and buffy coat layers that were removed separately and stored at −80°C. Muscle biopsy samples (~100-150 mg) of the vastus lateralis were removed under local anesthesia (1% lidocaine) using a 5-mm Bergstrom needle with manual suction, then immediately frozen in liquid nitrogen and stored at −80°C.

Mixed muscle protein synthesis rate

Mixed muscle protein synthesis (MPS) was determined from ~20 mg of the Post muscle using gas-chromatography mass-spectrometry (GC-MS) analysis of deuterium labeled alanine as previously described (Busch *et al.*, 2006). The newly synthesized fraction (f) of muscle proteins was calculated from the true precursor enrichment (p) using mass isotopomer distribution analysis [MIDA (Hellerstein & Neese, 1999; Busch *et al.*, 2006)]. MPS was calculated as the change in enrichment of deuterium labeled alanine (Busch *et al.*, 2007) bound in muscle proteins over the entire labeling period and expressed as the common unit for protein synthesis rates (%*hr^{-1}).

Muscle DNA extraction

Muscle DNA synthesis rate (DNA %F) was determined from DNA extracted from muscle biopsy samples. Total muscle DNA (~8 μg) was extracted from 50 mg tissue following manufacturers instructions (MiniDNA kit, Qiagen) and eluted in 200 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). A 20 μl aliquot of total DNA was used for PCR procedures (below). The remaining DNA was precipitated with cold ethanol, suspended into 200 μl nuclease free H_2O and hydrolyzed to free deoxyribonucleic acids. The deoxyadenosine fraction was separated and analyzed for deuterium content by GC-MS as previously described (Busch *et al.*, 2007).

Blood processing

Peripheral blood mononuclear cells (PMBC) were purified from frozen whole blood samples using magnetic beads following the manufacturer's protocol (Miltenyi Biotech Inc, Auburn CA). The buffy coat layer was used for one participant because the whole blood sample yielded insufficient DNA. Briefly, anti-CD^{14+} beads were added to

whole blood (1 ml) or buffy coat (~500 µl) and collected using Whole Blood Columns and MiniMacs separator (Miltenyi Biotech Inc, Auburn CA). The PMBC fraction was suspended in 200 µl phosphate buffer saline (3.2 mM Na_2HPO_4, 0.5mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.5). DNA was extracted from PBMC using a DNA Mini kit (Qiagen Inc, Valencia CA), eluted using 200 µl nuclease free H_2O, and processed for GC-MS analysis as described for DNA %F (Busch *et al.*, 2007).

Calculation of DNA %F

Deuterium labeling of DNA occurs exclusively through de-novo nucleotide synthesis and allows calculation of the rate of newly synthesized DNA (DNA %F) over extended periods (Neese *et al.*, 2002; Busch *et al.*, 2007). The enrichment of a synthesized product cannot exceed the enrichment of the true precursor; therefore the enrichment of DNA of a cell pool that is fully replaced during the labeling period (e.g. PMBC) is equal to the enrichment of the precursor ($EM1^*$). We determined $EM1^*$ using two methods: 1) PMBC DNA enrichment and 2) the relationship between body water deuterium enrichment and MIDA calculations of $EM1^*$. MIDA predicts $EM1^*$ based on the labeling patterns at a given body water D_2O enrichment as previously described (Hellerstein & Neese, 1999; Busch *et al.*, 2007). A generalized form of the relationship between $EM1^*$ and body water D_2O is $EM1^*=3.193*BW+0.0013$ where BW is the body water D_2O enrichment measured in saliva samples. There was no difference between the DNA %F calculated from body water and PMBC, and the reported values are from body water.

Muscle membrane phospholipid synthesis

Total phospholipids were extracted from muscle biopsy samples and the glycerol fraction was separated by liquid chromatography and analyzed by mass spectrometry (Turner *et al.*, 2003). Glycerol is enriched with deuterium exclusively through de-novo synthesis pathways and is used to form a variety of downstream products, including membrane phospholipids. The synthesis rate of glycerol within membrane phospholipids (GLY %F) is an index of membrane synthesis.

Real-time PCR

The ratio of mitochondrial DNA (mtDNA) to nuclear DNA (nDNA) copy number was determined using real-time PCR from an aliquot of total muscle DNA. Briefly, 10 ng of DNA was amplified in a 20 μl reaction with a pre-made master mix (Thermo Fisher, Rockford IL) and Taq-Man® primers and probes (Applied Biosystems, Carlsbad CA). Samples were amplified in triplicate for each primer/probe sequence (singleplex) and all plates included blank and internal controls. PCR conditions were a hot-start (15 min @ 95°C) followed by 40 cycles of denaturing (15 sec @ 95°C) and annealing (60 sec @ 60°C) in 96-well clear reaction plates using a 7300 Real-Time PCR System (Applied Biosystems, Carlsbad CA). One nuclear and one mitochondrial primer/probe set was custom designed using Primer Express® software (v. 3.0, Applied Biosystems, Carlsbad CA) and the other primers/probes were inventoried Taqman® Gene Expression Assays for genomic DNA manufactured by Applied Biosystems (**Table 2**). The inventoried assays include proprietary sequences that are not specified by Applied Biosystems.

mtDNA content

The relative copy number (RC) of mtDNA to nDNA (Szuhai *et al.*, 2001) adjusted for qPCR efficiency (Pfaffl, 2001) was calculated as $RC = E^{(\Delta C_t)}$ where

$$E = 10^{(-1/slope)}$$
$$\Delta C_t = C_t nDNA - C_t mtDNA$$

The slope of the linear regression line of diluted DNA samples covering the expected threshold cycle (C_t) for gene targets revealed equal efficiency (E) between gene targets (E= 75%). C_t values were determined automatically using the SDS v.1.4 software then manually verified that the threshold line was located early in the linear amplification phase. C_t values were consistent between the control samples run on separate plates.

Statistics

Statistical analysis was performed using PRISM v4.0c (GraphPad Software Inc, La Jolla CA). Differences in subject characteristics were compared using two-way ANOVA (group x time) with repeated measures. Changes in $VO_{2\ max}$ were calculated as the percentage increase over baseline and compared using an unpaired t-test. MPS and DNA %F were compared with unpaired t-test. Correlations were calculated using Pearson's r. Significance was set at p=0.05 and data are presented as mean±SD.

Results

Changes in subject characteristics

Absolute $VO_{2\ max}$ increased in the PRO but not CHO group following six weeks of aerobic training (change from baseline PRO: 12.2 ± 6.2%; CHO: 3.3 ± 8.7%, p=0.033, **Figure 2**). Average total body weight, fat free mass, and percent body fat did not change for either group (data not shown).

Deuterium labeling and turnover

Body water enrichment reached ~1.5-2.5% by week two and was maintained through week six (**Figure 3)**. Average MPS (**Figure 4A**) over six weeks of endurance exercise was not different between PRO and CHO groups (PRO: 0.039 ±0.006%*hr^{-1}; CHO: 0.043±0.006 %*hr^{-1}, p=0.22). Because MPS was not different between CHO and PRO groups, we combined the two groups into a single older-exercise group (Older-EX) and compared it to the younger-sedentary group (Yg-SED). Older-EX had a higher MPS than Yg-SED (Older-EX: 0.0044±0.006; Yg-SED: 0.033±0.006 %*hr^{-1}, p=0.0043, **Figure 4B**).

The average DNA %F (**Figure 5A**) over six weeks of endurance exercise was not different between PRO and CHO groups. Combining the two groups resulted in an average DNA %F of 4.2±2.8% (95% CI: 2.7-5.8% newly synthesized DNA). The change in DNA enrichment per week was lower in the young-sedentary group (Yg-SED) than the older-exercise group (Older-EX, **Figure 5B**). The final enrichment of the Yg-SED was similar to background samples and resulted in DNA %F that was not different than zero (p>0.05). DNA %F was correlated with MPS (Pearson r^2=0.25, p=0.054) and the slope of the linear regression line (**Figure 5C**) was different than zero (m=1.1±0.04, p=0.056).

The average GLY %F over six weeks of endurance exercise was not different between PRO and CHO groups (PRO=37.6±11.9 vs. CHO=42.2±11.2 % new in six weeks, **Figure 6A**). Combining the CHO and PRO groups into a single Older-EX group resulted in higher average GLY %F than the Yg-SED (Older-EX: 7.13±2.1, Yg-SED: 1.36±0.55 %*week^{-1}, $p<0.0001$, panel B). GLY %F was not correlated to MPS or DNA %F (data not shown).

mtDNA content

The ratio of mtDNA to nDNA was not different between groups at baseline or following six weeks of endurance exercise (**Figure 7**). The internal controls were consistent across different plates (C_t±SD [CV] for B2M=24.2±0.41 [0.017%]; TAF1-L=23.8±0.42 [0.018%]; Dloop=16.0±0.46 [0.029%]; mtRNR=16.4±0.46 [0.028%]).

Discussion

We used labeled water to simultaneously measure the synthesis rates of multiple processes in response to six weeks of endurance exercise training with either carbohydrate or carbohydrate plus protein consumption after exercise. Labeled water provides a method for measuring long-term synthesis processes that were previously limited to acute time frames. Our results showed that maximal aerobic fitness increased in the protein but not carbohydrate group. Counter to our hypothesis, the synthesis rates of mixed muscle proteins, DNA, and membrane phospholipid bound glycerol were not different between protein and carbohydrate groups during the six week endurance training protocol. We anticipated that the DNA synthesis rate would be near zero since skeletal muscle fibers do not undergo regular cell divisions unless stimulated by a strong anabolic stimulus such as growth during childhood or resistance training. However, we measured ~4% newly synthesized DNA in *adults* during *aerobic* training. We suggest that the DNA synthesis is due to satellite cell division. These results add to a growing body of evidence that shows aerobic exercise can stimulate skeletal muscle synthetic processes, which former paradigms had limited to occurring in response to resistance exercise.

DNA synthesis rate

We measured the synthesis rate of DNA in adult human skeletal muscle to be ~4% over six weeks. The value is intriguing because skeletal muscle is a post-mitotic tissue and therefore does not undergo regular cycles of DNA replication and cell division of myofibrils. The D_2O technique labels DNA through *de novo* synthesis pathways, thus the synthesis rate does not include DNA repair or base salvage pathways (Neese *et al.*,

2002). We consider three sources could contribute to the measured DNA synthesis: 1) mitochondria 2) cells that are continuously replicating in muscle tissue or 3) satellite cell recruitment.

We determined that the DNA synthesis could not be accounted for by mtDNA. First, the lack of mtDNA:nDNA change with training suggests that even if mtDNA changed nDNA would have changed in the same direction with equal magnitude. Second, deuterium incorporation into DNA happens at the deoxyribose moiety of deoxyribonucleotides during *de novo* synthesis (Busch *et al.*, 2007), therefore the absolute number of base pairs is relevant to the source of DNA turnover. The small size of mtDNA (~16.5 kb) and mtDNA copy number (~2500 per nuclear diploid genome) is less than 0.01% of total DNA (~3 Gb). Therefore, even a fully turned over mtDNA pool could not account for the ~4% newly synthesized DNA reported in our study.

Second, it is not likely that cells other than myofibrils are contributing to our measured DNA synthesis rate. The muscle biopsy is a heterogeneous sample of cell types in skeletal muscle tissue including fibroblasts, macrophages, neurons, and vascular cells. A cell type, or collection of multiple types, that contributes ~4% of total DNA could be fully turned over and could theoretically account for the newly synthesized DNA over six weeks. However, muscle biopsy samples predominately contain contractile cells that are multinucleated, therefore the majority of DNA content is from myofibrils. We recruited the SED group to determine the rate of DNA synthesis under sedentary and free living conditions to account for basal turnover of other cell types. The SED group had deuterium enrichments that were not different from background following four weeks of labeling and indicates the cells represented in the biopsy samples

have basal DNA synthesis rates near zero. Thus, the contribution of cells other than myofibrils is small and does not adequately account for our measured DNA synthesis rate during aerobic training.

The third potential source of DNA synthesis is satellite cell replication. Satellite cells are resident stem cells within skeletal muscle that are usually quiescent but can be activated to divide into two daughter cells. A portion of activated satellite cells is used to maintain the population of satellite cells, and the others fuse to existing myofibrils or form nascent myofibrils (Schultz, 1996; Kuang *et al.*, 2007). Early work showed isotopic labeling of DNA in skeletal muscle could be attributed to satellite cell replication (Moss & Leblond, 1971). Additionally, about 80% of satellite cells could be labeled within five days in growing rats (Schultz, 1996). Satellite cells make up ~2-5% of total nuclei in muscle samples from adult humans (Hawke & Garry, 2001; Thornell *et al.*, 2003), therefore it is possible that replication of satellite cells could account for ~4% newly synthesized DNA.

Satellite cells can be activated by resistance exercise and are thought to promote skeletal muscle adaptations including repair and hypertrophy (Crameri *et al.*, 2004; Olsen *et al.*, 2006; O'Reilly *et al.*, 2008). Aerobic exercise is not known to stimulate muscle hypertrophy, thus few studies have considered satellite cell activation with aerobic exercise. However, a recent report in humans showed a single bout of aerobic exercise increased markers of satellite cell activation (Roberts *et al.*, 2010), possibly to promote muscle repair or remodeling. If satellite cells are activated and differentiated, then an increase in muscle protein synthesis would be stimulated to support the new cell. Accordingly, we show a positive correlation (~25%) between MPS and DNA %F over

the six weeks of aerobic training. The coordinated synthesis of DNA and proteins in skeletal muscle can help maintain the posited myonuclear domain, which is shown to be maintained with aging (Cristea *et al.*, 2010). A previous report showed that DNA and protein synthesis are not correlated and may be regulated independently (Zhang *et al.*, 2004), however the study assessed skin cells over several hours. We measured skeletal muscle over several weeks of endurance training and the correlation between DNA and protein synthesis suggests satellite activation and tissue remodeling. The lower correlation may be due to MPS being an average value for all proteins in the muscle biopsy sample and is not specific to satellite cells or newly synthesized myofibrils. Recent advances in separation techniques are used to determine the synthesis rate of individual proteins (Jaleel *et al.*, 2008) and could be useful for future studies that examine satellite cell specific proteins.

Macronutrient consumption may influence satellite cell activation with exercise. Initial human studies indicate that the effects of nutrition on satellite cell activation develop over repeated exercise sessions and are not measurable after a single bout. For example, a recent study evaluating the several hours after aerobic exercise showed markers of satellite cell activation were raised similarly between groups who consumed protein, carbohydrates, or placebo before exercise (Roberts *et al.*, 2010). Differences in satellite cell number in response to macronutrient consumption were shown following twelve weeks of resistance training (Olsen *et al.*, 2006). People who consumed protein or creatine supplementation compared to carbohydrates had increased numbers of satellite cells and myonuclei (Olsen *et al.*, 2006). These gains were shown at four and six weeks of training and the greatest strength gains at twelve weeks were in the creatine

supplemented group. It should be noted that short-term activation of satellite cells is identified with qPCR of mRNA transcripts (Roberts *et al.*, 2010), while long-term markers of satellite cell content are identified using immunohistochemistry (Crameri *et al.*, 2004; Collins *et al.*, 2005; Olsen *et al.*, 2006). Future studies can investigate the synthesis rate and expression of specific proteins related to satellite cell activation to determine whether macronutrient intake can influence the translation of proteins that regulate satellite cell activation. Aging may impair the activation of satellite cells to myogenic stimuli (Beccafico *et al.*, 2010), therefore the ability for aging populations to regulate satellite cell activation in response to feeding and exercise should be considered.

Mixed muscle protein synthesis

Average MPS was not different between protein or carbohydrates groups and appears to contrast short-term studies. Post-exercise protein consumption has been repeatedly shown to increase muscle protein synthesis in the several hours following aerobic (Levenhagen *et al.*, 2001; Pennings *et al.*, 2011) and resistance exercise (Koopman *et al.*, 2005; Moore *et al.*, 2009). Additionally, the importance of timed protein consumption after exercise is supported by studies over several days and weeks that reported increased nitrogen retention (Jordan *et al.*, 2010) and strength gains (Esmarck *et al.*, 2001). Our results suggest such changes are not due to long-term changes in protein synthesis. Instead, they are consistent with results showing that the timing of protein intake can vary around exercise and still allow increased amino acid uptake over several hours (Tipton *et al.*, 2001; Tipton *et al.*, 2007) or muscle cross sectional area with training (Verdijk *et al.*, 2009).

There are important considerations between our results and previous findings. First, acute tracer studies are conducted in in-patient settings and commonly have strict dietary controls whereas our long-term measures are made on free-living people with fluctuations energy balance. It is known that negative energy balance leads to negative protein balance (Todd *et al.*, 1984) and it is likely that our participants went through cycles of positive and negative protein balance. Second, we used labeled water to directly measure skeletal muscle protein synthesis. Other studies have used non-specific methods that measure net changes such as nitrogen balance (Jordan *et al.*, 2010) or muscle cross sectional area (Esmarck *et al.*, 2001). Our results do not contradict these cumulative measurements but suggest that any short-term increases in protein synthesis following exercise and protein consumption may not increase the cumulative protein synthesis over several weeks compared to carbohydrates only. Perhaps the timing of protein intake is less critical in people who are consuming adequate calories and protein outside of a tightly controlled in-patient setting.

MPS was higher in our older exercise group compared to the younger sedentary group and provides support that aerobic training increases protein synthesis, despite the participants being older. Resistance exercise has been used to study skeletal muscle protein synthesis across age groups (Kumar *et al.*, 2009), however aerobic exercise is a common exercise among aging populations. Here we show that skeletal muscle protein synthesis is increased during a lifestyle intervention that is feasible for aging populations. Previously we have shown that consuming protein following aerobic exercise stimulates positive nitrogen balance in people who are in energy balance (Jordan *et al.*, 2010), however the same is not observed during negative energy balance (Heusinger, Minor,

Melanson, and Miller unpublished results). Importantly, our current measurements represent an increase in average protein synthesis in free-living conditions where energy balance may vary on a day-to-day basis.

Our long-term results support short-term studies showing that aerobic exercise can stimulate skeletal muscle protein synthesis throughout a variety of ages (Durham *et al.*, 2010). Others have reported decreased protein synthesis rates with aging measured over several hours under basal conditions (Rooyackers *et al.*, 1996; Henderson *et al.*, 2009). These are in contrast to studies showing no differences in basal protein synthesis with aging (Volpi *et al.*, 2001; Cuthbertson *et al.*, 2004) and could be attributed to slight differences in conditions (e.g. health status, dietary control) or lifestyle habits between groups (e.g. diet, exercise). The younger untrained group represents a maximal rate of long-term basal synthesis because previous reports show either similar (Volpi *et al.*, 2001) or lower skeletal muscle protein synthesis with aging (Henderson *et al.*, 2009). The average resting skeletal muscle protein synthesis we calculated over six weeks (~0.03%*hr^{-1}) was similar to acute studies under resting conditions [~0.03-0.06 %*hr^{-1} (Volpi *et al.*, 2001; Henderson *et al.*, 2009; Smith *et al.*, 2009)]. Therefore, the increase during aerobic training over resting may be greater if compared to an older non-exercising group. Aerobic exercise combined and maintenance of energy balance seem to increase long-term protein synthesis and may attenuate the gradual loss of muscle mass with age.

Membrane phospholipid glycerol

The synthesis of membrane phospholipid glycerol was not different between protein or carbohydrate groups and was not correlated to synthesis of skeletal muscle

DNA or proteins. Interestingly, the synthesis of membrane phospholipid glycerol was greater in the aerobic training group compared to sedentary controls and suggests remodeling within myofibrils. Membrane phospholipid glycerol synthesis represents the formation of new cell membranes because the incorporation of deuterium into glycerol within membrane bound phospholipids occurs only through *de novo* synthesis pathways. By area, cellular organelles contain most of the phospholipid content and likely represent the majority of membrane remodeling. In particular, the mitochondrial reticulum is a large portion (20-40%) of intracellular membranes with continuous remodeling (Liu *et al.*, 2009) and separation of the mitochondrial fraction could facilitate investigation of long-term adaptive changes. Deuterium labeling combined with sub-fractional analysis can provide unique insight into long-term adaptive changes to cellular membranes.

Mitochondrial adaptations

Mitochondrial DNA (mtDNA) copy number per nuclear diploid genome (nDNA) is an index of mitochondrial content (Menshikova *et al.*, 2007). We did not detect any differences in mtDNA:nDNA between groups at baseline or following training. Interestingly, the increase in $VO_{2\ max}$ in the protein group suggests that consuming protein post-exercise during endurance training can promote long-term aerobic adaptations independent of changes in mtDNA:nDNA. We do not expect that absolute nDNA content changed with endurance training; therefore it is likely that the aerobic adaptations occurred independently of changes in mitochondrial content. Our results are in contrast to Menshikova *et al*. who showed an increase in mtDNA:nDNA following 12-weeks of aerobic training in older humans (Menshikova *et al.*, 2006). It is possible that our six weeks of training (three sessions per week) compared to twelve weeks (four to six

sessions per week) was not sufficient to increase mtDNA copy number. We did not observe a decline in mtDNA content with age as others have (Short *et al.*, 2005), however our study was not designed or powered to detect age-related changes in mtDNA content. Others have reported that protein consumption following exercise can lead to greater strength gains during resistance training (Esmarck *et al.*, 2001). We chose to study aerobic exercise in aging adults since it is a common lifestyle recommendation to promote healthy aging and less is known about whether nutrition can modify aerobic adaptations. Each participant in the protein group increased both absolute and relative $VO_{2\ max}$ by ~10%, which is similar to other endurance training studies, while the carbohydrate group did not, contrasting previous studies without nutritional intervention (Menshikova *et al.*, 2006). Our results support that post-exercise nutrition can promote endurance exercise adaptations.

Limitations

We did not include an age-matched group who did not undergo training, thus we were not able to directly compare the effects of exercise on our long-term synthetic measurements. However, we do not expect protein synthesis or DNA synthesis to be increased with aging under sedentary conditions. On the contrary, previous reports showed depletion of satellite cells (Renault *et al.*, 2002) and decreased protein synthesis with age (Henderson *et al.*, 2009). Thus, our use of a younger sedentary group provides greater evidence that exercise increases DNA synthesis rates even in an aging population.

We chose treadmill training because brisk walking is a common exercise recommendation and we intended to study people using a simple lifestyle modification for aging (e.g. exercise followed by protein consumption). It should be noted that our

training protocol used treadmills while the $VO_{2\ max}$ used cycle ergometry. Cycling ergometry tends to yield lower $VO_{2\ max}$ values and perhaps our differences between pre and post testing would be greater if the same modality was used.

Conclusion

We conclude that long-term measurements of synthetic processes within skeletal muscle provide important insights into aging, exercise and nutrition that are missed in acute studies. Our results show that post-exercise protein consumption does not alter long-term measures of multiple synthetic processes but can improve maximal aerobic performance. The measured DNA synthesis (~4% over six weeks) could be due to satellite cell activation during aerobic exercise training. Further studies should consider the implications of aerobic exercise on satellite cell recruitment and skeletal muscle function with aging.

Author contributions

MMR assisted with study design, data collection, analysis and manuscript preparation. MKH and ST assisted with tissue analysis for deuterium labeling and manuscript preparation. KLH and BFM assisted with study design, funding, analysis and manuscript preparation.

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Tables

Table 1: Subjects were matched for sex, age, body mass index (BMI), and $VO_{2\ max}$ and then randomized to consume either CHO or PRO drinks following each exercise session. A third group did not undergo any training or dietary intervention and provided a younger and sedentary comparison group (Yg-SED).

Table 2: Sequence information of PCR gene targets. Primers and probes for MT-Dloop and B2M were custom designed and are reported. MT-RNR2 and TAF-1L were purchased as proprietary sequences from Applied Biosystems.

Table 1

	CHO (5F, 3M)	*PRO (5F, 3M)*	*Yg-SED (3F, 1M)*
Age (yr)	52±10	48±7	21±2
Height (m)	1.67±0.11	1.68±0.14	1.6±0.1
Weight (kg)	72.2±24.2	71.0±21.0	58.5±11.4
BMI ($kg*m^{-2}$)	25.3±4.8	24.7±3.3	22.6±1.9
Fat (%)	29.7±5.6	30.7±8.2	24.6±5.7
$VO_{2\ max}$ ($ml*kg^{-1}*min^{-1}$)	31.1±7.1	25.5±4.2	38.4±3.5
$VO_{2\ max}$ ($l*min^{-1}$)	2.2±0.77	1.83±0.62	2.25±0.5

Table 2

Name	*Gene*	*Chr*	*Forward*	*Reverse*	*Probe*
Displacement Loop	MT-Dloop	mt	AGCACATTACAGTCAAATCCCTTCTC	CACGGAGGATGGTGGTCAAG	CCCCATGGATGACCCC
β-2 Microglobulin	B2M	15	GTGCCTGATATAGCTTGACACCAA	TCGGGAAAAGACACATTAATATTGCCA	CCCCAAGTGAAATACC
Mitochondrial encoded 16S RNA	MT-RNR2	mt		Proprietary Sequence Assay ID: Hs02596860_s1	
TATA box binding protein	TAF-1L	X		Proprietary Sequence Assay ID: Hs00542346_s1	

Figures

Figure 1: Sixteen participants completed six weeks of progressive aerobic training while consuming either carbohydrates or carbohydrates with twenty grams of protein following each exercise session. Deuterium oxide (D_2O) was consumed three times daily (tid) or two times daily (bid) to isotopically label newly synthesized products obtained in muscle biopsy samples. Body composition and maximal aerobic fitness ($VO_{2\ max}$) were determined before and after training. Saliva and blood samples were used to determine steady state D_2O content in body water and precursor enrichments for synthesis rate calculations.

Figure 2: Absolute $VO_{2\ max}$ increased in PRO but not CHO following six weeks of aerobic training. Box plots indicate the mean, interquartile range, and sample range of the percentage increase from baseline.

Figure 3: Body water D_2O enrichment measured from saliva swabs was raised to 1.5-2.5% during the six week labeling period.

Figure 4: There were no differences in mixed muscle protein synthesis rates (MPS, panel A) between CHO or PRO nutrition with aerobic exercise training. The younger sedentary group (Yg-SED) had lower MPS than the older exercise group (Older-EX, panel B). *$p<0.005$

Figure 5: There were no differences in DNA synthesis rate (DNA %F, panel A) between CHO or PRO nutrition with aerobic training. The deuterium enrichment of DNA (EM1*) was lower in the younger-sedentary group (Yg-SED) than in the older-exercise group (Older-EX, panel B). The low enrichment of Yg-SED resulted in a DNA synthesis rate that was not different than zero. The data are expressed as enrichment % per week because the Yg-SED group was labeled for four weeks and the Older-EX group for six weeks. MPS and DNA %F were correlated over six weeks of aerobic training (panel C). *p=0.027

Figure 6: The synthesis rate of membrane phospholipid bound glycerol (GLY %F), an indication of membrane turnover, was not different between CHO or PRO nutrition groups (Panel A). GLY% F was lower in the Yg-SED than Older-EX (Panel B). *p<0.0001

Figure 7: There were no differences at baseline or following six weeks of aerobic training for relative copy (RC) number of mtDNA:nDNA using primers/probes for Dloop and B2M.

Figure 1

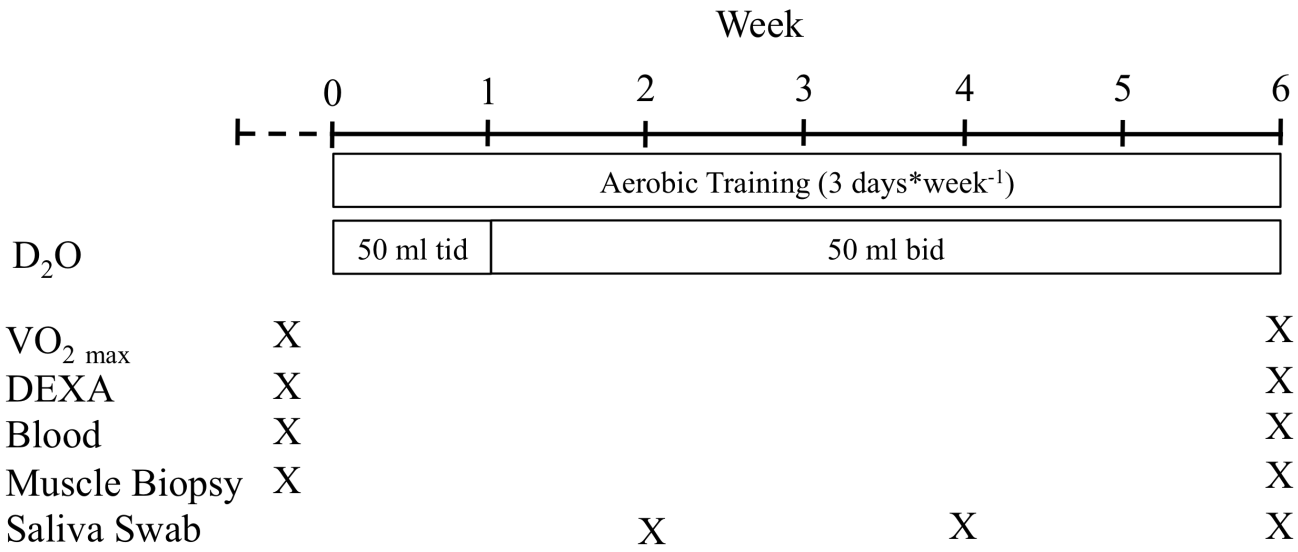

Week
0
1
2
3
4
5
6
Aerobic Training (3 days*week-1)
D2O
50 ml tid
50 ml bid
VO2 max
DEXA
Blood
Muscle Biopsy
Saliva Swab
X

Figure 2

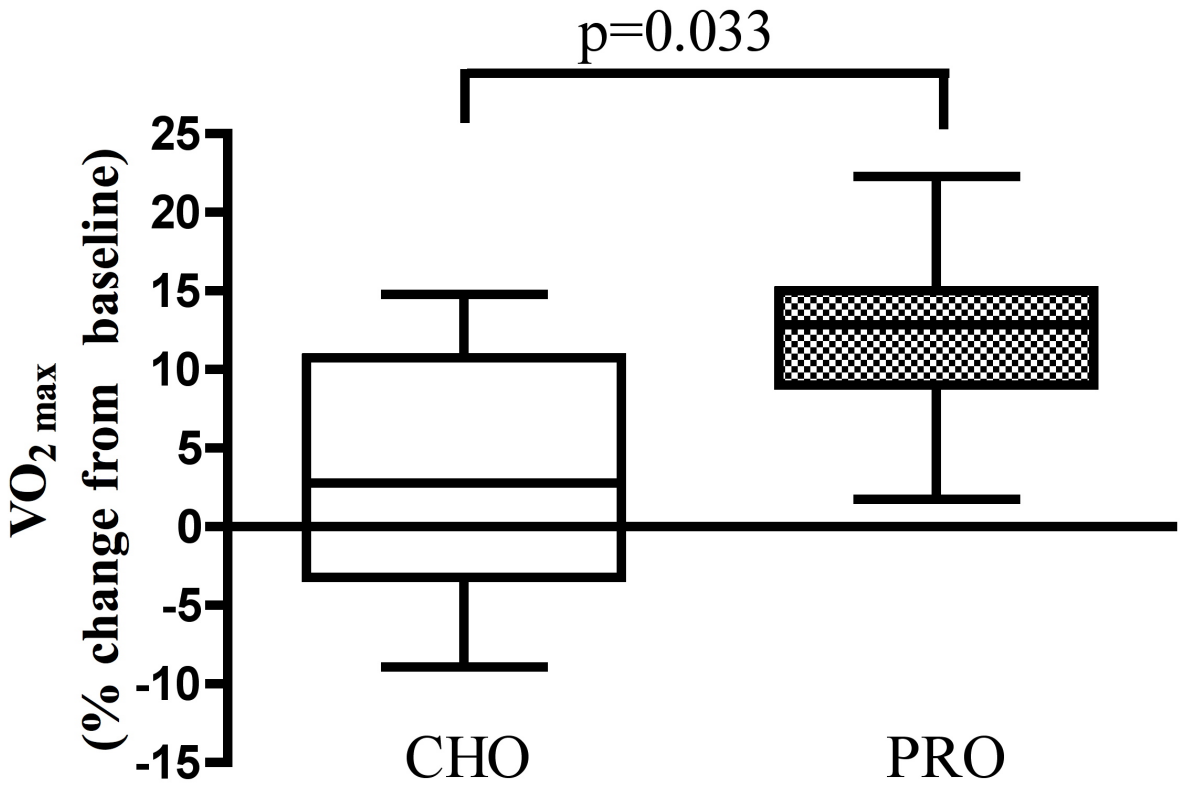

p=0.033
25
20
15
10
5
0
-5
-10
-15
$VO_{2\ max}$
(% change from baseline)
CHO
PRO

Figure 3

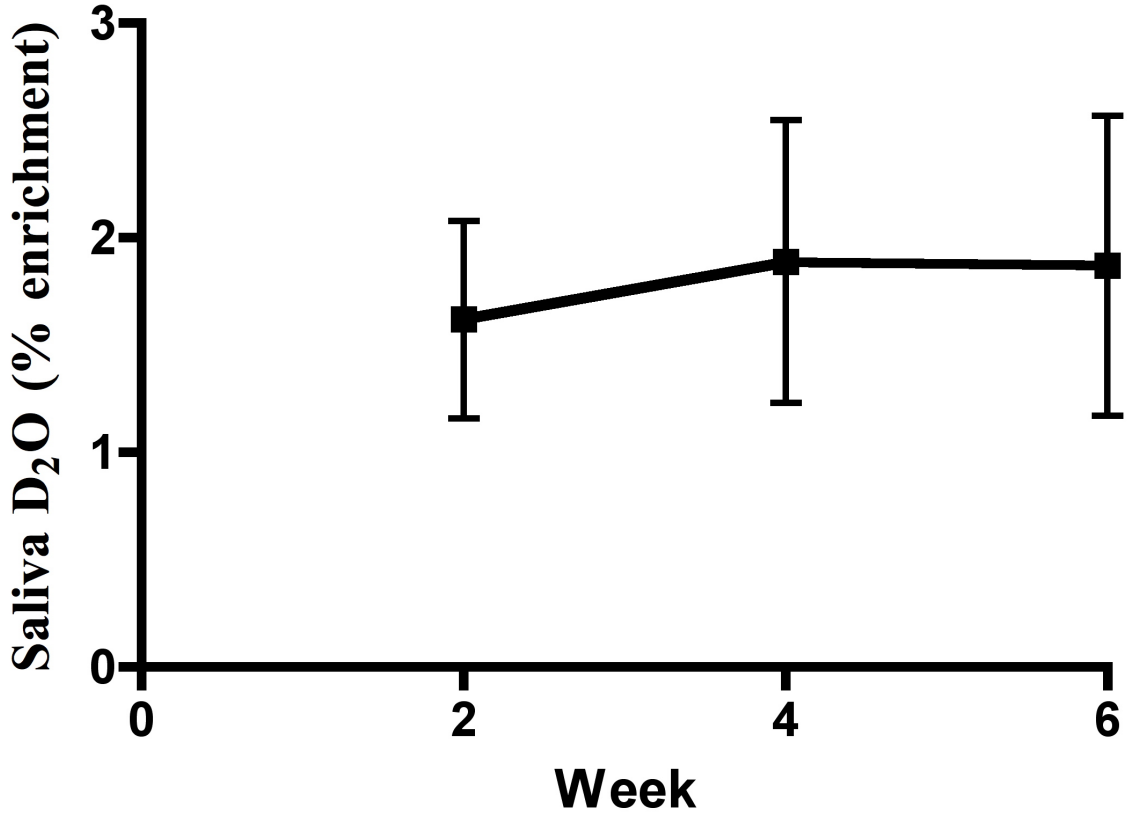
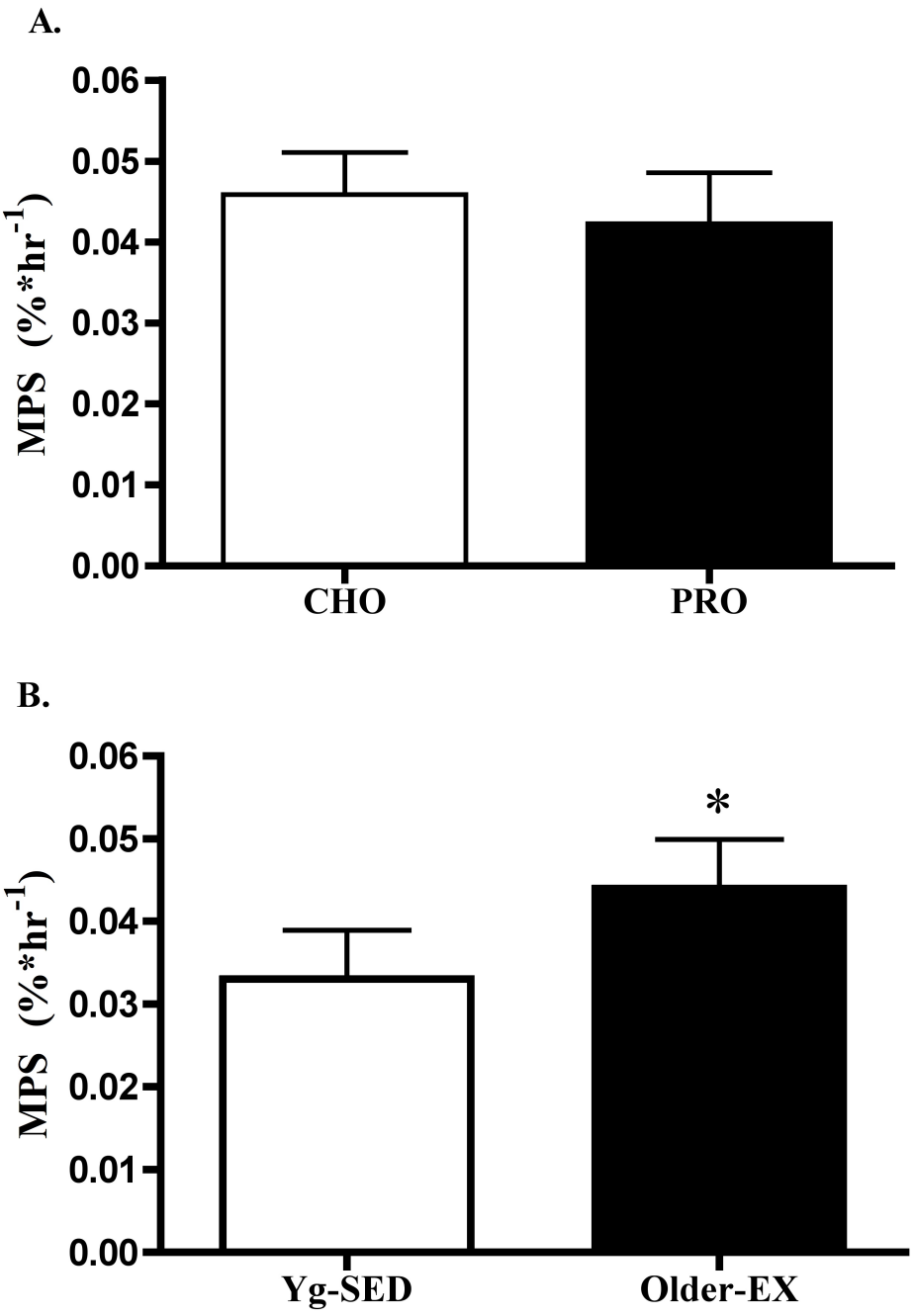


Figure 4


A.
0.06
0.05
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0.03
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0.01
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MPS (%*hr-1)
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0.06
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MPS (%*hr-1)
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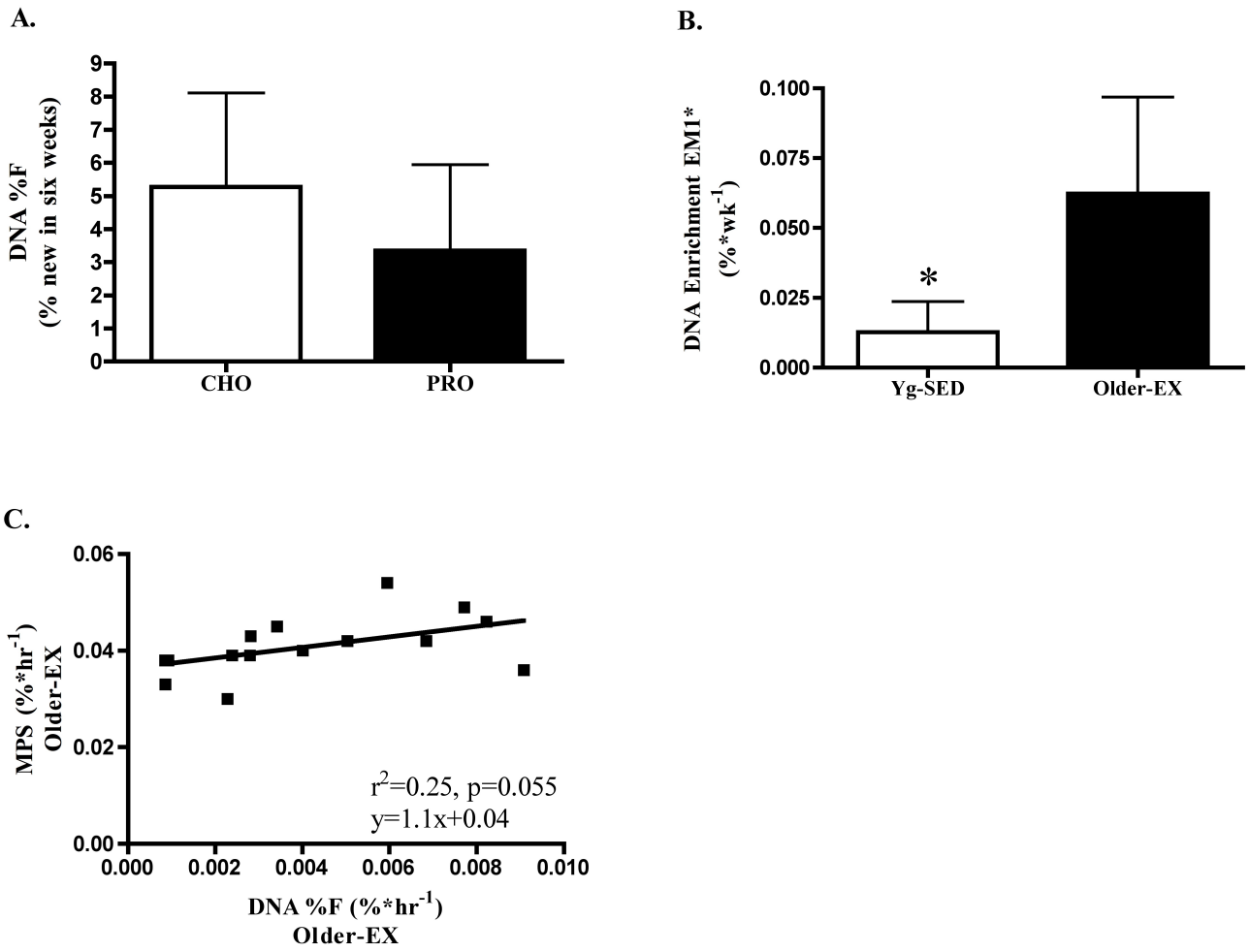
Yg-SED
Older-EX

Figure 5


A.
DNA %F
(% new in six weeks)
9
8
7
6
5
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3
2
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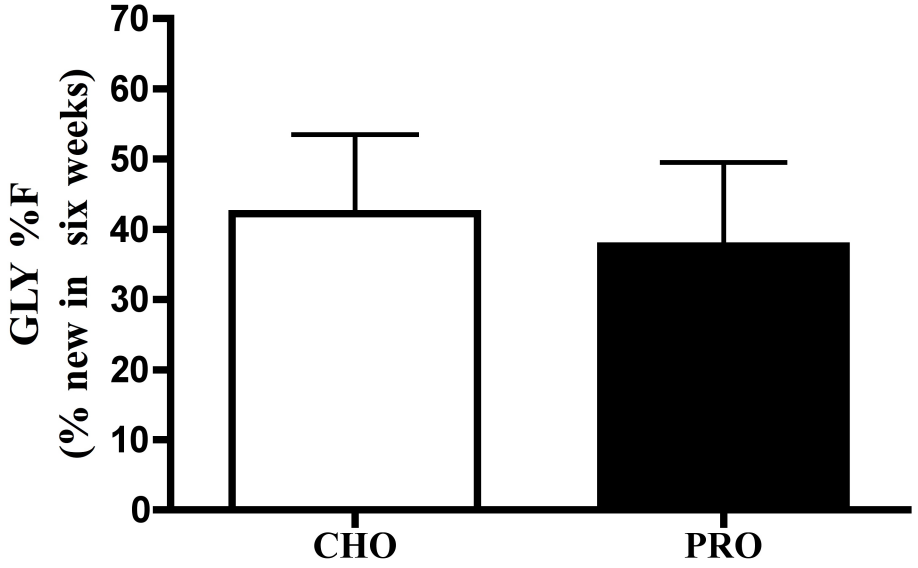
CHO
PRO
B.
DNA Enrichment EM1*
(%*wk-1)
0.100
0.075
0.050
0.025
0.000
*
Yg-SED
Older-EX
C.
MPS (%*hr-1)
Older-EX
0.06
0.04
0.02
0.00
r2=0.25, p=0.055
y=1.1x+0.04
0.000
0.002
0.004
0.006
0.008
0.010
DNA %F (%*hr-1)
Older-EX

Figure 6

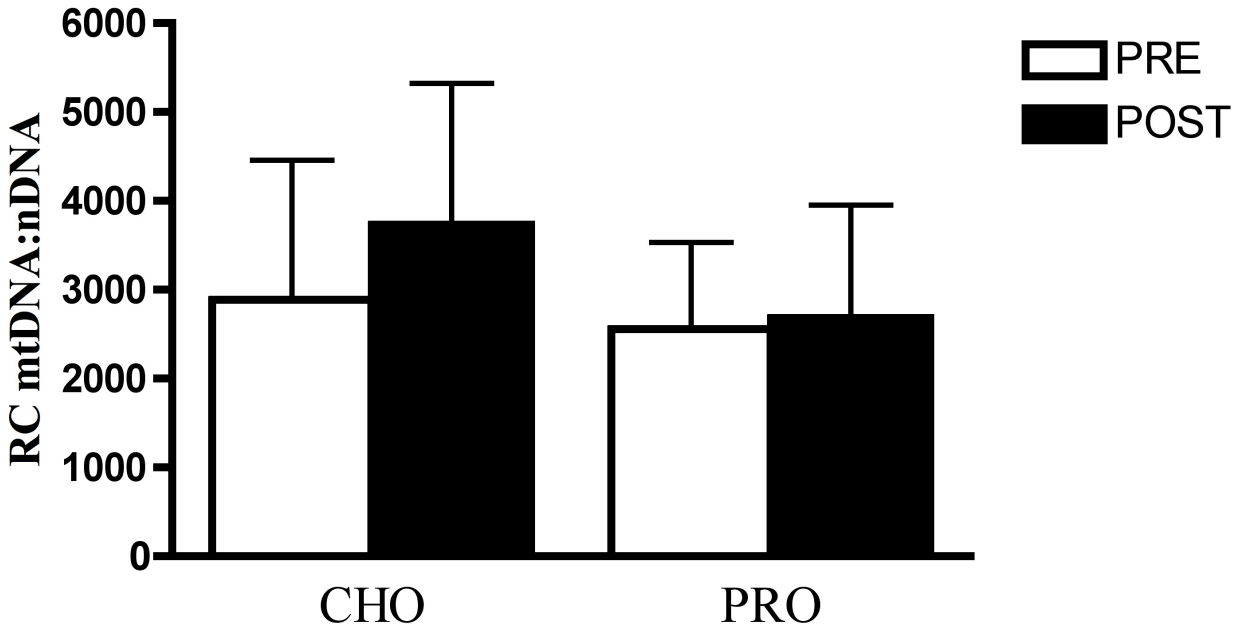
A.


GLY %F
(% new in six weeks)
70
60
50
40
30
20
10
0
CHO
PRO

B.


GLY %F
(% new per week)
10.0
7.5
5.0
2.5
0.0
*
Yg-SED
Older-EX

Figure 7



CHAPTER VI - OVERALL CONCLUSIONS

The loss of skeletal muscle mass and function with age is due to an imbalance between skeletal muscle protein synthesis and degradation pathways, yet the contributing mechanisms are not fully understood. Decreased mitochondrial protein synthesis resulting in decreased mitochondrial function occurs during aging and appears to contribute to the progression of chronic disease. Current evidence suggests that aging leads to a resistance to anabolic stimuli, such as exercise and protein consumption, and contributes to a net loss of skeletal muscle protein over time. Aging populations commonly consume a variety of drugs in combination with exercise and dietary recommendations, however specific drug classes can negatively affect skeletal muscle protein turnover during recovery from exercise. In particular, β-adrenergic antagonists are routinely prescribed drugs for cardiovascular disease, yet β-adrenergic signaling appears to regulate skeletal muscle protein turnover and mitochondrial adaptations to exercise. It is necessary to understand the regulation of skeletal muscle protein turnover following exercise and drug consumption to avoid potential negative interactions with skeletal muscle adaptations.

We evaluated skeletal muscle protein synthesis and mitochondrial biogenesis following β-adrenergic stimulation at rest and β-adrenergic blockade during exercise. Our results indicate that non-selective β-adrenergic stimulation does not increase skeletal

muscle protein synthesis or mitochondrial biogenesis under resting conditions. However, non-selective β-adrenergic blockade during exercise impaired post-exercise mitochondrial protein synthesis and suggests skeletal muscle adaptations are regulated in part through β-adrenergic signaling. Furthermore, we performed a training study to evaluate if post-exercise protein consumption could increase protein synthesis over several weeks. Contrary to short-term studies, consuming protein after exercise did not increase protein synthesis over the long-term. Additionally, we measured DNA synthesis during aerobic training. We attribute the DNA synthesis to satellite cell activation, which previous exercise paradigms have limited to occur during resistance exercise training. The activation of satellite cells could promote skeletal muscle remodeling by replacing older skeletal muscle cells will newly synthesized cells.

The potential regulation of mitochondrial protein turnover through β-adrenergic pathways has important implications for therapeutic recommendations for aging populations. β-adrenergic receptor blockers are routinely used to treat cardiovascular disease with aging, yet drugs that modulate β_2-adrenergic receptor signaling can regulate skeletal muscle protein turnover. Our results showed no stimulation of skeletal muscle protein synthesis or mitochondrial biogenesis in the several hours following non-selective β-adrenergic stimulation. It is possible that greater or repeated β_2-adrenergic stimulation is required to induce skeletal muscle adaptations, such as during exercise training. Our infusion of a non-selective β-adrenergic antagonist during a bout of cycling blunted the post-exercise increase in skeletal muscle mitochondrial protein synthesis. Our results indicate that combined treatment of a non-selective β-adrenergic blocker with aerobic exercise may impair skeletal muscle adaptations with training.

We demonstrated that consuming protein with carbohydrates, compared to carbohydrates only, following exercise does not increase skeletal muscle protein or DNA synthesis rates during aerobic training. Short-term studies have shown protein consumption following exercise stimulates skeletal muscle protein synthesis and support protein consumption and exercise as a lifestyle treatment for sarcopenia. Our results indicate that such short-term increases do not translate into long-term differences over several weeks of training. Deuterium labeling allows a direct measurement of skeletal muscle synthesis rates over several weeks and allows simultaneous measurements of adaptive processes in skeletal muscle.

The use of short and long-term kinetic measures can provide insight into exercise and β-adrenergic signaling on skeletal muscle protein turnover. Our investigations showed that decreasing β-adrenergic signaling during exercise blunted skeletal muscle protein synthesis in the several hours following aerobic exercise. We presented a method for measuring long-term synthesis rates and showed that increased protein synthesis during short-term studies does not extend to long-term changes in protein synthesis. Future investigations could use deuterium labeling to determine whether short-term decreases in skeletal muscle protein synthesis with β-adrenergic antagonists, or other drug classes, persist over long-term in aging humans. A further understanding of the ability for commonly consumed drugs to impair skeletal muscle adaptations to exercise could improve future lifestyle recommendations.

APPENDIX-MANUSCRIPT V

Measurement of skeletal muscle collagen breakdown by microdialysis

Benjamin F Miller[1], Darren Ellis[2], Matthew M Robinson[1], Jeffery D Rivera[1], Michael Kjaer[3], and Henning Langberg[3]

[1]Department of Health and Exercise Science, Colorado State University, 200 Moby B Complex, Fort Collins, CO 80523-1582
[2]Department of Sport and Exercise Science, University of Auckland, Auckland, NZ.
[3]Institute of Sports Medicine, Copenhagen, Bispebjerg Hospital, 2400 Copenhagen NV, Denmark

Running title: Collagen breakdown after exercise

Correspondence to:

BF Miller
Department of Health and Exercise Science
Colorado State University
Fort Collins, CO 80523-1582

Fax: 970-217-3291
Office: 970-217-0445
Email: bfmiller@cahs.colostate.edu

Key Words: Skeletal muscle, microdialysis, collagen

Abstract

Exercise increases the synthesis of collagen in the extracellular matrix (ECM) of skeletal muscle. Breakdown of skeletal muscle collagen has not yet been determined because of technical limitations. The purpose of the present study was to use local sampling to determine skeletal muscle collagen breakdown. Microdialysis fibers were tested *in vitro* to predict bath hydroxyproline (OHP) concentrations. We used a *N*-Methyl-*N*-[tert-butyldimethyl-silyl]trifluoroacetimide (MTBSTFA) derivative to analyze OHP by gas chromatography-mass spectroscopy (GC-MS) and compared the results to a colorimetric OHP assay. Ten young, healthy male subjects performed a bout of resistance exercise with one leg followed 17-21 hours later by *in vivo* skeletal muscle sampling by microdialysis in exercised (EX) and control (CON) legs. Microdialysis reliably predicted [OHP] *in vitro* ($R^2 = 0.90$). Analysis with GC-MS was strongly correlated to traditional analysis methods (CON: slope = 1.03, $R^2 = 0.896$, $p < 0.05$, EX: slope = 0.795, $R^2 = 0.896$, $p < 0.05$). We conclude that *in vitro*, microdialysis fibers were able to measure OHP concentrations and were sensitive to changes in concentrations, a strenuous bout of exercise did not increase skeletal muscle collagen breakdown 17-21 hours post-exercise, and our measurement of OHP by GC-MS was in agreement with traditional assays.

Introduction

Skeletal muscle is plastic and changes in response to exercise, feeding, inactivity and aging. In general, *in vivo* studies of skeletal muscle protein turnover have focused on the turnover (synthesis and breakdown) of mixed muscle samples, thus disregarding the turnover of individual protein components of skeletal muscle. Approximately 10% of skeletal muscle protein is collagen (Kjaer, 2004), the main protein component of the extracellular matrix (ECM). The body of research into collagen metabolism is not as common as that into skeletal muscle despite the fact that collagen is relatively abundant in skeletal muscle. In the past, it was assumed that collagen did not adapt to mechanical load or chemical stimuli (Neuberger & Slack, 1953). Evidence is now available that skeletal muscle ECM is metabolically active and as responsive as contractile protein to mechanical stimuli (Kjaer, 2004).

Since the ECM is the support structure that translates muscle fiber contraction into movement, it makes intuitive sense that remodeling of contractile units (muscle fibers) would be accompanied by remodeling of ECM. It is now apparent that collagen protein synthesis increases in skeletal muscle after an acute bout of exercise (Miller *et al.*, 2005; Mittendorfer *et al.*, 2005; Moore *et al.*, 2005; Miller *et al.*, 2006) and the time course of change mirrors that of myofibrillar and sarcoplasmic proteins (Miller *et al.*, 2005). More specifically, skeletal muscle collagen synthesis is increased six hours after exercise, peaks at 24 hours, and is still elevated 48 hours after an acute bout of exercise (Miller *et al.*, 2005). Less clear is how collagen protein breakdown, the other equally important component of collagen turnover, responds to exercise or other physiological

manipulations. Lack of information about skeletal muscle collagen breakdown mostly stems from the technical difficulty of its measurement.

The synthesis of collagen occurs in fibroblasts, which then export the molecule into its surrounding matrix. Several post-translation modifications occur after transcription and translation of the collagen gene. One of the first post-translational modifications is the hydroxylation of proline, which helps in the arrangement of the triple-helical structure. The hydroxylation of proline to hydroxyproline (OHP) is almost unique to collagen. Further, procollagen molecules are capped with terminal extension peptides that aid in their export and are subsequently cleaved in the ECM where the collagen assembles. ECM degradation is orchestrated by a balance between the activities of matrix metalloproteases (MMP) and the tissue inhibitors of MMP (TIMP), which are in turn regulated by such factors as mechanical tension, integrin stimulation, and cytokines (Kjaer, 2004). The complex balance between the activities of these two protein families ultimately determines the amount of ECM breakdown.

Previous measurements of collagen breakdown have used whole body measures such as plasma and urine markers of breakdown. For instance, Brown et al. measured plasma and urine OHP, and serum concentrations of type I collagen in subjects after eccentric resistance exercise (Brown *et al.*, 1997; Brown *et al.*, 1999). High serum concentrations of creatine kinase were measured after eccentric exercise, an indication of muscle damage, and increased serum levels of type I collagen, indicative of collagen breakdown. A second marker of collagen breakdown is the carboxy-terminal telopeptide region of type I collagen (ICTP), a polypeptide product of MMP-induced breakdown. Experiments using long-term training found increased ICTP after multiple weeks of

training (Langberg *et al.*, 2000), but not after a single exercise bout (Salvesen *et al.*, 1994), perhaps indicating that collagen is somewhat slower to turnover compared to skeletal muscle. In all of these experiments, OHP or ICTP were measured in plasma or urine thus representing contributions from all ECM pools, with bone and skin likely being the predominant sources.

Measurement by microdialysis represents a means to measure local concentrations of solutes in a variety of tissues such as adipose tissue (Enevoldsen *et al.*, 2001), muscle tissue (Miller *et al.*, 2005), and tendon (Langberg *et al.*, 2002) enabling *in vivo* studies of metabolism and blood flow (Hickner *et al.*, 1991; Boushel *et al.*, 2000). In principle, a microdialysis fiber acts similarly to a capillary bed by equilibrating a perfused solution with interstitial fluid in its area. In the peritendinous space of the Achilles' tendon, Langberg and others measured ICTP after an acute bout of exercise (Langberg *et al.*, 1999b) and after endurance training (Langberg *et al.*, 2001). We have made similar measurements in skeletal muscle and found a large variability between subjects. In a practical sense, OHP is representative of all collagen types (not just type I as for ICTP) and using a lower molecular weight metabolite (OHP at 131 Da versus ICTP at 9000 Da) could be used in conjunction with a small molecular weight cutoff probe to increase sample purity for subsequent analysis.

Recently, Trappe et al., reported a method by which the microdialysis technique was used to measure skeletal muscle interstitial concentrations of 3-methylhistidine (3-MH) (Trappe *et al.*, 2004). 3-MH is a modified amino acid found only in contractile proteins, and is distinctive in that it cannot be reutilized for protein synthesis once it has been released during the breakdown process. Because 3-MH can not be recycled, it acts

as a natural tracer of contractile protein breakdown. Hydroxyproline has similar properties as 3-MH as it is almost unique to collagen and, once released, cannot be recycled back into collagen (i.e. is irreversibly lost). Therefore, we proposed using skeletal muscle sampling of OHP by microdialysis as a means to measure skeletal muscle collagen breakdown.

Since changes in OHP were likely to be small, we did not want our method to be constrained by the sensitivity of analysis. The standard assay for OHP, and therefore collagen content, is the assay of Woessner (Woessner, 1961). This assay is a highly sensitive (capable of detecting a range of 3.8 - 19.1 μmol/L) colorimetric assay that has not been modified in over 40 years. However, to potentially increase sensitivity, and simplify sample preparation, we created a gas chromatography – mass spectroscopic (GC-MS) method for the analysis of OHP based on an amino acid derivative with reported sensitivities of 1.2 – 1.6 μmol/L (Deng *et al.*, 2002).

The purpose of this study was to determine whether we could get an index of skeletal muscle collagen breakdown by local sampling of OHP by microdialysis and analysis by GC-MS. We approached this problem with *in vitro* and *in vivo* experiments. Our hypotheses were that 1) *in vitro*, microdialysis fibers would be sensitive to changes in OHP concentrations and dialysis fluid would accurately reflect *in vitro* concentrations, 2) *in vivo* concentrations of OHP would increase 24 hours after an acute bout of exercise indicative of an increase in skeletal muscle collagen breakdown, and 3) a GC-MS method would provide reliable and accurate analysis of OHP.

Methods

In vitro experiment

In order to first determine the efficacy of using microdialysis to sample OHP an *in vitro* experiment was conducted. Two microdialysis probes were constructed with a single plasmaphoresis hollow fiber (0.4 mm diameter, 30 mm active length, molecular mass cut-off 3000 kDa (Asahi, Japan), glued to a gas-tight nylon inlet tubing with an 100 μm stainless steel inner wire to contribute to mechanical stability. The probes were placed in a series of baths containing various known amounts of OHP suspended in lactated Ringer's solution (0, 1.3, 4.5, and 10.5 μg/ml). Perfusate, was pumped through the probes from a 1 ml syringe (BD & Co, NJ, USA), using a high-precision syringe pump (Harvard Apparatus, MA, USA) at 2 μl/min. Each concentration was sampled for four hours. The individual microdialysis fiber sampled each concentration in series and one probe failed at the last high measurement and was excluded from analysis. The analysis of OHP concentration was by the method of Woessner (see below).

In vivo experiment

Subjects

Ten healthy recreationally active young males, aged 18-35 yr (**Table 1**), were recruited for study from The University of Copenhagen subject recruitment website. Potential subjects were screened to ensure that none of the subjects had a recent history or current lower limb injuries, were not on any medication, were non-smoking, had no adverse affects to anesthetic, and were free of coagulation or blood disorders. After passing the health and medical screening process, written informed consent was obtained

as approved by the Ethical Committee of Copenhagen and the University of Auckland Human Participants Ethics Committee (Ref#2006-030).

Exercise Protocol

The protocol for the *in vivo* study is presented in **Figure 1**. Subjects were asked to refrain from exercise 24 hours prior to the resistance exercise session, except for ordinary activities of daily living, which often includes cycling for transportation in Copenhagen. The subjects reported to the laboratory in the afternoon between 1600 and 1700 for determination of eight-repetition maximum (RM). Subjects worked up to a maximal-effort set of eight repetitions over three to five progressively heavier sets on the leg extension machine, using their dominant leg only. After the 8 RM was established, ten sets of eight repetitions were performed at that weight. The subjects aimed to complete eight reps on every set, but some subjects could perform only six or seven reps during the final two to three sets. If subjects were unable to complete at least six reps for two successive sets, the weight was lowered slightly for the remaining sets in order to keep work volume as consistent as possible between subjects. Failure to complete all repetitions was not a confounding variable of this study as the goal of the exercise was simply to provide a significant resistance-training stimulus. The rest period between sets was 90 sec and verbal encouragement was given. Following the exercise protocol, subjects were free to return home where they consumed a normal evening meal, and were instructed to return to the laboratory the next morning overnight fasted for the collection of samples by microdialysis.

Microdialysis Sample Collection

Subjects were instructed to arrive at either 0800 or 0900, depending on the time of their evening exercise to ensure sampling for all subjects was conducted 17-21 hours after exercise, corresponding to peak skeletal muscle collagen synthesis rates (Miller *et al.*, 2005). The area of the vastus lateralis of both legs was then shaved and 2.5 ml of local anesthetic (1% Lidocaine, no epinephrine). AstraZeneca, A/S, Albertslund, Denmark) injected subcutaneously and intramuscularly at the intended entry and exit points of the microdialysis guide cannula. After insertion of microdialysis catheters, subjects lay supine for four hours, during which sampling was conducted (**Figure 1**).

Microdialysis was performed according to the methods described by Lonroth et al (Lonroth *et al.*, 1987). Two sterilized (ethylene oxide sterilization, Maersk Medical A/S, Lynge, Denmark) custom made microdialysis catheters (the same as described for the *in vitro* experiment) were inserted into the distal area of vastus lateralis of each leg from the lateral side using a guide cannula, following muscle fiber orientation. The cannulae were then withdrawn and the catheters were taped in place and perfused with a sterile Ringer-acetate solution (Pharmacia & Upjohn, Copenhagen, Denmark) with the addition of 5 µl prostaglandin-E [15-^{3}H(N)]PGE_2 (specific activity, 3.7 Gbq/mmol) per ml of perfusate solution to determine probe recovery characteristics (Langberg *et al.*, 1999a). Two fibers were used to obtain duplicate samples and also to provide continued sampling in the event of a fiber malfunction. The perfusate was flushed through the tubing to the leg, and the rate was then set at 2 µl/min with a high-precision syringe pump (CMA 100; Carnegie Medicine, Solna, Sweden). Microdialysis collection vials attached to the outlet tubing collected 120 µl dialysate per vial at 1-hour intervals for 4 hours. The microvials were

weighed before and after collection to determine actual volume collected. A 10 µl sample was removed from each vial for the calculation of recovery, following the method of Scheller and Kolb (Scheller & Kolb, 1991) and then frozen at -80°C until further analysis.

Analysis

Samples from the *in vivo* study were first run using the assay of Woessner (Woessner, 1961). With OHP standards it was determined that Woessner's assay could be adapted to 50 µl samples with no change in performance. We adapted the assay for smaller volumes because of the relatively small volume of microdialysis samples, and for the convenience of running multiple samples at once on a plate reader (Molecular Devices SpectraMax M5, Sunnyvale, CA). Therefore, 50 µl samples were run in duplicate. Absorption was measured and compared with OHP standards ranging from 0 to 10 µg/ml.

Samples from the *in vivo* study were then run using a new method developed in our laboratory using GC-MS. The following materials for GC-MS were purchased: hydroxyproline (Fisher Scientific), 2,3,3,4,4,5,5-D_7 DL-Proline (PRO D7) (Cambridge Isotopes), *N*-Methyl-*N*-[tert-butyldimethyl-silyl]trifluoroacetimide (MTBSTFA) (Pierce Scientific), unlabeled amino acids (gift from Dan Wright at the Colorado Department of Health and Environment) and methanol and pyridine (Sigma).

Stock solutions of amino acids were prepared in 75% methanol 25% water at 1000 µg/mL and diluted to working concentrations (0, 0.75, 1.5, 3, and 7.5 µg/mL) in methanol. Twenty-five µl of PRO D7 at 10 µg/mL was added along with 25 µL of calibration standards, quality control standards, or samples. The tubes were vortexed for

20-40 sec and then evaporated to dryness under nitrogen at 80°C. To the dried tubes, 50 µL of pyridine and 50 µL of MTBSTFA were added. The tubes were vortexed a second time and incubated at 80°C for 60 minutes. After cooling, the standards, quality control standards and samples were transferred to auto sampler vials for analysis.

The analysis was performed on an Agilent gas chromatograph (GC) mass spectrometer (MS) system consisting of a 7890 GC equipped with a 7683B auto sampler and a 5975C MS. The column was an Agilent DB5MS 0.25mm X 30m capillary column with a 250 µm film thickness. The injection port was kept at 250°C for the 1 µL injection in splitless mode. The split vent was opened after 15 seconds. The system was operated with a constant flow of 1.8 ml/min. The column temperature started at 100°C with a 1-min hold. The temperature then ramped to 290°C at 35°C/min with a final hold of 1-min. The MS transfer line was kept at 280°C.

The MS operated in electron impact (EI) ionization with selected ion monitoring (SIM). Standard auto tune (AT) was used to optimize the MS source, quadrupole, and electron multiplier (EM). EM voltages were typically run 200 volts over the AT setting. Ions 293, 265, and 191 m/z were used for PRO D7 while ions 416, 388, and 314 m/z were used for OHP. A 50 msec dwell was used for all ions. Quantification used ions 293 and 416 m/z. Solvent delay was set to 3 min 15 sec.

Calculations

The *in vivo* recovery of OHP was determined by the internal reference method (Scheller & Kolb, 1991), via radioactive counting of prostaglandin-E [15-^{3}H(N)]PGE_2 as performed previously by Langberg et al (Langberg *et al.*, 1999a). The relative recovery was calculated for each microdialysis catheter using the formula:

$$\frac{(C_P - C_{Bl}) - (C_D - C_{Bl})}{C_P - C_{Bl}}$$

Where C = counts per minute, P = perfusate, Bl = blind sample, D = dialysate. The relative recovery was then applied to assay measured concentrations to get obtain a final *in vivo* concentration.

Statistics Subject characteristics are presented as means ± standard error (SEM), while all other data are presented as means ± standard deviation (SD). Differences between EX and CON and between methods were determined by a repeated measures analysis of variance (ANOVA). Agreement between methods was determined by Pearson's correlation analysis. Differences were considered significant when $p < 0.05$. All statistics were performed on SPSS 16.0.

Results

In vitro study

There was a linear relationship between measured bath OHP concentration and the perfusate measured from the dialysis fiber with a R^2 of 0.90 ($p = 0.01$) (**Figure 2**). Although the relationship was linear, there was a slope of 0.795 indicating that there was less recovered by the microdialysis fiber than present in the bath. Average coefficient of variation (%CV) from two microdialysis fibers over three concentrations (not including zero as to not bias) was 3.6%.

Development of GC-MS

The GC-MS assay was linear from 0.75 μg/ml to 75 μg/ml with an R^2 value of 0.9977. Daily calibration curves were run from 0.75 μg/ml to 7.5 μg/ml. The R^2 values for the four run days were 0.9956, 0.99812, 0.9972, and 0.9973. New standards of OHP and [$^2H_7$]proline were derivatized and analyzed via GC-MS using full scan electron impact ionization. The resultant spectra were interpreted and compared with published spectra to confirm proper identification of the target amino acids.

Intrarun precision was confirmed by running a single subject sample six times with a %CV of 2.46. Intraday precision and accuracy was confirmed with quality control samples of 1.5 μg/mL of OHP spiked into 25 μL of lactated Ringer's solution. Three samples were analyzed with the daily run, one at the beginning of the run (after the curve), one in the middle of the run, and one at the end of the run. Percent CVs for the four run days was, 2.22, 6.51, 4.82, and 7.04. Percent recovery for the four days was 90, 92, 98, and 91. Interday precision and accuracy was confirmed over four days of running with a %CV for the assay of 5.88 and a percent recovery of 93. Finally, interfering

compounds were investigated with a quality control sample at of 1.5 ug/mL of OHP in 25 μL of matrix spiked with leucine, valine, phenylalanine, citrulline, and methionine at 40 μg/mL and tyrosine at 37.6 μg/mL. There was no interference in the quantification of OHP.

In vivo study

In vivo samples were compared using the assay of Woessner and our GC-MS method. There was good agreement between the two analysis procedures in samples measured from the both legs (slope = 1.13, $R^2 = 0.887$, $p < 0.0001$) (**Figure 3 A**).

The relative recovery of radiolabeled PGE_2 averaged 72.79% ± 13.6%. This recovery value is similar to the 79% reported in a previous study (Langberg *et al.*, 2001). When analyzed by the traditional method, there was no significant difference between CON (7.67 ± 6.65 μg/ml) and EX (7.56 ± 5.34 μg/ml) (**Figure 3 B**). Similarly, GC-MS-determined values were not significantly different with a CON value of 6.22 ± 5.58 μg/ml and a EX value of 6.31 ± 5.81 μg/ml.

Discussion

In vitro, microdialysis fibers were able to measure OHP concentrations and were sensitive to changes in bath concentrations. We were also able to measure skeletal muscle interstitial OHP concentration that can be used as an index of skeletal muscle collagen breakdown. There was good agreement between a traditional assay and our new GC-MS approach. However, surprising to us was that a strenuous bout of exercise did not increase skeletal muscle collagen breakdown 17-21 hours post-exercise.

In vitro experiment

The *in vitro* experiment was intended to determine the suitability of the microdialysis method to sample free OHP in skeletal muscle interstitial fluid. It was therefore important to be sure that OHP was able to diffuse across the microdialysis membrane, that it accurately predicted local concentrations, and that it was sensitive to changes in local concentrations. The regression line had an R^2 of 0.90 (**Figure 2**). However, the slope of the line of 0.795 indicates that the dialysate measured from the *in vitro* bath underestimates the actual concentration. This underestimation was driven by the highest value where the bath (10.5 μg/ml) was underestimated by the dialysis fluid (8.0 μg/ml). Otherwise, the values were closely approximated (4.5 to 4.5, 1.1 to 1.3, and 0.0 to 0.0 in bath and dialysis fluid, respectively). Even though the slope indicates a slight underestimation due to the higher concentrations, our in vivo sampled values were on the mid to low range where we saw better matching. The results of the *in vitro* study provided confidence in the ability of the *in vivo* experiment to obtain reliable and repeated measurement of OHP from human interstitial fluid.

In vivo experiment

he *in vivo* study demonstrated a novel way to directly measure collagen breakdown in human skeletal muscle connective tissue. Whole body collagen protein breakdown has previously been estimated from breakdown markers such as ICTP (Langberg *et al.*, 2000), OHP (Brown *et al.*, 1997) and Type I collagen (Brown *et al.*, 1999) in serum and urine. As measurements of these breakdown markers provide collagen turnover for the entire body, an estimate of the contribution to this turnover rate from skeletal muscle collagen protein is not possible. Recently however, ICTP was successfully measured in the peritendinous space of the Achilles' tendon with microdialysis after bouts of endurance training (Langberg *et al.*, 1999b; Langberg *et al.*, 2001). In the current study we used the same principle applied to a small molecular weight metabolite of collagen breakdown.

Our report is the first to measure local skeletal muscle collagen breakdown after exercise. The concentration of OHP in dialysate was not significantly different between EX and CON using either method of analysis. The fact that OHP did not increase in EX was a surprise, as it was expected that strenuous exercise would increase collagen breakdown. We do not expect that potential differences in blood flow masked any changes, since we made our measurements overnight fasted, roughly 12 hours after exercise. The equality is reflected in recovery rates of the probes between the Control and Exercise leg of 71% and 74%. There are several possibilities for our observed lack of breakdown; 1) muscle collagen breakdown does not occur after single bout of exercise, 2) our timeframe of measurement was outside the time-course of increased breakdown, 3) the exercise stimulus was not sufficient to change collagen breakdown, or 4) the

microdialysis technique is not sufficiently sensitive to measure changes in muscle collagen breakdown. Each of these possibilities will be discussed below.

The possibility exists that muscle collagen breakdown does not occur after a single bout of exercise. In two recent studies on skeletal muscle breakdown post exercise using the microdialysis technique, skeletal muscle 3-MH concentration did not rise within the 24 hour period after an acute bout of resistance exercise (Trappe *et al.*, 2004) or within the 72 hour period after a bout of aerobic exercise (Haus *et al.*, 2007). As mixed muscle proteolysis has been shown to increase by 31-61% in the first 3-4 hour post resistance exercise (Phillips *et al.*, 1997) and remain increased through 48 hours (Phillips *et al.*, 1997), the combined results suggest that at the least, proteolysis of the myosin and actin component (of which 3-MH is derived) of skeletal muscle is not increased. As myosin and actin are the proteins necessary for force production in muscle, it may not be practical to let these particular proteins undergo a high degree of breakdown. Collagen may respond in a similar fashion as the contractile skeletal muscle as it would not be energetically favorable to let collagen break down after exercise. With ECM serving as the framework for the muscle, it makes sense that the body may protect against a significant degree of ECM breakdown. Chronic exercise training leads to tendon hypertrophy (Hansen *et al.*, 2003) indicating that synthesis of collagen can exceed breakdown over time. Experiments using both acute bouts of exercise (Takala *et al.*, 1989; Thorsen *et al.*, 1996), and long-term training (Hupli *et al.*, 1997; Langberg *et al.*, 2000) measured high concentrations of serum ICTP after multiple weeks of training, but not after a single exercise bout, perhaps indicating that ECM remodeling only takes place after repeated bouts of exercise. It does not seem likely that collagen breakdown is

completely absent since breakdown is necessary for tissue remodeling, and we know that exercise does not lead to tissue fibrosis.

The measurement of skeletal muscle OHP in this study was made during the time period of 17-21 hours post exercise. This time was chosen to correspond to peak muscle collagen synthesis rates (Miller *et al.*, 2005). Salveson et al. (Salvesen *et al.*, 1994) and Brahm et al. (Brahm *et al.*, 1996) have previously shown that whole body ICTP concentration showed no change immediately after exercise. Further, Langberg et al. (Langberg *et al.*, 1999b) showed peri-tendon ICTP concentration actually decreased immediately after exercise and at 72 hours post-exercise, had returned to baseline levels (Langberg *et al.*, 1999b). A follow-up study by Koskinen et al. found a significant decrease in MMP-2 immediately and 1 day after exercise followed by an increase 3 days after exercise in the area around the Achilles tendon (Koskinen *et al.*, 2004). However, a previous study that measured plasma OHP concentration pre-exercise, and 1,2,3,7,and 9 days after both concentric and eccentric exercise, found no changes in OHP concentration at any time point although plasma collagen was increased after eccentric exercise (Brown *et al.*, 1999). It is likely that in the present study we missed, either too early or too late, an elevation of collagen protein breakdown. Future studies will need to examine additional time points.

It is possible that our exercise intensity was not sufficient to stimulate muscle collagen breakdown. We believed that ten sets of 8 RM should have provided a high load (intensity) and volume. Brown et al. reported elevated urine (Brown *et al.*, 1997), but not plasma OHP (Brown *et al.*, 1999) after accentuated, eccentric (lengthening) contractions, which is likely a higher stimulus than ours. Further, since bicycles are a means of

transportation for most Danes, our subjects could be considered somewhat trained. In studies of mixed muscle protein synthesis it has been demonstrated that subjects that are trained have a significantly blunted effect to exercise. Phillips et al. reported that mixed muscle protein fractional synthesis rate is elevated 118% by a bout of resistance exercise in untrained subjects but only 48% in trained subjects (Phillips *et al.*, 1997). Further, Tipton et al. did not report an increase in muscle protein synthesis following resistance exercise in highly trained swimmers (Tipton *et al.*, 2001). We are inclined to discount the training effect though, since our previous studies demonstrating increased collagen synthesis after exercise were in similar subjects (Miller *et al.*, 2005; Miller *et al.*, 2006).

Finally, it is possible that the method used in this study was not sensitive enough to accurately measure collagen protein breakdown. Because of the large SD (95% CI: 2.9 – 12.4 CON TRAD, 3.7 – 11.4 EX TRAD, 2.2 – 10.2 CON GCMS, and 2.2 – 10.5 EX GCMS), it would take large differences to determine significant differences. Although this is the first time that microdialysis has been used to directly measure skeletal muscle collagen protein breakdown, the results from the *in vitro* experiment suggested that our technique was a reliable one. In addition, since the GC-MS provides a sensitive measure of OHP concentration, and our GC-MS method agreed with previous measures of analysis (**Figure 4**), we believe that we are not limited by the sensitivity of sample analysis. For example, consider that the average %CV for one subject injected six separate times was 2.46% (see results). If we use the mean value of approximately 7.6 μg/ml from the in vivo sampling, the assay itself would be able to distinguish concentration differences of 0.187 μg/ml. As the SD of the in vivo work (≈5.8 μg/ml) was well in excess of 0.187 μg/ml, we do not consider the GC-MS analytical method a

limitation. Further, directionality of differences were always the same between the two analysis methods with CON greater than EX in six trials, CON and EX same in one trial, and EX greater than CON in three trials. Finally, recovery of our sample was accounted for by a radiolabelled species thus excluding probe performance as a source of variability. If sensitivity of analysis and probe recovery are excluded, one has to conclude that the large SD is due to physiological variability between subjects.

In the report of Trappe et al. the lack of change in 3-MH concentration after exercise was confirmed by *a-v* differences across the leg. In our case, confirmation by *a-v* differences is not feasible since this would not eliminate the contribution of skin and bone. For now, we conclude that our 17-21 hour post-exercise sampling likely missed changes in interstitial OHP concentrations.

Finally, there are two other potential limitations to our proposed method. First, although this is the first measure of skeletal muscle collagen breakdown that we are aware of, our measurement must be considered an index since at this point it is not possible to determine a rate. Determination of a rate would require incorporation of blood flow measurements, for which now there are only indexes of blood flow changes (Hickner *et al.*, 1991), or isotopes with appropriate modeling assumptions. The equality is reflected in recovery rates of the probes between the Control and Exercise leg of 71% and 74%. Second, one could speculate that our method still does not completely eliminate the contribution of bone and skin since they are in the area of the musculature. However, Rodahl and others have calculated that microdialysis sampling is limited to the 100 mg of tissue in the vicinity of the probe (Rosdahl *et al.*, 1993), giving us some confidence that we are only sampling skeletal muscle tissue.

Perspectives

We have provided a new method to measure skeletal muscle collagen breakdown. In theory this method could be applied to other tissues such as tendon or cartilage that are accessible by microdialysis. Measurements of mixed skeletal muscle turnover do not provide information on the turnover of individual protein components. We have determined previously that synthesis of ECM collagen protein is rapid and responsive to exercise. The present study does not support that breakdown, the second component of turnover, acts the same way. It is not yet clear whether breakdown happens more rapidly or is delayed compared to our sampling time point, or if it simply does not occur after an acute bout of exercise and instead remodels after prolonged training. The balance of synthesis and breakdown of collagen in skeletal muscle tissue could provide insight into such observations as the decrease in muscle quality with aging or designation of exercise for the maintenance of muscle force in diseased or aged states.

Acknowledgements

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Table: Subject characteristics for *in vivo* trial (mean ± SEM).

Characteristic	Value
Age (yr)	23 ± 1.7
Wt (kg)	82 ± 4
Ht (cm)	182 ± 1
BMI (kg /m^2)	25 ± 1
8-RM (kg)	61 ± 4

Figures

Figure 1. Study design for *in vivo* study.

Figure 2. *In vitro* comparison of a known bath concentration versus measured dialysate concentration. Samples were collected from two separate microdialysis fibers in the same bath. Repeated samples were made over two hours at each concentration for a total n = 8. Values are means ± SD. Average CV for repeated measures was 3.6%.

Figure 3. *In vivo* measurement of interstitial hydroxyproline (OHP) concentration in control (CON) and exercise (EX) leg one day after a bout of strenuous resistance exercise. Presented are data using both the traditional assay of Woessner (Woessner, 1961) (TRAD) and our GC-MS analysis (GCMS). Individual samples were compared in the CON leg (**A**) and EX leg (**B**) with good agreement between analysis techniques. There was no difference between CON and EX legs by either analysis techniques (**C**). Values are means ± SD.

Figure 1

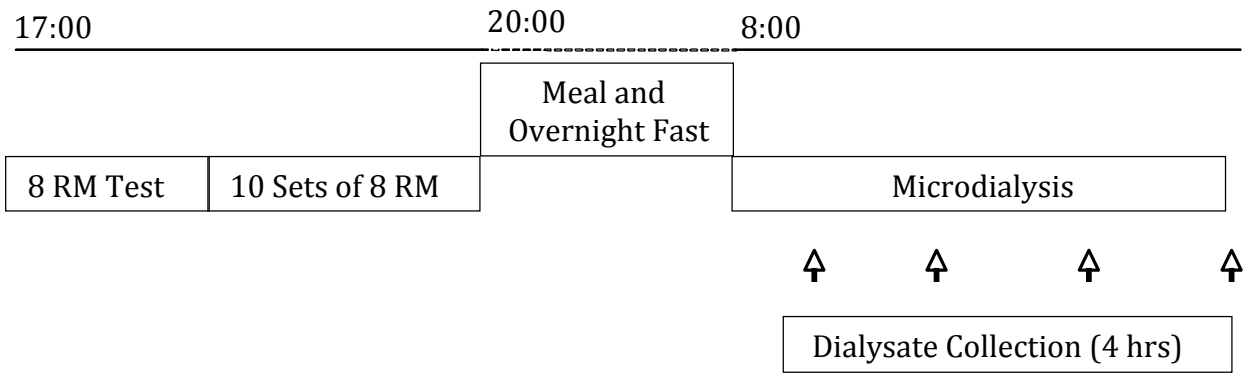

17:00
20:00
8:00
Meal and
Overnight Fast
8 RM Test
10 Sets of 8 RM
Microdialysis
Dialysate Collection (4 hrs)

Figure 2

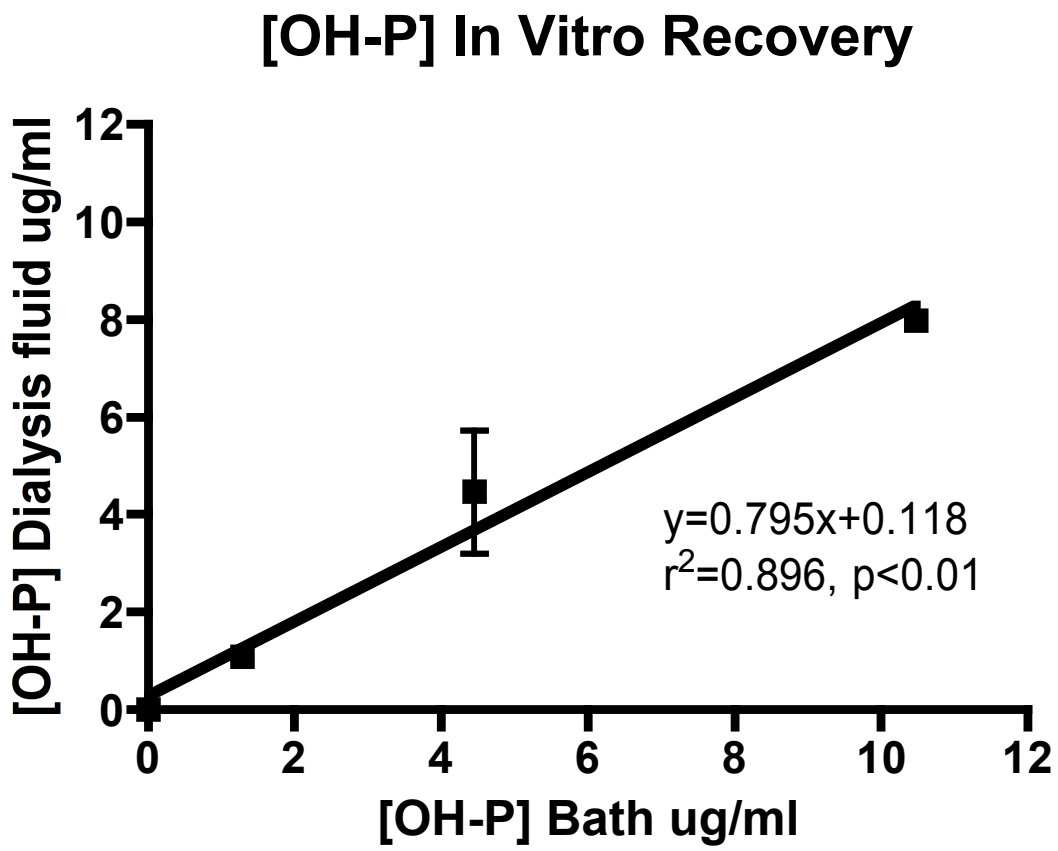
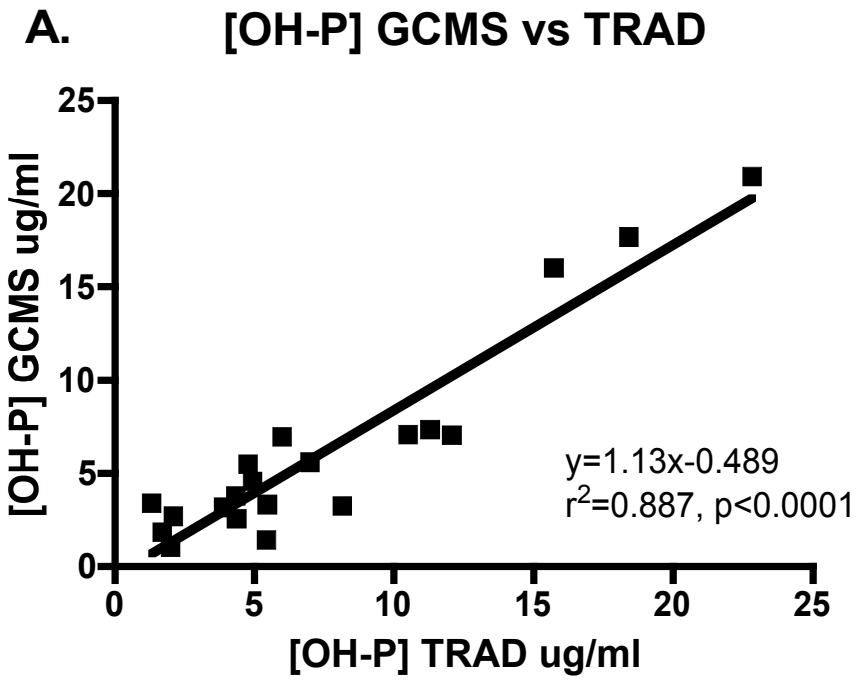
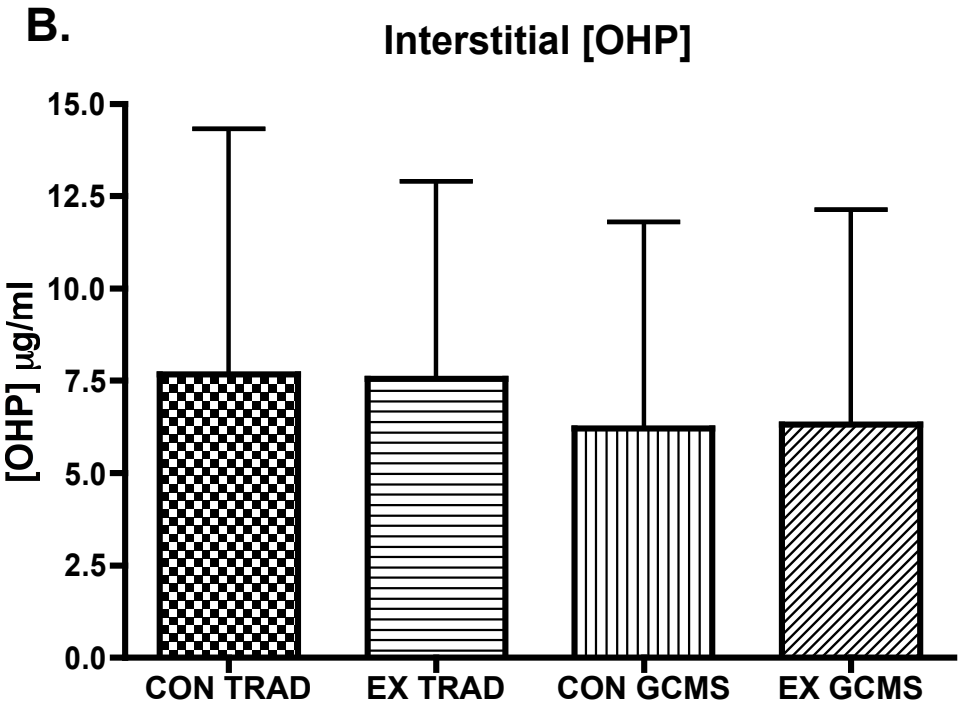

[OH-P] In Vitro Recovery
[OH-P] Dialysis fluid ug/ml
[OH-P] Bath ug/ml
0
2
4
6
8
10
12
y=0.795x+0.118
r2=0.896, p<0.01

Figure 3


A.
[OH-P] GCMS vs TRAD
[OH-P] GCMS ug/ml
[OH-P] TRAD ug/ml
y=1.13x-0.489
r2=0.887, p<0.0001
0
5
10
15
20
25


B.
Interstitial [OHP]
[OHP] μg/ml
0.0
2.5
5.0
7.5
10.0
12.5
15.0
CON TRAD
EX TRAD
CON GCMS
EX GCMS

SUPPLEMENTAL MATERIAL

Informed Consent for Experiment #1 and #2

COLORADO STATE UNIVERSITY

TITLE OF STUDY: Sympathetic Nervous System & Metabolism In Older Humans

Principal Investigator: Christopher Bell, Ph.D.
Department of Health and Exercise Science
220 Moby-B Complex, Campus Delivery 1582
physiology@cahs.colostate.edu
970-491-3495

Co-Principal InvestigatorS:

Matthew S. Hickey, Ph.D.
220 Moby-B, Campus Delivery 1582
970-491-5727

Benjamin F. Miller, Ph.D.
220 Moby-B, Campus Delivery 1582
970-491-3291

Brian L. Tracy, Ph.D.
220 Moby-B, Campus Delivery 1582
970-491-2640

Raoul F. Reiser II, Ph.D.
220 Moby-B, Campus Delivery 1582
970-491-6958

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? You are an adult man or women aged between 18 and 79 years. You do not smoke. You have normal blood pressure. You are not pregnant.

WHO IS DOING THE STUDY? Christopher Bell, Ph.D., Matthew Hickey, Ph.D., Brian L. Tracy, Ph.D., Raoul F. Reiser II, Ph.D. and Benjamin F. Miller, Ph.D. of the Health and Exercise Science Department will perform this research. Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. The National Institutes of Health are paying for this research.

WHAT IS THE PURPOSE OF THIS STUDY? The purpose of our studies is to understand the way in which we burn calories at rest and during activities, and how this might be influenced by aging and weight gain.

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW WELL EQUIPPED IS THE LOCATION FOR DEALING WITH AN EMERGENCY? All of the procedures (unless otherwise stated) will take place in the Human Performance Clinical Research Laboratory (HPCRL) in the Department of Health and Exercise Science (Moby Complex). Drug infusions will be supervised by a medical doctor. The HPCRL has an automated defibrillator with built in transcutaneous pacing and a “crash-cart” stocked with oxygen and emergency medications.

HOW LONG WILL THE STUDY LAST? This whole research project will take place over a period of approximately five years. However, your part of this study will be either: _____ 1) three or four visits over a period of several days, or _____ (your initials)
_____ 2) several visits over a few to several weeks. _____ (your initials)

WHAT WILL I BE ASKED TO DO?
This consent form applies to a large research project. You are only being asked to participate in one part of the total project. Depending on the part of the research project that you are involved in, you will be asked to participate in some of the following procedures. Many potential procedures are described in the section below. However, the procedures that you will be asked to do for this part of the study have a check mark next to them. The check marks were put there by one of the researchers. The time associated with each procedure reflects the amount of time you will spend performing or undergoing the procedure, not the total time of the study. A member of the research team will fully explain each checked procedure that applies to your participation and specifically how long each session (total time) in the laboratory will be.

_____Treadmill Stress Test
A treadmill stress test will be performed on your first visit to the laboratory. This test will tell us if your heart is healthy. You will be asked to walk on a motorized treadmill for approximately 10-12 minutes. The treadmill will be on a hill that is made steeper every 2 minutes. While you are walking we will measure your heart rate with an electrocardiogram (ECG) and your blood pressure with a cuff placed around your upper arm. Dr. Wyatt Voyles, a physician, will supervise the test. If we do not think your heart is healthy you will be referred to your primary care physician for further testing. There is a chance that you may not be allowed to take part in our study. You will be asked to do this test once; it lasts roughly 1 hour.
_____ (your initials)

_____Exhausting Exercise Test
This test will tell us how fit you are and is very similar to the treadmill stress test. You will be asked to walk or run on a motorized treadmill, or ride an exercise bike, until you are too tired to continue. The treadmill will be on a hill that is made steeper every 2 minutes. While you are walking or running we will measure your heart rate with an electrocardiogram (ECG). We will ask you to wear a nose clip (something that stops you breathing through your nose) and ask you to breathe through a mouthpiece. This will let us measure the gases you breathe in and out. We will also ask you to breathe (10 – 12 breaths) from a big balloon containing a special, certified pre-mixed gas mixture (Air Gas Intermountain) made up of 21% oxygen, 0.5% acetylene, 5% helium, and 73.5% nitrogen. Normal room air contains 21% oxygen, less than 1% carbon dioxide and approximately 78% nitrogen. We may also ask you to exercise at an intensity that is not exhausting while we make the same measures. These tests last roughly 1 hour.
_____ (your initials)

_____ Pregnancy Test. If you are female you will be required to have a sample of your urine tested for the presence of human chronic gonadotropin (HCG), a hormone that indicates whether you may be pregnant. This will require approximately 1 cup of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. (~10 minutes). _____ (your initials)

_____Blood Pressure
We will measure your blood pressure using a standard blood pressure cuff (the same as in a doctor's office). Blood pressure will be measured during all of the tests performed in the lab with the exception of body composition. There are no known risks associated with this procedure. _____ (your initials)

_____Body Composition
We will measure how much fat you have in your body using a test called dual energy x-ray absorptiometry (DEXA). The DEXA test requires you to lie quietly on a padded table while a small probe gives off low-level x-rays and sends them over your entire body. This test gives very accurate measurements of your body fat and bone mineral density. This test lasts approximately 30 minutes. _____ (your initials)

_____Calories Burnt at Rest
We will measure the amount of calories you burn at rest. This is called your resting metabolic rate. This test will take place while you are lying down on a bed. A plastic transparent bubble will be placed over your head and shoulders and room air will be pumped in through a pipe. We will measure the air that you breathe in and out. There are no known risks associated with this procedure. Sometimes we will do this test when injecting drugs into your veins: _____ (your initials)

_____Drugs – Placebo (Pills/injection/patch)
You may be asked to receive one or more of the drugs described below. There is a chance that instead of receiving these drugs you will receive a placebo. A placebo is a "drug" that has no effect on your body. You will not know if you are taking a placebo, only the research staff will know. Because a placebo has no effect then there are no risks associated with taking one. _____ (your initials)

_____Drugs – Propranolol (Injection)
Propranolol is a type of drug commonly called a beta-blocker. It is usually given to people who suffer from heart problems or high blood pressure. This drug will probably make your heart rate, blood pressure, and metabolic rate smaller. The drug will be injected into a vein in your arms or hands for roughly 45 minutes. During this time we will measure the calories you burn. We will also measure your heart rate and your blood pressure. This test will last roughly 2.5 hours. _____ (your initials)

_____Drugs – Isoproterenol (Injection)
Isoproterenol is a drug that has the opposite effects of a beta-blocker. This drug will probably make your heart rate, blood pressure, and metabolic rate bigger. The drug will be injected into a vein in your arms or hands for roughly 90 minutes. The drug will be injected first in small amounts for 30 minutes, then in bigger amounts for the next 30 minutes, and in even bigger amounts for the final 30 minutes. During this time we will measure the calories you burn. We will also measure your heart rate and your blood pressure. This test will last roughly 2.5 hours. _____ (your initials)

_____Drugs – Propranolol (Pills)
Propranolol is a type of drug commonly called a beta-blocker. It is usually given to people who suffer from heart problems. This drug will probably make your heart rate, blood pressure, and metabolic rate smaller. You may be given 2 propranolol pills per day for 21 days. You will not know if you are taking a placebo pill (a pill that has no physiological effects) or a propranolol pill. Only the research staff will know this. We will give you the pills to take away every 7 days at the Human Performance Clinical Research Laboratory. _____ (your initials)

_____Drugs – Vitamin C (Injection)
You may receive an injection of vitamin C on one or two days: once during one of the days you receive the drug called isoproterenol and/or once during an exercise test. The amount you receive will depend on your body composition. Vitamin C is an anti-oxidant, that is, it will reduce your level of oxidative stress. Oxidative stress is the damage done to your body as a result of everyday metabolic and chemical reactions taking place inside your body. _____ (your initials)

_____Drugs – Vitamins C and E (Pills)
You will be given vitamin C and E pills to take via mouth every day for 30 days. You will take 1000 mg of vitamin C per day and 400 I.U. of vitamin E per day. These vitamins are the same as those that are for sale over the counter from most pharmacies.
_____ (your initials)

_____Drugs – Clonidine (Patch)
Clonidine is a drug used to treat people with high blood pressure. You will be asked to wear a patch on your arm/shoulder that allows clonidine to slowly leak from the patch, through your skin and into your blood. You will be asked to wear two patches – one patch/week over three weeks. _____ (your initials)

_____Drugs – Trimetaphan Camsylate (Injection)
Usually, signals are generated in your brain that then travel along your spine and out through nerves that attach to different parts of your body (muscles, heart, lungs, etc.). Trimetaphan is a drug that stops some of the nerve signals from leaving the spine.
_____ (your initials)

_____Drugs – Phenylephrine (Injection)
To confirm that we have stopped the nerve signals traveling from the brain to your heart with trimetaphan camsylate (see above) we will inject a small dose of phenylephrine (25, 50, and/or 100 µg over 1 second). Phenylephrine will temporarily narrow your blood vessels, causing your blood pressure to increase for a short period of time (< 5 minutes).
_____ (your initials)

_____Microneurography
This test will measure the activity of your nerves while you rest on a bed. We will place a very thin needle just under the skin below your knee and record the electrical patterns of a nerve there. To help locate the area where we will need to place the needle, an electric stimulus will be used that may cause brief (1-2 seconds) feelings of "pins and needles" or a dull ache in your lower leg. When the needle is in the proper position, your foot will twitch by itself. The electric stimulus will then be turned off, and there will be no other feelings during the recording of your nerve activity. The stimulus could be used periodically for up to 1 hour, but not more than 1 hour. This test lasts roughly 2 hours.
_____ (your initials)

_____Blood Collection
We will be taking blood from you on different days while you are taking part in our study. When a person donates blood usually the amount donated equals approximately one pint. We will be taking less than one third of this amount. Your blood will be tested for various things that are involved with your nerves, the amount of calories you burn at rest, and the drugs that we will be giving you. Your blood will be taken from veins in your arms or hands using needles and hollow plastic tubes called catheters. In some instances these catheters may remain in your veins for several hours.
_____ (your initials)

_____Cutting Little Pieces of Muscle from Your Legs
This test is commonly called a muscle biopsy. During the muscle biopsy a drug (an anesthetic) will be injected into an area of your thigh to make it feel numb. A small incision (roughly 1/4 inch) will be made using a sharp sterile blade. A sterile probe will be inserted into your leg and a little piece of muscle (roughly the size of a sweet corn kernel) will be removed. Matthew Hickey, Ph.D., will perform this procedure. This procedure lasts approximately one hour. _____ (your initials)

_____Measurement of Stress-Hormone Release
Your body produces chemicals that help you to deal with stressful situations. Catecholamines are one example of these types of chemicals. So that we can measure how much of these chemicals are made we will place a catheter in one of your veins. Through this catheter we will inject catecholamines that have been treated with a very, very small amount of radiation. We will then sample a small amount of blood from a catheter that is inserted into a different vein to measure the concentration of these catecholamines. This procedure may last up to 90 minutes. _____ (your initials)

_____Metabolic Gene Tests
Genes are passed on to us from our parents and influence the way we look (for example, hair and eye color) and the way our bodies work. Some genes have been shown to influence how the nervous system helps our bodies to burn calories. We will collect approximately 10 ml of blood (less than 1 tablespoon) and analyze it for these genes, and only these genes. We will destroy your blood sample (burn/incinerate) after we have analyzed it for your genes. _____ (your initials)

_____Food Questionnaire
You will be asked questions about the types and amounts of food you eat. We will estimate how many calories you eat per day, and how many of those calories come from fat, carbohydrate, and protein. There are no known risks associated with this procedure. The questionnaires may take up to one hour to complete._____ (your initials)

_____Activity Questionnaire
You will be asked questions about the types of activities you perform every day. Some of these questions will be about the activities you perform at work and during exercise. There are no known risks associated with this procedure. The questionnaire may take up to 30 minutes to complete. _____ (your initials)

_____Urine Collection
You will be asked to collect all of your urine in a plastic container provided by the investigator over a 24-hour period. Your urine will be analyzed for levels of oxidative stress. There are no known risks associated with this procedure._____ (your initials)

_____Daily Exercise
You will be asked to report to the lab every day for 5-days to exercise on a stationary cycle for one hour. The exercise will be at an intensity determined by the results of your stress test. _____ (your initials)

_____ Steadiness test
You will sit in a special chair and perform muscle contractions with your hand, arm, or leg muscles. This test might occur in the Human Clinical/Research Laboratory or the Neuromuscular Function Laboratory in the Moby complex. (15 min – 1 hour)
_____ (your initials)

____ Measuring Muscle Activity
Sticky electrodes will be placed on the skin over the muscles involved and will remain in place until the end of the visit. Natural oil in the skin will be removed with rubbing alcohol, and the skin will be gently roughened with a fine abrasive paste or cloth.
_____ (your initials)

_____ Postural Sway test

You will stand as still as possible for up to one minute at a time. During this time forces under your feet will be measured by highly sensitive "bathroom scales". These measures determine how steady you can keep yourself while standing. This test will occur in the Clinical Biomechanics Research Laboratory. (15 – 30 min)

_____ (your initials)

_____ Stable Isotope (Leucine) Infusion

Stable isotopes are naturally occurring, biologically safe atoms that weigh a tiny amount more than usual. They are very useful in studies of metabolism. You will sit/lie with a hollow plastic tube inserted into a vein in your arm or hand. The stable isotope leucine, an essential amino acid (protein) made in plants, will be pumped into your vein such it mixes with your blood and other tissues. (Up to 6 hours)

_____ (your initials)

ARE THERE ANY RISKS ASSOCIATED WITH THESE PROCEDURES?
The Human Performance Clinical Research Laboratory keeps an automated defibrillator with built in transcutaneous pacing and a "crash-cart" stocked with oxygen and emergency medications. A medical doctor will supervise all of the drug infusions. The investigators have a great deal of experience with all of the procedures. Some of the procedures you are being asked to volunteer for have a number of associated risks:

Treadmill Stress Test
There is a very small chance of an irregular heartbeat during exercise (< 1% of all subjects). Other rare risks of a stress test are heart attack (< 5 in 10,000) and death (<2 in 10,000). Exercise can make you tired and uncomfortable.

Exhausting Exercise Test
There is a very small chance of an irregular heartbeat during exercise (< 1% of all subjects). Other rare risks of a stress test are heart attack (< 5 in 10,000) and death (<2 in 10,000). Wearing a mouthpiece and nose-clip can sometimes cause dryness in the mouth and mild discomfort. Acetylene takes the place of oxygen on hemoglobin and may cause suffocation if breathed continuously for an extended period (hours). To reduce this risk you will only breathe the acetylene gas mixture for approximately 12 breaths. This test has been used safely to study women during labor, intensive care patients, and very sick people with chronic pulmonary and cardiovascular diseases.

Body Composition
There is a small amount of radiation exposure (0.05 mRem) associated with the DEXA test that is less than 1/20 of a typical chest x-ray. The more radiation you receive over the course of your life, then the greater the risk of having cancerous tumors or of inducing changes in genes. The changes in genes possibly could cause abnormalities or disease in your offspring. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is unclear. Women who are or could be pregnant should receive no unnecessary radiation and should not participate in this study.

Drugs – Vitamins C and E – Pills
Oral administration of vitamins C and E is safe in the doses proposed in this investigation. A recent study found that oral administration of vitamin E (greater than 400 I.U. per day for longer than one year) increased the risk of all-cause mortality. In this investigation you will not take more than 400 I.U. per day for longer than 30 days. If you feel unwell while taking the vitamins we strongly recommend that you to stop taking them and contact the investigator as soon as possible.

Microneurography
There is a small chance of a "pins and needles" feeling off-and-on for 1 to 7 days after the test (20% or less of all subjects); there is also a very small chance (less than 1% of all subjects) of an aching feeling in your leg off-and-on for a period of several weeks or months after the test.

Blood Collection
When the needle goes into a vein, it may hurt for a short period of time (a few seconds). Also there may be minor discomfort of having the needle/plastic tube taped to your arm. In about 1 in 10 cases, a small amount of bleeding will occur under the skin that will cause a bruise. The risk of forming a blood clot in the vein is about 1 in 100, and the risk of significant blood loss is 1 in 1,000. Additionally, there is a risk that you may faint while having blood collected or having the catheter inserted in your vein.

Muscle Biopsy
During the procedure you may feel discomfort associated with the injection of the numbing drug (the anesthetic) but during the actual muscle removal the discomfort should be minimal. There is a risk that you may faint during the procedure. There is also a risk of muscle cramp, bleeding, of loss of feeling in your leg, and of damage to a skin (cutaneous) nerve. The risk of infection and bruising is extremely small if you follow the instructions for caring for the incision. A very small and minor scar will remain as a result of the incision, but may not be noticeable. Matthew Hickey, Ph.D. will perform these procedures under surgically clean conditions. Emergency medical equipment will be available. You will be screened prior to the procedure for history of allergic reactions to Novocain.

Measurement of Stress-Hormone Release
The risks associated with this procedure are the same as those described for blood sampling. In addition you will be exposed to a very, very small amount of radiation (approximately 2.3 μSv). This negligible dose is equivalent to approximately 9 hours of normal day-to-day background radiation.

Metabolic Gene Tests
The risks associated with this procedure are the same as those described above for blood sampling (fainting, bruising, blood clotting, blood loss, and minor discomfort when the needle enters your vein).

Drugs – Vitamin C – Injection
Vitamin C has been safely injected without any unfavorable side effects into the veins of patients with high blood pressure, high cholesterol, Behcet's Syndrome, smokers, and directly into the hearts of cardiac patients. However, too much vitamin C may lead to diarrhea and/or renal problems such as formation of kidney stones. In addition, intravenous administration of concentrated vitamin C may cause irritation to the area local to the infusion. To reduce these risks we will reduce the concentration of vitamin C by dissolving your dose in saline. Your dose will be calculated based on your body composition.

Drugs – Propranolol (Injection)
We will be giving you more propranolol than is usually given to sick people.
This drug will probably make your heart rate and blood pressure smaller. We will be measuring your heart rate and blood pressure very carefully when we are injecting this

drug. There is a small risk of hypotension (low blood pressure). The symptoms of blood pressure dropping too low are dizziness, fainting, or in rare instances heart block. If your systolic blood pressure falls below 90 mmHg, or if your heart rate falls below 40 bpm we will stop the injection immediately.

Drugs – Isoproterenol (Injection)
Isoproterenol can cause an increase in systolic blood pressure. It may also cause the heart to beat faster and harder which can give you a sensation of pounding in the chest or palpitations. Rarely, isoproterenol can cause heart block and fainting. Previous research participants who have received isoproterenol have made the following comments: "It's like having a work-out for your heart without exercising your muscles" and, "It's like the feeling you get in your heart when somebody sneaks up behind you and gives you a fright." Isoproterenol administration will be terminated immediately if: 1) your systolic blood pressure goes up by more than 35 mmHg; 2) your heart rate goes up by more than 50 bpm; or, 3) heart rate increases above 135 bpm (older adults) or 155 bpm (young adults).

Drugs – Propranolol (Pills)
We will be giving you a similar amount of propranolol than is usually given to sick people. This drug will probably make your heart rate and blood pressure smaller. There is a small risk of hypotension (low blood pressure). The symptoms of blood pressure dropping too low are dizziness, fainting, or in rare instances heart block. If your mean blood pressure falls below 90 mmHg, or if your heart rate falls below 40 bpm we will stop giving you the pills. There is also a small risk of depression (feeling sad), diarrhea, and bronchospasm (difficulty breathing). To help avoid any unpleasant side effects we will be taking a number of precautions:

We will be measuring your heart rate and blood pressure very carefully every 7 days when you are taking these pills, and 14 days after you have stopped taking them.
We will call you on the telephone at a mutually agreeable time to ask you questions about your health and how you are feeling. These calls will take place 3-4 days after each time you pick up your pills.
You will be given a list of people and their telephone numbers who you should contact if you feel unwell while you are taking the pills.
You will be given a credit card sized laminated tag to carry while you are taking the pills. The tag will read, "Beta-Blocker Alert. I am a participant in a clinical research investigation. I may currently consume 160 mg of propranolol per day. For information please call Dr. Christopher Bell 970-491-7522".

Drugs – Clonidine (Patch)
We will be giving you a similar amount of clonidine that is usually given to sick people. This drug will probably make your heart rate and blood pressure smaller. There is a small risk of hypotension (low blood pressure). The symptoms of blood pressure dropping too low are dizziness, fainting, or in rare instances heart block. If your mean blood pressure falls below 90 mmHg, or if your heart rate falls below 40 bpm we will stop giving you

the clonidine. There is also a small risk of dry mouth, tiredness or weakness, and irritation (itchy skin) at place where you wear the patch. To help avoid any unpleasant side effects we will be taking a number of precautions:

We will be measuring your heart rate and blood pressure very carefully every 7 days when you are wearing the clonidine patch, and 14 days after you have stopped wearing it. We will call you on the telephone at a mutually agreeable time to ask you questions about your health and how you are feeling. These calls will take place 3-4 days after each time you pick up your patch.
You will be given a list of people and their telephone numbers who you should contact if you feel unwell while you are wearing the patch.
You will be given a credit card sized laminated tag to carry while you are wearing the patch. The tag will read, "Clonidine Alert. I am a participant in a clinical research investigation. I may currently receive 0.2 mg of transdermal clonidine per day. For information please call Dr. Christopher Bell 970-491-7522".

Drugs - Trimetaphan Camsylate
Administration of trimetaphan camsylate is associated with acute low blood pressure, fast heart rate, dry mouth, and blurred vision. In addition, unlikely but possible events include slowed breathing, itching, difficulty urinating, constipation, diarrhea, and fainting. Trimetaphan camsylate will release a substance into your body called histamine; individuals with a history of histamine allergy should not be given trimetaphan camsylate. The actions of trimetaphan camsylate are typically very short duration (≈ 15 min). To reduce the risks associated with trimetaphan camsylate, we will stop giving the drug if: 1) your heart rate increases above 115 bpm; 2) if the rhythm of your heart becomes abnormal; or, 3) if your systolic blood pressure decreases below 70 mmHg. Because trimetaphan camsylate decreases blood pressure you will be advised not to leave the Human Performance Clinical Research Laboratory until your blood pressure has returned to within ± 5 mmHg of normal.

Drugs – Phenylephrine
Administration of phenylephrine is associated with increased blood pressure. Potential risks include headache, excitability, and restlessness. This drug is not thought to affect the function of the heart. The dose of phenylephrine that we propose to administer is smaller than that normally used clinically to treat hypotension (low blood pressure).

Muscle contractions
There is a slight risk of muscle strain and muscle soreness resulting from brief strong muscle contractions. There is no known risk from light muscle contractions. If it occurs, the soreness should not last more than two days or affect your normal function.

Measuring muscle activity
There is no known risk from this procedure. The skin might be slightly and temporarily irritated for no more than a day.

Postural Sway
The risks associated with this test include loss of balance with the potential for falling. This risk is extremely low because you will have both feet on the ground and be closely surrounded by a handrail and a research assistant.

Stable Isoptopes
There is a small risk of blood infection and the same risks apply as described under the heading "Blood Collection". To reduce the risk of infection stable isotopes will be sterile and prepared in a pharmacy using sterile procedures. Each isotope will be used once only. Isotopes will be infused through a special filter designed to protect against infection.

FUTURE USE OF BLOOD OR MUSCLE SAMPLES
It is possible that we may want to use any extra blood or muscle tissue for future research not described in this consent form. We will keep private all research records that identify you (to the extent allowed by law) for both current and future use (please refer to the section, "WHO WILL SEE THE INFORMATION THAT I GIVE" for more information regarding privacy). Future research will pertain to physiological function.

Only choose one of the following:

_____ I give permission for the use of my blood or muscle tissue collected as part of the current study only.

_____ (your initials)

_____ I give permission for the use of my blood or muscle tissue for the current study as well as for future studies.

_____ (your initials)

It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
You will not be allowed to participate in these studies for any of the following reasons:
You are not aged between 18 and 79 years.
You are pregnant.
You are a nursing mother.
You smoke or have smoked during the previous two years.
You have high blood pressure (greater than 145/90 mmHg).
You have asthma.

You are not free of overt disease as assessed by medical history, physical examination, fasting blood chemistry (fasting plasma concentrations of glucose > 125 mg·dL-1, cholesterol > 240 mg·dL-1, high-density lipoprotein cholesterol < 30 mg·dL-1, low-density lipoprotein cholesterol > 150 mg·dL-1, Chem 7 panel, thyroid panel) ECG and blood pressure at rest and during incremental exercise.
You are taking systemic vasoactive drugs (i.e. drugs that affect blood pressure and/or heart function).
Your participation has not been approved by a physician, Dr. Wyatt Voyles, or by a senior member of the research team.
You are taking medications that would confound interpretation of the results of the studies.
You should not receive trimetaphan camsylate if you have a history of histamine allergy.
You should not receive propranolol if you have a bronchospastic disease (e.g. asthma) or if you are diabetic.
You should not receive ascorbic acid injections if you are taking bishydroxycoumarin, or if you are already taking vitamin supplements.
You should not receive phenylephrine if you have a history of sulfite sensitivity.
You should not receive clonidine if you are currently receiving anticoagulant therapy or have a known or suspected bleeding disorder.

WILL I BENEFIT FROM TAKING PART IN THIS STUDY?
There are no direct benefits to you for participating in this study beyond receiving information on your body composition and metabolic and cardiovascular risk factors.

DO I HAVE TO TAKE PART IN THE STUDY?
Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHAT WILL IT COST ME TO PARTICIPATE?
There is no cost to you for participating except that associated with your transportation to our facilities.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law.

Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some

circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court.

CAN MY TAKING PART IN THE STUDY END EARLY?
Your participation in the study could end in the rare event of muscle strain, if you become pregnant, or if you miss an excessive number of appointments.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY?
For experiments that involve the blood sample, muscle sample, fine wire electrodes, and venous catheterization, you will be paid $15/hour.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH?
The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the investigator, Christopher Bell, Ph.D., at 970-491-3495 or physiology@cahs.colostate.edu . If you have any questions about your rights as a volunteer in this research, contact Janell Barker, Institutional Review Board, at 970-491-1655. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 14 pages.

_____ _____
Signature of person agreeing to take part in the study Date

_____
Printed name of person agreeing to take part in the study

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff

Informed Consent for Experiment #3

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: Nutritional strategy for increased mitochondrial biogenesis.

Principal Investigator: Dr. Benjamin F. Miller, Assistant Professor, Department of Health and Exercise Science, Colorado State University. bfmiller@cahs.colostate.edu

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH?
If you are 18-35 or 55-75 years old and physically healthy but sedentary, then we are interested in you taking part in this study. Sedentary means that you do not currently participate in more than two dedicated aerobic exercise sessions per week. Males and females are invited to participate. Eighteen subjects in each group that meet the criteria to participate will be selected.

WHO IS DOING THE STUDY? Dr. Miller is an Assistant Professor in the Department of Health and Exercise Science at CSU who is interested in using exercise and nutrition to combat muscle wasting with aging. Dr. Miller will be assisted by Dr. Matt Hickey and graduate students of the laboratory.

WHAT IS THE PURPOSE OF THIS STUDY?
As people age, they lose muscle function. We are interested in how to increase the amount of mitochondria (organelle responsible for making energy in the body) to maintain function in older subjects. We use exercise and nutritional interventions, rather than drugs, and study how these treatments might benefit older subjects.

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?
The study will take place at Colorado State University and will last one year.

WHAT WILL I BE ASKED TO DO?
The time commitment in full is explained below for each group followed by detailed procedures.

If you are in the younger group, you will be expected to:
Participate in an initial and exercise screening procedure (1 hr),
Provide two muscle samples twice (once before training and once after training) for a total of four muscle samples for the entire study (1 hr before and 1 hour after training period),
Consume a special water throughout the study (1 minute daily),
Participate in a 6-week training program of 5 days/week (1 hour/day).

If you are in the older group, you will be expected to:
Participate in an initial and exercise screening procedure that includes a monitored cardiac screening (2 visits of 1 hour each),

Provide two muscle samples twice (once before training and once after training) for a total of four muscle samples for the entire study (1 hr before and 1 hour after training period),
Consume a special water throughout the study (1 minute daily),
Participate in a 6-week training program of 3 days/week (1 hr/day).

We will ask you to answer some questions about your past and current participation in exercise, and will measure your body weight, height, and body composition (% body fat). Your gender and date of birth will also be recorded. A small (15mL) sample of blood will be taken for screening purposes. Before undergoing the sampling procedures, you will be asked a few questions relating to your present state of health, current medication and past medical history. This is to exclude the presence of any condition or medication that might prolong your bleeding time or make the blood sampling unsafe for you. If you are female you will be required to have a sample of your urine tested for the presence of human chronic gonadotropin (HCG), a hormone that indicates whether you may be pregnant. This will require approximately 1 cup of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. This visit will take approximately 30 minutes.

If you are older, you will also undergo cardiac screening in the presence of a cardiologist. This will involve the placement of ten collecting electrodes on your chest which will be connected to an electrocardiogram (ECG). You will then be asked to walk on a treadmill, slowly at first and progressively faster until the cardiologist asks you to stop. This test will take approximately 45 minutes.

If anything adverse is found in any of the medical screening, you will be advised.

If the initial screening is successful or you are a younger subject, you will then perform an exercise test. In the first, we will determine your maximal oxygen consumption (VO2 max). This involves riding on a stationary bicycle while breathing into a mouth piece that samples the breathed air. The exercise will start easy and get progressively more difficult until you indicate that you can not go further. We will also measure your heart rate throughout the test. This test will take approximately 45 minutes each.

In another visit a small (100mg, about the size of a pea) sample of muscle will be taken from both of your thighs (one in each). This will be done under a local anesthetic. A small (6-8mm) cut will be made in your skin and another cut of the same size in your muscle. The muscle sample will be taken under sterile conditions and will be carried out by an experienced investigator (Matt Hickey PhD) with medical oversight. Following this, pressure and ice will be applied, the incision will be secured by band-aids, then firmly bandaged with gauze. The biopsy procedure will not prevent you from performing any of your normal daily activities afterwards. After 12 hours you can remove the gauze, by three or four days the steri-strips, and after one week the incision will be inspected. The resultant scar will gradually fade and be hardly noticeable. This visit will last about half an hour.

The day of the muscle biopsy, you will be asked to consume a small cup of water (50 ml) in the laboratory and 5-6 more times the remainder of the day. In each day after this first day, you will be asked to consume one small glass (70 ml) each day for the remainder of the study. In most instances, you will consume the glass when you complete your exercise training (see below). The glass of water you will consume contains what is called an isotope of water. The water we provide you has a special label. This label is present naturally, but we add it in a higher concentration in order to follow reactions in the body. You will not notice this label, nor will it change anything in your body. We can only located it by special analysis procedures sample collection.

After the muscle biopsy and concomitant with the water consumption, you will begin a 6-week training program. You will be expected to exercise three times per week (if older) or five times per week (if younger) on a stationary bike at the training facility in the Department of Health and Exercise Science at Colorado State University. To begin with the sessions will be short (20 minutes) and you won't have to work very hard. As the program progresses, you will gradually be asked to work harder and for longer (up to one hour at a time). At three separate times during the 6-week training program we will ask you to provide a saliva sample using a cotton swab.

After each training session you will be given a drink. You will need to consume straight away, under supervision. The drink will contain 160 Calories, which will provide you with plenty of energy after your workout. You will not be allowed to eat or drink anything other than water and the drinks we provide you for 90 minutes before, and two hours after, each training session. The drinks will consist of a combination of any or all of the following: maltodextrin, whey protein, vegetable gum. If you are allergic to any of these things, you should not participate in this study.

You will be provided with a diary to use throughout the program. In this diary you will be asked to record your exercise, as well as how carefully you stuck to the timing of your eating and drinking around each exercise session. Periodically throughout the program you will also be asked to keep a detailed food record for three days at a time. The food record is to make sure you do not lose too much weight during the exercise program.

After the last training session, you will repeat the muscle biopsy procedure in which a small piece of muscle will be obtained from both of your thighs (one in each), and a small blood sample (approximately 10 ml) will be taken from an arm vein.

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
You must not have any heart disease, diabetes or other major illness. You also must not have or have had any problems with bleeding, or be on medication that prolongs bleeding time, or are on hormone replacement therapy or other medications known to alter metabolism. Because smoking is known to affect the factors being investigated, you can not be a smoker.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?
These procedures are all low risk in a healthy population.

During exercise testing (cardiac screen, VO2max) there is a small risk (less than 1 in 10 000) of morbidity or mortality due to a cardiac event. There are also risks of fatigue and muscle strains. You may experience temporary breathlessness or dizziness towards the end of the VO2max test. These feelings are transient and pass once the test is finished. During vigorous exercise (training, test of endurance capacity) there is also a risk of cardiac complications but in individuals with good cardiac health this risk is extremely low (1 in 1 000 000). There are also risks of fatigue, boredom, and muscle soreness. You are likely to experience temporary physical discomfort during the blood sampling procedure, the possibility of bruising or fainting, and an extremely slight risk of infection. Discomfort during the blood sampling is minor and brief (<30s) and bruising will not last more than one week.

If you have not participated in a regular exercise program before, you may experience some discomfort initially. In this instance, you will be encouraged to continue with the program as the feeling will be replaced with one of enhanced well-being as your fitness improves.

There is a small risk of infection at the site of the incision for the biopsy, and a small risk of the biopsy incision reopening or bleeding after you leave the lab. The risk of allergic reaction to lidocaine (the anaesthetic used for the biopsy incision and microdialysis insertion) is extremely low. You will have a small scar at the incision site. The rate and degree of healing varies considerably, but it is expected that scars will be difficult to see within 6-12 months after the procedure.

It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

ARE THERE ANY BENEFITS FROM TAKING PART IN THIS STUDY?

You will receive health and fitness benefits in the form of improved aerobic and endurance capacities. These improvements may have a positive effect on your functional independence, feelings of well-being, quality of life, and performance of work, sport and leisure activities. You may also experience decreases in body fat percentage, anxiety and depression, risk of cardiac arrest, and risk factors for coronary artery disease such as blood pressure and cholesterol.

DO I HAVE TO TAKE PART IN THE STUDY?

Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHAT WILL IT COST ME TO PARTICIPATE?

It will not cost you any money to participate in the study. Any treatment or medical costs that arise as a result of your participation in this study are your responsibility.

WHO WILL SEE THE INFORMATION THAT I GIVE?

We will keep private all research records that identify you, to the extent allowed by law.

Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.

In the researchers' records you will be identified according to either your initials or a number. For example, if John Smith was the first subject recruited his data will be labelled as either 1 or js. We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court.

CAN MY TAKING PART IN THE STUDY END EARLY?
If you find it difficult to keep to the eating schedule around your training sessions, or if you miss too many training sessions you may be removed from the study.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY?
You will receive free heart (if older) screening and maximal oxygen consumption tests, which would normally cost a substantial amount. You will be receiving an eight-week, supervised exercise program at no charge. Finally, at the completion of the study you will receive $300 for your participation, or $25 per muscle biopsy if you discontinue early.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH?
The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact our laboratory at 970-491-7913 or Dr. Benjamin Miller at 970-491-3291. If you have any questions about your rights as a volunteer in this research, contact Janell Barker, Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 4 pages.

_____ _____
Signature of person agreeing to take part in the study Date

_____
Printed name of person agreeing to take part in the study

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff

DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

MEDICAL AND EXERCISE HISTORY

NAME_____ GENDER_____ DATE_____

BIRTHDATE_____ AGE_____ HEIGHT_____ WEIGHT_____

ADDRESS_____

TELEPHONE_____ EMAIL_____

1. How often do you exercise? _____ times/week

2. Describe the intensity of your exercise (circle one)
   1 = none
   2 = light (e.g. casual walking, golf)
   3 = moderate (e.g. brisk walking, jogging, cycling, swimming)
   4 = heavy (e.g. running, high intensity sport activity)

3. What types of exercise do you engage in and how much do you do each session? (circle all that apply)
   1 = none
   2 = walking _____ km or minutes
   3 = jogging/running _____ km or minutes
   4 = swimming _____ meters or minutes
   5 = cycling _____ km or minutes
   6 = team sports (rugby, cricket, soccer, etc.) _____minutes _____ intensity
   7 = racquet sports _____minutes
   8 = weight training _____minutes _____# reps _____# sets
   9 = other _____

4. How much time per week do you spend exercising? _____ hours/week

5. Do you measure your heart rate during exercise? _____
   If yes:
   a. How high does it get during your typical workout? _____ beats/min
   b. What heart rate is maintained throughout most of your workout? _____beats/min

6. How long have you had a regular exercise program?_____

7. What condition or shape do you consider yourself to be in now (in terms of physical fitness)?
   1 = poor
   2 = fair
   3 = good
   4 = excellent

8. Do you or have you ever smoked? _____
   If yes: How long ago?_____ For how many years?_____ How many packs/day?_____

9. Has a close blood relative had or died from heart disease or related disorders (Heart Attack, Stroke, High Blood Pressure, Diabetes etc.)?
1=Mother
2=Father
3=Brother - Sister
4=Aunt - Uncle
5=Grandmother - Grandfather
6=None
If yes- Give ages at which they died or had the event and the problem they had.
_____
_____
_____

10. Have you ever had your cholesterol measured?
1=yes
2=no

If yes- write the date and value (or if it was normal or abnormal)
_____

11. Indicate which of the following apply to you (circle all that apply).
1 = high blood pressure
2 = high blood fats or cholesterol
3 = known heart disease or abnormalities
4 = stressful lifestyle at home or at work
5 = diabetes mellitus
6 = gout (high uric acid)

12. Any medical complaints now (illness, injury, limitations)?
1 = yes If yes, describe completely_____
2 = no _____
_____

13. Any bleeding disorders?
1 = yes If yes, describe completely_____
2 = no _____
_____

14. Are you allergic to lidocaine?
1 = yes
2 = no

15. Any major illness in the past?
1 = yes If yes, describe completely_____
2 = no _____
_____
_____

16. Any surgery or hospitalization in the past?
1 = yes If yes, describe completely_____
2 = no _____
_____
_____

17. Are you currently taking any medications (prescription or over-the-counter: including birth control)?
1 = yes If yes, list drugs and dosages _____

2 = no _____

_____

_____

18. Are you allergic to any medications?
1 = yes If yes, list medications_____
2 = no _____

_____

_____

19. Do you now have, or have you ever had, any of the following? (circle all that apply)
1 = heart murmurs
2 = any chest pain at rest
3 = any chest pain upon exertion
4 = pain in left arm, jaw, neck
5 = any palpitations
6 = fainting or dizziness
7 = daily coughing
8 = difficulty breathing at rest or during exercise
9 = any known respiratory diseases

Please describe fully any items you circled_____

_____

_____

_____

21. Do you now have, or have you ever had, any of the following? (circle all that apply)
1 = any bone or joint injuries
2 = any muscular injuries
3 = muscle or joint pain following exercise
4 = limited flexibility
5 = any musculoskeletal problems which might limit your ability to exercise

Please describe fully any items you circled_____

_____

_____

_____