MAPK Signaling and Intracellular Hormone Receptor Phosphorylation at Rest and Following Resistance Training

By

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ABSTRACT

Mitogen-activated protein kinases (MAPK) ERK, JNK, and p38 are a family of signal transduction proteins that respond to diverse forms of physiological stress including exercise. MAPK appear to mediate several adaptations to exercise skeletal muscle however most of their data are limited to cell culture, animal or endurance training study designs. Increasing evidence suggests they mediate translational efficiency and capacity. Further, recent data indicates they can regulate the steroid androgen (AR) and glucocorticoid (GR) receptors via phosphorylation. However, there are no data investigating AR and GR phosphorylation in humans or their response to resistance exercise (RE). Thus, understanding how MAPK, AR, and GR phosphorylation respond during resistance exercise will give us better insight to their specific roles in muscle adaptation. This dissertation investigated the activation of MAPK and epinephrine receptor signaling to a caffeine containing pre-workout supplement during resistance exercise. Further we also described MAPK expression and phosphorylated AR and GR in resting skeletal muscle between males and females. Finally we conducted a training study to determine differences in MAPK, AR, and GR phosphorylation following a period of stressful resistance training. The results of this dissertation indicates 1) pre-workout supplementation improves performance but does not enhance MAPK or epinephrine receptor signaling early after RE, 2) males and females express different expression of phosphorylated AR, GR, and p38 at rest, 3)

MAPK and phosphorylated AR and GR are activated after acute RE and are differentially regulated following a period of short-term stressful training.

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Chapter 1

INTRODUCTION

Resistance exercise (RE) increases skeletal muscle health, quality of life, and athletic performance. As such, decades of research have sought to determine strategies to maximize and optimize the adaptions to RE (Hawley et al 2011; Jäger et al. 2017). The adaptations in muscle plasticity from RE occur at the myocellular level (Spiering et al. 2008). A plethora of research has supported the use of protein supplementation to improve accretion of muscle mass to a resistance-training (RT) program (Hawley et al. 2011). Increasing muscle size and strength has implications for improved force-producing capacities for sport performance. In particular, acute supplementation with caffeine or caffeine containing pre-workout supplements has been shown to improve various components acute muscular performance (Gonzalez et al. 2011), but the molecular consequences of supplementation in humans after RE is largely unstudied.

Endurance (Watt et al. 2003) and RE studies indicate the use of caffeine-based supplements improve time to exhaustion, local muscular endurance (Beck et al. 2006; Duncan 2012), muscular power (Gonzalez et al 2011), mood (Duncan et al. 2012b), and indices of muscle damage (Hurley et al 2013). Many of these improvements in acute exercise performance might arise from increases in the catecholamines epinephrine (EPI) and

norepinephrine (NE). Catecholamines exert their effect on muscle via binding to β_2 adrenergic receptors (β_2-AR) at the surface of the sarcolemma, and influence molecular signaling cascades regulating anabolism (mitogen-activated protein kinases; MAPK) and metabolism (cyclic adenosine monophosphate; cAMP) (Sato et al. 2011). Activation of cAMP via β_2-AR leads to phosphorylation of protein kinase A (PKA), hormone sensitive lipase (HSL) and glycogen phosphorylase, subsequently increasing energy availability within contracting muscle tissue (Cargnello and Roux 2011). Thus intracellular signaling during and after exercise regulate performance and adaptation.

Mitogen-activated protein kinase (MAPK) molecules are known mechanisms of signal transduction, such that growth factors, cytokines, mechanical forces and cellular oxidative stress initiate parallel signaling cascades, and regulates muscle adaptation to exercise (Long et al. 2004). The three most studied MAPK are extracellular signal-regulated kinase 1 and 2 (ERK), c-jun NH_2-terminal kinase (JNK), and p38-MAPK (Cargnello and Roux 2011). ERK is involved in muscle contraction induced myofiber hypertrophy and maintenance of muscle mass (Haddad and Adams 2004; Shi et al. 2009; Wu et al. 2000). JNK and p38-MAPK are referred to as stress-activated protein kinases since these MAPK are activated by mechanical tension, inflammatory cytokines, and oxidative stress (Cargnello and Roux

2011). JNK phosphorylation is quantitatively related to the intensity and duration of RE load placed on muscle tissue during exercise (Boppart et al. 1999; Gehlert et al. 2015), and is required for cellular remodeling (Dhanasekaran and Reddy 2008; Khurana and Dey 2004). p38-MAPK is a mediator of PGC-1α activation, mitochondrial biogenesis, and improves substrate oxidation capacities of skeletal muscle (Gibala et al. 2009; Hawley et al. 2014). Thus, the MAPK family integrate numerous exercise stimuli to gene transcription and muscle adaptation (Spiering et al. 2008).

Recently, cancer (Koryakina et al. 2014) and C2C12 skeletal muscle myotube (Kim and Lee 2009) cell lines suggest androgen receptors (AR) are activated in the absence of testosterone. Ligand-independent activation of AR and glucocorticoid receptors (GR) occur in part via the mitogen-activated protein kinase (MAPK) signaling cascade activate AR and GRs by phosphorylation, even in absence of testosterone and cortisol (Galliher-Beckley et al. 2011; Kim and Lee 2009). The proteins ERK, JNK, and p38-MAPK modify the phosphorylation of the AR and alter AR protein expression in myotubes (Kim and Lee 2009). The phosphorylation of AR and GR at specific residues regulates receptor function and may influence eventual muscle phenotype response (Galliher-Beckley et al. 2011, Kim and Lee 2009, Koryakina et al. 2014). The modulation of the acute RE program variables could promote specified signaling and fiber adaptation following RE

(Figueiredo et al. 2015; Fry 2004, Gehlert et al. 2015; Spiering et al. 2008). However, there are no data investigating the MAPK influence on the AR and GR in humans despite their role initiating muscle growth, regeneration, and remodeling. Furthermore, increasing evidence suggest profound crosstalk between MAPK, Akt/mTOR, and AR signaling (Figueiredo et al. 2015, West et al. 2016; Liu et al. 2013). It is likely these pathways coordinate signaling responses to produce specified gene transcription and muscle adaption. Yet in humans, it is unclear how the cumulative activation of these pathways (MAPK, AR, GR, B2AR mTOR) converge and influence RE adaptions.

The integration of hormonal receptors and MAPK activation in non-canonical or ligand-independent signaling deserves further investigation. To date, most studies investigating cross-talk between these receptors (B2AR, AR and GR) and MAPK have done so using murine or cell culture models. It is unclear how the integration of these pathways (if any) occurs in exercising humans. Furthermore, even if caffeine or pre-workout supplementation improves RE performance, it is not known how is this reflected in the local myocellular level. If caffeine supplementation does indeed improve exercise performance via a catecholamine related mechanism, it is not clear how their respective receptors are regulated (β_2-AR).

Numerous cell culture and human models report the magnitude and duration of chronic stimulation of β_2-AR via agonists results in a receptor

down-regulation and desensitization (Fry et al. 2006, Sterczala 2017; Fan et al. 2016). This point relates to overtraining since stressful training down-regulates or decreases the sensitivity of β_2-ARs and could contribute to sustained performance decrements. Thus it is curious if chronic supplementation with caffeine or pre-workout supplements improves performance short-term, with potential detrimental effects with long-term supplementation. Decades of research has sought to determine if testosterone and cortisol are indicators of the anabolic/catabolic milieu during intense training. However, recent literature has implicated the testosterone cortisol ratio a marker of exercise intensity rather than overtraining. As mentioned earlier the primary actions of testosterone and cortisol occur via interaction with their intracellular receptors. A clearer understanding of the hormonal consequences of overtraining may be better understood by studying their respective intracellular receptors. We have shown resting expression, and activation of MAPK are differentially regulated following overreaching versus overtraining (Nicoll et al. 2016). Altered MAPK phosphorylation might regulate AR and GR both at rest and after exercise, but this has not yet been investigated in the literature.

Finally, RE studies primarily utilize male subjects for their investigations. There are very little data concerning sex-based differences in cell-signaling responses to resistance exercise. Acute measures of muscle

protein synthesis after RE are not different between males and females (West et al. 2012). Yet males respond with potentiated mTOR activation and AR expression post RE (West et al. 2012; Vingren et al. 2009). On the contrary, sprint interval training produced higher mTOR/p70s6K activation in females compared to males (Esbjörnsson et al. 2012). Most of these investigations have solely focused on the mTOR pathway, despite a growing body of evidence indicating MAPK converges on the mTOR pathway at numerous stages (Figueiredo et al. 2015, Gehlert et al. 2015; Martin et al. 2014; West et al. 2016). Moreover, although males express greater AR content, females have greater GR expression (Vingren et al. 2009). Thus, more research is needed to understand the physiological differences in muscle metabolism between males and females. Perhaps, the relative improvement in muscle adaptation (strength, hypertrophy, power) from RE is similar, but the pathways that influence the eventual adaptation may be different. The receptor pathways involved in sex-specific muscle adaptation to RE remain to be fully elucidated.

PURPOSE

The purpose of this dissertation was to investigate the integration of the MAPK signaling pathway (ERK, JNK, p38) on β_2-AR, AR, and GRs. To accomplish this, three studies were performed to investigate MAPK and receptor phosphorylation at rest (three studies) and within ten minutes of the end of a RE protocol (two studies). One of the studies was a training study to determine if there were changes in MAPK, AR, and GR expression following a resistance-training period.

Study I: **The effects Pre-workout Supplementation on β_2-Adrenergic and MAPK Signaling Pathways**

Purpose Study I

Determine the effect of a caffeine containing pre-workout supplement on B2-adrenergic receptor activation, anabolic signaling, and muscular performance.

Hypotheses Study I

Hypothesis #1: Pre-workout supplementation will significantly alter β_2 adrenergic receptor phosphorylation, and increase PKA and MAPK (ERK, JNK, p38) activation compared to the placebo condition.

Hypothesis #2: MusclePharm Assault™ supplementation will significantly increase muscular power, velocity, and repetitions until failure compared to the placebo condition

Dependent Variables Study I

1. Phosphorylated beta2-adrenergic receptors:

    p-β_2-AR (ser345/346; PKA site)

    p-β_2-AR (ser355/356; GRK site)

2. Total β_2AR expression

3. pPKA- Catalytic subunit (thr197)

4. MAPK

    Phosphorylated ERK

    Phosphorylated JNK

    Phosphorylated p38

5. Epinephrine

6. Lactate

7. Muscular performance

    Barbell Squat Power

    Barbell Squat Velocity

    Muscular Endurance (repetitions until failure)

8. Rating of Perceived Exertion

Study I Design

Randomized, counter-balanced, double-blind, placebo-controlled, within-subject crossover study

Delimitations

Ten resistance-trained males (mean±SD, age=22±2.4 yrs, hgt=175±7 cm, body mass=84.1±11.8kg) participated in this study. Subjects were between the ages of 18 and 30, healthy, non-obese (BMI <28 kg/m2), non-smoking, and free of metabolic cardiovascular, kidney diseases, as well as free of a history of seizures. Subjects were screened for participation using a health-history questionnaire for contraindications to exercise by American College of Sports Medicine (ACSM) guidelines. Subjects who were currently taking any medications or supplements that may interact with ingredients in the supplement/placebo involved in this study were excluded from the study. To participate in this study subjects were considered healthy and recreationally resistance trained defined as having performed resistance exercise 3 or more days /week for at least 1 year, with at least two years of training history and were familiar with barbell back squat technique and performed lower body resistance exercise at least once per week for the six months prior to the start of the study.

Assumptions

1. Subjects accurately answered the health history questionnaire
2. All equipment utilized for testing sessions functioned properly for all testing sessions.

Study II

Sex Based Differences in MAPK Steroid Receptor Phosphorylation in Human Skeletal Muscle

Purpose Study II

Determine if there is differential expression and phosphorylation of AR, GR, and MAPK at rest between males and females.

Hypotheses Study II

Hypothesis #1: Total androgen receptors will be greater in males. Total glucocorticoid receptors will be greater in females.

Hypothesis #2: Phosphorylated androgen receptors will be greater in males. Phosphorylated glucocorticoid receptors will be greater in females.

Hypothesis #3: Phosphorylated and total MAPK (ERK, JNK, and p38) will be similar between males and females.

Dependent Variables Study II

1. Androgen Receptors

    Total Androgen Receptor

    Phosphorylated Androgen Receptor at ser81

    Phosphorylated Androgen Receptor at ser213

    Phosphorylated Androgen Receptor at ser515

    Phosphorylated Androgen Receptor at ser650

3. Glucocorticoid Receptors

    Total Glucocorticoid Receptor

    Phosphorylated Glucocorticoid Receptor at ser134

    Phosphorylated Glucocorticoid Receptor at ser211

    Phosphorylated Glucocorticoid Receptor at ser226

4. MAPK

    Total and phosphorylated ERK

    Total and phosphorylated JNK

    Total and phosphorylated p38

Study II Design: Cross-sectional

Assumptions

1. Subjects accurately answered the health history questionnaire
2. All equipment utilized for testing sessions functioned properly for all testing sessions.

Study III

MAPK, Androgen, and Glucocorticoid Receptor Phosphorylation Following High-Frequency Resistance Exercise Overtraining

Purpose Study III

Determine if there is differential resting expression and phosphorylation of AR, GR, and MAPK and post exercise responses following normal training and stressful training

Hypotheses Study III

Hypothesis #1: Total androgen and glucocorticoid receptors will not change in either group. MAPK signaling will not change in the control group but will be higher in OT.

Hypothesis #2: Phosphorylated androgen receptors will be greater in higher in CON after the training period. Phosphorylated glucocorticoid receptors will be higher in OT after the training period.

Dependent Variables Study III

1. Androgen Receptors

Total Androgen Receptor

Phosphorylated Androgen Receptor at ser81

Phosphorylated Androgen Receptor at ser213

Phosphorylated Androgen Receptor at ser515

Phosphorylated Androgen Receptor at ser650

3. Glucocorticoid Receptors

   Total Glucocorticoid Receptor

   Phosphorylated Glucocorticoid Receptor at ser134

   Phosphorylated Glucocorticoid Receptor at ser211

   Phosphorylated Glucocorticoid Receptor at ser226

4. MAPK

   Total and phosphorylated ERK

   Total and phosphorylated JNK

   Total and phosphorylated p38

5. Hormonal Concentrations

   Testosterone

   Cortisol

6. Muscular Performance

   Barbell Back Squat Power

   Barbell Back Squat One Repetition Maximum Strength

   Bilateral Knee Extension One Repetition Maximum Strength

Study III Design: 2 X 2 X 2 (Group X Training X Time) Between group factorial

Assumptions

1. Subjects accurately answered the health history questionnaire
2. All equipment utilized for testing sessions functioned properly for all testing sessions.
3. Subjects will ensure maximal effort during all training sessions.

Chapter 2

Literature Review

MAP-Kinase signaling: "Mapping" the roles of MAPK phosphorylation to resistance exercise adaptation.

1. Introduction

Chronic resistance exercise (RE) increases muscular hypertrophy, strength, contraction velocity and structural architecture (Franchi et al. 2014). These phenotypic adaptations improve sport performance as well as quality of living and successful aging. While a plethora of research supports the use of RE to improve muscular adaptations for sport and quality of daily living, the molecular underpinnings of these adaptations remain less clear. Advancements in molecular biology, and their application to the field of exercise physiology have elucidated numerous cellular signaling pathways that may contribute to exercise adaption.

The foremost studied of these pathways is the protein kinase B (Akt) / mammalian target of rapamycin (mTOR) cascade (Spiering et al. 2008). Relevant data from the last two decades has established this pathway as a central regulator of nutrient sensitivity, protein synthesis, and mechanotransduction (Hawley et al. 2014). However, there is a growing body of literature suggesting another family signaling proteins called the mitogen-activated protein kinase (MAPK) pathway converges on the Akt/mTOR and regulates many of its downstream substrates (Figueiredo et al. 2015, Liu et al.

2013; Martin et al. 2014; Miyazaki et al. 2011; Roux et al. 2007; West et al. 2016; Williamson et al. 2006). Furthermore, MAPK are capable of directly entering the cell nucleus and regulate gene expression (Cargnello and Roux 2011; Long et al 2004). Thus, the activity of MAPK and their convergence on the Akt/mTOR pathway integrates numerous extracellular signals into a specific response to elicit a specified phenotype (Shi et al. 2008), and the activation of both mTOR and MAPK pathways may be required for a complete protein synthetic response post RE (Drummond et al. 2009)

In vitro and animal models suggest multiple nodes of regulation between MAPK and mTOR signaling cascades (Roux et al. 2007; West et al. 2016). However, investigation in humans and intact skeletal muscle requires further elucidation. Recent evidence indicates differential MAPK/mTOR signaling depends on the resistance exercise load, volume (i.e. number of contractions), and rest intervals (Ghelert et al 2015; Tersiz et al 2010; Burd et al. 2010; McKendry et al. 2016). Further, their responses also depend on the training status of the individual (i.e. untrained versus highly trained) (Coffey et al. 2006; Gonzalez et al. 2016). The differential activation of these pathways may be related to the altered differential rates of protein synthesis, recovery, and translational capacity reported between resistance training naïve and resistance training accustomed individuals (Tang et al. 2008, Wilkinson et al. 2008). Finally, the MAPK pathway has also been shown to integrate and

regulate steroid hormone receptors via phosphorylation (i.e. activation) independent of hormonal concentration (Kim and Lee 2009; Zheng et al. 2017).

Thus the purpose of this review is to 1) Synthesize the results of MAPK phosphorylation following RE with an emphasis on human subjects. 2) Highlight the role MAPK activation my have on RE skeletal muscle adaptation. 3) Integrate how MAPK activation may have differential roles depending on training history/stimulus (untrained→ recreational training→ highly trained→ stressful training). 4) Highlight emerging areas of research related to MAPK phosphorylation and expression in canonical and non-canonical signaling that may prove useful in understanding skeletal muscle adaptation.

2. Classical MAPK signaling cascades

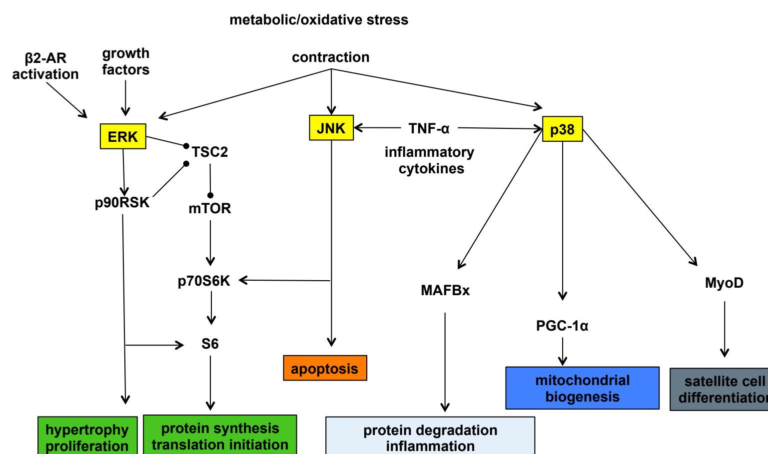

Figure 2-1 Representative diagram of the interaction of MAPK ERK, JNK, and p38 with their upstream extracellular stimuli, down stream substrates, and implications for muscle adaptation.

Mitogen-activated protein kinase (MAPK) molecules regulate signal transduction, such that growth factors, cytokines, mechanical forces and cellular oxidative stress initiate parallel signaling cascades, and regulates muscle adaptation to exercise (Long et al. 2004). The three most studied MAPK are extracellular signal-regulated kinase 1 and 2 (ERK), c-jun NH_2-terminal kinase (JNK), and p38-MAPK (Cargnello and Roux 2011). ERK is involved in muscle contraction induced myofiber hypertrophy and maintenance of muscle mass (Haddad and Adams 2004; Shi et al. 2009; Wu et al. 2000). JNK and p38-MAPK are referred to as stress-activated protein kinases, since these MAPK are activated by mechanical tension, inflammatory cytokines, and oxidative stress (Cargnello and Roux 2011). JNK phosphorylation is quantitatively related to the intensity and duration of RE load placed on muscle tissue during exercise (Boppart et al. 1999; Gehlert et al. 2015), and is required for cellular remodeling (Dhanasekaran and Reddy 2008; Khurana and Dey 2004). p38-MAPK is a mediator of PGC-1α activation, mitochondrial biogenesis, and improves substrate oxidation capacities of skeletal muscle (Gibala et al. 2009; Hawley et al. 2014). Thus, the MAPK family integrate numerous exercise stimuli to gene transcription and muscle adaptation (Spiering et al. 2008).

The ERK pathway is stimulated via growth factors, muscle contraction, and beta2-adrenergic receptor (β_2-AR) activation (Haddad and Adams 2004; Shi et al. 2007; Widegren et al. 2000). The down stream substrate of ERK is

p90RSK. The activation of ERK and p90RSK, in general have been related to increases in cell growth and proliferation (Anjum and Blenis 2008). Increasing evidence points to ERK signaling as a key component to the translation initiation (Williamson et al. 2006) and muscle cell growth (Haddad and Adams 2004; Shi et al. 2009).

JNK and p38 are typically referred to as stress activated protein kinases and are activated my similar extracellular stimuli since apoptosis signal-regulating kinase 1 (ASK1) is a common upstream signaling component (Ichijo et al. 1997). JNK is highly mechanosensitive (Boppart et al. 1999; Martineau et al. 2001); however JNK can also initiate apoptosis from excessive stretch (Tan et al. 2009). Conversely JNK is also required for myogenesis and muscle differentiation (Khurana and Dey 2004). Thus, the role of JNK in adaptation is pleiotropic in regulating growth and death cellular pathways. p38 is similarly activated via contraction; however it plays many roles in muscle adaptation including the production of cytokines, increases in mitochondria, and metabolic fluctuations during exercise (Combs et al. 2012).

All of the aforementioned responses of MAPK ultimately lead to the activation of the early immediate genes in the cell nucleus. Skeletal muscle cells poses early immediate genes c-fos, c-myc, and c-jun (Kami et al. 1995). Resistance training in the untrained and trained state activate early immediate

genes (Trenerry et al. 2011) although chronic training may attenuate some of these responses (Nader et al. 2014). The activation of ERK or its down stream substrates induces interactions transcription factors and early immediate genes including c-fos and c-myc. Similarly cyclical stretch induces the activation of early immediate genes and subsequent myogenic gene transcription (Ikeda et al. 2003)

3. Role in Fiber Adaptation

Protein synthesis/hypertrophy

Early studies indicated MAPK phosphorylation increases during muscular contraction (Widegren et al. 2000), and RE activates numerous transcription factors and early immediate genes (Trenerry et al. 2011). Since MAPK are activated by contraction, the logical conclusion suggested that they may be involved in protein synthesis in muscle hypertrophy. However, early studies have primarily utilized IGF-1 administration to investigate the role MAPK may contribute to adaptation (Haddad and Adams 2004). In particular, their role in mechanotransduction suggested that they might be involved in contraction mediated muscle hypertrophy (Boppart et al. 2001; Carlson et al. 2001; Martineau et al. 2001; Wretman et al. 2001). Of these, ERK has been the most investigated MAPK in relation to muscle hypertrophy. Phosphorylation increases from muscle contraction the presence of insulin like growth factor in metabolic byproducts (Haddad and Adams 2004; Ohno et

al. 2018; Yuan et al. 2017). While the primary regulator of protein synthesis appears to be mTOR downstream substrates including p70S6K and S6 protein, the ERK pathway can phosphorylate the aforementioned proteins numerous steps during signal transduction (Zhang et al. 2013). Although there have been correlations between p70S6K phosphorylation and muscle hypertrophy (Tersiz et al. 2008) there have been no direct correlation between the ERK pathway and muscle growth in humans. Instead it appears that contraction induced activation of ERK may “prime” the translational machinery for translation initiation and elongation (Williamson et al. 2006). Further, besides the mechanism of increasing ribosome assembly and translation initiation, the activation of early immediate genes from MAPK further regulates increases in ribosomal biogenesis (Brook et al. 2016; Stefonovsky et al. 2001). Thus, the short-term activation post exercise to increase indices and assembly of ribosomal proteins for protein synthesis, also activate translational capacity. This increase in ribosome biogenesis from mechanical overload appears to be mTOR independent (Goodman et al. 2011; West et al. 2016) and suggestive of ERK/MAPK involvement. Importantly the MAPK pathway appears necessary for the maintenance of muscle mass via ERK and JNK by sensitizing and integrating contraction and IGF-1 mediated stimuli into muscle hypertrophy (Haddad and Adams 2004; Martin et al. 2014; Shi et al. 2009). Although it is clear now that increased ribosome biogenesis from chronic RE supports enhanced translational

capacity (Brook et al. 2016; Figueiredo et al. 2015), in vitro data suggests ribosome biogenesis alone is not the sole determinant of IGF-1-mediated of muscle hypertrophy (Crossland et al. 2017).

Myogenesis

Recovery from muscle contraction in damage from resistance exercise activates a quiescent stem cell pool in skeletal muscle termed satellite cells (McCarthy et al. 2011). Satellite cells respond to acute resistance exercise and the satellite cells themselves are involved in muscle regeneration known as myogenesis (Bellamy et al. 2014). Studies investigating short term and long-term satellite cell responses to resistance exercise indicate an activation and expansion of satellite cells in the early 24 to 72 hours following and exercise bout (Damas et al. 2018). In addition, the degree of long-term muscle hypertrophy from resistance exercise training is in part genetically determined via the recruitment of and activation satellite cells (Petrella et al. 2008). Individuals who display the greatest degree of muscle hypertrophy following a period of RT have a larger satellite so pool prior to the training intervention, and can expand their satellite cell pool to a much greater extent following successes bouts of exercise (Petrella et al. 2008). Macaluso and colleagues (2012) showed that increasing aerobic capacity was also accompanied by an increase in satellite cell number in trained men. Further,

while those subjects had higher satellite cell content they also displayed lower resting levels of p38.

The activation of the satellite cells and the progression from proliferation to differentiation from myoblasts to myotubes are dictated by MAPK. In vitro data has indicated ERK, JNK, and distinct isoforms of p38 are differentially expressed throughout the time course of myogenesis (Wu et al. 2008). The most important MAPK in during myogenesis is p38. Following the activation of satellite cells, ERK and JNK regulate the proliferation of satellite cells, while p38 decreases the activity of ERK and JNK to allow differentiation of myoblasts into myotubes (Khurana and Dey 2002). The activation of p38 induces differentiation while inhibition of p38 prevents differentiation. On the contrary, ERK inhibits myogenic transcription in myoblasts, but activates transcription during hypertrophic growth after differentiation in myotubes (Khurana and Dey 2002; Wu et al. 2000). Thus, the activation of MAPK contribute differential roles during the course of muscle regeneration such that ERK increases proliferation prior to differentiation, while p38 increases throughout differentiation. Although the induction of satellite cells appears to be an important factor in the regeneration of muscle tissue following exercise murine an in vitro model suggests that satellite cells are not the only contributing factor that regulate regeneration and muscle hypertrophy. The depletion of satellite cells in in mouse cells indicates that fibers can still

hypertrophy in the absence of satellite cells following mechanical overload (McCarthy et al. 2011).

Fiber Type Composition

Specificity of resistance training elicits specific adaptations in skeletal muscle (Pareja-Blanco et al. 2017). Myosin heavy chain (MHC) isoform expression changes during chronic training (Fry 2004). Normally there is a shift from MHCIIx to MHCIIA following chronic training. Cell culture models indicate MAPK may mediate MHC isoform expression (Meissner et al. 2007; Meissner et al. 2011). Immobilization or decreased contractile activity increases MHCIIx expression. High velocity resistance exercise with increasing amounts of fatigue decreases MHCIIx expression (Pareja-Blanco et al. 2017). However, decreased MHCIIx expression also increases muscle hypertrophy due to additional total volume performed (Pareja-Blanco et al. 2017). Furthermore, evidence by Andersen et al. (2018) suggests only two successive training bouts can silence the MHCIIx gene for up to four days after loading. The presence of basal 38 phosphorylation is necessary for the maintenance of MHCIIx/d isoform expression in this mouse skeletal muscle cells (Meissner et al. 2007) and inhibition of p38 results in a slow isoform phenotype.

The role of ERK in skeletal muscle MHC expression is less clear. Murgia and colleagues (2000) concluded that the activation ERK promotes slow muscle phenotype in regenerating muscle. Conversely Shi et al. (2007) reported greater ERK expression in fast muscle fibers, and more importantly, the presence of ERK signaling actually promoted the fast phenotype (Shi et al. 2008). Thus, the role of ERK in muscle phenotype expression is not clear. Basal activity of p38 is necessary for MHCIIx expression and inhibition of p38 decreases MHCIIx expression in favor of MHCIIA a more oxidative phenotype. Similarly, increases in mitochondrial biogenesis occur through the AMPK/p38/PGC-1alpa signaling pathway (Zhang et al. 2014). Together, the limited evidence available on MAPK and fiber phenotype, indicate their role, at least partially in fiber adaptations.

4. Acute Resistance exercise Studies

Of the three MAPK investigated in RE adaptations, ERK is the most studied, likely because of evidence indicating the role of the ERK pathway's role in muscle growth (Shi et al. 2009, Haddad and Adams 2004), translation initiation (Roux et al. 2007), and ribosome biogenesis (Stefonovsky et al. 2001; Zhang et al. 2013). The activation of ERK and downstream substrates are dependent on contraction load, mode, volume, and metabolic stress accumulated during the RE bout (Ahtianen et al. 2015; Franchi et al. 2014; Gehlert et al. 2015; Hulmi et al. 2012; Holm et al. 2010; Popov et al. 2015).

Although any one of the acute RE program variables can influence ERK activation a combination of the acute program variables likely elicits an increase in phosphorylation. Indeed, there may only need to be minimum threshold of volume or intensity needed to produce and increases in phosphorylation from rest.

Taylor and colleagues (2012) reported an increase in post-exercise phosphorylated ERK after subjects performed four sets 8-10 repetitions at 80-85% 1RM (high intensity) or 18-20 repetitions at 60-65% 1RM (low intensity) in the leg press. While there was a pronounced ERK activation post-exercise, there was no difference between the high and low intensity conditions. This directly conflicts with Holm et al. who reported potentiated ERK phosphorylation in the high intensity (70 %1RM) versus low intensity (16% 1RM) in 10 sets of knee extensions. Thus, the disagreement between studies is not clear; however the additional volume performed by the low intensity protocol in Taylor et al. (2012) may have contributed to the increase in ERK.

Additional volume as an anabolic signaling cue may be supported by results from Burd et al. (2010), who reported an increase in ERK activation post exercise if knee extensions were taken to failure at 30%1RM, but not if they were performed at 30%1RM and work matched to a 90%1RM work load. Yet, Ahtianen et al. (2015) reported similar ERK phosphorylation after 10 sets

of 10RM compared to 5 sets of 10RM. Furthermore, we reported an increase in ERK activity in barbell back squats when performed at 90% 1RM (5 sets of 3 repetitions), but not at 30% 1RM (5 sets of 10 repetitions) (Kudrna et al. 2017). These data suggest the activation of ERK during RE requires a minimum load at controlled repetition ranges, and in this case, higher intensities and additional volume does not potentiate a greater activation of ERK. However, there may also be a minimum threshold of volume that must be performed at lower intensities (Burd et al. 2010; Holm et al. 2010; Hulmi et al. 2012; Tersiz et al. 2010), and the additional volume that contributes to the activation ERK and downstream substrates likely occurs through additional time under tension (Burd et al. 2012) or additional metabolic stress (Ohno et al. 2018; Popov et al. 2015; Wretman et al. 2001).

In reference to RE responses, JNK is the least studied of the three MAPKs. Following RE the phosphorylation of JNK increases substantially, and the exercise induced increase appears to be dependent on the external load applied during contraction (Gehlert et al. 2015). Of the three MAPKs, JNK is the most mechanosensitive, suggesting greater eccentric loading potentiates JNK activation (Martineau and Gardiner 2001). The potentiation of JNK post RE does not arise from greater time under tension, since there was greater JNK under heavy eccentric loading even when loads were equalized for time under tension (Gehlert et al. 2015). The JNK phosphorylation during

RE occurs early in the RE exercise session since Galpin et al. (2012) reported JNK activation increased midway through (8th set) an acute exercise session consisting of fifteen sets of three repetition clean pulls. Indeed the mechanosensitivity of JNK may be an essential component of adaptation to RE since overload induced JNK phosphorylation does not decrease with aging (Hornberger et al. 2005) or years of chronic training (Gonzalez et al. 2017).

Murine models also suggest JNK can modulate p70S6K phosphorylation at the auto-inhibitory domain (Thr421/Ser424) during contraction and in the presence of growth factors (Martin et al. 2014). Activation at Thr421/Ser424 is activated to a greater extent after maximal eccentric contractions (but not submaximal) compared to maximal concentric contractions (Eliasson et al. 2006). Indeed the phosphorylation of p70S6K at Thr421/Ser424 is sensitive to ERK, p38, JNK, and FAK activation via mechanical stimuli (Deldicque et al. 2008; Klossner et al. 2009; Martin et al. 2014), and perhaps MAPK (particularly JNK and ERK) “prime” the phosphorylation of p70S6K from loading during RE, that enhances MPS in and translation in the presence of amino acids and nutrition.

A study by Gonzales et al (2017) indicated there was similar JNK phosphorylation when trained subjects performed a high volume versus high

load RE bout. Although volume and loads were different between conditions, but JNK activation was similar. Perhaps one of the other functions of JNK activity after RE, aside from its mechanosensitive properties, is a signal for potential muscle damage. The exposure of rat muscles to a repeated bout of injurious exercise attenuated JNK activity compared to muscles that had not experienced injurious exercise (Takagi et al. 2018). Muscle damaging eccentric RE increases JNK to a greater extent than concentric RE (Boppart et al. 1999). Perhaps the acute JNK in trained individuals, who are more resistant to DOMS and muscle damage to RE, could contribute to hypertrophic signaling. In this case, adequate RE load or volume may be required to induce a mechanotransductive signal for hypertrophy. On the contrary, RE naïve subjects, who experience more severe symptoms of DOMS, may experience a potentiated JNK response from RE that may transduce a signal for muscle damage rather than hypertrophy (Boppart et al. 2006), and over time this response my shift from inflammatory/apoptotic to hypertrophic. Despite this hypothesis, additional work in humans is required.

The most well described roles of p38 has been it's involvement in mitochondrial and myogenesis adaptations (Brien et al. 2013; Irrcher et al. 2003; Zhang et al. 2014). As mentioned earlier in this review, p38 regulates proteins involved in mitochondrial biogenesis and improving the oxidative and glucose transport characteristics in skeletal muscle (Hawley et al. 2014).

While these adaptions have been most frequently observed in aerobic metabolism, the role of p38 following muscle injury and regeneration has garnered considerable attention in its ability to regulate satellite cell differentiation (Wu et al. 2000). p38 activation and it's contribution to satellite cell differentiation and injury regeneration have been well described; however these roles likely occur over many days post exercise. Thus, discerning the role of acute p38 activation during RE is difficult due to its multi-faceted role and responses during muscle contraction.

p38's sensitivity to muscle contraction has been reported in both human (Holm et al. 2010) and rodent (Carlson et al. 2001; Wretman et al. 2001) models. In particular, RE volume does not influence the degree of p38 phosphorylation post-exercise. Performing additional sets of RE at 6RM (Tersiz et al. 2010) and 10RM (Ahtiainen et al. 2015) in lower body RE did not produce additional increases in p38 phosphorylation after exercise. Similar to ERK and JNK, p38 is sensitive to the degree external load applied during RE, indicating greater activation during heavy loads compared to light loads (Holm et al. 2010). Post exercise increases in p38 are greater in type II muscle fibers compared to type I muscle fibers (Tannerstedt et al. 2009). Unlike, ERK and JNK the influence of RE intensity on p38 activation is less investigated; however there appears to be a critical role in eccentric contractions as a strong stimulus for activation of this kinase (Franchi et al. 2014; Tannerstedt

et al. 2009). When untrained subjects performed 5 sets of 10RM leg press versus 15 sets of 1RM leg press, the alpha isoform of p38 increased similarly in both conditions; however the gamma isoform increased in both conditions but was greater in the 5 X 10RM protocol (Hulmi et al 2012). This point is unclear since the 5X10RM protocol produced an ~3 fold higher lactate response compared to 15X1RM. Wretman and colleagues (2001) reported p38 activation increased following eccentric contractions, but not metabolic perturbation in mouse muscle. Indeed, high-force contractions can couple p38-mTOR interactions that may occur separately from metabolic stress (Rahnert and Burkholder 2013).

While mechanical signals transmitted via MAPK onto the mTOR pathway may be expected, recent evidence from Willkomm et al. (2017) provided evidence lactate accumulation from a single set of unilateral high intensity knee extensions (HIT; no rest) attenuated p38 activation following exercise compared to three sets of knee extensions with rest between sets (STD). Lactate was higher in the first ten minutes of recovery in the HIT compared to STD, and resulted in attenuated p38 up to 4 hours post exercise in the HIT condition only. Follow-up analyses in muscle cell culture indicated lactate attenuates p38 activation and reduces muscle specific gene expression in the following hours of exposure. Thus, the contractile and

metabolic stress experienced by skeletal muscle during resistance exercise may cause specific muscle phenotypes.

5. Chronic Resistance Exercise Studies

The role ERK contributes to muscle adaptation has garnered considerable attention in recently, since continued evidence points to its role in ribosome biogenesis and potential role in contraction mediated regulation of modulating protein synthesis (Miyazaki et al. 2011). Resistance exercise increases MAPK activity in the immediate hours of recovery. However, the chronic changes in these responses and changes in total expression have not been frequently reported. Following chronic exercise training, the acute phosphorylation after exercise attenuates (Coffey et al. 2006; Ogasawara et al. 2013; Walker et al. 2013; Yu et al. 2003). The change in total MAPK expression remains unclear, might arise from resting total MAPK (overall capacity) and the amount phosphorylated (activity) fine tune their expression to regulate long-term skeletal muscle adaptations. In a cross-sectional study, Galpin et al. (2016) reported the amount of total ERK expression moderately increased after 12 weeks of circuit weight training compared to untrained controls, while high-level powerlifters and weight lifters who had >10 years of RT history showed lower total ERK expression relative to untrained controls. Ten days of overload in mice increased resting total ERK expression with no changes in phosphorylation status (Steiner et al. 2015). In chronically

endurance trained mice (Lee et al. 2002) and humans (Yu et al. 2001), total ERK expression is higher, and total p38 is lower than untrained controls respectively. This might suggest the conclusion that voluminous exercise (resistance or aerobic) leads to an increase in total ERK expression, however 21-weeks of RT does not alter total ERK expression in young or old men (Ahtianen et al. 2016). In winstar rats performing heavy voluminous hypertrophic RE, there were no changes in total ERK or p38 expression. However, phosphorylated ERK was greater in the hypertrophic RE than muscular endurance and heavy strength RE groups. Walker and colleagues (2013) reported no changes in total ERK expression in young men after 20 weeks of variable or constant resistance RE. They did report phosphorylated ERK was elevated at rest in the constant resistance RE group. Thus, while there were no changes in total content, the resting cellular environment was activated to a greater extent following training.

Acute exercise phosphorylation of exercise induced p38 and ERK phosphorylation is attenuated after chronic training (Coffey et al. 2006; Walker et al. 2013). Figueiredo et al. (2015) did not report attenuated ERK phosphorylation post exercise in men following 8 weeks (16 training sessions) of RT at 70% and 90% 1RM. On the contrary Brook et al. (2016), reported attenuated ERK signaling after only three weeks (9 training sessions) of RT at 75% 1RM. While, both studies (Brook et al. 2016; Figueiredo et al. 2015)

progressively increased loads during the RT programs, thus ensuring a progressive stimulus to training, only Brooks et al. reported attenuated signaling, likely because Brooks et al. (2016) utilized unilateral knee extensions, while Figueiredo et al. (2015) utilized bilateral leg press, knee extension, and knee flexion exercises. The additional exercises (leg press and knee flexion) likely added the volume necessary to elicit a signaling response even after 8 weeks of training. Thus, while an exercise novice may experience increased intracellular protein signaling (Coffey et al 2006), training accustomed individuals experience attenuated signaling responses (but highly specified; Karagounis et al. 2010), likely due to enhanced muscular adaptions reflected at rest.

There are limited data concerning JNK expression and phosphorylation following chronic RE. JNK is increased substantially after muscle damaging exercise (Touchberry et al. 2012). Repeated bouts of injurious exercise attenuates JNK phosphorylation post exercise (Takagi et al. 2018). However, in humans, the mechanosensitive qualities may be preserved following chronic training. Intense and stressful endurance exercise attenuates JNK phosphorylation in mice (Kim et al. 2015) and humans (Hinkley et al. 2017). However, while exercise induced attenuation of ERK and p38 have been observed in trained subjects (Coffey et al. 2006; Kudrna et al. 2017), JNK phosphorylation in RT subjects is maintained (Gonzalez et al. 2016; Gehlert

et al. 2015). More importantly, in trained subjects, JNK activation post RE is highest within the first 10-60 minutes post exercise, and subsequently declines to resting values there after (Galpin et al. 2012; Gonzalez et al. 2016; Gehlert et al. 2015). Yet in RE naïve, JNK activity increases immediately post exercise and may stay elevated up to 5 (Townsend et al. 2018) and 48 (Thompson et a. 2003) hours post exercise. Recreationally active men who performed 7.5 days stressful high power overreaching RE reported increased resting total JNK expression (Nicoll et al. 2016). However, changes in JNK total expression have not been frequently reported. Further, to our knowledge, there have been no long-term (>8 weeks) training studies investigating changes in JNK phosphorylation or expression to RE using a within subjects design. Although several studies have investigated JNK in reference to aerobic exercise (Hinkley et al. 2017; Boppart et al. 2000), further research is necessary in humans performing chronic RT to completely understand the pleiotropic role of JNK in skeletal muscle adaptation. Collectively, there may be differential roles for acute exercise induced activation of JNK following chronic training. Trained subjects who are more resistant to muscle damage maintain mechanosensitive responsiveness of JNK early in the recovery period. Untrained muscle may require extensive remodeling do to extensive exercise trauma and may therefore be elevated early in the post exercise period, and remain elevated at later time points to facilitate inflammatory processes required for recovery and remodeling.

Chronic RT attenuates the acute activation of p38 after RE. Despite attenuated acute exercise responses to familiar exercise, the resting phosphorylated expression of p38 appears to be highly responsive to chronic training. Chronic aerobic training appears to have a large influence of decreasing p38 activity, which might be related to enhanced oxidative or satellite cell capacities in chronically trained muscle (Ljubic and Hood 2009; Lee et al 2002; Macaluso et al. 2012; Yu et al. 2001). Macaluso et al. (2012) reported lower resting phosphorylated p38 in subjects who had higher satellite cell content. Conversely, the same subjects who had higher satellite cell content also had higher aerobic capacities. Those data suggest that on an individual level, chronic aerobic training increases satellite cell number and aerobic capacity, while concomitantly decreasing resting p38 phosphorylation. In reference to RE, 8 weeks of hypertrophy training in humans did not report changes in resting p38 phosphorylation immediately following training or after a period of detraining (Léger et al. 2006).

We have shown that following stressful short-term overreaching decreased resting phosphorylation of p38 (Nicoll et al. 2016). Finally, chronically trained runners with more than 14 years of training history actually showed increased p38 activity at rest compared to untrained controls (Nicoll et al. 2017). Of note, p38 activity post exercise is highly transient, and the

response may depend on the recovery strategies employed post exercise (Figueiredo et al. 2016) and the exercise modality itself (RE vs aerobic). p38 regulates satellite cell activity and differentiation (Segalés et al. 2016; Willkomm et al 2017). Chronic RT increases satellite cell content during myogenesis; however the time course in humans is not well defined. Further, the activity of p38 at different stages of myogenesis dictate satellite cell kinetics and commitment, during the days undergoing differentiation (Wu et al. 2000). Thus, the recovery responses of p38 post exercise, and the days following, may differ between chronic aerobic and resistance exercise.

Repeated bouts of exercise attenuate exercise-induced phosphorylation of MAPK proteins (Walker et al. 2013; Yu et al. 2003). While training decreases exercise induced responses, there are beneficial adaptations that remain in muscle chronically and training specifies intracellular signaling activation while concomitantly specifying muscle protein synthesis responses (Tang et al. 2008). Cumulatively, exercise induced responses in the trained state appear different relative to the untrained state (Tang et al. 2008; Wilkinson et al. 2008). Further, muscle metabolism at rest in the trained state also indicates enhanced resting rates of muscle protein synthesis and reduced protein breakdown (Holwerda et a. 2018; Reidy et al. 2017). Chronic resistance exercise increases in ribosome biogenesis (Figueiredo et al. 2015). At the same time, satellite cells proliferate and are

incorporated into the muscle fibers as new myonuclei following chronic RE (Bellamy et al. 2014; Petrella et al. 2008). Data are not clear as to the role of increased total protein expression; however increase total protein expression at the same relative phosphorylation ratio may indicate lesser requirement exercise induced to elicit translational efficiency responses. In addition, murine models suggest that a period of detraining can re-sensitize skeletal muscle signaling proteins to respond to resistance exercise (Ogasawara et al. 2013). For muscle hypertrophy, numerous intracellular adaptations may sustain enhanced hypertrophy. Further, changes in satellite cell activation, ribosome biogenesis, and translational efficiency may occur independently of each other at different time-courses as one goes from untrained to trained. Increased phosphorylation and a generalized protein synthetic response may occur early in resistance training history followed by increases in ribosome biogenesis to sustain enhanced translational capacity. Finally, satellite cell expansion and incorporation into the myofiber may ultimately sustain muscle hypertrophy and enhance the regeneration properties following in the trained state.

6. Excessive / Stressful Training

Normal resistance training (RT) decreases protein degradation and inflammatory signaling (Karagounis et al. 2010). In addition, repeated bouts of RT elicit beneficial adaptations that allow an increase in translational capacity and regeneration, including ribosome biogenesis and satellite cell expansion (Brook et al. 2016; Petrella et al. 2008). However, while adequate amounts of resistance exercise can prove fruitful for muscle adaptation, the molecular responses to excessive exercise is not well understood. Overreaching and overtraining are typically determined by a decrease or stagnation and muscular performance (Meeusen et al. 2013). While in the past it is been hypothesized that increases in circulating cytokines are indicative of overtraining, actual evidence at the skeletal muscle level in humans remains lacking (Halson et al. 2003; Main et al. 2010; Smith 2000; Smith 2004). To date, only one study has investigated intracellular signaling protein activation in humans following short-term stressful RT (Nicoll et al. 2016).

Mouse models have primarily utilized endurance training to induce muscular damage on downhill running protocols (Pereira et al. 2014; Da Rocha et al. 2017). The two studies that utilized squat like or heavy resistance exercise in mice have indicated an increase in inflammatory signaling in skeletal muscle (Coffey et al. 2007; Alves Souza et al. 2014). Two key proteins elevated in excessive exercise with insufficient recovery are TNF

alpha and a chronically elevated increase in p38 phosphorylation. Following normal training, there is decrease in atrophic and inflammatory proteins, and concomitant increase hypertrophic signaling, followed by subsequent increases in myofibrillar protein content (Karagounis et al. 2010). However, with insufficient recovery, there is an increase in atrophy and inflammatory protein expression and may create an environment where anabolic capacities are insufficient to overcome atrophy signaling.

Alves Souza et al. (2014) used a mouse model to show that excessive exercise can decrease the cross-sectional area of muscle tissue and is accompanied by decreased IGF-1, and MyoD expression and increased MAFbx expression. Considering TNF alpha can chronically activate p38 and lead to inflammation, protein degradation, and atrophy (Li et al. 2005), data by Nicoll et al. (2016), might suggest the elevated p38 activation at rest following overtraining may in some way be related to maladaptation. In humans, it is unlikely to see substantial decreases in muscle hypertrophy since the time course of changes between animal and human models are likely different. Instead, excessive exercise likely attenuates adaptation in muscle size as opposed to definite muscle atrophy.

Moreover, the use of signaling proteins as indicators of hypertrophy or atrophy is complicated by data suggesting that with adequate periods rest

between training bouts there is an increase in muscle protein synthesis with lower acute signaling. However, periods of insufficient recovery between training bouts actually increases protein phosphorylation more than long recovery periods. but blunts muscle protein synthesis (Takegaki et al. 2017). Although adequate rest or detraining following chronic training may re-sensitize signaling proteins to anabolic stimuli, (Ogasawara et al. 2013), data in humans on this topic remains lacking.

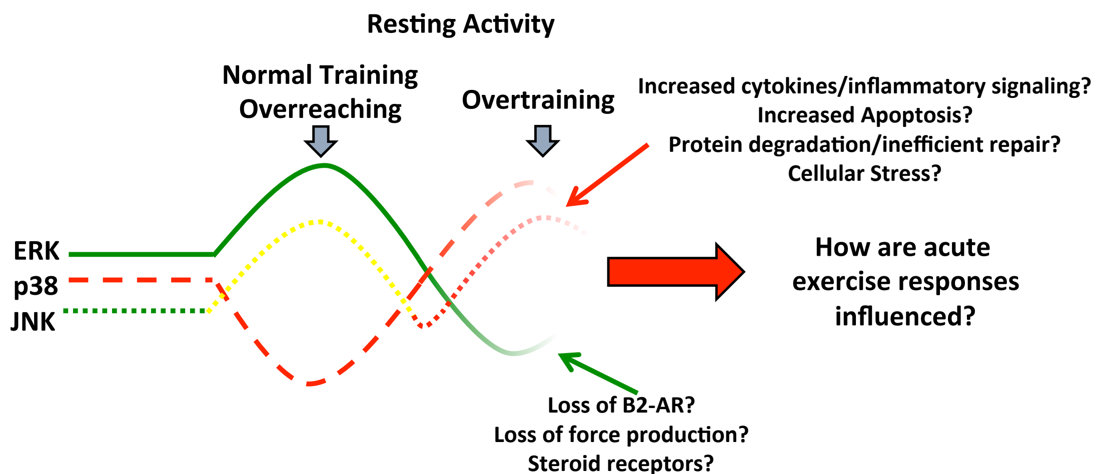

Figure 2-2 Figure depicting results from Coffey et al. (2007), Karagounis et al. (2010), and Nicoll et al. (2016). Chronic training with sufficient rest maintains an overall anabolic cellular environment. However, during overtraining the role of p38 becomes more inflammatory and potentially impedes successful skeletal muscle adaptations.

7. Future Directions

Recently, cancer (Koryakina et al. 2014) and C2C12 skeletal muscle myotube (Kim and Lee 2009) cell lines suggest the androgen receptors (AR) is activated in the absence of testosterone. The MAPK signaling cascade can activate AR and glucocorticoid receptors (GR)s by phosphorylation, even in absence of testosterone and cortisol (Galliher-Beckley et al. 2011; Kim and Lee 2009). The ERK, JNK, and p38 modify the phosphorylation of the AR and alter AR protein expression in myotubes (Kim and Lee 2009). The phosphorylation of AR and GR at specific residues regulates receptor function and likely influence eventual muscle phenotype response (Galliher-Beckley et al. 2011; Gioeli et al. 2006). The modulation of the acute RE program variables could promote specified signaling and fiber adaptation following RE (Spiering et al. 2008). However, there are no data investigating MAPK influence on the AR and GR in humans despite their role initiating muscle growth, regeneration, and remodeling. These two pathways (steroid receptors and MAPK) likely coordinate signaling responses to produce specified gene transcription and adaption responses. It is unclear how MAPK pathways converge and influence AR and GR function to RE in human muscle.

Current RE guidelines suggest performing moderate intensity, high volume, short rest periods during training to promote an increase in anabolic hormones, such as testosterone, 22kDa growth hormone, and insulin-like

growth factor-1 (IGF-1) (ACSM et al. 2009). A recent study (McKendry et al. 2016) reported that short rest (1-minute) RE using moderate loads (75%1RM) blunts the muscle protein synthetic (MPS) response and early phase signaling responses in recreationally trained males compared to the same protocol using longer rest intervals (5-min). Akt/mTOR signaling and MPS were attenuated in the short rest condition compared to long rest despite an increase in anabolic hormones. The authors hypothesized metabolic stress induced by the short rest between sets blunts early signaling processes (0-3hrs) but does not influence late MPS responses (16-24hrs) once metabolic stress has cleared (McKendry et al. 2016).

Spiering at al. (2008) also reported decreased p70S6K phosphorylation following RE despite an increase in anabolic hormones and the authors hypothesized that the decreased p70S6K response arose from elevated cortisol in the high hormone condition. Interestingly, in that same cohort but a different analyses, Spiering et al. (2009) reported that the RE induced increase of testosterone in the high hormone condition potentiated and sustained AR content whereas the low hormone condition saw a decrease in AR content. Taken together, in conditions where metabolic stress and cortisol are high, testosterone may potentiate AR signaling to compensate for decreased Akt/p70S6K activity. Conversely, in conditions where metabolic stress is low, but intensity (%1RM) is high, signaling is

maintained through mechanosensitive events, as high load, low volume, long rest RE does not usually produce increases in testosterone or cortisol.

We have recently shown that total protein and phosphorylated expression of MAPK change with chronic (Galpin et al. 2016; Nicoll et al. 2017) and stressful training (Nicoll et al. 2016). Androgen receptor content is up-regulated after repeated bouts of RE (Willoughby and Taylor 2004, Mitchell et al. 2013). However, this response is highly individualized and it is likely that an individual's respective ratio of testosterone:AR and cortisol:GR provide a clearer picture of physiological processes and adaptation in the muscle than mean changes. In fact, the individual up-regulation of AR following 12 weeks of training was positively associated ($r=0.60$, $p=0.003$) with increases in muscle hypertrophy (Mitchell et al. 2013). Greater AR content increases the likelihood of hormone receptor interactions and may enhance and specify translation initiation, translational capacity, and eventual MPS in trained individuals. Furthermore, there are limited data concerning the role of GR following RE as only Vingren et al. (2009) and Willoughby et al. (2003) have described GR responses following RE. Further Da Rocha et al. (2017) reported phosphorylated AR and GR change following a overtraining treadmill protocol. Further administration of exercising mice with androgens increases phosphorylation of the androgen receptor at ser213 (Zang et al.

2017). Thus, hormonal and contractile signaling processes likely contribute to eventual muscle adaptation.

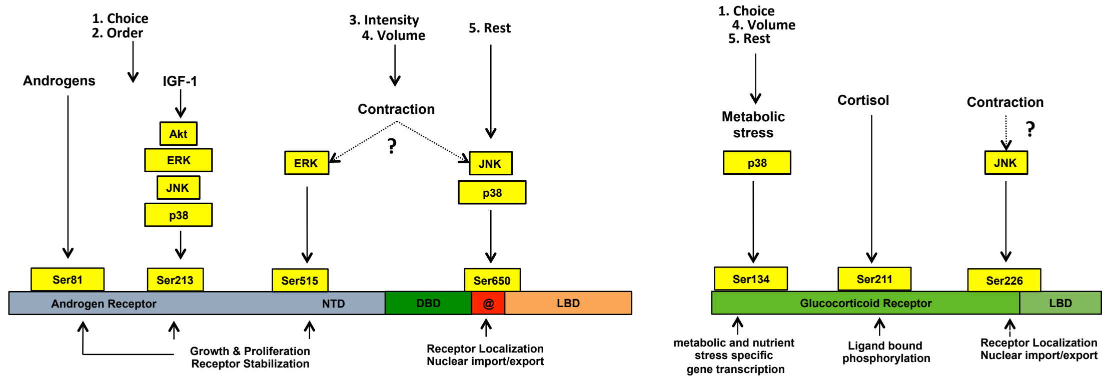

Figure 2-3 Representative figure of the acute program variables of exercise choice, load, volume, order, and rest period and how they could influence MAPK and AR and GR phosphorylation at specific serine residues that influences receptor function. Modified from Daniels et al. (2013).

8. Conclusion

In conclusion, MAPK represent an intricate signaling network to relay extracellular stimuli to a biochemical response and adaption to exercise. Novice exercise skeletal muscle often a produces activation following RE and this response decreases over the course of many training session. Although exercise induced responses attenuate, skeletal muscle no has chronic adaptations that enhance adaptation in the trained state including ribosome biogenesis, fiber type conversions, and satellite cell expansion. Thus, an acute exercise responses in MAPK phosphorylation may not be needed in the trained state. Excessive exercise training results in an increase in

inflammatory signaling that may in part be due to altered JNK and p38 activity in the resting state. The activation of these proteins from acute exercise in the overtrained state warrants further investigation. Finally, increasing evidence points to the integration of MAPK signaling and steroid receptor phosphorylation, which may indicate an alternative mode of muscle adaption via AR and GR that may work synergistically or independent of circulating hormones.

The effects Pre-workout Supplementation on B_2-Adrenergic and MAPK Signaling Pathways

ABSTRACT

The synergistic effects of caffeine containing pre-workout supplements on exercise performance has been reported, however no studies have specifically investigated the potential myocellular mechanisms during resistance exercise. β_2 adrenergic receptors are a likely target of the stimulatory effects of pre-workout supplements and stimulate signal transduction pathways regulating energy production and anabolism, including protein kinase A (PKA), and the mitogen-activated protein kinase (MAPK). **PURPOSE**: To elucidate the role of pre-workout supplementation on myocellular responses to an acute resistance exercise (RE) bout. **METHODS**: Subjects consumed a caffeine containing multi-ingredient pre-workout supplement 60 minutes prior to an acute RE bout using barbell back squats. Muscle biopsies were obtained prior to supplementation and 10 minutes after exercise. Tissue samples were analyzed for the phosphorylation of β_2 adrenergic receptors, protein kinase A (PKA), and three MAPK (ERK, JNK, p38). Epinephrine was determined prior to supplementation (baseline; BL), after supplementation but prior to RE (PRE), and immediately after RE (POST). Muscular power, velocity, and repetitions until failure were assessed during the RE bout. **RESULTS**: Epinephrine increased at PRE in the SUPP condition only. Post-exercise epinephrine was increased from BL in PL and SUPP, but the response was higher in SUPP. Muscular power and velocity were higher in SUPP across four sets of back squats compared to PL.

p- β_2AR and p-MAPK increased post exercise with no differences between conditions. There were relationships between epinephrine, p- β_2AR, and ERK, but the relationships were dependent on the whether the PL or SUPP was consumed. **CONCLUSION**: Consumption of a caffeine containing pre-workout supplement improves performance, possibly through increases in pre-exercise catecholamines. β_2-adrenergic and mechanosensitive protein signaling were similar post-exercise. These data provide insight to the myocellular responses to pre-workout supplementation and RE performance.

INTRODUCTION

Resistance exercise (RE) is well known to increase skeletal muscle health, quality of life, and athletic performance. As such, decades of research has sought to determine strategies to maximize and optimize the adaptions to RE (Hawley et al 2011; Jäger et al. 2017). Many adaptations in muscle plasticity from RE occur at the myocellular level (Spiering et al. 2008). A plethora of research has supported the use of protein supplementation to improve accretion of muscle mass to a resistance-training (RT) program (Hawley et al. 2011). Increasing muscle size and strength has implications for improved force-producing capacities for sport performance. In particular, acute supplementation with caffeine or caffeine containing pre-workout supplements has been shown to improve various components acute muscular performance, but the molecular consequences of supplementation in humans after RE is understudied in humans (Lowery et al. 2013; Shelmadine et al. 2009).

Endurance (Watt et al. 2003) and RE studies indicate the use of caffeine-based supplements improve time to exhaustion, local muscular endurance (Beck et al. 2006; Duncan 2012), muscular power (Gonzalez et al 2011), mood (Duncan et al. 2012b), and indices of muscle damage (Hurley et al 2013). Similarly, pre-workout supplementation alters endocrine responses following RE (Hoffman et al. 2008). It has been hypothesized many of these

improvements in acute exercise performance are in large part due to increases in the catecholamines epinephrine (EPI) and norepinephrine (NE). Catecholamines exert their effect on muscle via binding to β_2 adrenergic receptors (β_2-AR) at the surface of the sarcolemma, and influence molecular signaling cascades regulating anabolism (mitogen-activated protein kinases; MAPK) and metabolism (cyclic adenosine monophosphate; cAMP) (Sato et al. 2011). Activation of cAMP via β_2-AR leads to phosphorylation of protein kinase A (PKA), hormone sensitive lipase (HSL) and glycogen phosphorylase, subsequently increasing energy availability within contracting muscle tissue (Cargnello and Roux 2011). β_2-AR signaling can activate PKA which subsequently initiates the mobilization of substrates during exercise. The phosphorylation of PKA via β_2-AR can then feed back to the β_2-AR by phosphorylating the receptor at ser346 (Fan et al. 2016). Furthermore, phosphorylation at ser355 regulates β_2-AR internalization and desensitization (Fan et al. 2016). Thus, despite clear direct pathways in β_2-AR signaling, the regulation of the receptor itself in response to exercise is unclear and understudied in humans.

Mitogen-activated protein kinase (MAPK) molecules are known mechanisms of signal transduction, such that growth factors, cytokines, mechanical forces and cellular oxidative stress initiate parallel signaling cascades, and regulate muscle adaptations to exercise (Long et al. 2004).

The three most studied MAPK are extracellular signal-regulated kinase 1 and 2 (ERK), c-jun NH_2-terminal kinase (JNK), and p38-MAPK (Cargnello and Roux 2011). ERK is involved in muscle contraction induced myofiber hypertrophy and maintenance of muscle mass (Haddad and Adams 2004; Shi et al. 2009; Wu et al. 2000). JNK and p38-MAPK are referred to as stress-activated protein kinases since these MAPK are activated by mechanical tension, inflammatory cytokines, and oxidative stress (Cargnello and Roux 2011). JNK phosphorylation is quantitatively related to the intensity and duration of RE load placed on muscle tissue during exercise (Boppart et al. 1999; Gehlert et al. 2015), and is required for cellular remodeling (Dhanasekaran and Reddy 2008; Khurana and Dey 2004). p38-MAPK is a mediator of PGC-1α activation, mitochondrial biogenesis, and improves substrate oxidation capacities of skeletal muscle (Gibala et al. 2009; Hawley et al. 2014). Thus, the MAPK family integrates numerous exercise stimuli to gene transcription and muscle adaptation (Spiering et al. 2008).

The integration of hormonal receptors and MAPK activation in non-canonical or ligand-independent signaling deserves further investigation. To date, most studies investigating cross-talk between these receptors (β_2-AR), and MAPK have done so using murine or cell culture models. It is unclear how the integration of these pathways (if any) occurs in exercising humans. Furthermore, if caffeine or pre-workout supplementation improves RE performance, how is this reflected at the local skeletal muscle level? If

caffeine supplementation does indeed improve performance via a catecholamine related mechanism, it is not clear how are their respective receptors (β_2-AR) are regulated during exercise. Therefore, the purpose of this study was to determine the effect of a caffeine containing pre-workout supplement on β_2-adrenergic receptor activation, MAPK signaling, and muscular performance.

METHODS

Experimental Approach to the Problem

To determine the effect of a pre-workout supplement (SUPP) on molecular signaling responses, a within-subject, placebo controlled, crossover design was implemented. Given the importance of catecholamine response on signal transduction and muscle performance, an intravenous catheter was used to determine the immediate effects of supplementation and RE, and to avoid anticipatory responses from repeated blood draws from a needle. Literature reports peak absorption of caffeine in circulation occurs at 60 minutes post consumption, and is sustained for approximately 3-5 hours thereafter. For this reason we chose to obtain a muscle biopsy at rest, determine epinephrine pre- and post- supplementation and immediately post-exercise, and a muscle biopsy 10 minutes post exercise. The POST muscle biopsy was chosen due to the rapid and transient responses of ERK, which has been shown to return to resting values within one hour, with highest

values being reported closer to the cessation of exercise (~10 post exercise) but not immediately post (Creer et al. 2005). In addition, obtaining the post exercise biopsy sample at 10+ exercise allowed for the determination of temporal delay between elevated systemic catecholamine responses, β_2-AR activation, and phosphorylation of signal transduction proteins. Muscle performance was assessed via power and velocity output during the barbell back squat exercise. Similarly, muscular endurance (repetitions until failure) was assessed during the final set of the barbell back squat exercise.

Participants

Subjects refrained from physical activity other than that required by the experimental trials, and withdrew from alcohol, tobacco and any kind of caffeine intake 4 days before testing. Each participant was evaluated by a researcher to ensure the subjects all had safe and consistent barbell back squat technique prior to the maximal squat test. Subjects also demonstrated their ability and comfort in achieving the minimum parallel squat depth for the study. All subjects in the study had at least 2 years of resistance training experience, and had been resistance training consistently for at least 3 months leading up to the study. Finally all subjects must have squatted at least once per week in the six months prior to the study. All subjects signed an informed consent statement as approved by the University Institutional Review Board and in accordance with the Helsinki Declaration.

Supplementation

Dietary Recall – All subjects were asked to record food and drink consumption (including portion sizes) for two consecutive days prior to their first experimental visit. Subjects were then asked to repeat this same diet in the two days prior to the remaining experimental visit.

Supplementation/Placebo condition – All subjects consumed either the multi-ingredient pre-workout supplement (MusclePharm Corp™, Denver, CO or an iso-caloric flavor and color matched placebo drink 60 minutes prior to each exercise session. The supplement/placebo mix containers were labeled as either "x" or "z", and mixed with 12 oz of water in a black (non-transparent) shaker. The experiment was double-blind in nature as neither the research staff nor the subjects knew which drink was the placebo and which was the supplement. Questionnaires were provided to each subject at each exercise session to record any adverse effects they perceived as a result of taking the placebo or supplement. All subjects were queried at the end of the experimental day as to whether they knew which condition they were given or if they had no idea. Chi-squared analyses indicated the blinding was successful. (i.e., those who had no idea or were incorrect were not different from those who were correct)

Table 1: Supplement ingredients

Supplement Facts	
Serving size: 18.2g	
	Amount Per Serving
Calories	20
Total Carbohydrate	6g
Sugars	5g
Vitamin C	300mg
Vitamin E	40IU
Vitamin B6	20mg
Vitamin B12	50mcg
Calcium	62mg
Creatine Monohydrate	5g
Beta-Alanine	2g
Betaine Anhydrus	1.5g
Isomaltulose	5g
L-Glycine	1g
L-Taurine	1g
Caffeine Anhydrus	250mg
Theacrine	50mg
Black Pepper Fruit Extract	5mg

Table 1 Supplement ingredient list.

1RM testing

The one-repetition maximum (1-RM) strength tests were completed for the barbell squat exercise according to previously described methods (Kraemer et al. 2006). Subjects were familiarized with proper back squat technique according to the National Strength and Conditioning Association guidelines (Baechle and Earle 2008), were instructed to stand with feet approximately shoulder width apart, and to descend until the femur is parallel to the ground (as assessed by a Certified Strength and Conditioning Specialist). Once proper depth was attained, subjects were instructed to ascend to the starting position. Briefly, subjects performed a light warm-up of

5 to 10 repetitions at 40% to 60% of perceived maximum. After 1 minute, subjects performed 3 to 5 repetitions at 60% to 80% of perceived maximum. Conservative increases in weight were made, and the subject attempted a 1-RM lift. If the lift was successful, a rest period of 3 minutes was allowed prior to the next attempt. The 1-RM was attained within three to five sets as to avoid excessive fatigue (Kraemer et al. 2006).

Acute Resistance Exercise Bout.

To warm up, all subjects cycled lightly on a cycle ergometer for 5 minutes. Subjects then performed two warm-up exercise sets with the barbell back squat at 35 and 55% of their 1-RM. The subjects then performed four sets of barbell back squats for 8 repetitions at 75% of their 1- RM with two minutes of rest in between sets. Once all four sets were completed, subjects received 4 minutes of rest and completed a fifth set of barbell back squats at 60% of 1-RM until concentric failure. Upon completion of the final set to failure, subjects gave a post-exercise biopsy (POST) 10 minutes after the cessation of exercise.

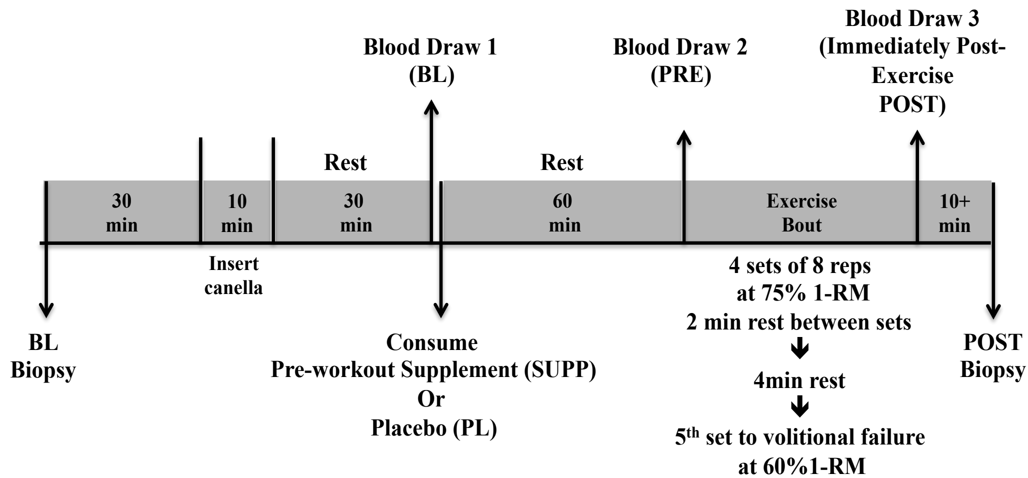

Figure 3-1 Baseline (BL) biopsies and blood draws were obtained. An additional blood draw was obtained 60 minutes after supplementation with a pre-workout supplement (SUPP) or placebo (PL), but immediately prior to the acute resistance exercise bout (PRE). Subjects performed the acute resistance exercise bout of barbell back squats. A blood sample and muscle biopsy was obtained immediately post and 10-minutes post exercise (POST) respectively.

Muscle Biopsies

All vastus lateralis muscle biopsies were performed as previously described (Fry et al. 2016; Galpin et al. 2016; Nicoll et al. 2016). On the first day of the exercise protocol, muscle biopsies and blood samples were taken before, and after the acute RE bout. Subjects reported to the lab following an eight-hour overnight fast. Subjects were asked to rest comfortably for 30 minutes to return to resting state following arrival at the lab.

All muscle biopsies were obtained from the vastus lateralis by percutaneous needle biopsy prior to supplementation (BL) and 10 minutes after the final set of barbell back squat (POST). To prepare the subject's leg

for the muscle biopsy, the thigh was shaved and cleaned with Betadine solution. Three mL of 2% lidocane (without epinephrine) solution was injected into the skin and the surrounding tissues of the leg. Next, a 1 cm incision was made through the skin and fascia. A Bergstrom needle was used for tissue extraction, using the double-chop technique (Staron, 1991) and suction (Evans et al. 1982). Following the pre-exercise biopsy, sterile and flexible pressure wraps were placed over the incision site. Bandages were securely wrapped and the volunteers began their warm-up and exercise protocol. All samples were extracted from the same incision, and orientation of the biopsy needle was rotated approximately 45° for each biopsy to insure samples were obtained from a different region. Immediately following extraction, muscle samples were cooled to –159° C by liquid nitrogen to halt metabolic processes, and stored at –80° C.

Blood Measures

An intravenous catheter was inserted into an antecubital vein, and a normal saline lock was attached. During each blood draw, the first 3 ml of blood (with saline from the catheter lock) was collected into a discard tube preceding each serial sample. Approximately 5-10 mL of blood was collected and divided into EDTA Vacutainer™ test tubes for plasma. The tubes were subsequently centrifuged for 15 minutes at 2000 RPM. After which the plasma was pipetted into eppendorf tubes and stored at -80° C. Blood was

drawn 30 minutes after the first muscle biopsy (immediately before supplementation), prior to the start of the barbell back squat exercise (60 minutes post supplementation), and within 30 seconds after the end of the final set of the barbell back squat. Standard blood processing procedures for determination of hematocrit and hemoglobin were determined to account for plasma volume shifts during exercise (Dill and Costill 1974).

Enzyme-linked Immunosorbent Assay (ELISA)

Serum samples were analyzed in duplicate for plasma epinephrine via commercially available enzyme-linked immunosorbent assays (Eagle Biosciences; Nashua, NH). All samples from the same subject were analyzed on the same assay plate, and by the same investigator in one run to avoid inter-assay variation. Plasma samples were thawed only once before analysis. Intra- and inter-assay variance was 9.8% and 10% respectively. Hormonal values were not corrected for plasma volume shifts since this is the molar concentration the receptors are actually exposed to during and post-exercise.

Tissue Sample Preparation for Western Blots

Ten to fifteen mg of sample was placed in 1 ml of lysing buffer containing 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, and 2.3% (w/v) SDS in 62.5 mMTris·HCL buffer (pH 6.8), with a 1% solution of Halt Protease

and Phosphatase Inhibitor Cocktail to inhibit protease and phosphatase activity (Thermo Fisher Scientific Inc., Waltham, Mass.). Samples were homogenized in a test tube (3 X 10 s) using a Tissue Miser homogenizer. The resulting suspensions were then heated for 10 min at 60° C, and subsequently frozen at –80° C.

Western Blotting

Muscle homogenate samples were assayed for total protein concentration using a micro Lowry method with Peterson's modification (Sigma Aldrich, Saint Louis, MO. No P5656). Following protein determination, 20 µg of protein was loaded onto 4-15% pre-cast gels (Bio-Rad) and electrophoresed at 200 V for 30 min. Once resolved, proteins were transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Burlington, NH) at 15 V for 90 minutes using an Idea Scientific Co. wet transfer apparatus (Minneapolis, MN). After transfer, PVDF membranes were blocked in Odyssey® blocking buffer (TBS without Tween 20) for 1 h at room temperature. Following blocking, membranes were then incubated with primary antibodies for total β_2-AR total (1:500, mouse monoclonal, no. MCA2784, Bio-Rad), p-β_2-AR ser346 (1:1,000, rabbit polyclonal, no. PA5-38728; ThermoFisher), p-β_2-AR ser355/356 (1:1,000, rabbit polyclonal, no. PA5-38403; ThermoFisher), total α/β catalytic subunit of PKA (PKA-C) (1:1000, mouse monoclonal, no. MAB5908; R&D Systems),

phosphorylated α/β/γ catalytic subunit of PKA (Thr197) (1:1000, rabbit polyclonal, no. 4781S; Cell Signaling), ERK1/2 (1:2000, mouse monoclonal, no. 4696s, Cell Signaling), p-ERK1/2 (Thr202/Tyr204) (1:1000, rabbit monoclonal, no. 4376s; Cell Signaling), total JNK (1:500, mouse monoclonal, no. sc-7345; Santa Cruz BioTech), p-JNK (Thr183/Tyr185) (1:1000, rabbit monoclonal, no. 4671s; Cell Signaling), total p38-MAPK (1:1000, mouse monoclonal, no. MABS1754; EMD Millipore), and p-p38 (Thr180/Tyr182) (1:1000, rabbit monoclonal, no. 9211s; Cell Signaling) overnight at 4° C with gentle agitation. Following overnight incubation with agitation, membranes were washed (3 X 5 min) with TBS plus Tween-20 (TBST), and then probed with infrared (IR) secondary antibodies (1:10,000) specific to the host animal (700 nm anti-mouse, 800 nm anti-rabbit) for 1 h at room temperature to label the respective total and phospho-receptor/MAPK primary antibodies. After secondary antibody incubation, membranes were washed again (3 x 5 min) in TBST, rinsed once with TBS, and then scanned with an Odyssey Infrared Imaging System and accompanying software (v1.2, LI-COR Biosciences, Lincoln, NE) to quantify IR intensity for each labeled protein band. Infrared-labeled secondary antibodies were obtained from Li-COR Inc. (LI-COR Biosciences, Lincoln, NE). Total and phosphorylated bands were identified on the same membrane with IR markers scanned at two different wavelengths (700 nm; total- β_2-AR, total-PKA and total-MAPK, and 800 nm; p-β_2-AR, p-PKA, p-MAPK). Phospho-β_2-AR, p-PKA, total-β_2-AR, total-PKA, phospho-

MAPK, and total-MAPK signals were normalized to total protein abundance loaded per lane via total protein stain (REVERT; Li-COR). Phospho- signals were then normalized to the total-receptor or MAPK signal and are expressed as the ratio of phospho- signal : total signal (i.e. p-β_2-AR:total-β_2-AR or phospho-MAPK : total-MAPK. All statistical analyses were performed on the total protein adjusted ratios.

Statistical Analyses

All data were assessed for normality with the Shapiro-Wilks test. All hormonal, lactate, and performance data were normally distributed, however there were random violations in normality for β_2-AR, PKA, and MAPK variables. Thus, all receptor (β_2-AR) and signaling (PKA, MAPK) data were analyzed using non-parametric methods. Hormonal, performance, and RPE data were analyzed using parametric methods. Paired sample t-tests determined differences in mean power and repetitions to failure between SUPP and PL conditions. A 2 x 4 (condition x time) RMANOVA examined differences in MP, MV, and RPE between sets 1 - 4. A sequential Holm-Bonferroni correction was applied across the 6 comparisons between sets (critical p-values: 1. p=0.0083; 2. p=0.01; 3. p=0.0125; 4. p=0.0.0167; 5. p=0.025; 6. p=0.05). A 2 x 3 (condition x time) RMANOVA determined differences in epinephrine between conditions at BL, PRE, and POST. Lactate was analyzed with a 2 x 2 (condition x time) RMANOVA. Follow-up

post hoc analyses for both epinephrine and lactate were performed with a protected Fisher's LSD.

All receptor and signaling data were analyzed using Wilcoxon signed-rank tests for pairwise comparisons between BL and POST for both SUPP (first comparison) and PL (second comparison) conditions. A third comparison of the POST values between conditions was also performed. Since repeated pairwise comparisons do not provide an omnibus test similar to ANOVA and a protected Fisher's LSD, post-hoc analyses on MAPK analyses utilized Holm's sequential Bonferroni procedure. Briefly, the p-values obtained from the pair-wise comparisons were ordered from smallest to largest. Critical p-values to be deemed statistically significant were sequentially determined at $p \leq 0.016$, $p \leq 0.025$, and $p \leq 0.05$ for the first, second, and third comparisons respectively. Pearson product-moment correlations were determined between epinephrine, β_2-AR and ERK phosphorylation to determine relationships between endocrine and molecular responses.

All hormonal, performance, and lactate data are expressed as mean $\pm$ standard error (SE). All receptor and signaling data are presented as box and whisker plots with the maximum (top whisker), minimum (bottom whisker), median, and interquartile range (25th-75th %) to provide a representation of

the spread of the data. The black dot at each time-point indicates the mean. Alpha-level was $p \leq 0.05$ for all analyses.

RESULTS

Endocrine responses

There was a significant condition X time interaction for epinephrine (F(2,18)= 11.61, p=0.001). Post-hoc analyses for PL indicated epinephrine did not increase from BL to PRE (p=0.165). However, epinephrine at POST was greater than BL and PRE (p<0.001). In SUPP epinephrine significantly increased from BL to PRE (p=0.028), and further increased at POST (p<0.001). In SUPP, epinephrine was greater at POST compared to BL and PRE, and was significantly greater than PL at POST (p=0.006).

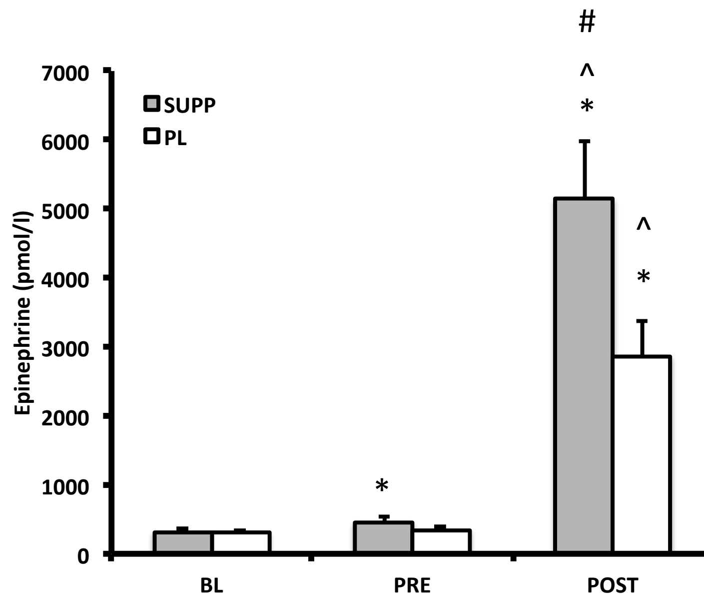

Figure 3-2 Epinephrine concentration at rest prior to supplementation (BL) with a pre-workout supplement (SUPP; grey bars) or placebo (PL; white bars), following 60-minutes of supplementation but prior to exercise (PRE), and immediately post exercise (POST). * indicates significantly different from BL. ^ indicates significantly different from PRE. # indicates response in SUPP significantly different from PL at corresponding time point. Data are presented as mean ± SE.

Lactate did not display a condition X time interaction (F(1,9)=4.35; p=0.067). There was no main effect of condition (F(1,9)=0.08; p=0.78), but there was a main effect of time (F(1,9)=203.9; p<0.001). Lactate was significantly higher at POST compared to PRE (11.5 ± 0.77 vs 0.86 ± 0.13 mmol/l; p<0.001).

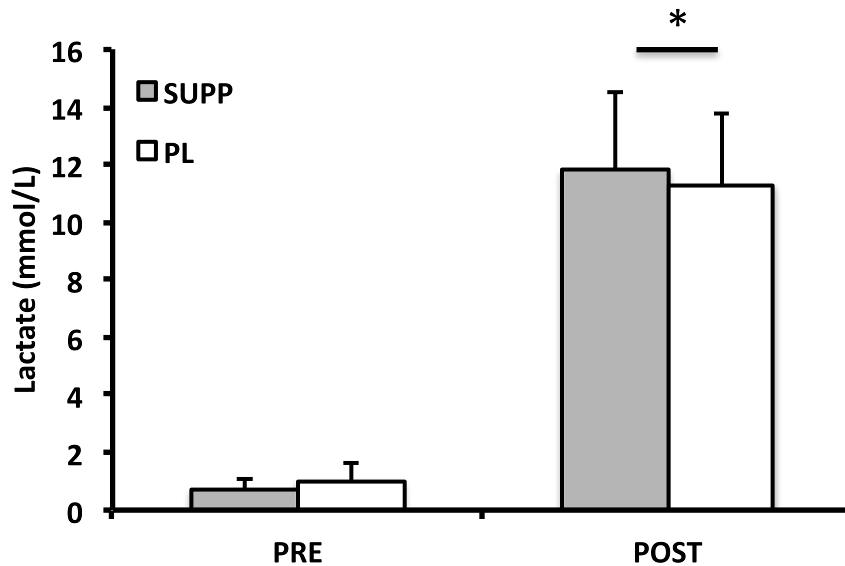

Figure 3-3 Lactate response immediately prior to exercise (PRE) and five minutes post exercise (POST). White bars indicate placebo (PL) condition, grey bars indicate supplemented (SUPP) condition. * indicates main effect of time such that lactate was higher at POST compared to PRE for both conditions. Data are presented as mean ± SE.

Barbell Back Squat Power and Velocity

There was no condition X time interaction on set mean power across the first four sets of barbell back squats ($F(3,27)=0.34$; $p=0.79$). There were main effects of condition ($F(1,9)=73.7$; $p<0.001$) and time ($F(3,27)=8.84$; $p<0.001$). The main effect of condition indicated mean set power was higher in SUPP compared to PL (1023 ± 57 vs 940 ± 56; $p<0.001$). Mean set power was lower on set 4 compared to sets one ($p=0.006$), and two ($p=0.011$). Mean power on set three was lower than set two ($p=0.003$) but was not different than set one ($p=0.022$). There was no difference between power on sets one and two ($p=0.282$).

There was no condition X time interaction for barbell set velocity ($F(3,27)=2.06$; $p=0.129$). There were main effects of condition (($F(1,9)=14.47$; $p=0.004$) and time (($F(3,27)=24.90$; $p<0.001$). Barbell velocities were significantly higher in the SUPP condition compared to PL (Main effect: 0.54 ± 0.02 vs 0.50 ± .02; $p=0.004$). Barbell velocity was significantly decreased across all sets, such that all comparisons between sets were significantly different ($p<0.009$ for all comparisons).

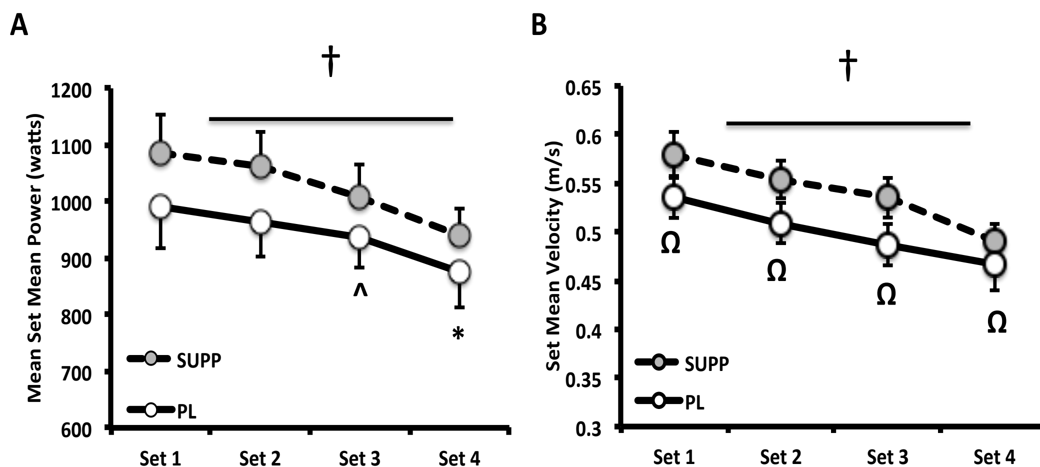

Figure 3-4 Muscular performance during four sets of barbell back squats. A) Mean set power, and B) mean set velocity during four sets of eight repetitions of barbell back squats. White circles indicate placebo (PL) condition, grey circles indicate supplemented (SUPP) condition. Dagger symbol indicates main effect of condition, for SUPP significantly different than PL. * indicates significantly different from sets 1 and 2. ^ indicates significantly different from set 2. Omega symbol indicates significantly different from all other sets. Data are presented as mean ± SE.

Muscular Endurance (repetitions to failure)

Repetitions to failure tended to favor the SUPP (19.8 ± 1.4) condition compared to PL (17.7 ± 1.5) although it was not significantly different ($t(9)=2.024$; $p=0.074$). Mean power during the final set to failure was not different between conditions ($t(9)=1.462$; $p=0.178$)

Rating of Perceived Exertion (RPE)

There was no condition X time interaction for RPE (F(3,27)=0.130; p=.941). There was no main effect of condition (F(1,9)=0.005; p=0.946). There was a main effect of time (F(3,27)=71.234; p <0.001). Post hoc analyses indicated RPE increased significantly after each set, such that RPE between all sets were significantly different from each other (p<0.001).

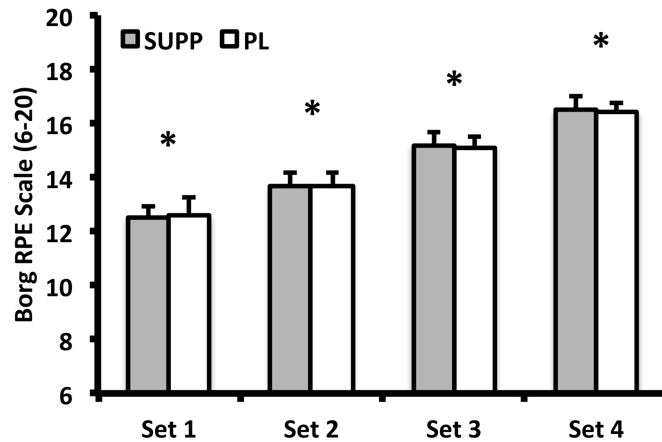

Figure 3-5 Rating of perceived exertion (RPE) at the end of each set of barbell back squats. White bars indicate placebo (PL), grey bars indicate supplemented (SUPP) condition. * indicated significantly different from all other sets. Data are presented as mean ± SE.

β_2-AR signaling

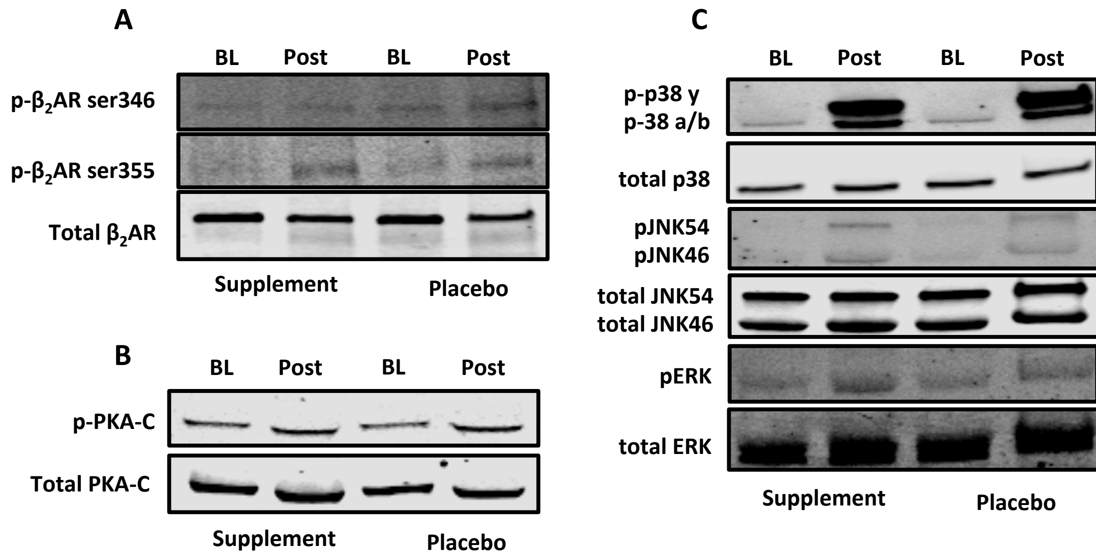

Figure 3-6 Representative western blot images of A) Total and phosphorylated β_2-adrenergic receptors (β_2-AR) at ser346, ser355 at rest prior to supplementation (BL) and ten minutes post exercise (Post). B) Total and phosphorylated catalytic subunit of protein kinase A (PKA-C) at Thr197 rest (BL) and ten minutes post exercise (Post). C) total and phosphorylated p38, JNK, and ERK at rest (BL) and ten minutes post exercise (Post). The left side (lanes 1 and 2) of representative blots indicate supplemented (SUPP) responses, while the right side (lanes 3 and 4) indicate placebo responses (PL).

p-β_2-AR at ser346 increased from BL to POST after SUPP ($z = -2.803$; $p=0.002$) and PL ($z = -2.803$; $p=0.002$) with no differences between conditions at POST ($z = -1.070$; $p=0.322$). p-β_2-AR at ser355 increased from BL to POST in PL ($z = -2.599$; $p=0.006$) and tended to increase in SUPP ($z = -2.191$; $p=0.027$) with no differences between conditions at POST ($z = -0.764$; $p=0.492$). Total β_2-AR expression decreased from BL to POST after SUPP ($z = -2.599$; $p=0.006$) and PL ($z = -2.803$; $p=0.002$) with no differences between conditions at POST ($z = -0.968$; $p=0.375$).

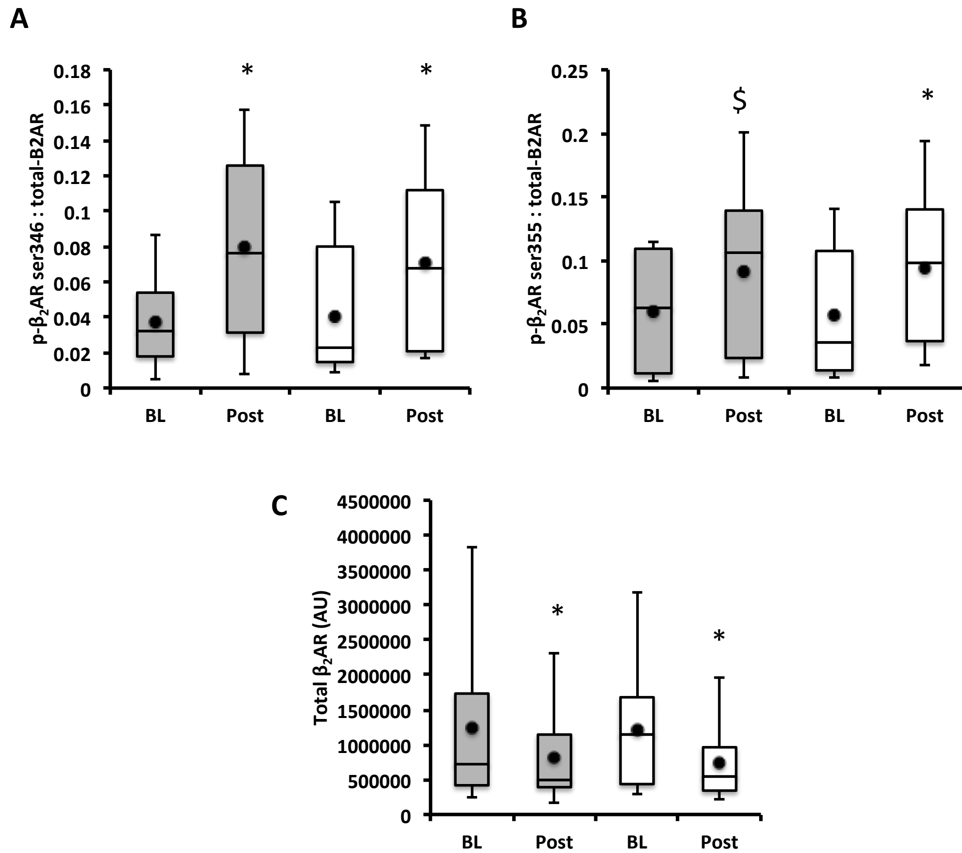

Figure 3-7 A) Phosphorylated β_2-AR ser346 **B)** Phosphorylated β_2-AR ser355, **C)** Total β_2-AR expression. Resting (BL) and post-exercise (Post) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates placebo condition (PL), grey box indicates supplemented condition (SUPP). * indicates significantly different from resting value at corresponding time-point. $ indicates trend for significant difference from BL in corresponding condition (p=0.027).

pPKA-C did not change from BL to POST after SUPP (z = -0.357; p=0.770) and PL (z = -0.663; p=0.557) with no differences between conditions at POST (z = -1.478; p=0.160). Total PKA-C expression did not change from BL to POST after SUPP (z = -0.968; p=0.375) and PL (z = -0.459; p=0.695) with no differences between conditions at POST (z = -1.376; p=0.193).

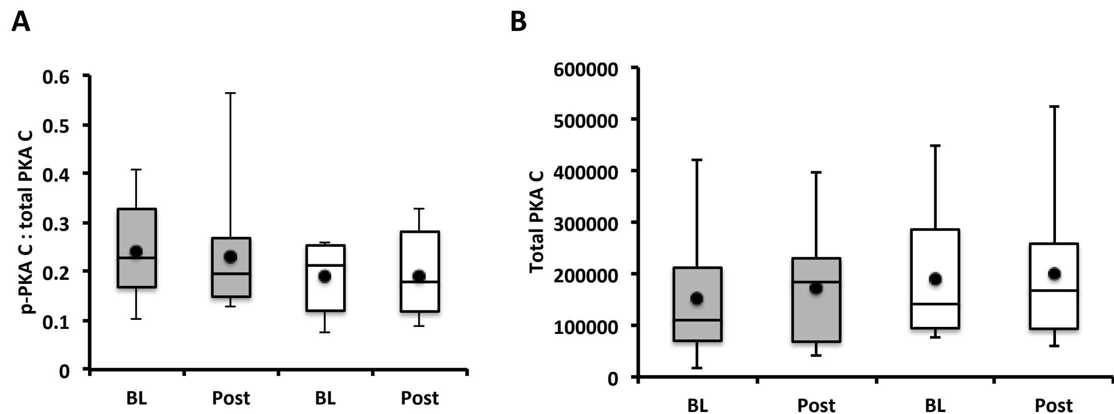

Figure 3-8 A) Phosphorylated catalytic subunit of protein kinase A (PKA Thr197) **B)** Total catalytic subunit of protein kinase A (PKA). Resting (BL) and post-exercise (Post) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates placebo condition (PL), grey box indicates supplemented condition (SUPP).

MAPK Signaling

p-ERK increased from BL to POST after SUPP (z = -2.803; p=0.002) and PL (z = -2.803; p=0.002) with no differences between conditions at POST (z = -0.663; p=0.557). p-JNK increased from BL to POST after SUPP (z = -2.803; p=0.002) and PL (z = -2.803; p=0.002) with no differences between conditions at POST (z = -1.070; p=0.322). p-p38 increased from BL to POST after SUPP (z = -2.803; p=0.002) and PL (z = -2.803; p=0.002) with no differences between conditions at POST (z = -1.580; p=0.131).

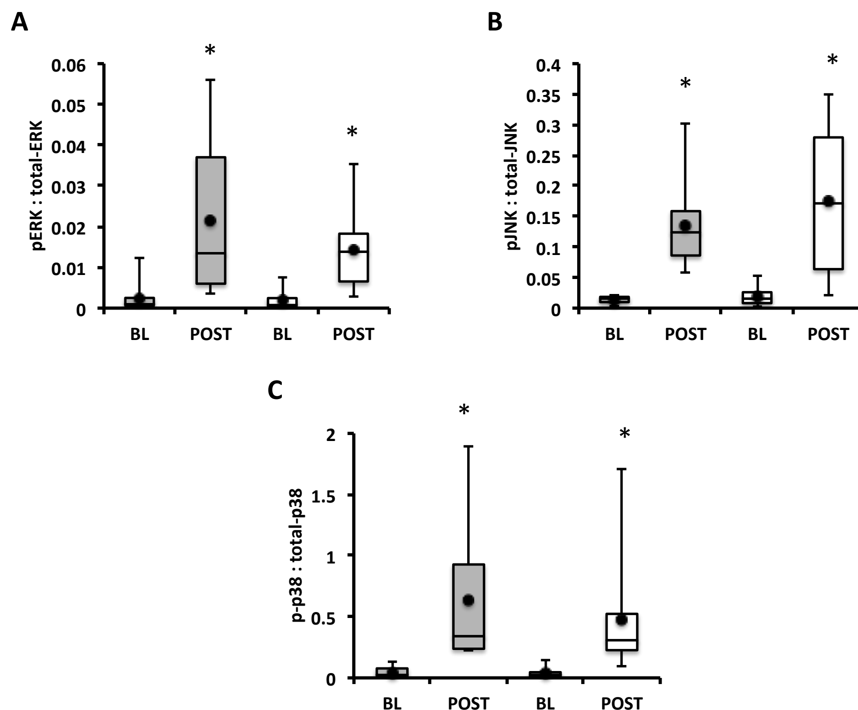

Figure 3-9 A) Phosphorylated ERK **B)** phosphorylated JNK, and **C)** phosphorylated p38. Resting (BL) and post-exercise (Post) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates placebo condition (PL), grey box indicates supplemented condition (SUPP). * indicates significantly different from resting value at corresponding time-point.

Correlations

The percent change from baseline in the PL condition indicated relationships between EPI and β_2-AR ser346 (n=10; R^2=0.339; r=-0.582; p=0.077). There was one value that contributed to approximately 50% of the variance in this relationship. When this value was removed the percent of variance explained between these two variables increased considerably (R^2 0.34 v 0.65) (n=9; R^2=0.656; r=-0.810; p=0.008).

In SUPP there was a correlation between the change in pERK and the change in β_2-AR ser346 (n=10; R^2=0.462; r=0.679; p=0.031). It should be noted there was one value that contributed to approximately 50% of the variance in this relationship. When this value was removed the percent of variance explained between these two variables increased considerably (R^2 0.46 vs 0.88) (n=9; R^2=0.886; r=0.941; $p<0.001$).

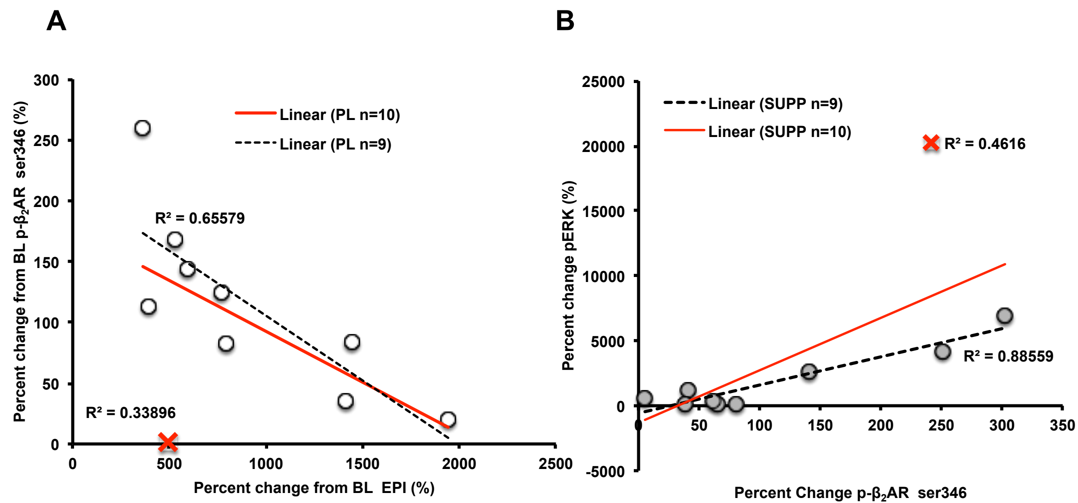

Figure 3-10 A) Correlation between the percent change from baseline in epinephrine and p-β_2-AR ser346 in the placebo condition. The red x indicates the one value that contributed more than 50% to the explained variance (n=10). The red line indicates the regression line for the entire sample in the placebo (PL) condition. The white circles indicate (n=9) when the one outlier was removed. Black dashed line indicates the regression line with one subject removed. **B**) Correlation between the percent change from baseline in pB2ARser346 and percent change in baseline in pERK in the supplemented condition (SUPP). The red x indicates the one value that contributed more than 50% to the explained variance (n=10). The red line indicates the regression line for the entire sample in the supplemented (SUPP) condition. The grey circles indicate (n=9) when the one outlier was removed. Black dashed line indicates the regression line with one subject removed.

DISCUSSION

The use of caffeine and caffeine containing pre-workout supplements has increased in recent years. While several reports have indicated improvements in resistance exercise performance, there are no studies investigating the myocellular response to caffeine supplementation to an acute exercise bout. The results of this study indicate pre-workout supplementation improves muscular power and velocity during an acute exercise bout as compared to PL, while concomitantly producing similar perceptions of exertion in SUPP and PL conditions. The improvement in performance is likely driven by the elevated pre-exercise epinephrine and potentiated post-exercise responses that were only evident in the SUPP condition. Despite different epinephrine concentrations, the molecular responses at the local skeletal muscle level were largely similar between SUPP and PL conditions. However, SUPP may alter the temporal activation or magnitude of these responses since there was a strong relationship in early β_2-AR phosphorylation events in the PL condition that was not seen in SUPP. Conversely, there was a strong relationship in later β_2-AR phosphorylation events in the SUPP condition that was not seen in PL. In this case elevated pre-exercise and enhanced post-exercise epinephrine may have enhanced signaling to ERK in SUPP, whereas the lower epinephrine or null pre-exercise response only facilitated signaling between epinephrine and ser346 in PL.

Resistance exercise increased the phosphorylation of β_2-AR at two sites. The ser346 site is phosphorylated by activated PKA. Phosphorylation of the receptor at ser346 by PKA activates a receptor G_s to G_i "switch" that eventually increases the phosphorylation of ERK (late signaling). G_s signaling is most often observed from β_2-AR agonist stimulation that increases cAMP accumulation, PKA activity, and subsequent ser346 phosphorylation. Conversely, phosphorylation at ser355/356 mediates receptor desensitization and internalization following agonist stimulation via a G-coupled receptor kinase 2 (GRK2) mechanism (Fan et al. 2016). While PKA did not increase in our investigation, phosphorylation at ser346 did increase, indicating we may have missed peak activation of PKA. Phosphorylation of PKA at Thr197 is considered a critical step for increasing PKA-C activity (Steinberg et al. 1993) In vitro work has shown β_2-AR increases cAMP accumulation within milliseconds of agonist stimulation (Lohse et al. 2008). Moreover, the activation of PKA from cAMP accumulation occurs in as little as 3 seconds (Zamir et al. 2009). Thus, increased phosphorylation at ser346 would suggest that PKA increased during our RE bout.

Phosphorylation at ser355 increased as well, suggesting the receptors may have been internalized during RE. Finally, there was also a down-regulation of the receptors following an acute bout of RE. In cardiomyocytes

receptor internalization occurs within 10 minutes of epinephrine exposure, and are recycled back to the membrane following 60 minutes of epinephrine withdrawal (Fan et al. 2016). Prolonged epinephrine exposure (>60 min) prevented these receptors from relocating back to the cell membrane and remained they desensitized (Fan et al. 2016). Chronic β-blockade increases β_2-AR density and muscle contractile forces (Murphy et al. 1997). Prolonged internalization can tag receptors for down-regulation (Gagnon et al. 1998). The strength and duration of agonist stimulation influences PKA and GRK2 site directed phosphorylation (Fan 2016; Liu et al. 2009). Given the length of the exercise intervention was ~26 minutes, down regulation of the receptor may have occurred due to the length of receptor stimulation.

Of note, even though β_2-AR may be internalized, evidence suggests they can still signal in the internalized endosomal state (Bowman et al. 2016). Thus, the decrease in receptor content following exercise may be a normal consequence of RE and receptors may be resynthesized back to pre-exercise levels in the hours to days following exercise, since the BL total β_2-AR expression were not different between experimental visits, despite down regulation from the exercise intervention from the previous experimental visit. Fry et al. (2006) and Sterczala et al. (2017) have reported down-regulation and desensitization of resting expression of β_2-ARs following a period of stressful overreaching and overtraining. The acute exercise response in

human skeletal muscle has not yet been investigated, and further research is necessary to determine exactly what variables influence β_2-AR phosphorylation and total protein expression following an acute RE bout.

Exercise induced MAPK phosphorylation were similar between SUPP and PL conditions. This may be expected since MAPK are sensitive to muscle contraction (Martineau et al. 2001; Boppart et al. 1999). ERK signaling and p38 phosphorylation depends on total exercise volume (Ahtiainen et al. 2015; Tersiz et al. 2010). JNK phosphorylation depends on the degree of the load eccentric load (Gehlert et al. 2015). Given that the load, repetition, and set schemes were the same for both SUPP and PL conditions, this may be why MAPK responses were similar. The final set to failure tended to be greater for the SUPP condition, however a single set to failure is unlikely to facilitate greater signaling responses as opposed to if all four sets were performed to failure (Burd et al. 2010; Tersiz et al. 2010).

Interestingly, there were relationships between the exercise induced epinephrine response and β_2-AR signaling, but the observed relationships were dependent on the condition in which they were performed. For instance, in the placebo condition the negative relationship between p β_2-AR ser346 and epinephrine may represent a temporal delay between elevated circulating catecholamines and β_2-AR ser346 phosphorylation due to receptor sensitivity.

For example, some individuals may be more sensitive to the epinephrine response during exercise. This would lead to signaling to PKA and feedback to β_2-AR ser346 phosphorylation faster. In this case, greater ser346 phosphorylation occurs at lower changes in epinephrine (faster peak ser346), while greater epinephrine in less sensitive individuals takes longer to "signal" to PKA (lower peak ser346). On the contrary, SUPP may accelerate this process (elevated epinephrine at PRE, potentiated epinephrine at POST), leading to strong positive relationship observed between ERK and ser346 in the SUPP only. Though highly speculative, we do observe relationships between adrenergic signaling and ERK post-RE in humans. In vitro work has corroborated mechanistic links between PKA signaling and mitogenic ERK signaling (Fraser et al. 2000). Future studies are required to determine the time course of these relationships.

Muscular power and velocity across all four sets of barbell back squats was greater in the SUPP condition compared to PL. Furthermore, muscular endurance tended to favor the supplement condition, which agrees with previous studies (Duncan and Oxford 2012a and 2012b). Faster movement velocities in the supplement condition may have been due to the elevated pre-exercise epinephrine. Improvement in muscle performance may have occurred via numerous mechanisms including calcium handling (Allen and Westerblad 1995), motor unit recruitment and conduction velocity (Bazzucchi

et al. 2011). Alternatively, β_2-AR agonist stimulation also improves sodium potassium pump kinetics, and may have enhanced the excitability of Na^+/K^+ pumps, and attenuated exercise-induced losses in Na^+/K^+ pump activity (Hostrup et al. 2014).

While we have elucidated the molecular response to pre-workout supplementation following RE, there are several limitations that must be considered. First, the timing of the biopsies limits the scope of the molecular responses reported in this investigation. The peak activation of phosphorylated protein expression depends on the design of the resistance exercise bout implemented (Gehlert et al. 2015). Since we only obtained biopsies at 10 minutes post-exercise, it is unknown what signaling events could have occurred at later time-points. Similarly, it is unclear what the responses would have been if biopsies were taken immediately post exercise considering the fast effects of epinephrine on receptor responses. While we did obtain a blood sample 60 minutes post supplementation and verified elevated circulating epinephrine, we do not know if this affected skeletal muscle as well, since we did not obtain a biopsy post-supplementation but pre-exercise. Further, the responses reflected here indicated the responses in the fasted state, and it is not clear if hormonal or receptor signaling would have produced different responses if subjects performed the RE bout in the

post-prandial state. Future studies should investigate the time-course response of β_2-AR signaling following in the fed and fasted states.

CONCLUSION

In conclusion, the consumption of a pre-workout supplement improves performance and perceptions of exertion. The improvements in performance may have been due to elevated pre-exercise epinephrine. Despite the elevated neuroendocrine response, there were no differences in β_2-AR and MAPK signaling between supplemented and placebo conditions. The improvement in performance was also accompanied by similar perceptions of effort as compared to placebo. We also report relationships between β_2-AR signaling and the ERK pathway highlighting a new topic of investigation on upstream regulators of MAPK activation following RE.

Sex-Based Differences in Resting MAPK, Androgen, and Glucocorticoid Receptor Phosphorylation in Human Skeletal Muscle.

ABSTRACT

Previous research has indicated males express greater androgen receptor (AR) and lower glucocorticoid receptor (GR) content compared to females. In addition, males have greater testosterone concentrations compared to females. Recent evidence suggests AR and GR can be regulated in the absence of their ligands by phosphorylation (pAR & pGR) from mitogen activated protein kinases (MAPK) ERK, JNK, and p38. It is not known if there is differential phosphorylation of the AR and GR between sexes such as has been reported for their ligands (i.e. testosterone). **PURPOSE**: To determine if there is differential expression and phosphorylation of AR, GR, and MAPK in skeletal muscle at rest between males and females. **METHODS**: Ten college aged males (mean±SD; age=22±2.4 yrs, hgt=175±7 cm, body mass=84.1±11.8 kg) and ten females (mean±SD; age=20±0.9 years; hgt=169±7 cm; body mass=67.1±8.7 kg) reported to the laboratory following an overnight fast. Resting muscle biopsies were collected from the vastus lateralis and analyzed for total and phosphorylated GR (ser134, ser211, and ser226), phosphorylated AR (ser81, ser213, ser515, ser650), and MAPK (ERK, JNK, p38) via western blotting. A phosphorylation index (PI) was calculated to determine phosphorylated receptor expression after accounting for differences in total receptor content. Results are reported as mean±SE. Significance was determined at alpha-level $p \leq 0.05$. **RESULTS**: Males had more total AR compared to females

(+42±4%; p<0.001). Females had higher phosphorylation of AR at ser81 (+87 ± 11%; p=0.001) and ser515 (+55 ± 13%; p=0.019). However, when the phosphorylated ratios were corrected for differences in total AR expression (i.e. our PI), the overall phosphorylation at these sites were similar between sexes (ser515, males=100% vs females 92%; ser81, males=100% vs females=107%). pGR ser134 was higher in males compared to females (+50±15%; p=0.016). There was also a trend for higher pGRser211 in men (+34 ±15%; p=0.082). Phosphorylated p38 was higher in females compared to males (+5050±16%; p<0.001). In males, there was a relationship between pARser650 and pGRser134 (r=0.63; p=0.05), and there tended to be a relationship between total AR and pGRser211 (r=-0.59; p=0.068). These relationships were not observed in females (p>0.05). In females, there was a relationship between pARser213 and pGRser226 (r=-0.78; p=0.007), and there tended to be relationships between pARser81, and pGRser211 (r=0.58; p=0.07) and pGRser226 (0.61; p=0.059) respectively. These relationships were not observed in males (p>0.05). **CONCLUSION**: At rest, ARs and GRs are differentially phosphorylated at some, but not all sites between males and females. Differential regulation of phosphorylated AR, GR, and p38 between males and females may have implications on the degree of muscle adaptations observed following resistance training.

INTRODUCTION

Resistance exercise (RE) increases skeletal muscle mass and strength (Seynnes et al. 2007). The adaptations to skeletal muscle occur at the myocellular level (Spiering et al. 2008). RE studies primarily utilize male subjects for their investigations. Despite well-known differences in hormonal concentrations between males and females (Vingren et al. 2009), there is a paucity of data concerning the role hormonal receptors contribute to adaptations in these two populations. Currently, there are very few data concerning sex-based differences in cell-signaling responses to resistance exercise.

Acute measures of muscle protein synthesis after RE are not different between males and females (West et al. 2012), yet males respond with potentiated mTOR activation and AR expression post RE (West et al. 2012; Vingren et al. 2009). On the contrary, sprint interval training produced higher mTOR/p70s6K activation in females compared to males (Esbjörnsson et al. 2012). Most of these investigations have solely focused on the mTOR pathway, despite a growing body of evidence indicating mitogen-activated protein kinases (MAPK) converge on the mTOR pathway at numerous stages (Figueiredo et al. 2015; Gehlert et al. 2015; Martin et al. 2014; West et al. 2016). Mitogen-activated protein kinase molecules are known mechanisms of signal transduction, such that growth factors, cytokines, mechanical forces

and cellular oxidative stress initiate parallel signaling cascades, and help regulate muscle adaptations to exercise (Long et al. 2004). The three most studied MAPK are extracellular signal-regulated kinase 1 and 2 (ERK), c-jun NH_2-terminal kinase (JNK), and p38-MAPK (Cargnello and Roux 2011). Ligand-independent activation of AR and glucocorticoid receptors (GR) can occur in part via MAPK signaling, and can activate AR and GRs by phosphorylation, even in absence of testosterone and cortisol (Galliher-Beckley et al. 2011; Kim and Lee 2009).

Moreover, although males express greater AR content, females have greater GR expression (Vingren et al. 2009), it is not clear if ligand-independent activation of AR and GR contribute to sex-differences in muscle physiology. Thus, further research is needed to understand the physiological differences in muscle physiology between males and females. Perhaps, the relative improvement in muscle adaptation (strength, hypertrophy, power) from RE is similar, but the pathways that influence the eventual adaptation may be different. The receptor pathways involved in sex-specific muscle adaptation to RE remain to be fully elucidated. Therefore, the purpose of this study was elucidate differential expression and phosphorylation of AR, GR, and MAPKs at rest between males and females.

METHODS

Participants

Ten college aged males (mean±SD, age=22±2.4 yrs, hgt=175±7 cm, body mass=84.1±11.8 kg) and ten females (age=20±0.9 years; hgt=169±7 cm; body mass=67.1±8.7 kg) participated in this study. To participate in the study subjects were required to be healthy as determined by a health history questionnaire, be between the ages of 18-35 (females) or 18-30 (males), non-smoking, non-obese (BMI <28 $kg \cdot m^{-2}$), and free of any metabolic or cardiovascular disease. Subjects refrained from physical activity for 48 hours prior to testing. All male subjects reported they had at least 2 years of resistance training experience and they had been resistance training consistently for at least 3 months leading up to the study. Previous data suggests recreationally active men do not have different ERK expression compared to untrained controls (Galpin et al. 2016). All subjects signed an informed consent statement as approved by the University Institutional Review Board and in accordance with the Helsinki Declaration.

Muscle Biopsies

All vastus lateralis muscle biopsies were performed as previously described (Galpin et al., 2016; Fry et al., 2016; Nicoll et al., 2016). Subjects reported to the lab following an eight-hour overnight fast between the hours of 6am and 9am to account for variations in circadian rhythms in circulating

hormonal concentrations. Subjects were asked to rest comfortably for 30 minutes to return to resting state following arrival at the lab. A muscle sample was collected from each subject under resting conditions

All muscle biopsies were obtained from the vastus lateralis by percutaneous needle biopsy. To prepare the subject's leg for the muscle biopsy, the thigh was shaved and cleaned with Betadine solution. Three mL of 2% lidocane (without epinephrine) solution was injected into the skin and the surrounding tissues of the leg. Next, a 1 cm incision was made through the skin and fascia. A Bergstrom needle was used for tissue extraction, using the double-chop technique (Staron, 1991) and suction (Evans et al. 1982). All samples were extracted from the same incision, and orientation of the biopsy needle was rotated approximately 45° for each biopsy to insure samples were obtained from a different region. Immediately following extraction, muscle samples were cooled to –159 °C by liquid nitrogen to halt metabolic processes and stored at –80 °C.

Tissue Sample Preparation for Western Blots

Ten to fifteen mg of sample was placed in 1 ml of lysing buffer containing 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, and 2.3% (w/v) SDS in 62.5 mMTris·HCL buffer (pH 6.8), with a 1% solution of Halt Protease and Phosphatase Inhibitor Cocktail to inhibit protease and phosphatase

activity (Thermo Fisher Scientific Inc., Waltham, Mass.). Samples were homogenized in a test tube (3 X 10 s) using a Tissue Miser homogenizer. The resulting suspensions were then heated for 10 min at 60° C, and subsequently frozen at –80° C.

Western Blotting

Muscle homogenate samples were assayed for total protein concentration using a micro Lowry method with Peterson's modification (Sigma Aldrich, Saint Louis, MO. No P5656). Following protein determination, 20-μg of protein were loaded onto 4-15% pre-cast gels (Bio-Rad) and electrophoresed at 200V for 30 minutes. Once resolved, proteins were transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Burlington, NH) at 15V for 90 minutes using an Idea Scientific Co. wet transfer apparatus (Minneapolis, MN). After transfer, PVDF membranes were blocked in Odyssey® blocking buffer (TBS without Tween 20) for 1 h at room temperature. Following blocking, membranes were then incubated with primary antibodies for p-AR [(ser^{515}) (1:1000, rabbit polyclonal, no. ab128250; ABCam Cambridge, MA), p-AR (ser^{81}) (1:1000, rabbit polyclonal, no. PA5-64617), p-AR (ser^{213}) (1:1000, rabbit polyclonal, no. PA5-37478), p-AR (ser^{650}) (1:1000, rabbit polyclonal, no. PA5-37479)], total AR (1:1000, mouse monoclonal, no. MA5-15598), p-GR (ser^{134}) [(1:1000, rabbit polyclonal, no. ABS1008); MilliporeSigma (Burlington, MA)], p-GR (ser^{211})

[(1:1000, rabbit polyclonal, no. 4161S); Cell Signaling Technologies Inc. (Danvers, MA)], p-GR (ser^{226}) [(1:1000, rabbit polyclonal, no. ABS994); MilliporeSigma (Burlington, MA)], total GR (1:1000, mouse monoclonal, no. MA5-15801). total ERK1/2 (1:2000, mouse monoclonal, no. 4696s, Cell Signaling), p-ERK1/2 (Thr202/Tyr204) (1:1000, rabbit monoclonal, no. 4376s; Cell Signaling), total JNK (1:500, mouse monoclonal, no. sc-7345; Santa Cruz BioTech), p-JNK (Thr183/Tyr185) (1:1000, rabbit monoclonal, no. 4671s; Cell Signaling), total p38-MAPK (1:1000, mouse monoclonal, no. MABS1754; EMD Millipore), and p-38 (Thr180/Tyr182) (1:1000, rabbit monoclonal, no. 9211s; Cell Signaling) overnight at 4° C with gentle agitation. p-AR (ser^{81}), (ser^{213}), (ser^{650}), total AR, and total GR antibodies were obtained from ThermoFisher (Walthham, MA). p-GR (ser^{134}) and (ser^{226}) antibodies were obtained from MilliporeSigma (Burlington, MA). Following overnight incubation with agitation, membranes were washed (3 X 5min) with TBS plus Tween-20 (TBST). Following washing, membranes were then probed with infrared (IR) secondary antibodies (1:10,000) specific to the host animal (700 nm anti-mouse, 800 nm anti-rabbit) for 1 h at room temperature to label the respective total and phospho-receptor primary antibodies. After secondary antibody incubation, membranes were washed again (3 x 5min) in TBST, rinsed once with TBS, and then scanned with an Odyssey Infrared Imaging System and accompanying software (v1.2, LI-COR Biosciences, Lincoln, NE) to quantify IR intensity for each labeled protein band. Total and phosphorylated bands

were identified on the same membrane with IR markers scanned at two different wavelengths (700nm; total-AR and total-GR and 800nm; p-AR and p-GR). Infrared-labeled secondary antibodies were obtained from Li-COR Inc. (LI-COR Biosciences, Lincoln, NE). Phospho-AR, p-GR, p-MAPK, total-AR, total-GR, and total-MAPK signals were normalized to total protein abundance loaded per lane via total protein stain (REVERT; Li-COR). Phospho-receptor signals were then normalized to the total-receptor signal and are expressed as the ratio of phospho-target protein : total-target protein (i.e. pAR/total-AR, pGR/total-GR, and pMAPK/total-MAPK). All statistical analyses were performed on the total protein adjusted ratios.

Statistical Analyses

Independent samples t-tests were performed to compare total and phosphorylated receptor expression between males and females. Since total and phospho-MAPK expression were not normally distributed, non-parametric statistics were utilized on these variables. Mann-Whitney U tests were performed to compare phospho- and total expression of ERK, JNK, and p38 between males and females. Pearson correlations were performed between AR, GR, and MAPK variables to determine if there were relationships that may explain regulation of the anabolic-catabolic milieu. A phosphorylation index (PI) was calculated to determine phosphorylated receptor expression after accounting for differences in total receptor content. Results are reported

as mean±SE for parametric analyses. Results are presented as median±SE for non-parametric analyses.

RESULTS

Total Receptor Expression

Males had more total AR compared to females ($t(18)=8.889$; $p<0.001$). Total GR expression did not differ between sexes ($t(18)=1.474$; $p=0.158$).

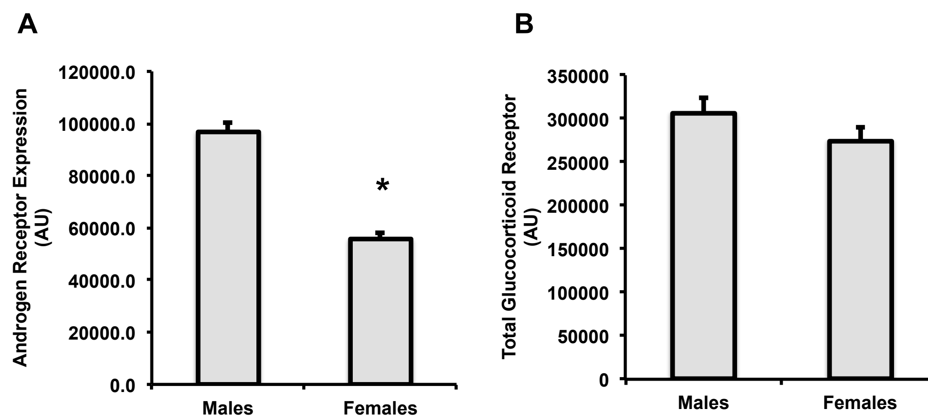

Figure 4-1 Total expression of **A)** androgen (AR) and **B)** glucocorticoid receptors (GR). Data expressed as mean ± SE as a percent of males (%). Dotted line indicates Phosphorylation Index (PI). * indicates significantly different from males.

Androgen Receptor (AR) signaling

Females had higher phosphorylation of AR at ser81 (+87 ± 11%) (t(18)=-4.121; p=0.002) and ser515 (+55 ± 13%) (t(18)=-2.574; p=0.026). However, when the phosphorylated ratios were corrected for differences in total AR expression (i.e. our PI), the overall phosphorylation at these sites were similar between sexes (ser515, males=100% vs females 92%; ser81, males=100% vs females=107%). There was no difference in phosphorylation of AR at ser213 (t(18)=1.290; p=0.216) or ser650 (t(18)=-1.206; p=0.245) between males and females.

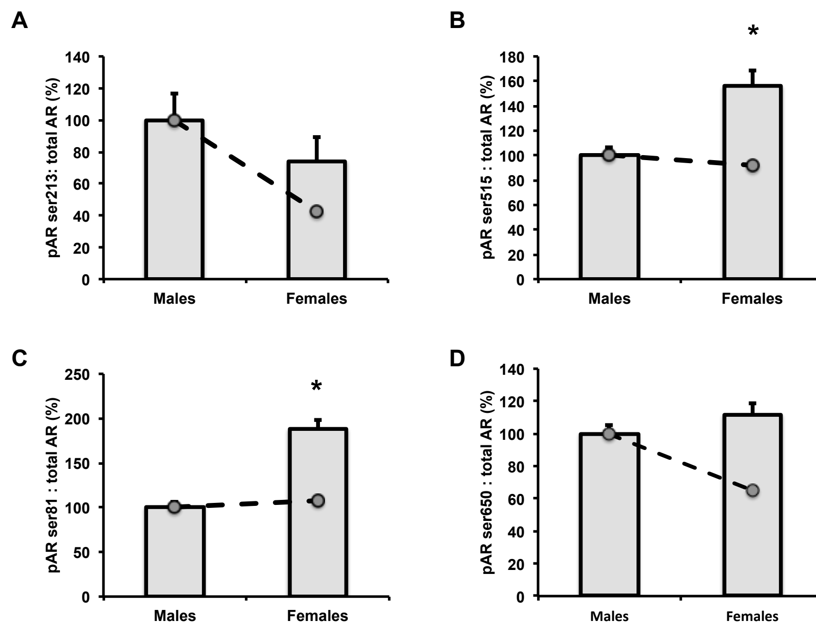

Figure 4-2 Phosphorylated androgen receptor (AR) at **A)** ser213, **B)** ser515, **C)** ser81, **D)** ser650. Data expressed as mean ± SE as a percent of males (%). Dotted line indicates Phosphorylation Index (PI). * indicates significantly different from males.

Glucocorticoid Receptor (GR) signaling

Phosphorylated GR at ser134 was higher in males compared to females (t(18)=2.652; p=0.016) There was a trend for higher pGRser211 in men (t(18)=1.843; p=0.082). Phosphorylation at ser226 was not different between sexes (t(18)=1.299; p=0.210).

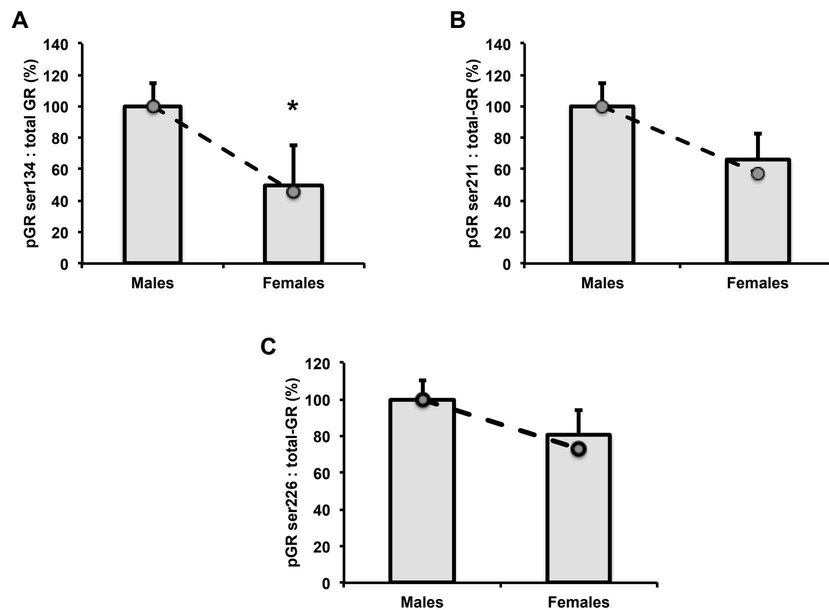

Figure 4-3 Phosphorylated glucocorticoid receptor (GR) at **A)** ser134, **B)** ser211, and **C)** ser226. Data expressed as mean ± SE as a percent of males (%). Dotted line indicates Phosphorylation Index (PI). * indicates significantly different from males.

MAPK signaling

Phosphorylated ERK (z=-0.529; p=0.631) and JNK (z=-0.378; p=0.739) were not different between males and females. Phosphorylated p38 was significantly higher in females compared to males (z=-3.780; p<0.001). Total expression of ERK (z=-0.832; p=0.436), and JNK (z=-0.983; p=0.353) was not different between groups. Total p38 expression tended to be higher in females (z=-1.890; p=0.063).

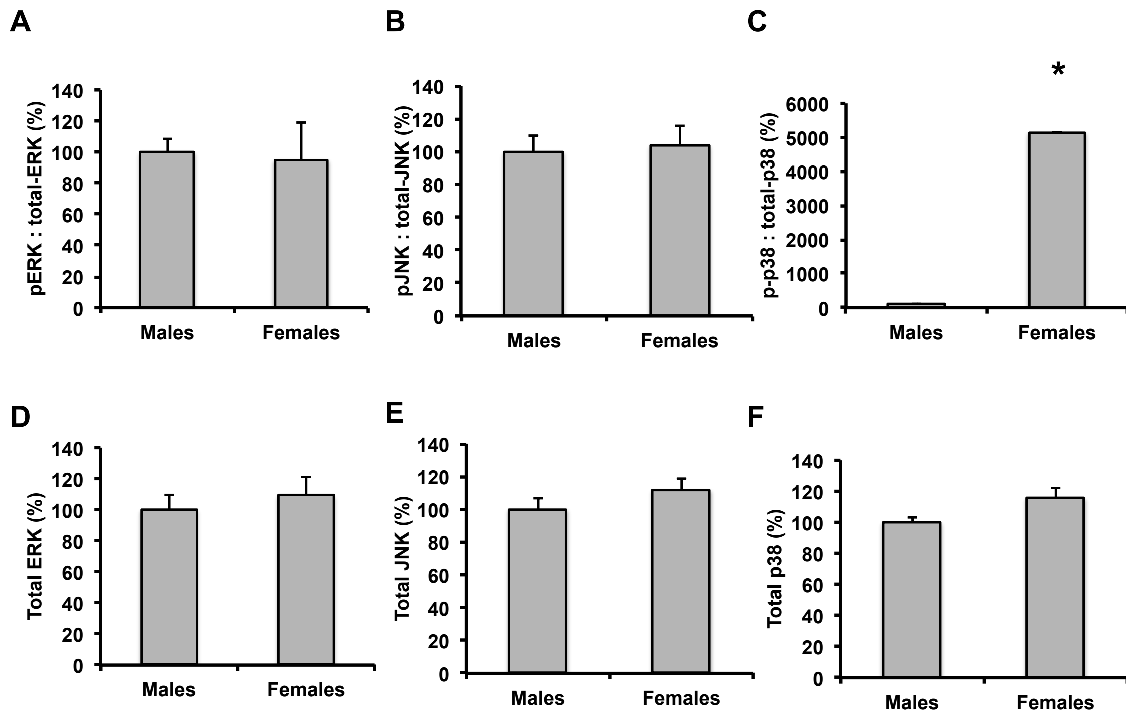

Figure 4-4 Phosphorylated MAPK **A)** ERK, **B)** JNK, and **C)** p38 Total expression of MAPK **D)** ERK, **E)** JNK, and **F)** p38. Data expressed as median ± SE as a percent of males (%). * indicates significantly different from males.

Correlations

In males, there was a relationship between pARser650 and pGRser134 (r=0.63; p=0.05), and tended to be a relationship between total AR and pGRser211 (r=-0.59; p=0.068). These relationships were not observed in females (p>0.05). In females, there was a relationship between pARser213 and pGRser226 (r=-0.78; p=0.007), and there tended to be relationships between pARser81 and pGRser211 (r=0.58; p=0.07), and pGRser226 (0.61; p=0.059) respectively. These relationships were not observed in males (p>0.05).

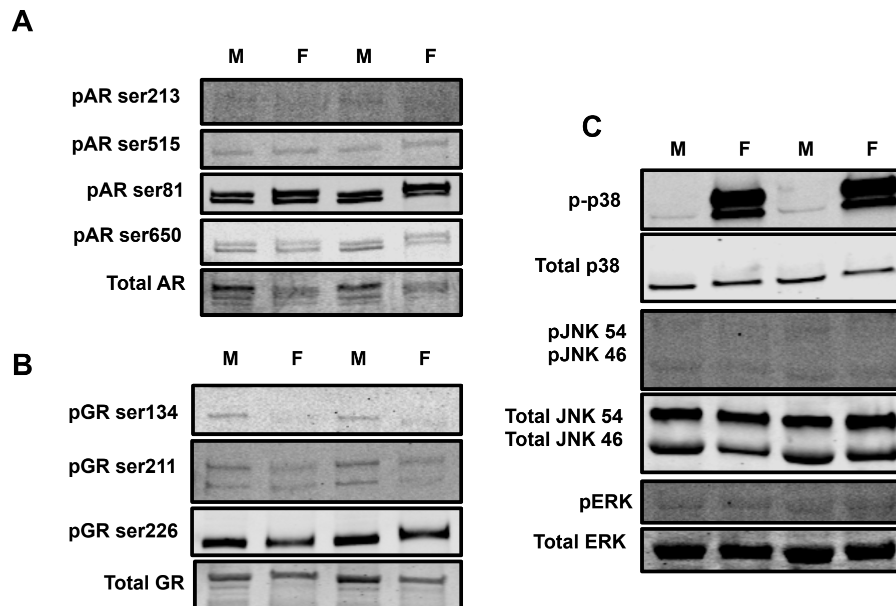

Figure 4-5 Representative western blot images for resting samples from males (M) and females (F) of **A)** Total and phosphorylated androgen receptor (AR) at ser515, ser81, ser213, and ser650 **B)** Total and phosphorylated glucocorticoid receptor (GR) at ser134, ser211, and ser226. **C)** Total and phosphorylated p38, JNK, and ERK.

DISCUSSION

Females present different sensitivities in mitochondrial respiratory volume, density, and function in skeletal muscle (Miotto et al. 2018; Montero et al. 2018). Furthermore, females also have higher protein content of enzymes related to beta-oxidation in skeletal muscle (Maher et al. 2010) which could be related to higher rates of fat oxidation during aerobic exercise (Montero et al. 2018). In reference to resistance exercise, males display a prolonged gene expression following RE compared to females (Liu et al. 2010), but do not display differences in MPS in the early recovery period (West et al. 2012). Here we present data suggesting that despite differences in the total expression of AR between males and females, site specific phosphorylation could be maintaining a similar cellular milieu between sexes at rest. In addition there was substantially greater phosphorylation of p38 in resting muscle of females compared to males.

Males display greater AR expression compared to females (Vingren et al. 2009; West et al. 2012), and this effect may arise from the potentiating effects of androgen exposure on androgen receptor stability (Chen et al. 2006). Indeed, the post-exercise testosterone response to RE has been shown to be a precursor to down regulation of the AR after RE (Spiering et al. 2009; Vingren et al. 2009). In the present study we provide evidence the ratio of phosphorylated AR at ser81 and ser515 is greater in females compared to

males. However, by adjusting the ratio based on differences in total AR expression (i.e. our PI), the overall cellular environment is similar. The phosphorylation of AR and GR at specific residues regulates receptor function, both in hormone dependent (Wang et al. 2002; Gioeli et al. 2002) and independent (Galliher-Beckley et al. 2011; Gioeli et al. 2006) manners. Of all the phosphorylation sites located on the AR, ser81 has the highest phosphorylation stoichiometry for ligands (Gioeli 2002). Furthermore, phosphorylation at this site is required for transcription of AR target genes (La Montagana et al. 2012) and sensitizes the AR is the presence of low androgens (Chen et al. 2006). Further, ser515 phosphorylation regulates nuclear/cytoplasmic shuttling and responds synergistically with androgen ligands and other growth-factors (Ponguta et al. 2008). Thus, the AR in females may phosphorylate to a greater extent to maintain nuclear localization and transcriptional activity despite lower overall AR expression and low circulating androgens. More research is needed to substantiate this hypothesis.

Contrary to previous research (Vingren et al. 2009), we did not observe greater GR expression in females. Instead total GR expression was similar between sexes, and tended to be higher in males. The phosphorylation at ser211 and ser226 were similar between males and females. The activation of GR at ser226 influences nuclear/cytoplasmic shuttling (Itoh et al. 2002).

Phosphorylation at ser211 increases when stimulated by glucocorticoids (i.e., dexamethasone) in vivo (Wang et al. 2002, Galliher-Beckley et al. 2011). Although resting differences in cortisol do not differ between males and females (West et al. 2012), post-exercise responses are higher in females post RE compared to males (West et al. 2012). If there were potential sex-based differences in ser211 and ser226 phosphorylation, it may not be manifested until the post-exercise period.

Resting phosphorylation of GRser134 was higher in males compared to females. Galliher-Beckley et al. (2011) reported ligand-independent phosphorylation at ser134 is regulated by the p38 MAPK pathway, and phosphorylation at this site specifies hormone stimulated GR gene transcription (Galliher-Beckley et al. 2011). Furthermore, GR phosphorylation at ser134 acts as a metabolic sensor in cells, and integrates a variety of cellular stress signals including glucose starvation, UV exposure, and oxidative stress (Galliher-Beckley et al. 2011). In our study, phosphorylated p38 was higher in females and not males, so the direct link between p38 and GR ser134 phosphorylation in intact human skeletal muscle is clearly more complex than what has been reported in in vitro work. More research is necessary to understand the role this site may contribute to skeletal muscle physiology and adaptation.

There were no differences in total or phosphorylated ERK and JNK between males and females. However, phosphorylated p38 was substantially higher in females compared to males. The reason for elevated p38 is not clear, as a previous study investigating sex-specific MAPK responses to sprint interval exercise did not report differential p38 phosphorylation at rest or after exercise (Fuentes et al. 2012). Thus, the discrepancy between our results and theirs are not clear. Macaluso and colleagues (2012) reported decreasing phosphorylation of resting p38 as the length of training history increased. Among our females, five of the subjects were part of the University's athletics teams, and although they did not exercise for 24 hours prior to the biopsy visit they, had 2-fold higher p38 phosphorylation compared to the non-athletes. This finding further compounds the differences between sexes we reported because it would be expected chronic exercise training decreases resting phosphorylation. However, resting phosphorylated p38 may increase slightly in females at rest and contribute to the enhanced fat oxidation observed during aerobic exercise. p38 is a key component of PGC-1a activation (Ikeda et al. 2008), and regulates increases in mitochondrial and oxidative capacities of skeletal muscle. More research is necessary to determine the cause of differences in phosphorylated p38 expression between the sexes.

While we provide evidence of differential phosphorylation of steroid receptor and MAPK signaling in skeletal muscle between sexes, there are several limitations that must be mentioned. While our male subjects were well trained, the female subjects in our study included university athletes (n=5) and recreationally active or untrained subjects (n=5). Thus, the subject characteristics were more varied in the female subjects. However, as a whole, the differences between sexes were greater than the differences within females suggesting there differences observed were inherent. Second, we also note that the biopsies were obtained in the fasted state, and it is not known if post-prandial expression and phosphorylation of these receptors differs between sexes. Androgen receptor expression does not differ in skeletal muscle throughout the menstrual cycle (Ekenros et al. 2017), however it is not known if glucocorticoid and MAPK expression also differs. Finally, while none of the females in our study were pregnant, it is unknown how pregnancy may affect receptor or MAPK expression and phosphorylation.

CONCLUSION

In conclusion we present novel data of differential phosphorylation of AR, GR, and MAPK between males and females. The site-specific phosphorylation between sexes may partially explain the differences in hypertrophy, or the ability to hypertrophy between the sexes. Alternatively the differences the phosphorylation at rest may also explain the differences in substrate oxidation and recovery strategies that have been previously observed. While we only report the expression and phosphorylation at rest in the fasted state, future research should investigate the responses to RE and aerobic exercise in the fed and fasted state.

MAPK, Androgen, and Glucocorticoid Receptor Phosphorylation Following High-Frequency Resistance Exercise Overtraining

ABSTRACT

Stressful training with insufficient recovery can attenuate or decrease muscle performance. Expression of MAPK proteins have been reported at rest following overreaching and over-training. The acute myocellular exercise responses to stressful training has not been investigated. **PURPOSE:** To investigate MAPK, androgen, and glucocorticoid receptor phosphorylation following a period of stressful training. **METHODS**: 16 men were matched on barbell squat 1 RM strength and randomized into a group that performed normal training or stressful training with insufficient recovery. The control group (CON) performed three speed-squat training sessions on non-consecutive days, while the overtraining group (OT) performed 15 training sessions over 7.5 days. Resting and post-exercise biopsies were obtained prior to (T1) and after the training period (T2). Samples were analyzed for total and phosphorylated androgen receptor (AR), glucocorticoid receptor (GR), and MAPKs (ERK, JNK, and p38). REULTS: Total AR were down-regulated post-exercise at T2 in overtrained group only. Phosphorylated AR at ser515 increased in both groups post-exercise at pre-training; however ser515 increased at post-training only in the OT group. ERK, JNK, and p38 increased post-exercise in CON and OT groups, however, only p38 post-exercise was attenuated at T2 in OT. Resting p38 was decreased in CON and increased OT at T2. At T2, phosphorylated GR at ser226 was attenuated post-exercise, and increased resting levels in OT only. **CONCLUSION:** These

data indicate that steroid receptors are phosphorylated after acute resistance exercise, and are differentially regulated after short-term stressful training. MAPKs may influence steroid receptor phosphorylation following normal and stressful training.

INTRODUCTION

Normal resistance training (RT) induces a decrease in protein degradation and inflammatory signaling (Karagounis et al. 2010). In addition, repeated bouts of RT elicit beneficial adaptations that allow an increase in translational capacity and regeneration, including ribosome biogenesis and satellite cell expansion (Brook et al. 2016; Petrella et al. 2008). However, while adequate amounts of resistance exercise can prove fruitful for muscle adaptation, the molecular responses to excessive exercise is not well understood. Overreaching and overtraining are typically determined by a decrease or stagnation of muscular performance (Meeusen et al. 2013). While in the past it is been hypothesized that increases in circulating cytokines are indicative of overtraining, actual evidence at the skeletal muscle level in humans is lacking (Halson et al. 2003; Main et al. 2010; Smith 2000; Smith 2004). To date, only one study has investigated intracellular signaling protein activation in humans following short-term stressful RT (Nicoll et al. 2016).

Mouse models have primarily utilized endurance training to induce muscular damage on downhill running protocols (Da Rocha et al. 2017; Pereira et al. 2014). The two studies that utilized squat-like or heavy resistance exercise in mice have reported an increase in inflammatory signaling in skeletal muscle (Alves Souza et al. 2014; Coffey et al. 2007). Two

key proteins elevated in excessive exercise with insufficient recovery are TNF-alpha and a chronically elevated increase in p38 phosphorylation. Following normal resistance training there is decrease in atrophic and inflammatory proteins, and a concomitant increase in hypertrophic signaling, followed by subsequent increases in myofibrillar protein content (Karagounis et al. 2010). However, with insufficient recovery there is an increase in atrophy and inflammatory protein expression which may create an environment where anabolic capacities are insufficient to overcome atrophy signaling.

Recently, cancer (Koryakina et al. 2014) and C2C12 skeletal muscle myotube (Kim and Lee 2009) cell lines suggest androgen receptors (AR) are activated in the absence of testosterone. Ligand-independent activation of AR and glucocorticoid receptors (GR) occur in part via the mitogen-activated protein kinase (MAPK) signaling cascade by activating AR and GRs via phosphorylation, even in absence of testosterone and cortisol (Galliher-Beckley et al. 2011; Kim and Lee 2009). The proteins ERK, JNK, and p38-MAPK modify the phosphorylation of the AR and alter AR protein expression in myotubes (Kim and Lee 2009). The phosphorylation of AR and GR at specific residues regulates receptor function and may influence eventual muscle phenotype response (Galliher-Beckley et al. 2011, Kim and Lee 2009, Koryakina et al. 2014). The modulation of the acute RE program variables could promote specified signaling and fiber adaptation following RE

(Figueiredo et al. 2015; Fry 2004, Gehlert et al. 2015; Spiering et al. 2008). However, there are no data investigating the MAPK influence on the AR and GR in humans despite their role initiating muscle growth, regeneration, and remodeling.

Numerous cell culture and human models report the magnitude and duration of chronic stimulation of β_2-AR via agonists results in receptor down-regulation and desensitization (Fry et al. 2006; Fan et al. 2016; Sterczala et al. 2017). This point has particular relevance to overtraining since stressful training down-regulates or decreases the sensitivity of β_2-ARs and could contribute to sustained performance decrements, but little information is available concerning the myocellular responses to acute RE in the OT state. Decades of research has sought to determine if testosterone and cortisol are indicators of the anabolic/catabolic milieu during intense training. However, recent literature has implicated the testosterone cortisol ratio a marker of exercise intensity and/or volume rather than overtraining (Fink et al. 2017; Fry et al. 1994). As mentioned earlier the primary actions of testosterone and cortisol occur via interaction with their intracellular receptors however there are also fast acting and non-genomic effects in skeletal muscle after hormone exposure (Boncompagni et al. 2015; Estrada et al. 2003; Hughes et al. 2016). A clearer understanding of the hormonal consequences of overtraining may be better understood by studying their respective intracellular receptors. We

have shown resting expression, and activation of MAPK are differentially regulated following overreaching versus overtraining (Nicoll et al. 2016). Furthermore Da Rocha et al. (2017) investigated phosphorylated AR and GR expression to a treadmill OT protocol in mice, and reported down-regulation of total receptor expression and phosphorylation, but they did not investigate MAPK Reponses. Altered MAPK activity could have implications on the regulation of AR and GR both at rest and after exercise, but this has not yet been investigated in humans. Therefore, the purpose of this study was to determine if there was differential resting expression and exercise induced phosphorylation of AR, GR, and MAPKs following normal training and stressful training with insufficient recovery.

METHODS

Experimental approach to problem

We have previously utilized a lower body resistance exercise to induce significant performance decrements, which resulted in altered muscle receptor and MAPK expression (Fry et al. 2006; Nicoll et al. 2016, Sterczala et al. 2017). The aforementioned studies examined resting expression of muscle signaling but did not investigate the responses to an acute exercise bout. Studies in molecular signaling responses in trained individuals show an attenuated signaling response to resistance exercise (Coffey et al. 2006; Gonzalez et al. 2016; Kudrna et al. 2017). Resistance training with barbell back squats produces significant transfer to sport-specific tests and movements (Wirth et al. 2016), but does not isolate the vastus lateralis to the same degree as bilateral knee extensions. Thus, unlike our previous studies that only utilized barbell back squats (or squat-like devices), we also included knee extensions in each resistance exercise session to sufficiently target the vastus lateralis for examination of acute exercise molecular responses. Since we were looking to avoid inducing significant muscle damage, we reduced from previous studies the load of the barbell speed squats from 70% 1RM to 60% 1RM to account for the added knee extensions in the training program. Furthermore, we chose the biopsy time at 10 minutes post-exercise, because previous research indicates MAPK phosphorylation occurs closer to the cessation of exercise (Gehlert et al. 2015), but not immediately after (Creer et

al. 2010). Since hormonal responses to barbell speed squats sometimes increases testosterone and cortisol, we chose to collect serum blood samples at rest (Pre-ex), immediately after barbell speed squats (Mid-ex), and five minutes after the last set of knee extensions (Post-ex) to determine if there were any additional or differential increases in hormonal variables after the back squats compared to knee extensions.

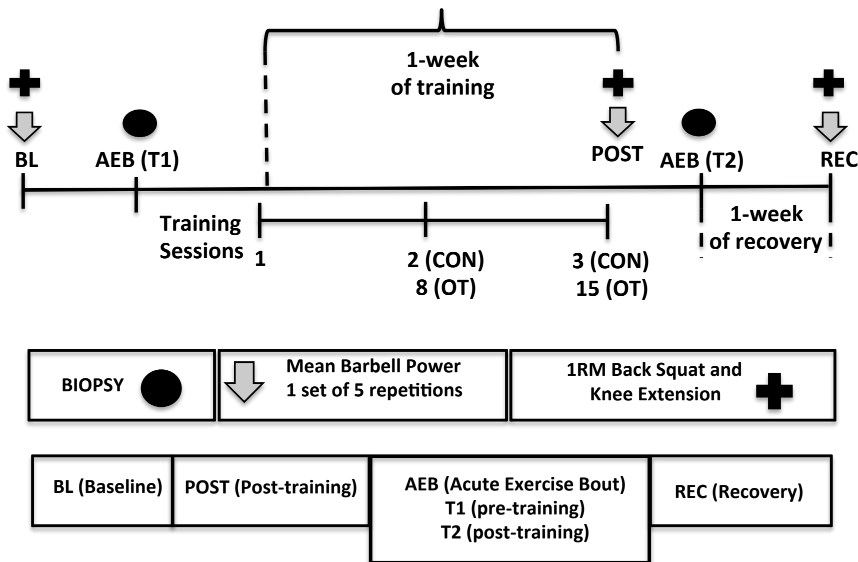

Figure 5-1 Study timeline. Barbell squat power (PWR; grey arrow) and one repetition maximum (1RM; black cross) was determined prior to training (BL). Following determination, subjects were matched on one-repetition maximum (1-RM) strength and randomized into a normal training control group (OT) or a high-frequency, with insufficient recovery, overtraining (OT) group. Subjects them participated in an acute resistance exercise (AEB; T1) in which 5 sets of 5 barbell speed squats at 60% of 1-RM was performed, followed by 3 sets of 10 repetition knee extensions at 70% 1-RM. Muscle biopsies (black circle) were performed at rest and at ten minutes post exercise. Following 2 days of rest subjects completed 1 week of training wherein CON participated in 3 training sessions over non-consecutive days and performed 5 sets of 5 barbell speed squats at 60% of 1-RM, followed by 3 sets of 10 repetition knee extensions at 70% 1-RM. The OT performed 10 sets of 5 repetitions barbell speed squats at 60% of 1-RM, followed by 3 sets of 10 repetition knee extensions at 70% 1-RM. OT group performed this training protocol twice per day (once in morning, once in evening), for 7.5 days for a total of 15 training sessions. 1-RM and PWR (POST) was obtained on the day of (but prior to) the final exercise training session. CON performed their post-training AEB visit 48 hours after the final training session. OT performed their post-training AEB visit 24 hours after their final training session. After the post-training AEB, all subjects ceased all exercise training for 1 week. After one full week of recovery (REC) subjects reported to the lab for a final test of PWR and 1-RM.

Participants

Subjects refrained from physical activity other than that required by the experimental trials. Each participant was evaluated by the researcher to ensure the subjects all have safe and consistent barbell back squat technique prior to the maximal squat protocol. Subjects also demonstrated their ability and comfort in achieving the minimum parallel squat depth for the study. Specifically, participants were instructed to stand with feet approximately shoulder width apart, and descend until the femur was parallel to the ground (as assessed by a Certified Strength and Conditioning Specialist). Participants in this study had at least 2 years of resistance training experience, had been resistance training consistently for at least 3 months leading up to the study, and must have squatted at least once per week in the six months prior to the study. Each subject signed an informed consent statement as approved by the University Institutional Review Board and in accordance with the Helsinki Declaration. All participants were recruited in a block randomization manner. Briefly subjects were recruited 8 at a time (four per group) and assessed for proper back squat technique. Subjects completed 1 RM strength testing and were match-paired on 1 RM strength and approximate years of training history into an arbitrarily named group 1 or 2. Group 1 was allocated to a normal training control group (CON) or to a high-frequency, insufficient recovery overtraining group (OT) via simple

randomization (coin-flip). Subjects were informed of their group allocation prior to the initial acute exercise biopsy visit (T1).

Strength testing

The 1 RM strength tests were completed for the barbell squat exercise according to previously described methods (Kraemer et al. 2006). Subjects were familiarized with proper back squat technique according to the National Strength and conditioning guidelines (Baechle and Earle 2008), participants were instructed to stand with feet approximately shoulder width apart, and descend until the femur is parallel to the ground (as assessed by a Certified Strength and Conditioning Specialist). Once proper depth was attained, subjects were instructed to ascend to the starting position. Briefly, subjects performed a light warm-up of 5 to 10 repetitions at 40% to 60% of perceived maximum. After 1 minute, subjects will perform three to 5 repetitions at 60% to 80% of perceived maximum. Conservative increases in weight were made, and the subject attempted a 1RM lift. If the lift was successful, a rest period of 3 minutes was allowed prior to the next attempt. The 1RM was attained within three to five sets as to avoid excessive fatigue (Kraemer et al. 2006).

Acute Resistance Exercise Bout.

Prior to the training intervention, all subjects participated in an exercise bout where blood and muscle biopsies were obtained to determine acute molecular responses in the pre-training state (T1). To warm up, all subjects cycled lightly on a cycle ergometer for 5 minutes. Subjects then performed two warm-up exercise sets with the barbell back squat at 35% and 55% of their 1-RM. The subjects then performed five sets of barbell speed squats for 5 repetitions at 60% of their 1-repetion maximum (1-RM) with two minutes of rest in between sets. Once all 5 sets were completed, subjects then performed 3 sets of 10 repetitions of knee extensions at 70% of 1-RM. Upon completion of the final set of knee extensions, a post exercise biopsy (POST) was obtained 10 minutes after the cessation of exercise.

Control (CON) and Overtraining Protocols (OT)

All subjects participated in a controlled training program, supervised and documented by a Certified Strength & Conditioning Specialist, an NSCA-Certified Personal Trainer, or a U.S.A.-Weightlifting regional- or national-level coach. For the CON group, subjects performed three training sessions over the 7.5 days on non-consecutive days. Each CON session included 5 sets of 5 repetitions of the barbell back squat exercise at 60% of 1-RM, followed by 3 sets of 10 repetitions of knee extensions at 70% of 1-RM. For the OT group, subjects lifted two times per day for 7.5 days for a total of 15 training

sessions. Each OT session included 10 sets of 5 repetitions of the barbell back squat exercise at 60% of 1-RM, followed by 3 sets of 10 repetitions of knee extensions at 70% of 1-RM. During training sessions, both groups subjects were instructed to perform each repetition at maximum concentric velocity. When barbell velocity dropped below 90% for at least two repetitions of a set, the barbell weight was decreased approximately 4.5 kg. to permit barbell velocities to be maintained. As a result, speed of movement was maintained throughout all training sessions for all subjects. An external dynamometer (Fitrodyne, Bratislava, Slovakia) was attached to the barbell via a tether to a monitor bar velocity and power for all training repetitions, with barbell power used as the criteria for decreased performance in the OT subjects.

Following the training period, the post-training acute-exercise biopsy visit (T2) was scheduled for 48 hours after the final training session in CON, and within 24 hours in OT. The timing of these biopsies at these time points were chosen because the acute exercise session in the CON required the subjects to experience a period of recovery between exercise sessions, while the OT group experienced insufficient recovery in between training bouts (similar to the training protocol). While the training protocols were very different between groups, the acute exercise protocol performed on the biopsy visits were exactly same, and was the same as the training protocol

performed by the CON group. Thus, we could investigate signaling responses to repeated exposures to the same training protocol when sufficient recovery was allowed (CON), and signaling responses to the same training protocol in the overtrained state when there was high-frequency of training and insufficient recovery between training bouts (OT).

Muscle Biopsies

All vastus lateralis muscle biopsies were performed as previously described (Galpin et al., 2016; Nicoll et al., 2016). On the day of the pre-training exercise protocol, muscle biopsies and blood samples were taken before, and after the acute RE bout. Subjects reported to the lab following an eight-hour fast. Subjects were asked to rest comfortably for 30 minutes to return to resting state following arrival at the lab. For the post-training exercise biopsy visits, subjects were required to report to the lab at the same time of day as the pre-training acute biopsy visit following an eight-hour fast.

All muscle biopsies were obtained from the vastus lateralis by percutaneous needle biopsy at rest (PRE) and 10 minutes after the final set of knee extensions (POST). To prepare the subject's leg for the muscle biopsy, the thigh was shaved and cleaned with Betadine solution. Three mL of 2% lidocane (without epinephrine) solution was injected into the skin and the surrounding tissues of the leg. Next, a 1 cm incision was made through the

skin and fascia. A Bergstrom needle was used for tissue extraction, using the double-chop technique (Staron, 1991) and suction (Evans et al. 1982).. Following the pre-exercise biopsy, sterile and flexible pressure wraps were placed over the incision site. Bandages were securely wrapped and the volunteers began their warm-up and exercise protocol. All samples were extracted from the same incision, and orientation of the biopsy needle was rotated approximately 45° for each biopsy to insure samples were obtained from a different region. Immediately following extraction, muscle samples were cooled to –159° C by liquid nitrogen to halt metabolic processes, and stored at –80° C

Blood Measures

An intravenous catheter was inserted into an antecubital vein, and a normal saline lock was attached. During each blood draw at rest (PRE), immediately after barbell back squats (MID), and five minutes post exercise (POST) the first 3 mL of blood (with saline from the catheter lock) was collected into a discard tube preceding each serial sample. Approximately 5-10 mL of blood was collected and divided into untreated Vacutainer™ test tubes for serum. The blood samples were allowed to clot in untreated Vacutainer™ test tubes. The tubes were subsequently centrifuged for 15 minutes at 2000 RPM, after which the serum was pipetted into eppendorf tubes and stored at -80°C. Blood was drawn immediately prior to the first

muscle biopsy prior to the start of the barbell back squat exercise, and 5 minutes after the end of the final set of the knee extensions. Standard blood processing procedures of determination of hematocrit and hemoglobin were recorded to account for plasma volume shifts during exercise (Dill and Costill 1974).

Enzyme-linked Immunosorbent Assay (ELISA)

Serum samples were analyzed in duplicate for total testosterone and cortisol via commercially available enzyme-linked immunosorbent assays (ALPCO; Salem, NH). All samples from the same subject were analyzed on the same assay plate, and by the same investigator in one run to avoid inter-assay variation. Serum samples were thawed only once before analysis. Intra-assay variance was 3.5% and 5.4% for testosterone and cortisol respectively, and inter-assay variance was 3.4% and 7.8% for testosterone and cortisol respectively. Hormonal values were not corrected for plasma volume shifts since this is the molar concentration the steroid receptors are actually exposed to during and post exercise.

Tissue Sample Preparation for Western Blots

Ten to fifteen mg of sample was placed in 1 ml of lysing buffer containing 10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, and 2.3% (w/v) SDS in 62.5 mMTris·HCL buffer (pH 6.8), with a 1% solution of Halt Protease

and Phosphatase Inhibitor Cocktail to inhibit protease and phosphatase activity (Thermo Fisher Scientific Inc., Waltham, Mass.). Samples were homogenized in a test tube (3 X 10 s) using a Tissue Miser homogenizer. The resulting suspensions were then heated for 10 min at 60° C, and subsequently frozen at –80° C.

Western Blotting

Muscle homogenate samples were assayed for total protein concentration using a micro Lowry method with Peterson's modification (Sigma Aldrich, Saint Louis, MO. No P5656). Following protein determination, 20-μg of protein were loaded onto 4-15% pre-cast gels (Bio-Rad) and electrophoresed at 200V for 30 minutes. Once resolved, proteins were transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (MilliporeSigma, Burlington, NH) at 15V for 90 minutes using an Idea Scientific Co. wet transfer apparatus (Minneapolis, MN). After transfer, PVDF membranes were blocked in Odyssey® blocking buffer (TBS without Tween 20) for 1 h at room temperature. Following blocking, membranes were then incubated with primary antibodies for p-AR [(ser^{515}) (1:1000, rabbit polyclonal, no. ab128250); ABCam (Cambridge, MA), p-AR (ser^{81}) (1:1000, rabbit polyclonal, no. PA5-64617), p-AR (ser^{213}) (1:1000, rabbit polyclonal, no. PA5-37478), p-AR (ser^{650}) (1:1000, rabbit polyclonal, no. PA5-37479)], total AR (1:1000, mouse monoclonal, no. MA5-15598), p-GR [(ser^{134}) (1:1000, rabbit

polyclonal, no. ABS1008); MilliporeSigma (Burlington, MA), p-GR (ser^{211}) (1:1000, rabbit polyclonal, no. 4161S); Cell Signaling Technologies Inc. (Danvers, MA), p-GR (ser^{226}) (1:1000, rabbit polyclonal, no. ABS994); MilliporeSigma (Burlington, MA)], total GR (1:1000, mouse monoclonal, no. MA5-15801). Total ERK1/2 (1:2000, mouse monoclonal, no. 4696s, Cell Signaling), p-ERK1/2 (Thr202/Tyr204) (1:1000, rabbit monoclonal, no. 4376s; Cell Signaling), total JNK (1:500, mouse monoclonal, no. sc-7345; Santa Cruz BioTech), p-JNK (Thr183/Tyr185) (1:1000, rabbit monoclonal, no. 4671s; Cell Signaling), total p38-MAPK (1:1000, mouse monoclonal, no. MABS1754; EMD Millipore), and p-38 (Thr180/Tyr182) (1:1000, rabbit monoclonal, no. 9211s; Cell Signaling) overnight at 4° C with gentle agitation. p-AR (ser^{81}), (ser^{213}), (ser^{650}), total AR, and total GR antibodies were obtained from ThermoFisher (Walthham, MA). p-GR (ser^{134}) and (ser^{226}) antibodies were obtained from MilliporeSigma (Burlington, MA). Following overnight incubation with agitation, membranes were washed (3 X 5 min) with TBS plus Tween-20 (TBST), then probed with infrared (IR) secondary antibodies (1:10,000) specific to the host animal (700 nm anti-mouse, 800 nm anti-rabbit) for 1 h at room temperature to label the respective total and phospho-receptor primary antibodies. After secondary antibody incubation, membranes were washed again (3 x 5 min) in TBST, rinsed once with TBS, and then scanned with an Odyssey Infrared Imaging System and accompanying software (v1.2, LI-COR Biosciences, Lincoln, NE) to quantify IR intensity for each labeled protein

band. Total and phosphorylated bands were identified on the same membrane with IR markers scanned at two different wavelengths (700 nm; total-AR, total-GR, total MAPK, and 800 nm; p-AR, p-GR, p-MAPK). Infrared-labeled secondary antibodies were obtained from Li-COR Inc. (LI-COR Biosciences, Lincoln, NE). Phospho-AR, p-GR, p-MAPK, total-AR, total-GR, total-MAPK signals were normalized to total protein abundance loaded per lane via total protein stain (REVERT; Li-COR). Phospho- signals were then normalized to the total-receptor/MAPK signal and are expressed as the ratio of phospho:total (i.e. pAR/total-AR and pGR/total-GR). All statistical analyses were performed on the total protein adjusted ratios.

Statistical Analyses

All data were assessed for normality with the Shapiro-Wilks test. Independent sample t-tests were used to examine differences in subject characteristics, 1-RM, and relative 1-RM at baseline prior to the training intervention. All hormonal and performance data were normally distributed, however there were random violations in normality for phosphorylated AR, GR, and MAPK. Thus, all receptor and signaling data were analyzed using non-parametric methods. Hormonal data were analyzed using parametric methods. A 2 x 2 x 3 (group x training x time) RMANOVA determined differences in testosterone and cortisol between groups (group) at PRE, MID, and POST (time) during the acute exercise biopsy visit prior to training and

after the training intervention. Follow-up post hoc analyses were performed with a protected Fisher's LSD for testosterone and cortisol for within subject contrasts (time). Independent t-tests were performed to determine between groups at PRE, MID, and POST.

For performance, set mean power, 1-RM back squat, and 1-RM knee extension were analyzed using a 2 X 3 (group x time) RMANOVA to examine changes in these variables prior to the training intervention (BL), immediately after the training intervention (POST) and following one week of recovery (REC). The percent change from BL was calculated from BL to POST, and BL to REC for set mean power, 1-RM back squat, and 1-RM knee extension. A 2 x 2 (group x time) RMANOVA determined differences in the individual percentage change from BL in each group. Follow-up post hoc analyses were performed with a protected Fisher's LSD.

All receptor and data were analyzed using Wilcoxon signed-rank tests for pairwise comparisons between resting (Rest) and POST at pre-training (T1), and post-training (T2). Comparisons of the resting and post-exercise values between T1 and T2 in each respective group was also performed. For between-group analyses, Mann-Whitney U tests were implemented to examine differences in resting and post exercise responses pre-training (T1) and post training (T2). Post-hoc analyses on receptor and MAPK data used

Holm's sequential Bonferroni procedure. Briefly, the p-values obtained from the pair-wise comparisons were ordered from smallest to largest. Critical p-values to be deemed statistically significant were sequentially determined at $p \leq 0.0125$, $p \leq 0.0167$, $p \leq 0.025$, and $p \leq 0.05$ for the first, second, third, and fourth comparisons respectively.

RESULTS

Subject characteristics

There was no difference between groups at baseline for age (t(14)= -0.558; p= 0.586), height (t(14)= 1.091; p=0.294), body mass (t(14)= 1.117; p=0.283), 1RM (t(14)= -0.307; p=0.763), relative 1RM (t(14)= -1.525; p=0.150), and years of resistance training history (t(14)= -0.010; p=0.992).

Subject characteristics at baseline

	CON	OT
N	8	8
Age (yrs)	20.75 ± 1.16	21.25 ± 2.25
Height (cm)	180 ±10.6	155 ± 62.5
Body mass (kg)	83.9 ± 10.7	77.8 ± 11.3
One-Repetition (1RM)	137.2 ± 18.4	140.6 ± 18.56
Relative 1RM (body mass / 1RM)	1.64 ± 0.2	1.82 ± 0.2
Training History (yrs)	5.09 ± 1.64	5.10 ± 2.36

Table 2 subject characteristics between control (CON) and overtrained (OT) groups

Muscular Performance

Mean power for the barbell back squat indicated a group X time interaction (F(2,28)=3.545; p=0.042). Independent t-tests did not indicate differences between groups at BL prior to training (t(14)=-0.630; p=0.539), at POST after the training intervention (t(14)=0.221; p=0.828), and after one

week of recovery (REC) (t(14)=1.113; p=0.285). Comparisons over time within each group indicated mean power did not change at any time-point in the OT group (main effect of time (F(2,14)=0.916; p=0.423). However, the CON group tended to display a main effect of time ((F(2,14)=2.90; p=0.08), and tended to indicate that power at recovery was higher than BL (p=0.06

Independent sample t-test on the percent change from BL values indicated no difference between groups at POST (t(14)=1.368; p=0.193). However, percent increase in mean power after one week of recovery (REC) was higher in CON compared to OT (p=0.03).

One-repetition maximum in the barbell back squat did not show an group X time interaction (F(2,28)=0.228; p=0.798). There tended to be a main effect of time when both groups were collapsed (F(2,28)=2.70; p=0.08). The percentage change from BL in 1-RM back squat did not display an interaction (F(1,14)=0.0287, p=0.60) or a main effect of time (F(1,14)=0.63; p=0.63).

One-repetition maximum in the knee extension did not display a group X time interaction (F(2,28)=0.237; p=0.790), although there was a main effect of time (F(2,28)=9.520; p=0.001). Post-hoc analyses indicated 1-RM knee extension strength was higher at POST compared to BL (p=0.007) and at REC compared to BL (p=0.006). There was no difference in 1-RM knee extension between POST and REC (p=0.362).

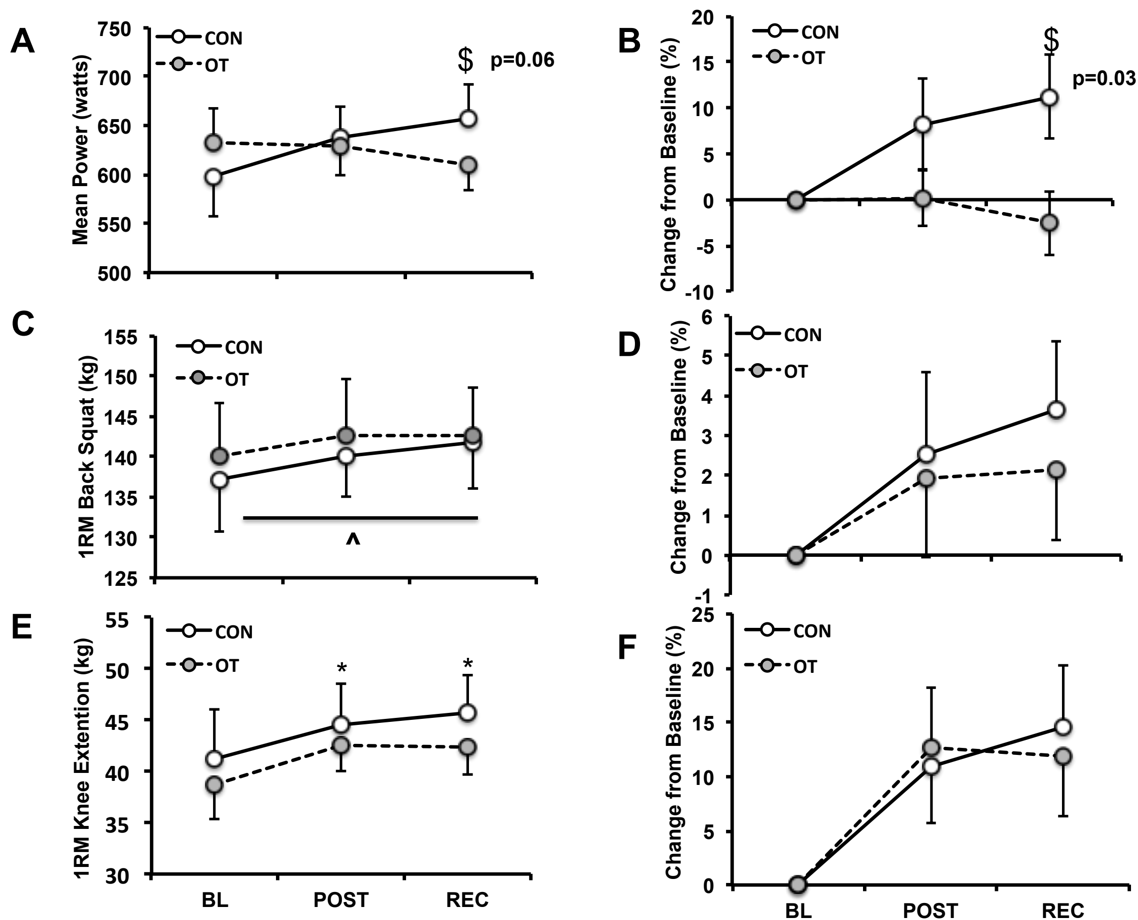

Figure 5-2 Measures of performance prior to training (BL), immediately after the training intervention (POST), and following one-week of recovery (REC). White circles indicate control croup (CON). Grey circles indicate overtraining group (OT). **A)** Mean barbell back squat power during one set of back squats at 60% 1-repetition maximum (1-RM). **B)** Percent change from baseline (BL) in mean power. **C)** One-repetition maximum (1-RM) in barbell back squat. **D)** Percent change from baseline (BL) in 1-RM back squat. **E)** One-repetition maximum (1-RM) in bilateral knee extensions. **F)** Percent change from baseline (BL) in 1-RM knee extension. $ indicates trend for significance at recovery (REC) compared to baseline (BL). ^ indicates trend for main effect of time (p=0.08). * indicates significantly different from baseline at corresponding time-point. Data are presented as mean ± SE.

Endocrine Responses

For testosterone, there was no training X group (($F(1,14)=0.192$; $p=0.668$) interaction, or main effect of training ($F(1,14)=0.866$; $p=0.368$). There tended to be a time X group interaction ($F(2,28)=2.772$; $p=0.07$), and there was a significant main effect of time ($F(2,28)=3.334$; $p=0.050$). Follow up post-hoc analyses of time (Pre-ex, Mid-ex, Post-ex) with both groups, and T1 and T2 values collapsed failed to provide significant differences between Pre-ex versus Mid-ex (45.6 ± 5.1 v 46.9 ± 5.6 nmol/l; $p=0.058$), Pre-ex versus Post-ex (45.6 ± 5.1 v 46.8 ± 5.4 nmol/l; $p=0.064$), and Mid-ex versus Post-ex (46.9 ± 5.6 v 46.8 ± 5.4; $p=0.808$) testosterone values. There tended to be a main effect of group ($F(1,14)=4.421$; $p=0.054$), with the OT group tending to have higher testosterone values compared to CON (57.7 ±7 v 35.15 ± 7.5 nmol/l; $p=0.054$).

Cortisol exhibited a training X group interaction ($F(1,14)=4.852$; $p=0.045$). Follow up analyses indicated there were no changes in CON in cortisol following training ($p>0.05$). However, the OT did indicate a main effect of training ($F(1,7)=6.375$; $p=0.040$), such that cortisol values were lower at T2 compared to T1. Independent t-tests between CON and OT groups indicated that OT group had higher cortisol values at Mid-ex ($t(14)=-2.615$; $p=0.020$) and Post-ex ($t(14)=-2.485$; $p=0.026$) compared to CON at T1. However at T2

cortisol values at Pre-ex, Mid-ex and Post-ex were not different between groups (p>0.05).

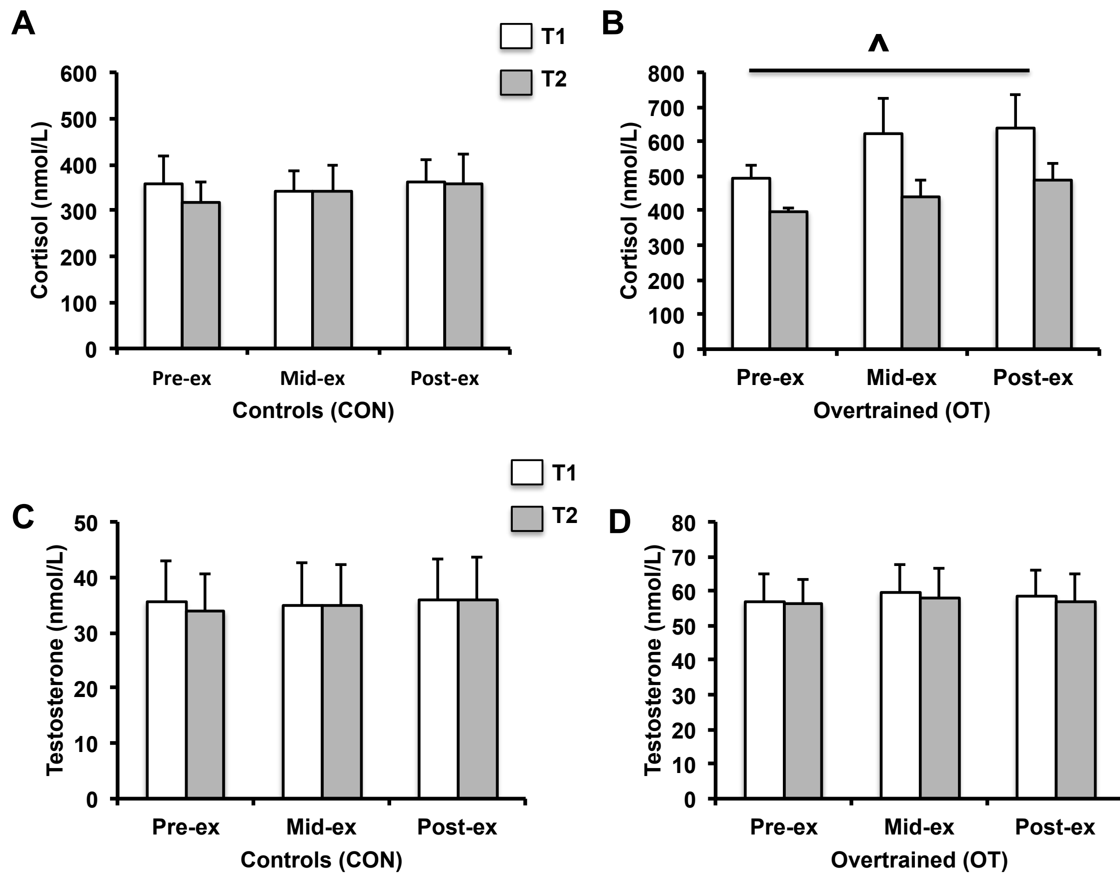

Figure 5-3 Endocrine responses at rest (Pre-ex) immediately after barbell speed squats (Mid-ex), and 5 minutes after the final set of knee extensions (Post-ex). **A & B)** Cortisol concentrations in control (CON) and overtrained (OT) groups pre-training (T1; white bars) and post-training (T2; grey bars). ^ indicates a main effect of training in the OT group only. Cortisol concentrations were lower following the training intervention. **C & D)** Total testosterone concentrations in control (CON) and overtrained (OT) groups pre-training (T1; white bars) and post-training (T2; grey bars). Data are presented as mean ± SE.

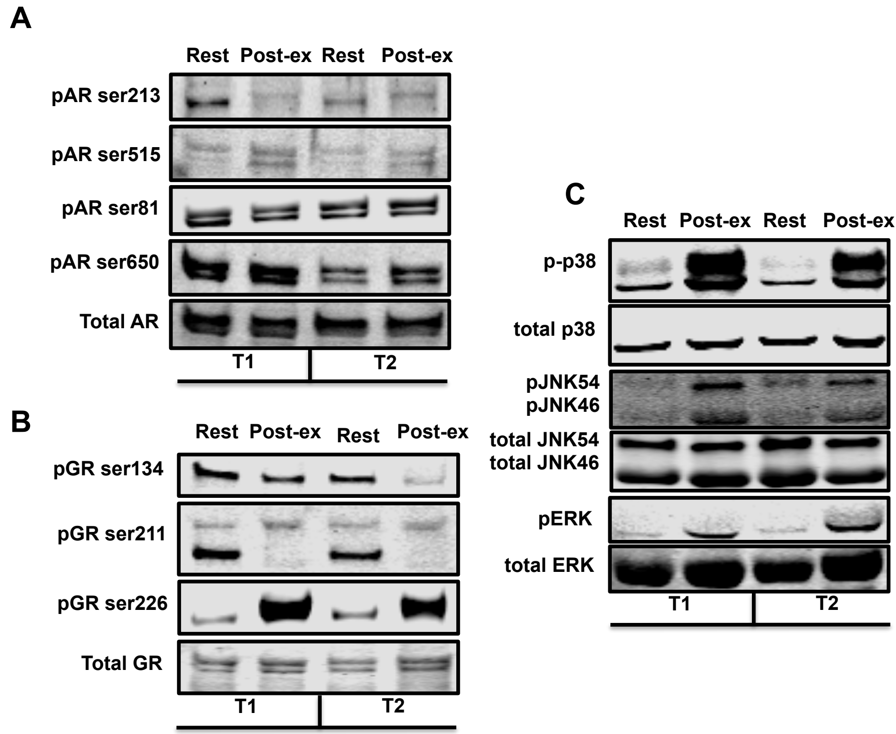

Figure 5-4 Representative western blot images for the control group (CON). **A)** total and phosphorylated androgen receptor (AR) at ser515, ser81, ser213, and ser650 at rest (Rest) and ten minutes post exercise (Post-ex). **B)** Total and phosphorylated glucocorticoid receptor (GR) at ser134, ser211, and ser226 at rest and ten minutes post exercise. **C)** Total and phosphorylated p38, JNK, and ERK at rest and ten minutes post exercise. The left side (lanes 1 and 2) of representative blots indicate pre-training responses (T1), while the right side (lanes 3 and 4) indicate post-training responses (T2).

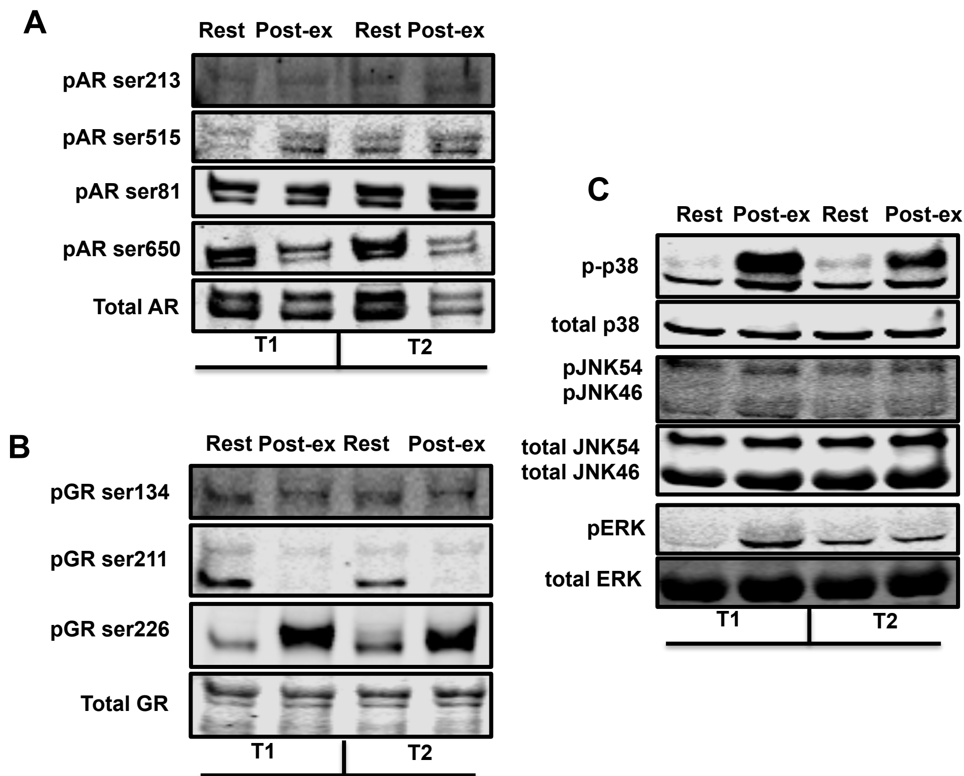

Figure 5-5 Representative western blot images for the overtraining group (OT) of **A)** Total and phosphorylated androgen receptor (AR) at ser515, ser81, ser213, and ser650 at rest (Rest) and ten minutes post exercise (Post-ex). **B)** Total and phosphorylated glucocorticoid receptor (GR) at ser134, ser211, and ser226 at rest and ten minutes post exercise. **C)** Total and phosphorylated p38, JNK, and ERK at rest and ten minutes post exercise. The left side (lanes 1 and 2) of representative blots indicate pre-training responses (T1), while the right side (lanes 3 and 4) indicate post-training responses (T2).

Androgen Receptor Expression and Phosphorylation

CON total AR expression did not change from rest to POST at pre-training (T1; z=-1.540; p=0.148) or post-training (T2; z=-1.960; p=0.055). Total AR at rest (z=-0.980; p=0.838) or post exercise (z=-1.680; p=0.109) did not differ between pre-training or post training. In OT, total AR expression did not change from rest to POST at pre-training (T1; z=-1.820; p=0.078). However, in the post-training state total AR did decrease from rest to post-exercise (T2; z=-2.521; p=0.008). Resting (z=-0.700; p=0.547) and post-exercise (z=-1.960; p=0.055) total AR were not different between pre-training and post training. Total AR expression did not differ between groups at rest (Mann-Whitney U =15.00; Z = -1.78; p=0.083) or post exercise (Mann-Whitney U =14.00; Z = -1.89; p=0.065) at pre-training (T1). Total AR expression also did not differ between groups at rest (Mann-Whitney U =22.00; Z = -1.05; p=0.328) or post exercise (Mann-Whitney U =13.00; Z = -1.995; p=0.050) post-training (T2).

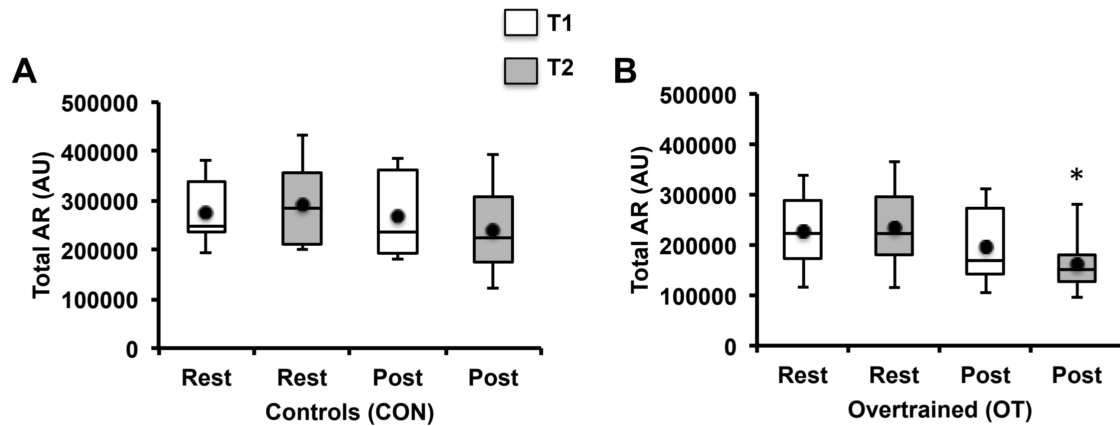

Figure 5-6 Total androgen receptor (AR) expression in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). Rest indicates pre-exercise value, Post indicates post-exercise value. * indicates significantly different from resting value at corresponding time-point.

pARser213 did not change with exercise or after the training period in the CON group. pARser213 increased post exercise in the OT group only at post-training (T2; z= -2.521; p=0.008). pARser213 did not differ between groups at any time point.

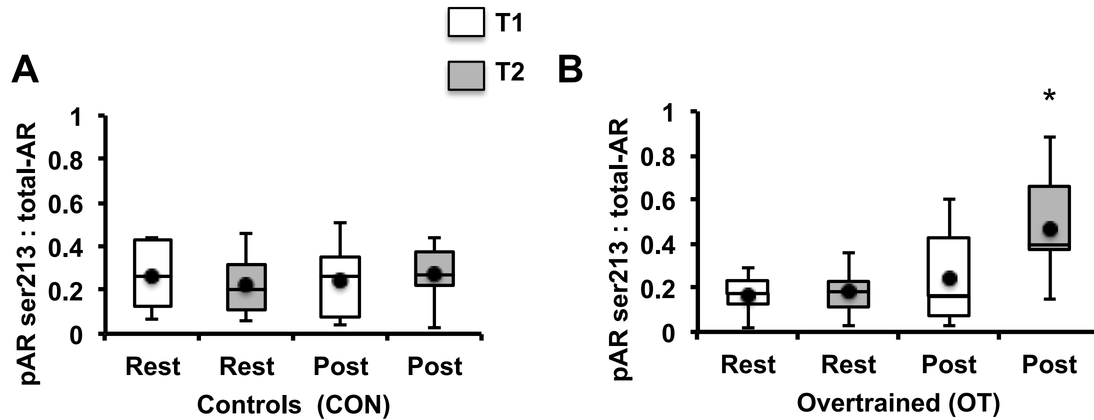

Figure 5-7 Ratio of phosphorylated androgen receptor (AR) when phosphorylated at ser213 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). * indicates significantly different from resting value at corresponding time-point

In CON pARser515 tended to increase post exercise at pre-training (T1) only ($z=-2.380$; $p=0.016$). In OT pARser515 increased post-exercise both pre-training (T1; $z=-2.380$; $p=0.016$), and at post-training (T2; $z=-2.521$; $p=0.008$). The post exercise increase at post-training (T2) was greater than pre-training (T1; $z=-2.380$; $p=0.016$). pARser515 did not differ between groups at any time point.

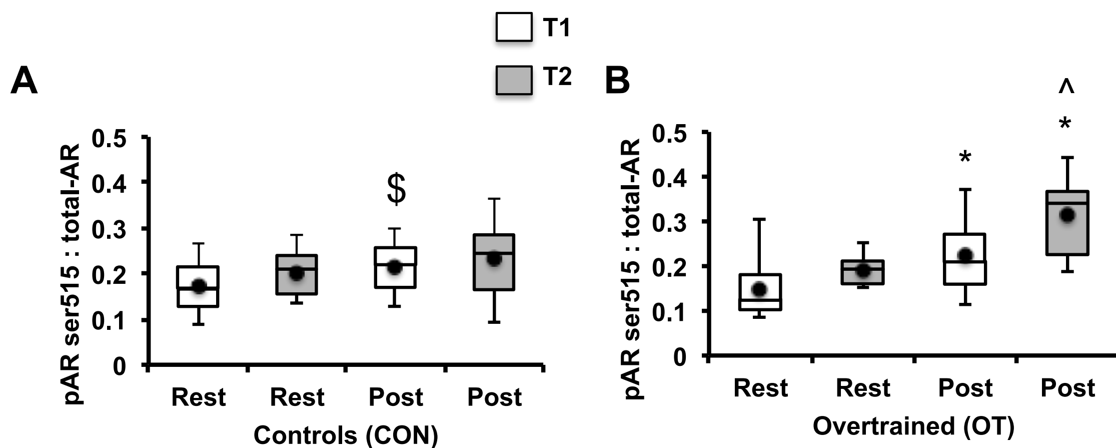

Figure 5-8 Ratio of phosphorylated androgen receptor (AR) when phosphorylated at ser515 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). * indicates significantly different from resting value at corresponding time-point. ^ indicates significantly different from pre-training value at corresponding time-point. $ indicates trend for significant difference from rest at corresponding time-point (p=0.016).

pARser81 tended to be lower at rest (z =-2.100; p=0.039) and post exercise (z = -1.960; p=0.055) in the post-training (T2) state only. However, these decreases did not reach the critical p-values from the holm-Bonferroni procedure to reach statistical significance. pARser81 did not differ between groups at any time point.

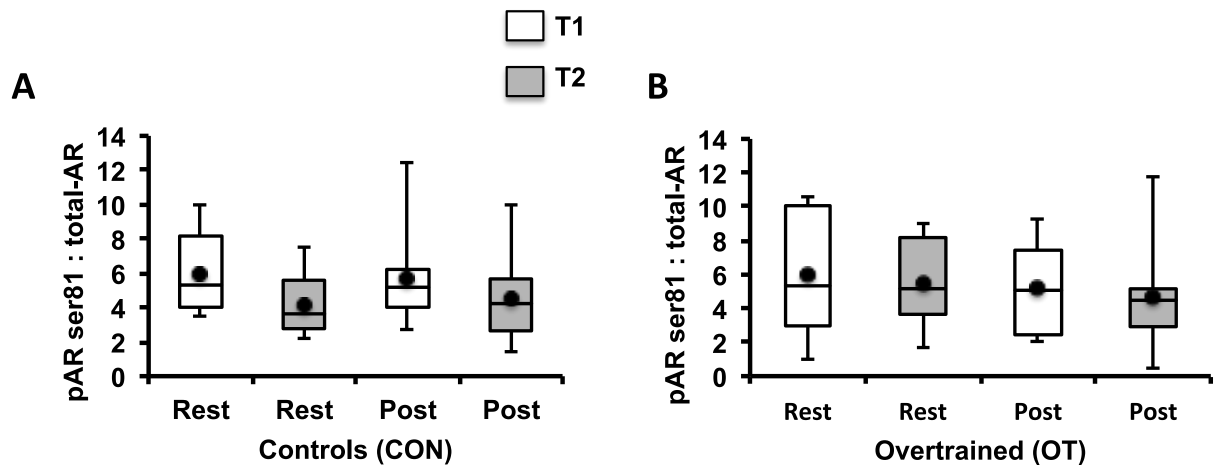

Figure 5-9 Ratio of phosphorylated androgen receptor (AR) when phosphorylated at ser81 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2).

pARser650 did not change overtime between either group at any time point. pARser650 did not differ between groups at any time point.

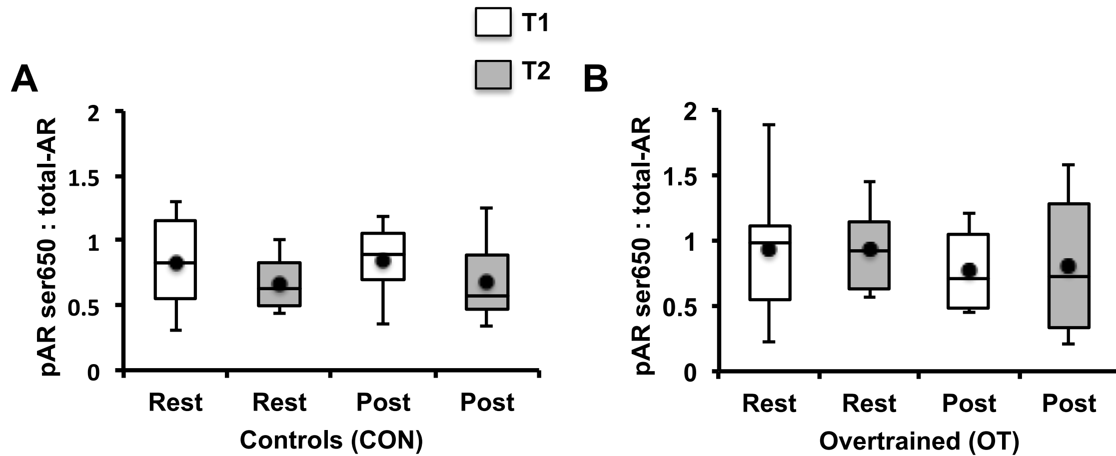

Figure 5-10 Ratio of phosphorylated androgen receptor (AR) when phosphorylated at ser650 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2).

Total GR did not differ between groups at any time point. Total GR did not change at post-exercise in either group pre- (T1) or post-training (T2).

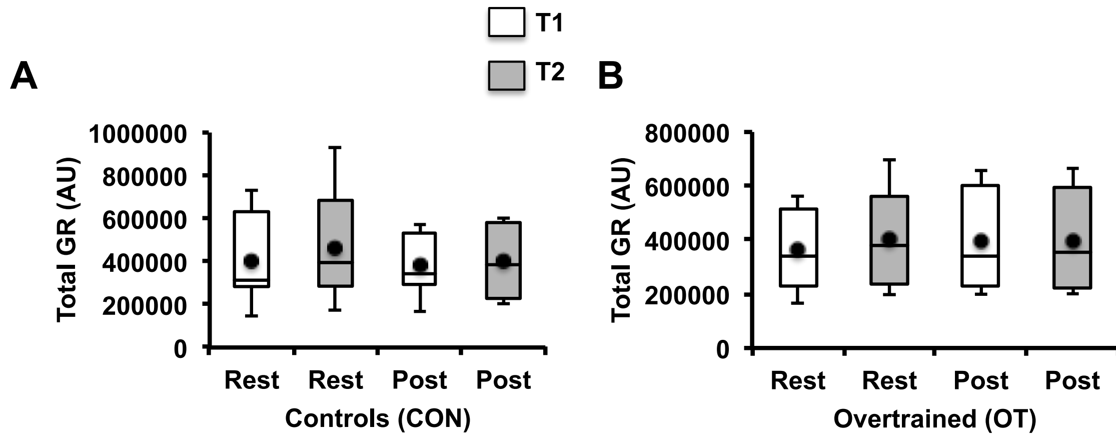

Figure 5-11 Total glucocorticoid receptor (GR) expression in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2).

pGRser134 higher in CON at rest (Mann-Whitney U=14.00; z =-1.890; p=0.065) and post exercise (Mann-Whitney U= 12.00; z = -2.100; p=0.038) compared to OT in the pre-training (T1) state only. However, these differences did not reach the critical p-values for the Holm-Bonferroni procedure to reach statistical significance.

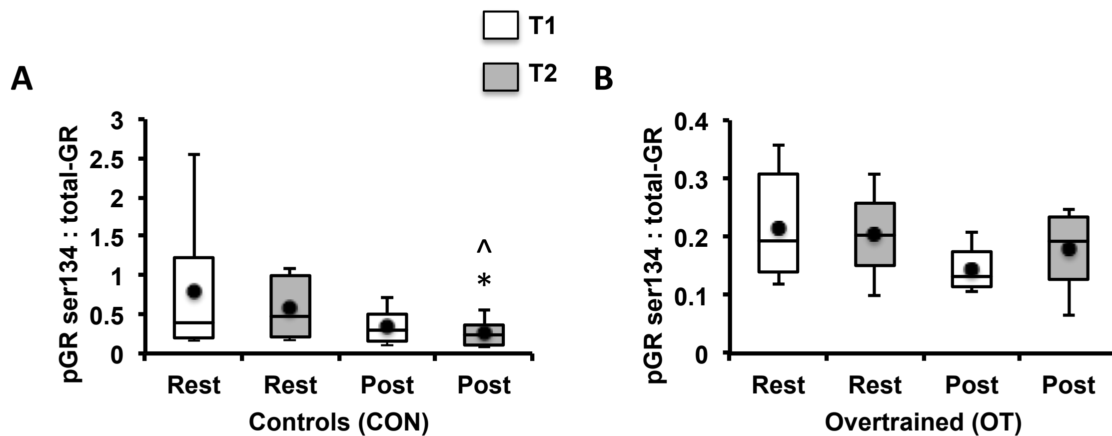

Figure 5-12 Ratio of phosphorylated glucocorticoid receptor (GR) when phosphorylated at ser134 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). ^ indicates significantly different from pre-training value at corresponding time-point. * indicates significantly different from resting value at corresponding time-point.

pGRser226 increased at post-exercise in CON at pre-training (T1; z=-2521; p=0.008) and post-training (T2; z=-2.521; p=0.008). In OT group, pGRser226 increased post exercise at pre-training (T1; z=-2.521; p=0.008) and post training (T2; z=-2.521; p=0.008). However in the OT group, pGRser226 at rest was higher at post-training (T2) compared to pre-training (T1; z=-2.521; p=-.008). The post exercise response in OT at post-training (T2) was less than the post-exercise response at pre-training (T1; z=-2.380; p=0.016). pGRser226 was greater in CON compared to OT at post exercise in the post-training (T2) state only (Mann-Whitney U=3.000; z =-3.046; p=0.001).

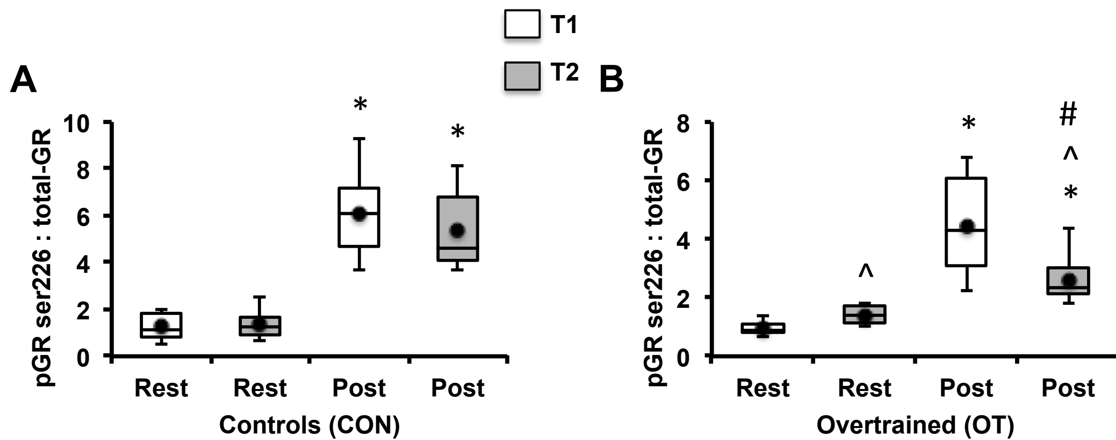

Figure 5-13 Ratio of phosphorylated glucocorticoid receptor (GR) when phosphorylated at ser226 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). ^ indicates significantly different from pre-training value at corresponding time-point. * indicates significantly different from resting value at corresponding time-point. # indicates significantly different from CON group at corresponding time-point.

pGRser211-alpha did not change at any time point in either group. pGRser211-beta decreased at post exercise in CON at pre-training (T1; z=-2.521; p=0.008) and post-training (T2; z=-2.521; p=0.008). Resting and post exercise response in CON did not differ between pre- (T1) and post training (T2). In OT group pGRser211-beta decreased post exercise at pre-training (T1; z=-2.521; p=0.008) and at post-training (T2; z=-2.521; p=0.008). Resting and post-exercise values did not differ between pre- (T1) and post-training (T2). pGRser211-alpha and pGRser211-beta were not different between groups at any time point.

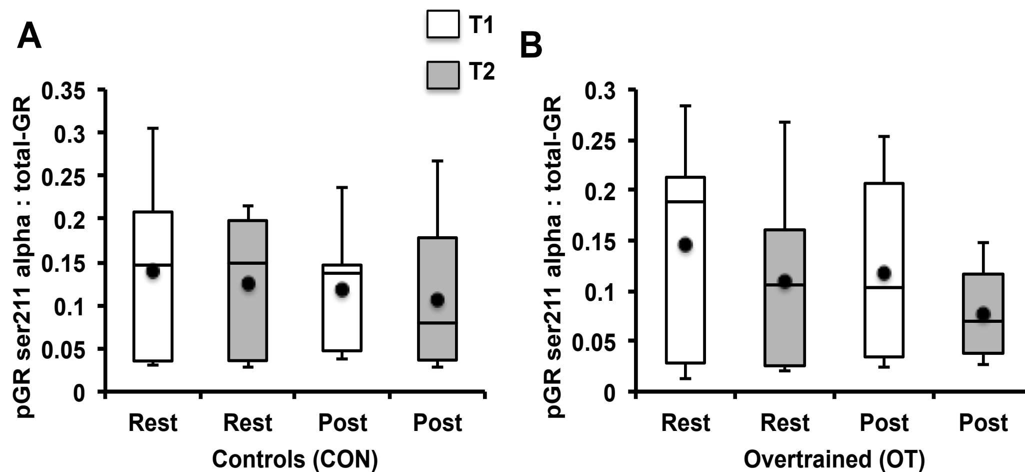

Figure 5-14 Ratio of phosphorylated glucocorticoid receptor (GR) when phosphorylated at ser211-alpha in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2).

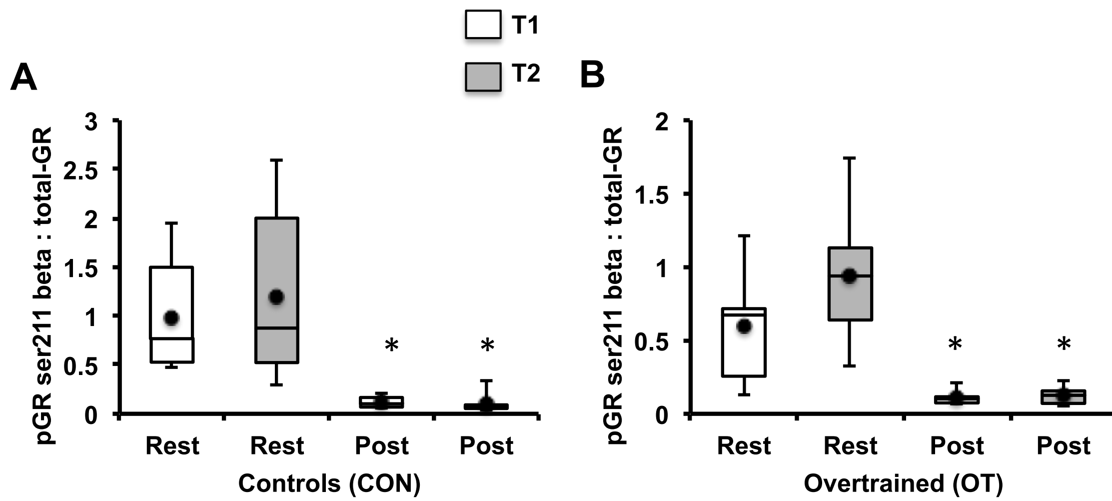

Figure 5-15 Ratio of phosphorylated glucocorticoid receptor (GR) when phosphorylated at ser211-beta in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). * indicates significantly different from resting value at corresponding time-point.

MAPK

In CON p-p38 increased post exercise at pre-training (T1; z=-2.521; p=0.008) and post-training (T2; z=-2.521; p=0.008). Resting p-p38 decreased from pre- (T1) to post-training (T2; z=-2.240; p=0.023). Post exercise values did not differ between pre- (T1) and post-training (T2). In OT p-p38 increased post exercise pre-training (T1; z=-2.521; p=0.008) and post-training (T2; z=-2.521; p=0.008). Resting p-p38 increased from pre- (T1) to post-training (T2; z=-2.521; p=0.008). Post exercise values were lower at post-training (T2) compared to pre-training (T1; z=-2.521; p=0.008). Phosphorylated p38 differed between CON and OT at rest pre-training (T1; Mann-Whitney U=8.00; Z=-2.521; p=0.010). Phosphorylated p38 differed between CON and OT at post exercise post-training (T2; Mann-Whitney U=5.00; Z=-2.8361; p=0.003). Post-exercise values at pre-training were not different (T1; Mann-Whitney U=12.00; Z=-2.100; p=0.038). Resting p-p38 at post training was not different between groups (T2; Mann-Whitney U=21.00; Z==1.155; p=0.279)

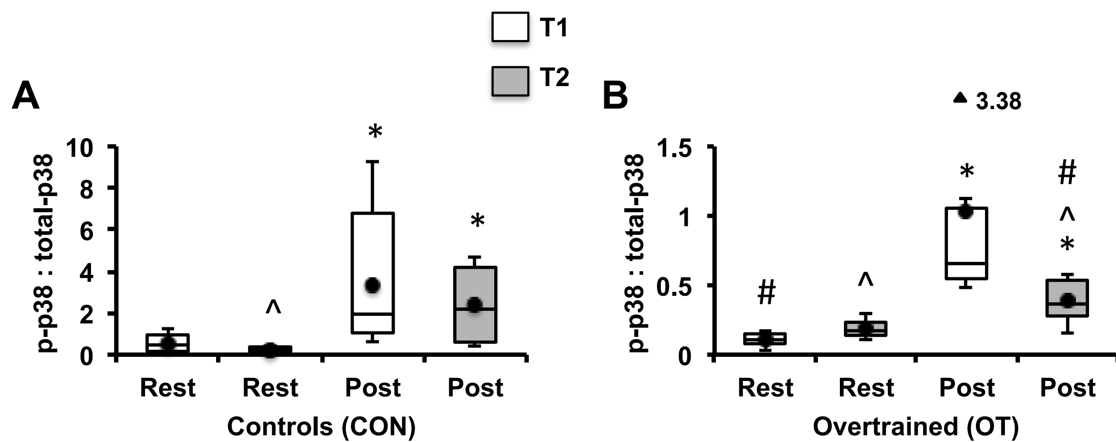

Figure 5-16 Ratio of phosphorylated p38 in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1). grey box indicates post-training values (T2). ^ indicates significantly different from pre-training value at corresponding time-point. * indicates significantly different from resting value at corresponding time-point. # indicates significantly different from CON group at corresponding time-point. Black triangle in OT group at pre-training indicates the maximum value for one subject at corresponding time point for graphical purposes.

Phosphorylated JNK increased post-exercise in CON at pre-training (T1; z=-2.521; p=0.008) and post training (T2; z=-2.521; p=0.008). Resting pJNK was higher at post training (T2) compared to pre-training (T1; z=-2.380; p=0.016). Post exercise values between pre-training (T1) and post-training (T2) were not different. In OT group, pJNK increased post-exercise at pre-training (T1; z=-2.521; p=0.008) and post-training (T2; z=-2.521; p=0.008). Resting and post-exercise values did not differ between pre-training (T1) and post-training (T2). Phosphorylated JNK did not differ between groups at any time point.

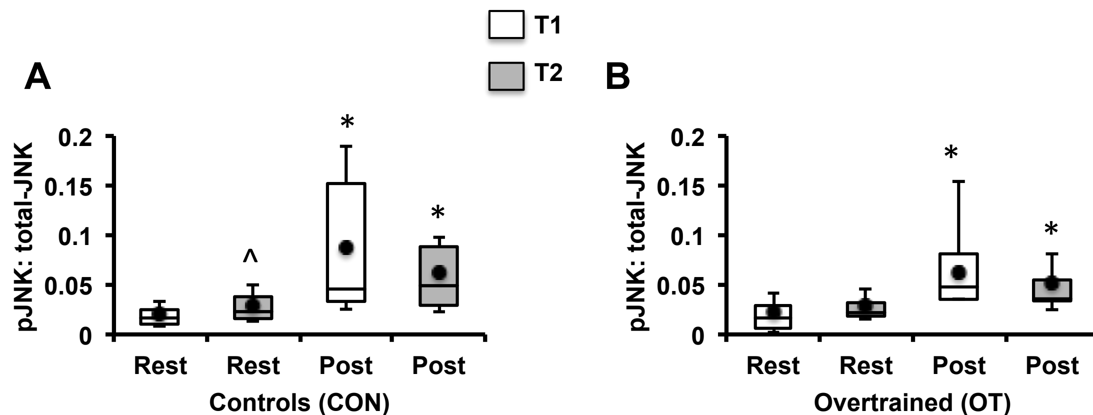

Figure 5-17 Ratio of phosphorylated JNK in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). ^ indicates significantly different from pre-training value at corresponding time-point. * indicates significantly different from resting value at corresponding time-point.

Phosphorylated ERK increased post exercise in CON at pre-training (T1; z=2.521; p=0.008) and at post training (T2; z=-2.38; p=0.016). However resting and post exercise values did not differ between pre- and post-training. In OT group, pERK increased post exercise at pre-training (T1; z=-2.521; p=0.008) and tended to increase post-exercise at post-training (T2; z=-1.960; p=0.055). Resting pERK tended to be different between pre- (T1) and post training (T2; z=-2.100; p=0.039). Post-exercise values between pre-(T1) and post-training (T2) were not different (z=-0.140; p=0.945). Resting pERK tended to be higher in CON compared to OT at pre-training (T1; Mann-Whitney U= 9.00; Z=-2.425; p=0.015). pERK did not differ between groups at any other time point.

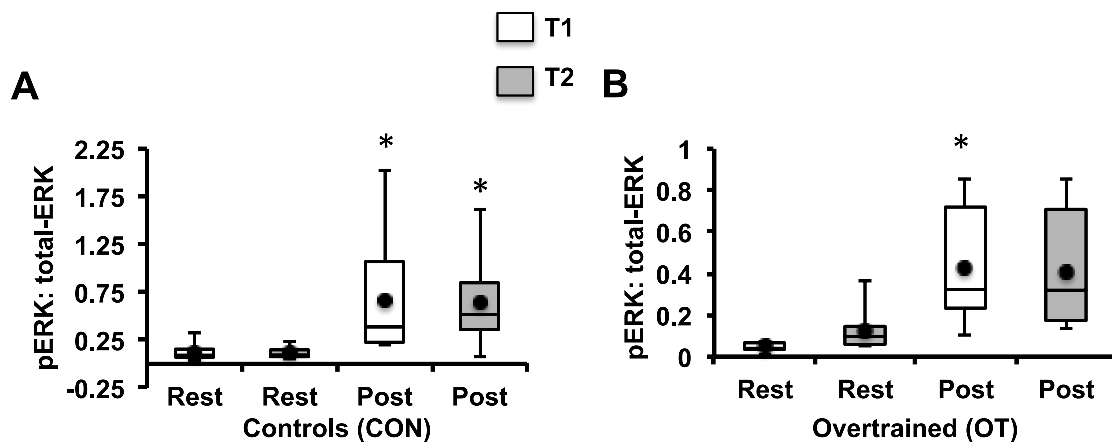

Figure 5-18 Ratio of phosphorylated ERK in **A)** control group (CON) and **B)** overtrained group (OT). Resting (Rest) and post-exercise (POST) time points are indicated on the x-axis. Boxes indicate median and interquartile range (25th - 75th percentile). Whiskers indicate maximum and minimum values. Black dot indicates mean. White box indicates pre-training values (T1), grey box indicates post-training values (T2). * indicates significantly different from resting value at corresponding time-point.

Total MAPK (p38, JNK, ERK) expression did not change in CON following the training intervention. Total JNK increased in OT group at post-training (T2; $z=-2.100$; $p=0.039$). There were no between group differences in total p38, JNK, or ERK expression at pre-training (T1) or post-training (T2).

DISSCUSSION

The results of this study elucidate several key factors associated with overtraining (OT) or overreaching (OR) RE. Firstly, the stagnation or decrease in performance following excessive RE with insufficient recovery is specific to the velocity (high power speed squats vs maximal strength back squats) and the modality (barbell back squats vs knee extensions) in which the stressful training stimulus was applied. Secondly, these data indicate steroid receptors (AR and GR) can be phosphorylated within 10 minutes following resistance exercise, and that their phosphorylation response may occur independently or in the absence of exercise induced increases in testosterone and cortisol. Thirdly, resting, and exercise induced increases in phosphorylated AR and GR are augmented following an OT stimulus with insufficient recovery, and these changes are distinctly different than what occurs during the non-OT state. The aforementioned changes in AR and GR phosphorylation may occur via specific activation of select proteins in MAPK pathway. Together, the acute exercise recovery strategies following OT may confer a "resistance" to repeated stressful exercise stimuli that are eventually reflected in altered resting activity.

Of the three MAPKs investigated in this study, p38-MAPK appeared to be most influenced by RE, and specifically OT. Following normal training with sufficient recovery between bouts, resting phosphorylated p38

decreased, and exercise induced phosphoryltion was maintained. The decrease in resting p38 phosphorylation is similar to previous findings in mice (Yu et al. 2001, Lee et al. 2002; Vichaiwong et al. 2009, Karagounis et al. 2010), and humans (Nicoll et al. 2016; Macaluso et al. 2012). However, in the overtrained state resting phosphorylated p38 increased, and the exercise induced increases are attenuated compared to pre-training. Elevated phosphorylated p38 at rest following RE with insufficient recovery has been reported in both murine (Coffey et al. 2007) and human (Nicoll et al. 2016) studies. The increase in resting p38 following stressful training may shift the roll of p38 from anabolic (myogenesis) to inflammatory/catabolic (Alves Souza et al. 2014; Coffey et al. 2007, Li et al. 2005, Nicoll et al. 2016). Furthermore, exercise induced p38 phosphorylation which may sensitize muscle cells to mechanical load, is attenuated with high frequency RE (15 sessions over 7.5 days), and may further impair recovery strategies.

JNK is the most mechanosensitve of the three MAPK (ERK, JNK, p38), and the exercise-induced response is not attenuated following OT. Total JNK increased slightly following OT, and previous evidence in an *ex vivo* murine model, reports JNK overexpression decreases the resting activation of other anabolic signaling proteins including ERK (Fujii et al 2004). JNK overexpression decreased resting ERK activity, but did not blunt contraction induced ERK phosphorylation (Fujii et al 2004). Thus, in relation to our

results, high frequency RE may maintain most of the mechanically induced signals for protein synthesis (JNK, ERK), but the resting state becomes inflammatory/apoptotic and impairs the protein synthetic and recovery strategies during late recovery (>24 hrs). RE with insufficient recovery between bouts may potentiate inflammatory/apoptotic signaling in spite of the phosphorylation of exercise induced anabolic signaling proteins (mTOR, p79s6K, ERK). This hypothesis may be supported by previous research in mice showing greater mTOR, p70s6K and S6 phosphorylation with shortened periods of recovery between RE bouts. While longer periods of recovery (72hrs vs. 24hrs vs 8 hrs) between RE bouts showed lower mTOR and p70s6K phosphorylation, longer rest periods enhanced protein synthesis, such that muscle protein synthesis with 72 hours of recovery was greater than after only 8 hours of recovery between bouts (Takegaki et al. 2017).

There was a down-regulation of total AR expression after exercise in the overtrained state. Down-regulation of AR has been reported at 60, 70, and 180 minutes post-exercise (Ratamess et al. 2005; Vingren et al. 2009, Spiering et al. 2009). Furthermore an exercise protocol that increases testosterone increases the stability of AR in the hours following RE (Spiering et al. 2009). In the non-OT, state AR expression was maintained at 10-min post in both CON and OT groups, yet after overtraining there was a down regulation of the receptor quickly after exercise. Down-regulation of the

receptor in the OT state was also accompanied by increases in phosphorylation at ser515 and ser213. Both ser515 and 213 have been implicated in cellular growth in various tissues (Willder et al. 2013; Ponguta et al. 2008; Zeng et al. 2017) and are phosphorylated by MAPK and Akt (Kim and Lee 2009, Yeh et al. 1999). It is curious if the increases in phosphorylation at ser213 and 515 in the OT are a compensatory response to down regulation of the receptor. In the absence of elevated hormones, stressful training may utilize other locally mediated mechanisms (MAPK, Akt) to sustain anabolic signals.

Several studies (Willoughby and Taylor 2004, Mitchell et al. 2013) but not all (Mobley et al. 2018), have reported increased total androgen expression following repeated exposure to RE. Here, although the OT group performed more training compared to CON, there was no increase in AR expression at rest. Despite the fact that repeated bouts of exercise increases AR expression, high frequency training with insufficient recovery may attenuate this response and impair successful adaptation to exercise. To date, only Da Rocha et al. (2017) have investigated phosphorylated AR in mice in the overtrained state, and reported down-regulation of resting total and phosphorylated AR following overtraining. However, Da Rocha et al (2017) employed an aerobic training OT model and did not state which phosphorylation site on the AR was decreased following OT. Direct

comparisons between studies is difficult because 1) the mechanical load on muscle tissue is substantially different between training modalities (aerobic vs resistance training), 2) we assessed four separate phosphorylation sites on the AR as opposed to just reporting p-AR, and 3) use of human versus murine models. Together these results indicate faster down-regulation of the AR in the overtrained state with potentiated site-specific phosphorylation.

Glucocorticoid receptor phosphorylation was sensitive to stressful training. Several sites at on the GR (ser134 and ser226) were altered with OT. Galliher-Beckley et al. (2011) reported ligand-independent phosphorylation at ser134 is regulated by the p38 MAPK pathway, and phosphorylation at this site specifies hormone stimulated GR gene transcription (Galliher-Beckley et al. 2011). GR phosphorylation at ser134 acts as a metabolic sensor in cells, and integrates a variety of cellular stress signals including glucose starvation, UV exposure, and oxidative stress (Galliher-Beckley et al. 2011). Following normal training there was a decrease in the post-exercise response of ser134, while in the overtrained state phosphorylation at ser134 was maintained post-exercise. Phosphorylation of GR at ser226 influences nuclear/cytoplasmic shuttling (Itoh et al. 2002). While activation of ERK and JNK decrease the transcriptional activity of GR, specifically the increase in phosphorylation on ser226 (mediated by JNK) decreases GR transcriptional activity via regulating nuclear export (Itoh et al.

2002). The post-exercise response of ser226 phosphorylation was maintained after normal training but was significantly decreased after OT. Moreover, the there was an increase in resting ser226 in the overtrained state. Together, decreased post-exercise ser226 phosphorylation might indicate a more transcriptionally active receptor post-exercise after overtraining. Consequently an increase in phosphorylation at ser226 at rest may represent a “resistance” to the receptor such that more is phosphorylated at rest to attenuate transcriptional (catabolic) activity. Given that p38 and JNK modulate activity at ser134 and ser226 respectively, and both of these kinases were altered at rest (p-p38, total JNK) and post exercise (p-p38) we speculate that these kinases may modulate site specific GR phosphorylation after OT. Indeed site-specific GR phosphorylation differentially affects target gene expression (Chen et al. 2008; Galliher-Beckley et al. 2011), and the data presented here shed light on potential cross-talk between MAPK and GR phosphorylation in skeletal muscle.

Da Rocha et al (2017) also investigated phosphorylated GR after an aerobic OT stimulus, and reported decreased total and phosphorylated GR. The authors did not state which phosphorylation sites were examined, so it is difficult to extrapolate our results to theirs. While they (De Rocha et al. 2017), and others (Willoughby et al. 2003, Coutinho et al. 2006) have reported decreased total GR expression following repeated exercise training, we did

not observe a decrease in total GR expression. Differences in findings may arise from differing exercise protocols (Willoughby et al. 2003 (heavy eccentric RE vs aerobic), da Rocha et al. 2017, Coutinho et al. 2006). Vingren et al (2009) reported no change in total GR expression up to 70 minutes post exercise, although females expressed greater GR expression than men. The RE protocol utilized by Willoughby et al. (2003) was specifically utilized to induce muscular damage. Thus, our protocol (60% 1-RM, non-failure) may not damage skeletal muscle sufficiently to alter total GR expression at rest or after exercise.

In the present study, barbell power was the only performance marker that indicated maladaptation to the overtraining stimulus. CON experienced a significant increase in barbell power, but only after a week of recovery. This point emphasizes that 1) increases in muscular power can occur quickly after a training program, 2) the increase in power was fully realized following a period of recovery, 3) excessive power training attenuates an improvement in power adaptation. The OT group did not decrease muscular power, instead there were no changes in their performance if assessed by a single set of barbell speed squats. Their stagnation in adaption might be classified as non-functional overtraining (where excessive training stagnates or attenuates performance adaptations) (Meeusen et al. 2013).

Maximum strength in the back squat and knee extension increased similarly in both groups.

Collectively these results suggest the performance decrements (or attenuation) from excessive training with insufficient recovery are specific to the training stimulus utilized to induce overtraining (Fry et al. 2006; Sterczala et al. 2017). Decreased power and force has been reported with previous overtraining protocols, but the decrements occurred only at the intensities and velocities closest to the overtraining stimulus (Fry et al. 2006; Sterczala et al. 2017). Two weeks of voluminous heavy RT also resulted in attenuated rates of strength adaptation when compared to a control group that had adequate rest between training bouts (Raastad et al. 2001; Raastad et al. 2003). Raastad et al. (2003) reported a 12% increase in leg press 6-RM strength in a high frequency training group compared to the 5% increase observed in the a normal training frequency group. Increases in 1-RM strength were similar between groups. Here we report similar findings that 1-RM strength can be improved despite attenuated performance in other components of muscle adaptation such as power.

Finally, while we did not see performance decrements per se, rather attenuated adaption, excessive exercise intensity is likely a greater driver of long-term performance decrements than exercise volume. Fry et al. (2006)

utilized 10 sets at 1-RM loads on a squat machine and produced long-term decreases in strength and power at 100% 1RM. Sterczala utilized high-power speed squats at 70% 1-RM and reported decreased barbell squat power after the training program, that returned to pre-training values with one week of recovery (overreaching). In that study, Sterczala et al. (2017) reported 1-RM squat strength was unaffected. Considering the current study's results, we utilized 60% 1-RM squats and 70% 1-RM knee extensions, and report no performance decrements in power, but instead attenuation when compared to a normally trained control. 1-RM in the back squat and knee extension increased similarly in both groups. It appears increasing exercise intensity (100%>70%>60% 1-RM) with insufficient recovery as the primary driver of long-term RE performance decrements, and the decrements observed are specific to the overtraining stimulus. Recent evidence suggests increases in muscular strength occurs with very little volume during a training program (Mattocks et al. 2017), and the short term increase in muscular strength in our study may have been due to neural adjustments (Seynnes et al. 2007).

Although the OT group increased muscular strength, they achieved the same adaptations while performing substantially more training. The concept that excessive exercise attenuates adaptation is essential (Moore and Fry 2007), since athletes, strength and conditioning coaches, and fitness enthusiasts seek to determine the minimum exercise stimulus needed to elicit

maximum or optimal adaptation. Although muscular strength may increase, much of the time invested to elicit adaptation was “wasted” since it did not contribute to enhanced adaptations. In the case of training frequency and volume, there may be a zone of optimal training frequencies or volumes for positive adaptation (Amirthalingam et al. 2017; Schoenfeld et al. 2015); however reports on this topic are conflicting (Brigatto et al. 2018; Schoenfeld et al. 2015; Ribeiro et al. 2015). Although the concept of the general adaptation syndrome (GAS) and its application to periodization has been challenged recently (Buckner et al. 2017; Buckner et al. 2018), the data here clearly highlight that in trained individuals, the fruition of power adaptions occur after a period of de-loading, and therefore periodization of training programs to allow for adequate recovery and accommodation of non-training stressors may be required to maximize training adaptations.

The contributions of the endocrine system during the onset or duration overtraining has been reviewed extensively (Meeusen et al. 2013; Fry and Kraemer 1997; Cadegiani and Kater 2017). However, there is little consensus on the usefulness of steroid hormones as indicators of overtraining. An acute bout of high volume RE requires longer recovery of performance compared to high intensity resistance exercise (Bartolomei et al. 2017), and was also accompanied by increases in post-exercise cortisol. And although high volume RE produces substantial increases in cortisol and

testosterone, to date long-term decrements (i.e., weeks to months) in performance from RE were only observed in high intensity OT protocols (Fry et al. 1994, Fry et al. 2006). As such, the influence of testosterone and cortisol on adaptation and maladaptation remains unclear. The decrease in post-exercise cortisol in the OT is similar to previous studies on stressful training (Fry et al. 1998; Raastad et al. 2001).

Only the OT produced an exercise-induced cortisol response at pre-training. After the overtraining protocol, cortisol values were lower than pre-training. Testosterone tended to be higher in the OT group at pre- and post-training. In general, the acute exercise protocol in this study was not a potent stimulus to increase testosterone. A previous study using 5 sets of 5 repetitions of maximal velocity speed squats at 50% 1-RM also reported increases in cortisol post exercise, with no change in testosterone (Kudrna et al. 2017). On the contrary, a study utilizing 10 sets of 5 repetitions at 70% 1-RM of speed squats reported a large effect size of increased testosterone post exercise, with no change in cortisol (Fry and Lohnes, 2010). The acute resistance exercise program variables influence post-exercise endocrine responses (McCaulley et al. 2009) and negligible differences in the post exercise testosterone response are likely due to the low total exercise volume and absolute intensity utilized.

The endocrine and receptor responses in this study deserves mention. In particular, while the exercise induced increase in cortisol decreased with OT, phosphorylation on the GR (ser134 and 226) displayed divergent responses such that there was an increase in ser134 phosphorylation in the OT post exercise. Ser226 phosphorylation was elevated at rest after OT and showed an attenuated response post exercise in the OT state. Attenuated ser226 phosphorylation post exercise relative to pre-training would suggest more GR was located in the nucleus. However if this increased nuclear translocation, it happened without an increase in cortisol from exercise. Moreover the phosphorylation of the AR increased after OT without a concomitant increase in testosterone from exercise. These results indicate AR and GR can be regulated independently of an acute exercise-induced hormonal response, possibly through contraction related mechanisms. Furthermore, AR and GR are differentially regulated by repeated bouts of resistance training, which suggests that endocrine and phosphorylation mediated activation of steroid receptors may play distinct roles in adaptation as one goes from sedentary to well-trained. Indeed Spillane et al. (2015) reported increased AR/DNA binding 3 and 24 hours post exercise despite no change in circulating androgens in well-trained subjects. Of note, muscle testosterone and DHT were constant at all time points, suggesting testosterone plays a permissive role in initiating AR activity and subsequent muscle adaptation in trained subjects such that any

presence of testosterone post-exercise (regardless of magnitude) participates in muscular adaptations (Mangine et al. 2017).

Limitations

The novel data presented in this study indicates altered MAPK and steroid receptor phosphorylation after non-stressful and stressful RE training. There are several limitations that must be considered. First, the primary criterion variable for the definition of overtraining is a decrease in performance (Meeusen et al. 2013). Thus, the subjects in out OT group did not experience performance decrements in muscular power. Instead, they experienced stagnation in adaptation as compared to the improved performances that were observed in the CON group. Stagnation of performance could also be considered a form of overtraining; however it is more often termed non-functional overreaching (NFOR) (Meeusen et al. 2013; Moore and Fry 2010). This group was performing RE of high-frequency and insufficient recovery, so although we did not observe a performance decrease, rather an attenuation, these data provide insight to the role insufficient recovery may contribute to maladaptation in performance and skeletal muscle physiology in humans.

The subjects also performed acute RE bouts where the biopsies were obtained in the fasted state. Since the phosphorylation of AR and GR in response to RE has not been studied in humans before, we were unsure the role nutrition may contribute to these responses. Thus, the molecular responses observed here are indicative of adaptions that occur in the fasted state. The expression of AR post-exercise are potentiated with nutritional supplementation provided post-exercise (Kraemer et al. 2006; Hooper et al. 2017). This could certainly influence the cellular milieu and provide a more anabolic environment for AR action and antagonize circulating cortisol concentrations (Kraemer et al. 2006).

We also acknowledge the results here are limited to the early post-exercise period (10 minutes post exercise). It is not clear what the activation time-course of these phosphorylation sites are over the hours (or days) following RE. Further, it is not clear if the time-course of phosphorylation of these receptors differs between trained and untrained subjects, as previous research on total AR kinetics indicate potentially different responses between training statuses (Spiering et al. 2009; Spillane et al. 2015). Moreover, it is not clear if the type of RE protocol implemented (i.e. 90% 1-RM vs 70% 1-RM vs 50% 1-RM) influences phosphorylation responses. Determining if AR and GR responses differ between RE training paradigms may provide rationale for the

observed hypertrophy between powerlifters and bodybuilders despite these two groups performing very divergent training programs (Fry 2004).

Finally, the attenuation of post exercise phosphorylation in some of the observed variables may have been the effect of repeated bouts of training and may not be indicative of overtraining. For example, RE naïve subjects maintain acute signaling responses of hypertrophic proteins while training accustomed individuals do not increase phosphorylation post-exercise (Coffey et al. 2006). Attenuation of phosphorylated signaling proteins have been observed in a RE murine model and the attenuated phosphorylation was recovered after a period of detraining (Ogaswara et al. 2013). It is not clear what degree or time-course is necessary to observe attenuation of signaling responses. While Coffey et al. (2006) and Gonzalez et al. (2017) observed attenuated signaling post-exercise in trained subjects, and Brooks et al. (2016) observed attenuation of select hypertrophy proteins after only 9 training sessions, Figueiredo et al. (2015) did not observe attenuated signaling after 8 weeks of resistance training. Further, Galpin et al. (2016) observed successive decrements in total and phosphorylated ERK expression as the length of RE training history increased (i.e. untrained→12 weeks→ 3 yrs→10 yrs). It is likely there may be negative feedback mechanisms to regulate the transcriptional efficiency and capacity (Figueiredo

et al. 2015) of muscle protein synthesis as one goes from sedentary to trained (Tang et al. 2008, Wilkinson et al. 2008)

Additionally, of note there were several physiological markers that were observed in the OT group that may have contributed to the attenuation in performance rather than decrease. Subjects in the OT group had lower p-p38, higher circulating testosterone, and higher exercise-induced cortisol response at pre-training compared to the CON group. While these molecular and endocrine differences were observed at baseline, it is difficult to control for these biomarkers. Subjects were matched on 1-RM and training history prior to randomization into CON or OT groups. Because of this, there were no differences in any of the subject characteristics or training history prior to training. Thus, the observed differences in p-p38, testosterone, and cortisol, between groups at baseline, might have contributed to the stagnation of adaptation (as opposed to decrease). As such, despite efforts to match and randomize, the OT group may have had physiological attributes that could have contributed to their resiliency to the stressful training program. Despite this, it is clear the OT did not adapt in the same manner as CON.

CONCLUSION

In conclusion, the results of this study indicate altered MAPK, AR, and GR phosphorylation following a period of normal and stressful training. We provide evidence AR and GR are phosphorylated in the early recovery period after RE and these responses are altered after chronic training. More importantly, these responses occurred in spite of unaltered circulating hormonal concentrations, indicating hormonal and receptor interactions after RE are more complex than previously suggested, and it is likely hormonal responses and local contraction mediated mechanisms work in tandem to regulate eventual skeletal muscle adaptations (Zeng et al. 2017). More research is required to elucidate how these responses could be influenced during the fed state as the data presented here are limited to post-exercise responses in the fasted state.

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APPENDICES

APPENDIX A

The effects of a pre-workout supplement on $beta_2$-adrenergic receptor activation and anabolic signaling pathways.

Informed Consent

INTRODUCTION

You are invited to participate in a research study examining the effectiveness of multi-ingredient a pre-workout dietary supplement combined with resistance exercise on various physiological variables. Fifteen subjects are being sought to participate in this study at the University of Kansas. The Department of Health, Sports, and Exercise Sciences at the University of Kansas supports the practice of protection for human subjects participating in research. The following information is provided to help you make an informed decision on whether or not to participate in the present study. Please feel free to ask any questions. This study was requested and is funded by MusclePharm Inc.

PURPOSE OF THE STUDY

The purpose of this study is to examine the effect of a multi-ingredient pre-workout dietary supplement combined with a resistance exercise bout on physiological measures. This supplement contains large amounts of caffeine (250 mg) the equivalent of 2.5 cups of coffee. The supplement is not currently commercially available and is not approved by the Food and Drug administration (FDA), as no supplements are approved by the FDA. Aside from examining the effectiveness of the supplement, the resistance exercise component of this study could provide information concerning the role of resistance training in various states of adaptations by using supplements. The physiological variables that will be measured include body weight, body composition, strength and power, as well as blood and muscle markers of skeletal muscle growth (hypertrophy) and metabolism response to resistance exercise. The findings of this study will determine if the use of this multi-ingredient pre-workout supplement in addition to resistance exercise results in changes in molecular changes that influence muscular strength and hypertrophy that are different from the changes in these variables that result from training without supplementation

BASIS FOR SUBJECT SELECTION

In order to participate in this study you must be male, between the ages of 18 and 30, healthy, non-obese (BMI <28 kg/m_2), non-smoking, and free of metabolic cardiovascular, kidney diseases, as well as free of a history of seizures. You will be screened for participation using a health-history questionnaire for contraindications to exercise by American College of Sports Medicine (ACSM) guidelines. If you are currently taking any medications or supplements that may interact with ingredients in the supplement/placebo involved in this study you will be excluded from the study.

In order to participate in this study you must be recreationally trained (exercise 3 or more days / week for at least 1 year) in order to mimic those who are generally more likely to use a supplement of this kind. In addition, if you currently use drugs that reduce inflammation (aspirin, ibuprofin, NSAIDs, etc.) on a regular basis you will be excluded from this investigation. You will also be asked to refrain from using these drugs during the course of this study. Due to gender differences in hormonal fluctuations and responses to resistance training only males will be recruited.

Participants with high blood pressure will be excluded from the study. Participants with a measured blood pressure of 140/90 will be considered as having high blood pressure. Assessment of high blood pressure will be taken on the first day of testing, prior to familiarization.

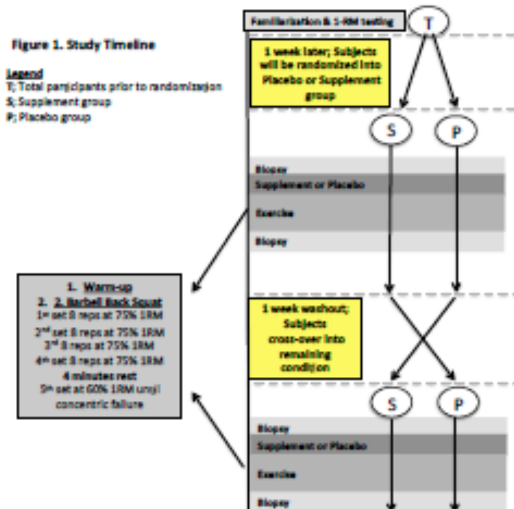
Participants will not participate in this study if you are currently taking nitrates for chest pain or if you are taking medication for antidepressants such as MAOI (Monoamine Oxidase Inhibitor) or SSRI, blood thinners, nonsteroidal anti-inflammatory drugs (NSAIDs), pseudoephedrine, or you are taking prescriptions drug or over-the-counter medication; or if, you suspect you have or have been treated for, or diagnosed with any medical condition, including but not limited to: high or low blood pressure, diabetes, glaucoma, anxiety, cardiovascular, psychiatric or seizure disorders, cardiac arrhythmia, stroke, heart, liver, kidney or thyroid disease. This product contains caffeine and should not be used by individuals wishing to eliminate caffeine from their diet or in combination with caffeine or stimulants from other sources including but not limited to, coffee, tea, soda, or other dietary supplements and medications. Subjects currently taking other exercise pre-workout supplements will be asked to refrain from their use for the duration of the study. Failure to comply with non-consumption of other dietary supplements (other than whey protein), will result in withdrawal from the study.

You are allowed to consume caffeine containing foods during the study. However, you will be required to abstain from any caffeine consumption for at least 3 days prior to familiarization, and experimental visits. If caffeine products are consumed and anytime during the study within 3 or less days from the experimental trials you will be excluded from the study.

This product contains artificial sweeteners. Subjects with a known history of allergies or who are sensitive to artificial sweeteners should refrain from participating in this study. Therefore, participants who have the aforementioned sensitivity to artificial sweeteners will be excluded from participation in this study.

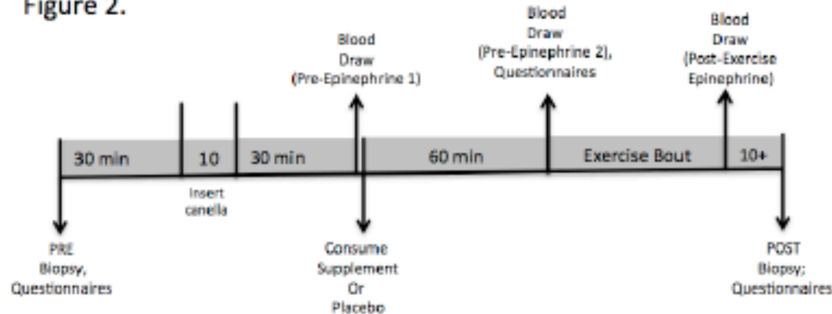
PROCEDURES

A time-line of the testing procedures and an overview of the exercise bout for the present study are presented below. All procedures will be conducted in the Applied Physiology Laboratory (APL) at the University of Kansas and will be supervised by trained personnel. It is important for you to note that this study will require your presence in the Applied Physiology Laboratory for testing and assessment of follow-up procedures.



KU Lawrence IRB # STUDY00002625 | Approval Period 6/2/2016 – 6/3/2017

Figure 2.



1) **Pre-testing Protocol** – Health history, physical activity, caffeine intake and tolerance, and pre-biopsy questionnaires, and anthropometric data, including a skinfold measurements, will be obtained approximately 1 week prior to Testing Day 1. These procedures will take approximately 1 hour. Resting blood pressure will be assessed prior to strength testing and familiarization. Subjects who record a resting blood pressure of 140/90 or higher will be excluded from participation in the study. You will be provided with a list of beverages and substances which contain caffine, and you will be asked to avoid these food for three days prior to familiarization, and both experimental trials.

2) **Exercise Testing** - All strength testing will be done using free weights or weight machines and following standard repetition maximum strength testing guidelines. You will be asked to warm-up for five minutes using a stationary leg cycle. Following this you will perform warm-up sets followed by maximal effort lifts for the barbell back squat.

3) **Dietary Recall** – All subjects will be asked to record food and drink consumption including portion sizes) for three consecutive days prior to their first experimental visit and will be asked to repeat this same diet in the days prior to the second exercise visit. Subjects will be given an explanation of portion sizes prior to completion of the dietary recall.

4) **Supplementation** – All subjects will be asked to consume either a supplement or placebo drink 60 minutes prior to each exercise session. The supplement/placebo mix containers will be labeled as either A or B, however the experiment will be double blind in nature as neither the research staff or the subjects will know which drink is the placebo and which is the supplement. An individual associated with the Applied Physiology Lab, but not directly involved in this research project, will be assigned the duty of randomizing subjects into placebo and supplement groups. They will also be responsible for mixing the supplement drinks, and monitoring the subjects as they consume the supplement. As this experiment is double blind, no other individuals associated with the study (research staff or subjects) will know which mix is the supplement and which is the placebo until after the study is completed. Questionnaires will be provided to you at each exercise session to record any adverse effects you might have as a result of taking the placebo or supplement.

5) **Resistance Exercise Session**. All subjects will warm up on a cycle ergometer at 0.5 % of their mass in kg of resistance. After 5 minutes, you will perform a series of warm-up exercise sets with the barbell back squat. Once at the target load has been determined you will perform four sets of barbell back squats for 8 repetitions at 75% of your 1-repetion maximum (1-RM). Once all four sets are completed, you will then perform a fifth and final set to concentric failure at 60% of 1-RM. Upon completion of the fifth and final set of the leg barbell back squat exercise you will then immediately partake in the post exercise biopsy.

6) **Muscle Biopsies** – You have been informed that one of the purposes of this study is to measure the cellular markers of muscle growth from the muscle samples that we collect. By obtaining a small sample of your muscle tissue (size of a pencil eraser or small finger nail), the different types of proteins in your muscle may be determined which will be helpful in the evaluation of health and exercise performance. All muscle tissue samples (biopsies) will be taken from the outer portion of the front of the thigh using a needle biopsy technique by Andy Fry, PhD. Andy Fry, PhD, Professor of HSES, has performed over 100 muscle biopsies over the past year and has assisted on over 500 biopsies over the past six years on various populations (athletes, sedentary people, elderly etc.) with no significant complications and nothing more than minimal adverse reactions. The procedure is being overseen by Dr. Jeffrey Burns M.D., who is a medical doctor in Neurology at the KU Medical Center in Kansas City, Kansas supervises the procedure, but will not be physically present for the biopsies. The total size of the muscle biopsy will be approximately the size of a pea. You will be placed on an examination table lying down on your back (supine) so that the muscles of the leg are relaxed. The skin will be thoroughly cleaned with antiseptic solution (Betadine) using sterile cotton swabs after which a surgical cover will be placed around the sampling site. If you are allergic to Betadine, an alternative antiseptic solution will be used to clean the skin. A small amount (2ml or 2cc) of a local aesthetic (2% Lidocaine) will be injected into the tissue under the skin around the site to be sampled. During this injection you may feel a slight burning sensation. If you are allergic to the local anesthetic, or have had allergic reactions to other anesthetics (i.e.: Novocain) then you will be disqualified from the study. Following the injection of the numbing agent into your thigh, a minimum of five (5) minutes will be allowed to pass to ensure adequate time for the agent to take effect in the area where the incision will be made. A small incision (1 cm) will then be made in the skin overlying the muscle and the biopsy needle inserted into the middle of the muscle (muscle belly) at a depth of 3 cm (about 1 inch). During the time that the sample is being taken (about 5 seconds) you may feel some deep pressure and cramping that will be moderately painful. You have been informed that if you have been previously diagnosed as having a bleeding disorder, a blood clotting problem, take blood thinning medication or have sensitivity to scarring, you will tell the researcher and not participate in the biopsy procedure.

   Sterile disposable instruments and sterile gloves will be used for the preparation of the site and the reusable biopsy needle will be thoroughly sterilized (via steam and heat autoclave) after each biopsy. Approximately 100 mg (size of a pencil eraser or small finger nail) of skeletal muscle tissue will be removed. With the invasive skeletal muscle biopsy technique there is the possibility of a blood related infection (HIV, Hepatitis B), but the reusable needles will be cleaned and sterilized using an autoclave, a generally accepted method of cleaning surgical instruments.

   A total of **four (4)** biopsies will be performed over the course of the study (see timeline, above).

7) **Blood Draws** – We will measure hormonal markers found in your blood prior to supplementation, after supplementation but prior to exercise, and at the end of exercise. Six blood draws will be performed during this study (see timeline, above). In this procedure, a small amount of blood will be taken from a vein for each blood draw by a trained phlebotomist using a needle and a syringe. Approximately 10 cc of blood (~ 2 teaspoons) will be drawn for each time-point. The blood draw will take about 5 minutes.

8) **Questionnaires** – We will administer questionnaires assessing your perceived mental and physical preparedness to exercise. The questionnaires will be administered prior to supplementation, after supplementation but prior to exercise, and after the exercise bout. In addition, we will assess your rating of perceived exertion during each set of the acute exercise bout. The questionnaires should take about 5 minutes to complete each time they are administered. In addition, we will also administer a questionnaire concerning any potential side effects of supplement consumption.

RISKS

1. **Skinfold Measurements** – There may be mild discomfort from the skinfold assessment. The pressure form the skinfold calipers may cause a slight pinching sensation at the site of measurement. However, this sensation is mild and brief (1-2 seconds). The site of measurement may appear red for 15-20 min after the assessment. This measurement is non-invasive, and it is unlikely there will be bruising.

2. **Muscle biopsies** – The use of local anesthetics will result in a slight burning sensation, lasting approximately 5 seconds. There is a risk of allergic reactions to the local injection (1 in 1 million). There is a small chance of bleeding from the biopsy site. The principle concern would be prolonged bleeding which would produce a bruise in the area. This would extend the muscle soreness, but is adequately treated with rest, ice, a compression bandage, and keeping the leg elevated as much as possible. Nausea, dizziness, and fainting can occur (1 in 100) during the biopsy process. As a result, the subjects will be in a supine position during the biopsy procedure. There is a risk of infection (1 in 1000) and irritation associated with the biopsy procedure. The use of aseptic techniques, careful cleaning of the skin and keeping the area dry will minimize the risk of infection. There is a risk of bruising (1 in 100) from the biopsy procedure. This will be minimized by placing ice over the site following the procedure and by applying a compression bandage on the site for the 24 hours following the biopsy. In some instances (1 in 200), some motor nerves may be damaged which may cause local muscle atrophy (decrease in the size of muscle fibers, with a small dimple on the skin). There is likely to be a small scar where the biopsy is performed. This scar usually dissipates over a period of 6-12 months at which time the scaring is very modest.

3. **Blood Draws** – The blood sample has a small risk of infection and bruising of the area. The needle stick for the blood draw will be mildly painful and only lasts a couple of seconds. Blood draws will be performed by HSES staff trained in blood draws and blood borne pathogens and bio-hazardous waste.

4. **Supplement/Placebo**— A single serving of this supplement contains 18.2g of pre-workout or placebo. The 18.2g of the supplement or placebo will be mixed with 8-12oz of water prior to consumption in both the placebo and supplement trials (experimental visit 1 and

2).You will be asked to ingest a supplement (18.2 grams) or a placebo (18.2 grams) drink sixty minutes before each exercise bout. This variation of the supplement is currently not available for commercial sale and is not approved by the Food and Drug Administration (FDA), as no supplements are approved by the FDA. You will asked to refrain from alcohol, caffeine, and cigarettes for 3 days prior to the experimental and familiarization visits. Alcohol is a known depressant and will influence the outcomes of the dependent variables in the study. Similarly, cigarettes will impact the efficacy of the exercise testing, therefore subjects who smoke 3 days prior to exercise sessions will be excluded/withdrawn from the study. Caffeine is the primary ingredient in the supplement used for this study. Similarly the consumption of the supplement is the primary independent variable for the study. Therefore, as to not influence the findings of our study we request that you not consume caffeine for 3 days prior to the experimental and familiarization visits.

The ingredients found in this supplement and information concerning the safety of their ingestion is listed below. Due to the possibility that other supplements may contain similar ingredients that might interact with ingredients in the study placebo/supplement and place you at risk or confound the results of this study, you are asked to refrain from using all other nutritional supplements or ergogenic aids during the course of this study.

Each ingredient in the supplement is listed below, we do not anticipate any adverse side effects from consumption of this supplement.

Vitamin C. This supplement contains 300 mg of vitamin C. Vitamin C is a naturally occurring vitamin found in citrus fruits. Taking too much vitamin C can cause diarrhea, nausea, and stomach cramps. The upper limit of vitamin C ingestion in adults is 2,000mg. Due to the small amount of this ingredient in the supplement, no side effects are anticipated due to this ingredient

Vitamin E This supplement contains 40 IU of vitamin E. Vitamin E is a vitamin that dissolves in fat. It is found in many foods including vegetable oils, cereals, meat, poultry, eggs, fruits, vegetables, and wheat germ oil. It is also available as a supplement. Some research shows that taking vitamin E in doses of 300-800 IU each day may increase the chance of stroke. High doses can also cause nausea, diarrhea, stomach cramps, fatigue, weakness, headache, blurred vision, rash, and bruising and bleeding. The amount of vitamin E in this supplement is well below the upper tolerable limit. Thus, we do not anticipate adverse side effects from consumption.

Vitamin B6. This supplement contains 20mg of vitamin B6. Vitamin B3 is essential in protein and fatty acid metabolism. Vitamin B6 is found in many fortified foods. Given that this vitamin is essential in metabolism there are few side effects associated with it's consumption at recommended levels. Sensory neuropathy (numbness) may develop when consumption exceeds 1000mg per day. The upper limit of vitamin B6 is 100mg per day. Given that the vitamin B6 in this supplement is well below the upper limit we do not anticipate any adverse effects form consuming this supplement.

Vitamin B12. The current supplement contains 50 mcg of vitamin B12. Vitamin B12 is a vitamin that helps keep the body's nerve and blood cells healthy. It is found in many

types of food such as fish, meat, poultry, eggs, milk, and other types of fortified foods. To date, there are no known adverse side effects associated with vitamin B12 consumption. Therefore, no side effects are anticipated due to this ingredient.

Calcium. This supplement contains 62 mg of calcium. Calcium is a mineral found in many types of food. Calcium maintains bone and important in muscle function. Taking too much calcium may increase constipation or incidence of kidney stones if over the suggested limit (more than 2,500 mg). Due to the small amount of this ingredient in the supplement and well below the upper limit, no side effects are anticipated due to this ingredient.

Creatine monohydrate. The supplement contains 5 g of creatine monohydrate, a substance that is produced naturally in the body and stored in the musculature of animals to contribute to the production of energy. Doses of creatine used to improve training intensity and recovery range from 1 to 24 g per day. Large doses of creatine may cause dehydration and gastrointestinal distress. However, because the combined dose of creatine monohydrate is at the lower end of the typical creatine dose, few side effects are anticipated.

Beta-Alanine. This supplement contains 2 g of beta-alanine, a naturally occuring amino acid derivative. Beta-alanine has been shown to decrease fatigue and increase work production in humans. No side effects have been reported for beta alanine doses of .800 g per day, and only tingling has been reported in extremely high doses. Typical dosage of beta-alanine is 3.2 - 6.4 g per day. Due to the small amount of this ingredient in the supplement, no side effects are anticipated due to this ingredient.

Betaine Anhydrous This supplement contains 1.5 g of betaine anhydrous, a naturally occuring amino acid derivative. Betaine anhydrous has been shown to be lipotropic - i.e. fat loss causing - by promoting the oxidization of lipids. It has also been noted to increase appetite, improve digestive efficiency, and in animals it has been shown to promote lean mass and reproductive abilities. Betaine anhydrous can be used for up to 20 g per day.. Possible side effects can include diarrhea, stomach upset (gastrointestinal irritation) and nausea. Typical dosage of beta-alanine is 3 - 6 g per day. Due to the small amount of this ingredient in the supplement, no side effects are anticipated due to this ingredient

Caffeine anhydrous. The experimental exercise supplement contains 250 mg of caffeine. This amount is the equivalent of 2.5 eight-ounce cups of coffee. Caffeine is a known stimulant and diuretic. Side effects of caffeine ingestion can include increased mental concentration, increased physical work capacity, 'jitters', nervousness, and dizziness or light headedness. These symptoms increase with the amount of caffeine consumed so we ask that you refrain from consuming more than one additional serving of caffeinated beverages per day, (coffee, tea, energy drinks, colas) during the study. You will be included in this study if you have previously consumed moderate amounts of caffeine (300mg/day or more) without experiencing any uncomfortable negative side effects.

Theacrine This supplement contains 50mg of theacrine, a naturally compound produced from plants and is similar in structure and effects as caffeine. Theacrine has been shown stimulatory effects in humans. While there are no official side effects for theacrine, at too

high of a dose, side effects would be similar to caffeine such as nervousness, anxiety, and jitters. Due to the small amount of this ingredient in the supplement, no side effects are anticipated due to this ingredient.

BioPerine Black Pepper Fruit Extract The supplement contains 5 mg of black pepper fruit extract. Black pepper is a flowering vine usually dried and used as a spice or for seasoning on food. There are no known side effects associated with black pepper fruit extract supplementation consumption. No side effects are anticipated due to this ingredient.

Isomaltulose, The supplement contains 5 g of isomaltulose, a substance that is an artificial sweetener. To date, there are no known adverse side effects of ismaltulose supplementation, therefore few side effects are anticipated.

L-Glycine. The supplement contains 1 g of glycine, an amino acid and neurotransmitter. Doses up to 45 g/day have been used with no adverse side effects. As such, glycine seems to be safe for most people when taken by mouth or applied to the skin. Most people do not experience side effects, although occasionally there have been a few reports of nausea, vomiting, stomach upset, and drowsiness.

L-Taurine. The supplement contains 1 g of creatine monohydrate, an amino acid produced by the body, and found in meats and fish. Doses of taurine supplementation 1 to 30 g per day have been reported with no adverse side effects.

Acesulfame potassium. Acesulfame potassium is a calorie-sugar substitute. This product contains 50 mg of Acesulfame potassium. It is commonly added to ensure shelf life of the product and there are no known adverse side effects of its consumption.

Sucralose. Sucralose is a non-nutritive sweetener and is considered non-caloric. This product contains 250 mg of sucralose. Sucralose is accepted by the FDA and is considered safe for consumption. We do not expect adverse side effects of consumption in this product.

Silicon Dioxide. Silicon dioxide is used in the manufacturing process. Silicon dioxide is not absorbed in the digestive tract; therefore there is no risk of toxicity. It is used to absorb moisture and prevent caking during packaging. This product contains 200 mg of silicon dioxide. There are no known adverse side effects of its consumption, and given the low amount in the product we do not expect complications from taking this supplement.

Red beet root juice powder. This supplement contains 80mg of red beet root juice powder. It is commonly used as a coloring additive in foods and beverages.

Natural & Artificial Flavors. This supplement contains 650 mg of natural and artificial flavors. It is used as flavoring for the supplement and is commonly used in many sports drinks and beverages. We do on expect any adverse side effects from it's consumption in this supplement,

Citric Acid. This supplement contains 350 mg of citric acid. It is found in many tart and sour fruits. It is also used as in many sports drinks and beverages. Many people report few, if any, side effects of citric acid consumption However diarrhea; loose stools; nausea; upset stomach; vomiting may be potential side effects. We do on expect any adverse side effects from it's consumption in this supplement.

Placebo. The placebo supplement contains maltodextrin (sugar), sucralose (stated above), natural & artificial flavors (stated above). The placebo drink will consist of a low-glycemic flavor and color matched drink sweetened with maltodextrin, sucralose, and natural & artificial flavors. Persons known to be allergic to one of the ingredients will be excluded from the study. There are no known risks to consuming a beverage of this sort except in those who have rare food allergies to the artificial colors or flavoring ingredients. No information on the occurrences rate are estimated to be less than 1 per 100 people, and lower in adults than children

INGREDIENT SUPPLEMENT LABEL

Supplement Facts

Serving Size: 18.2g

Servings per Container: *(see packaging specifications)*

	Amount Per Serving	% Daily Value
Calories	20	
Total Carbohydrate	6 g	2%*
Sugars	5 g	**
Vitamin C (as Ascorbic Acid)	300 mg	500%
Vitamin E (as D L-Alpha Tocopheryl Acetate)	40 IU	133%
Vitamin B6 (as Pyridoxine Hydrochloride)	20 mg	1000%
Vitamin B12 (as Methylcobalamin)	50 mcg	833%
Calcium (as Calcium Silicate)	62 mg	6%
Strength & Performance		
Creatine Monohydrate	5 g	**
Beta-Alanine	2 g	**
Betaine Anhydrous (Trimethylglycine)	1.5 g	**
Muscle Fuel		
Isomaltulose (as Palatinose™)	5 g	**
L-Glycine	1 g	**
L-Taurine	1 g	**
Energy & Focus		
Caffeine Anhydrous	250 mg	**
Theacrine (as TeaCrine®)	50 mg	**
Absorption Enhancer		
Bioperine® Black Pepper (*Piper nigrum*) Fruit Extract	5 mg	**

*Percent Daily Value Based on a 2000 Calorie Diet **Daily Value Not Established

Other Ingredients: Natural & Artificial Flavors, Citric Acid, Sucralose, Silicon Dioxide, Red Beet Juice Powder (for Color), Acesulfame Potassium

5. **Exercise Testing and Training** - During the strength testing sessions test there is a risk potential even though no health problems exist. The risk and discomforts that are associated with this type of test include muscle fatigue, lightheadedness, chest discomfort, and very rarely death. The potential for death during or immediately

following the test (or any vigorous exercise) is approximately 0.5 per 10,000 tests, according to the American College of Sports Medicine. Following these tests however you will experience muscle soreness due to the eccentric component of the resistance exercise. This soreness generally referred to as delayed on-set muscle soreness as it is most likely to occur in the days following the exercise bout. All exercise training will be prescribed relative to each subject and the principles of proper exercise progression and overload will be applied so as to reduce the risk of injury to the subject. Laboratory personnel will stay in contact with you after a testing/training session to ensure that you are comfortable. You will be given a 24 hr contact number for the Applied Physiology Laboratory personnel to convey any type of unusual discomfort.

In all of these procedures, care will be taken to employ "universal precautions" for the handling of blood and infectious materials to ensure your safety.

FOLLOW UP CARE

Following the procedure you will be provided with a biopsy care sheet, extra bandages and contact information for Andy Fry, Ph.D. After 24-hrs you must report back to the testing coordinator to check the wound. At this time the bandage will be removed and properly disposed of and a new sterile dressing placed over the wound. You will again be asked to report to the test coordinator after 3-days and be contacted via phone one week after the biopsy or whenever necessary to ensure normal recovery. The biopsy procedure often results in a small fine scar at the site of the incision, however, all care post-treatment will aid in reducing the potential for scarring. All care will be taken to aid in the healing of the wound. The entire biopsy procedure will be performed under sterile conditions. All testing staff and associated personnel will be trained in first aid and will be familiar with emergency procedures.

There have been no other major complications reported in the scientific literature as a result of taking small tissue samples from the skeletal muscle using the percutaneous needle biopsy technique described above. This procedure has been performed on numerous subjects by qualified personnel in many institutions worldwide with only slight discomfort being reported. During the muscle biopsy it is common to feel a strong cramping sensation in the muscle while the biopsy is being performed. However, muscle function is not impaired. In fact, subjects have been reported to continue participation in sporting events immediately following a muscle biopsy. It is common for subjects to experience mild soreness, moderate pain and bruising near the biopsy site, similar to a "Charlie-horse" the day after the procedure. In order to allow the incisions to heal properly and minimize the risk of infection, you should not get the biopsy site wet for 24-hours and avoid prolonged exposure to water for 4-days. Daily showers are acceptable (after the first 24-hours), but baths, swimming, sauna's etc. should be avoided for 4-days following the biopsy procedure.

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BENEFITS

Over the course of this study you will receive information concerning your session. You will also have trained staff monitoring your exercise sessions. You will also gain an increased understanding of resistance training and physiological changes that occur. A copy of all personal data from the tests will be provided to you and your data will be completely explained to you by a member of the investigation team.

The results of this investigation will provide a greater knowledge of the influence of exercise training and use of this supplement muscle markers of skeletal muscle growth (hypertrophy) and metabolism. These findings will not only have implications on those healthy individuals that are trying to gain muscle mass and exercise more efficiently but also those whose focus is to improve muscular strength and power.

PAYMENT TO SUBJECTS

You will receive a $125 honorarium for your participation in this three week study. Although not anticipated, if you need to discontinue the study due to an adverse event associated with the study, you will not receive compensation. In the event that you only complete half the study, thirty dollars ($30.00) compensation will be awarded. You will be withdrawn from the study if there is noncompliance with study procedures such as consumption of other dietary supplements (except whey protein). Investigators will ask for your social security number in order to comply with federal and state tax and accounting regulations.

COMPENSATION FOR INJURY

The following information is provided in accordance with HEW regulations: "In the event of injury, the Kansas Tort Claims Act provides for compensation if it can be demonstrated that the injury was caused by the negligent or wrongful act or omission of a state employee acting within the scope of his/her employment."

IN CASE OF EMERGENCY CONTACT PROCEDURE

In the event of a research related injury or adverse reaction, please contact Andrew Fry, Ph.D. at 785-864-4656 (office) or 785-865-6711 (cell), or the Applied Physiology Laboratory at 785-864-0773.

PARTICIPANT CONFIDENTIALITY

To perform this study, researchers will collect information about you. This information will be obtained from the health history and physical activity questionnaires, muscle biopsy, and muscle function evaluation. Your name will not be associated in any way with the information or tissue collected from you or with the research findings from this study. The researchers will use a study identification number or initials in place of your name. Any data stored electronically will be stored on a password protected laboratory computer (not a laptop). Any paperwork associated with you will be stored in a locked file cabinet secured in the Applied Physiology Laboratory. All tissue collected will be stored securely in the Applied Physiology Laboratory. All tissue collected will be used to analyze markers of muscle metabolism.

All tissue and blood samples will be de-identified. Tissue and blood samples will be kept for a maximum of 10 years, after which time samples will be destroyed via disposal into biohazard waste receptacles. However, due to the nature of experiments, we expect all amount of tissue and blood samples to be used during experimental analyses. All hard copy data will be destroyed after 5 years time, and electronic data will be kept indefinitely. All electronic data will remain de-identified and will in no way be traced back to you, the individual participant.

Only information that is relevant to the study will be shared with MusclePharm. Any and all data collected and sent to MusclePharm will be de-identified. Therefore, in no way will your health information, muscle performance, or results of tissue analyses be traced back to you. Information about current medications or potential disease reported on the health history questionnaire will NOT be forwarded to MusclePharm, and this information will strictly be collected to determine if you are eligible to participate in the present study. The information that will be sent to MusclePharm will be height, body mass, age, percent body fat, measures of muscle performance (strength, power, velocity), muscle physiology (beta-receptors and signaling proteins), mood responses, and any information regarding adverse effects of the supplement (nausea, vomiting, poor taste, etc.). The researchers will not share information about you with anyone outside of the Applied Physiology Laboratory (other than MusclePharm) personnel unless required by law or unless you give written permission.

Permission granted on this date to use and disclose your information remains in effect indefinitely. By signing this form you give permission for the use and disclosure of your information for the purposes of this study at any time in the future.

REFUSAL TO SIGN CONSENT AND AUTHORIZATION

You are not required to sign this Consent and Authorization form and you may refuse to do so without affecting your right to any services you are receiving or may receive from the University of Kansas or to participate in any programs or events of the University of Kansas. However, if you refuse to sign, you cannot participate in this study.

CANCELLING THIS CONSENT AND AUTHORIZATION

You may withdraw your consent to participate in this study at any time. You also have the right to cancel your permission to use and disclose information collected about you, in writing, at any time, by sending your written request to: Andrew Fry, Ph.D., University of Kansas, 1301 Sunnyside Avenue, Robinson Center Room 101, Lawrence, Kansas 66045. If you cancel permission to use your information, the researchers will stop collecting additional information about you. However, the research team may use and disclose information that was gathered before they received your cancellation, as described above.

PARTICIPANT CERTIFICATION

I have read this Consent and Authorization form. I have had the opportunity to ask, and I have received answers to, any questions I had regarding the study and the use and disclosure of information about me for the study. I understand that if I have any additional questions about this study I may call Prof. Andrew Fry (785-864-4656) or e-mail: acfry@ku.edu , or Justin Nicoll, justin.nicoll@ku.edu. I understand that if I have any additional questions about my rights as a research participant, I may call 785-864-7429 or write the Human Subjects Committee Lawrence Campus (HSCL), University of Kansas, 2385 Irving Hill Road, Lawrence, Kansas 66045-7563, email irb@ku.edu.

I agree to take part in this study titled 'The effects of a pre-workout supplement on beta2-adrenergic receptor activation and anabolic signaling pathways.' as a research participant. I further agree to the uses and disclosures of my information as described above. By my signature I affirm that I am at least 18 years old and that I have received a copy of this Consent and Authorization form.

____________________ Print Subject's Name

____________________ Signature of subject

________ Date

____________________ Print Name of Person Obtaining Consent

____________________ Signature of Person Obtaining Consent

________ Date

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RESEARCHER CONTACT INFORMATION

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APPENDIX B

PRE-EXERCISE TESTING HEALTH & EXERCISE STATUS QUESTIONNAIRE

Name ____ Date_____________

Home Address____

Phone Number ______________________ Email _______________________

Person to contact in case of emergency____

Emergency Contact Phone _____________________

Personal Physician ___________________________ Physician's Phone______________

Gender________ Age ______(yrs) Height ______(ft)______(in) Weight______(lbs)

Does the above weight indicate: a gain____ a loss____ no change____ in the past year?
If a change, how many pounds?__________(lbs)

A. JOINT-MUSCLE STATUS (✓Check areas where you currently have problems)

Joint Areas
( ) Wrists
( ) Elbows
( ) Shoulders
( ) Upper Spine & Neck
( ) Lower Spine
( ) Hips
( ) Knees
( ) Ankles
( ) Feet
( ) Other____________________

Muscle Areas
( ) Arms
( ) Shoulders
( ) Chest
( ) Upper Back & Neck
( ) Abdominal Regions
( ) Lower Back
( ) Buttocks
( ) Thighs
( ) Lower Leg
( ) Feet
( ) Other___________________

B. HEALTH STATUS (✓Check if you currently have any of the following conditions)

( ) High Blood Pressure
( ) Heart Disease or Dysfunction
( ) Peripheral Circulatory Disorder
( ) Lung Disease or Dysfunction
( ) Arthritis or Gout
( ) Edema
( ) Epilepsy
( ) Multiply Sclerosis
( ) High Blood Cholesterol or Triglyceride Levels
( ) Allergic reactions to rubbing alcohol

( ) Acute Infection
( ) Diabetes or Blood Sugar Level Abnormality
( ) Anemia
( ) Hernias
( ) Thyroid Dysfunction
( ) Pancreas Dysfunction
( ) Liver Dysfunction
( ) Kidney Dysfunction
( ) Phenylketonuria (PKU)
( ) Loss of Consciousness

* *NOTE: If any of these conditions are checked, then a physician's health clearance will required.*

C. PHYSICAL EXAMINATION HISTORY

Approximate date of your last physical examination______________________________

Physical problems noted at that time____

Has a physician ever made any recommendations relative to limiting your level of physical exertion? _________YES __________NO

If YES, what limitations were recommended?___________________________________

____

D. FEMALE REPRODUCTIVE HISTORY

If you are male, skip to Section E.

Did you begin menses within the past year? _________YES _________NO

Have you had consistent menstrual periods for the last 3 months?

YES_________ NO_________

Date of onset of last menstrual period____

Have you used a hormonal contraceptive within the last 3 months?

YES_________ NO_________

E. CURRENT MEDICATION USAGE (List the drug name, the condition being managed, the length of time used)

MEDICATION	CONDITION	LENGTH OF USAGE
______________________	______________________________	_________________
______________________	______________________________	_________________

F. PHYSICAL PERCEPTIONS (Indicate any unusual sensations or perceptions. ✓Check have recently experienced any of the following during or soon after *physical activity* (PA) during *sedentary periods* (SED))

PA	SED		PA	SED	
( )	( )	Chest Pain	( )	( )	Nausea
( )	( )	Heart Palpitations	( )	( )	Light Headedness
( )	( )	Unusually Rapid Breathing	( )	( )	Loss of Consciousness
( )	( )	Overheating	( )	( )	Loss of Balance
( )	( )	Muscle Cramping	( )	( )	Loss of Coordination
( )	( )	Muscle Pain	( )	( )	Extreme Weakness
( )	( )	Joint Pain	( )	( )	Numbness
( )	( )	Other______________________	( )	( )	Mental Confusion

G. FAMILY HISTORY (✓Check if any of your blood relatives . . . parents, brothers, sisters aunts, uncles, and/or grandparents . . . have or had any of the following)

( ) Heart Disease
( ) Heart Attacks or Strokes (prior to age 50)
( ) Elevated Blood Cholesterol or Triglyceride Levels
( ) High Blood Pressure
( ) Diabetes
( ) Sudden Death (other than accidental)

H. EXERCISE STATUS

Do you regularly engage in aerobic forms of exercise (i.e., jogging, cycling, walking, etc.)? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

What is your fastest 5 km time? ______________

What is your fasted 10 km time? _____________

What is your fasted mile time? _______________

What is your fasted times at other distances not listed? ___________________________________

Do you regularly lift weights? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

What is your back squat 1 repetition maximum (RM)? _____________

What is your deadlift 1 RM? ____________

What is your power clean 1 RM? ____________

What are your other 1 RMs that are not listed? ____

Do you regularly play recreational sports (i.e., basketball, racquetball, volleyball, etc.)? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

APPENDIX C

SUBJECTS WANTED!!

PRE-WORKOUT SUPPLEMENT STUDY

Eligibility: The University of Kansas is conducting a research study looking to recruit healthy recreationally active males between the ages of 18-30. You must also be able to squat at least 1.5 times your body weight, and have no current or recent (within the past 3 months) hip, knee, or hand injuries. The study consists of a familiarization visit (1hr) and two experimental visits (3hrs each). Participants will receive information about the response to a pre-workout supplement during resistance exercise and monetary compensation for completion of the study.

The University of Kansas is an equal opportunity institution.

If you are eligible and interested, please contact:

Andrew Fry, Ph.D.
Or
Justin Nicoll, M.S.
101 Robinson Center
kusupplementstudy@gmail.com

Purpose: The objective of this study is to examine the performance, molecular, and hormonal responses to a caffeine containing pre-workout exercise supplement.

APPENDIX D

Supplement Facts

Serving Size: 18.2g
Servings per Container: *(see packaging specifications)*

	Amount Per Serving	% Daily Value
Calories	20	
Total Carbohydrate	6 g	2%*
Sugars	5 g	**
Vitamin C (as Ascorbic Acid)	300 mg	500%
Vitamin E (as D L-Alpha Tocopheryl Acetate)	40 IU	133%
Vitamin B6 (as Pyridoxine Hydrochloride)	20 mg	1000%
Vitamin B12 (as Methylcobalamin)	50 mcg	833%
Calcium (as Calcium Silicate)	62 mg	6%
Strength & Performance		
Creatine Monohydrate	5 g	**
Beta-Alanine	2 g	**
Betaine Anhydrous (Trimethylglycine)	1.5 g	**
Muscle Fuel		
Isomaltulose (as Palatinose™)	5 g	**
L-Glycine	1 g	**
L-Taurine	1 g	**
Energy & Focus		
Caffeine Anhydrous	250 mg	**
Theacrine (as TeaCrine®)	50 mg	**
Absorption Enhancer		
Bioperine® Black Pepper (*Piper nigrum*) Fruit Extract	5 mg	**

*Percent Daily Value Based on a 2000 Calorie Diet **Daily Value Not Established

Other Ingredients: Natural & Artificial Flavors, Citric Acid, Sucralose, Silicon Dioxide, Red Beet Juice Powder (for Color), Acesulfame Potassium

APPENDIX E

Subject: **Condition:** **Date:**

Caffeine Questionnaire

1. How many cups of caffeinated coffee, tea, soda, and energy drinks (red bull, full throttle etc.) do you consume on a typical weekday?

2. How many cups of caffeinated coffee, tea, soda, and energy drinks (red bull, full throttle etc.) do you consume on a typical weekend day?

3. Do you consider yourself addicted to caffeine? YES NO

4. Do you ever get headaches or feel unwell because you did not get caffeine or coffee at your usual time? YES NO

5. Do you feel that you need a caffeinated beverage to wake up in the morning? YES NO

6. If you take part in this study will you be able to reduce or eliminate caffeine from your diet? YES NO

7. Have you ever had too much caffeine such that it caused negative side effects like rapid heart rate, anxiety, rapid breathing, or jitters? YES NO

a. IF yes, approximately how much caffeine (or how much soda, coffee, tea) did you consume? (you will find caffeine amounts for common foods on the next page)

____

____

b. If NO, What is the most caffeine you have ever consumed in a 3 hour period? (or how much soda, coffee, tea) did you consume? (you will find caffeine amounts for common foods on the next page)

____

____

Caffeine Content of Popular Drinks 12-ounce beverage	**milligrams**
Red Bull (8.2 oz)	80.0
Jolt	71.2
Pepsi One	55.5
Mountain Dew / Diet Mountain Dew / Code Red	55.0
Mellow Yellow / Surge	52.8
Tab / RC Cola / Diet RC	46.8
Diet Coke	45.6
Dr. Pepper/ Diet Dr. Pepper / Mr. Pibb	41.0
Pepsi-Cola / Diet Pepsi / Wild Cherry Pepsi	37.5
Coca-Cola Classic	34.0
Snapple Flavored Teas (Reg. or Diet)	31.5
Nestea Sweet Iced Tea	26.5
Nestea Unsweetened Iced Tea	26.0
Lipton Diet Green Tea with Citrus (16.9 oz)	23.0
Barq's Root Beer	23.0
Lipton Brisk, All Varieties	9
Diet Rite Cola	0
Sprite /7-Up / Slice / Sierra Mist / Fresca	0
Mug / Diet Barq's Root Beer /A&W Root Beer	0
Sundrop Orange / Minute Maid Orange	0

8 Ounce Beverage	**milligrams**
Coffee, Drip	115-175
Coffee, Brewed	80-135
Coffee, Espresso (2 ounces)	100
Tea, iced	47
Tea, brewed, imported brands (avg.)	50
Tea, instant	30
Tea, green	15
Hot cocoa	14
Coffee, Decaf,	3-4

Chocolate	**milligrams**
Dark chocolate	12 per/oz
Milk chocolate	9 per/oz
Coffee/Chocolate ice-cream	2 per/3.5oz

APPENDIX F

RPE Scale

rating of perceived exertion

rating	description
6	NO EXERTION AT ALL
7	
	EXTREMELY LIGHT
8	
9	VERY LIGHT
10	
11	LIGHT
12	
13	SOMEWHAT HARD
14	
15	HARD (HEAVY)
16	
17	VERY HARD
18	
19	EXTREMELY HARD
20	MAXIMAL EXERTION

for more information see http://www.topendsports.com/testing/rpe-scale.htm

APPENDIX G

FOOD RECORD

Accurately determining portion sizes and amounts

The most accurate way to determine portion size is to weigh and measure foods and beverages using measuring cups and spoons. The following guidelines will assist you in choosing how to describe and measure portion sizes.

Guidelines for describing and measuring foods

FOODS	MEASURE & DESCRIBE WITH:
Vegetables, fruit cup, pasta, rice, casseroles, ice cream, pudding, margarine, and all liquids including beverages, soups, gravies, salad dressing	measuring cups (c) teaspoons (tsp) Tablespoons (Tbs)
Any solid food such as meat, cheese or frozen entrees	weight in grams (gm) or ounces (oz)
Pie, cantaloupe, other melons	fraction of the whole (Ex: 1/8 of 9" pie, ¼ of 6" melon)
Liquids	Fluid ounces or cups

"Guesstimating" Guidelines

Since measuring is not always possible or practical, there are times when "guesstimating" will suffice. Use the following guidelines to help you determine portion sizes when you're not able to weigh or measure.

- A woman's fist is about a cup.
- A man's fist is about 1-1/2 cups.
- The cupped palm of an adult's fist holds about one-half cup.
- 3 ounces of cooked meat is similar in size to a standard deck of cards.
- A one-ounce meatball is approximately the size of a golf ball.
- A McDonald's plain hamburger patty is 2 ounces of cooked meat.
- The average cooked chicken breast weighs between 3 and 4 ounces.
- A Kraft American single is a one-ounce slice of cheese.
- The standard slice of bologna is one ounce.
- A package of peanuts that you'd get on an airplane is a one-ounce package.
- A frozen Lender's bagel is two ounces. Most other bagels are three to four ounces.

Measurement conversion information

Common household measures

3 teaspoons	= 1 Tablespoon
4 Tablespoons	= ¼ cup
5-1/3 Tablespoons	= 1/3 cup
8 Tablespoons	= ½ cup
10 2/3 Tablespoons	= 2/3 cup
12 Tablespoons	= ¾ cup
16 Tablespoons	= 1 cup

½ fluid ounce	= 1 Tablespoon
1 fluid ounce	= 2 Tablespoons
2 fluid ounces	= ¼ cup
4 fluid ounces	= ½ cup
6 fluid ounces	= ¾ cup
8 fluid ounces	= 1 cup
16 fluid ounces	= 2 cups
2 cups	= 1 pint

1 ounce (oz.)	= 28.4 grams
1 pound (lb.)	= 16 ounces
¼ pound	= 4 ounces

Abbreviations

teaspoon	= tsp
Tablespoon	= Tbsp
cup	= C
ounce	= oz
fluid ounce	= fl oz
pound	= lb
grams	= gm

FOOD INTAKE RECORD FORM *EXAMPLE*

Name: __*Volunteer*__________ **Date:** _*10/04/09, Thursday*_ **Visit:** 1 ② (circle)

Time	Amount	Full Description of Food or Beverage Consumed
7:10 am	8 fl-oz	Orange juice, from frozen concentrate, prep
	1 cup	Kellogg's Crispix cereal
	½ cup	2% milk (vitamin A & D fortified)
	2 teaspoons	sugar
	1 cup	Black coffee
10:00am	12 fl-oz can	Diet Coke
12:15pm	2 slices	Pepperidge Farm whole grain oatmeal bread
	2 leaves	Iceberg lettuce
	1 tablespoon	Mayonnaise, Cain's regular
	1 medium	Fresh pear
	1 cup	2% milk (vitamin A & D fortified)
2:30 pm	12 oz can	Orange Fanta, regular
6:30pm	6 oz	Pork chop, broiled
	1 medium	Baked potato
	2 tablespoons	Margarine, Blue Bonnet Soft, Low-Sodium
	5 inner leaves	Romaine lettuce
	4 slices	Fresh red tomato (1/4" thick)
	2 tablespoons	Ranch Dressing, Hellman's Creamy Lite
	½ cup	Frozen peas, cooked in microwave (Shaw's)
	1 cup	Whole milk (vitamin A, D & calcium fortified)
	1/8 of 9" diam.	Homemade cherry pie
9:10 pm	1 large	Fresh Granny Smith Apple
	1 –12 ounce glass	Mineral water, Adirondack Sparkling
	1 each	Bagel, plain (3 oz-wt size)

Name:____________________Date: _____Day: ___________ 1 2 3 4 5 (circle)

Time	*AMOUNT*	**Complete** *DESCRIPTION* **of food/beverage**

Name:____________________Date: _____Day: ___________ 1 2 3 4 5 (circle)

Time	*AMOUNT*	**Complete *DESCRIPTION* of food/beverage**

Name:____________________Date: _____Day: ___________ 1 2 3 4 5 (circle)

Time	*AMOUNT*	**Complete *DESCRIPTION* of food/beverage**

APPENDIX H

Effects of dietary forskolin on skeletal muscle cAMP

INTRODUCTION

The Department of Health, Sport and Exercise Sciences at the University of Kansas supports the practice of protection for human subjects participating in research. The following information is provided for you to decide whether you wish to participate in the present study. You may refuse to sign this form and not participate in this study. You should be aware that even if you agree to participate, you are free to withdraw at any time. If you do withdraw from this study, it will not affect your relationship with this unit, the services it may provide to you, or the University of Kansas.

PURPOSE OF THE STUDY

cAMP is a chemical in muscle cells that helps control cell function.. Previous studies suggest that forskolin, a plant extract, may increase the amount of cAMP in cells, but it is not known whether this occurs in human muscle cells. The purpose of this investigation is to determine the effects of a forskolin dietary supplement on the concentration of cAMP in human skeletal muscle cells.

BASIS FOR SUBJECT SELECTION

In order to participate in this study you must be female, between the ages of 18 and 35, healthy, non-obese (BMI $<$28 kg/m2), non-smoking, and free of any cardiovascular, metabolic or musculoskeletal conditions that may affect the results of the investigation. You must not have asthma, low blood pressure (systolic $\leq$ 100 mmHg or diastolic $\leq$ 60 mmHg), gastric ulcers, or glaucoma or be pregnant. Additionally, you must not be taking any prescription medications (excluding birth control), especially anti-platelets, anti-hypertensives, or anti-asthmatics. You may take over the counter medication, with the exception of aspirin, ibuprofen (Advil) or naproxen (Aleve). In addition, you must be free of any bleeding or blood clotting disorders as these conditions represent an increased risk for adverse events due to the muscle biopsy procedure. You will be screened for participation using a health-history questionnaire for contraindications to exercise by American College of Sports Medicine (ACSM) guidelines.

PROCEDURES

Study Design – This investigation will consist of two testing visits at applied physiology laboratory in which you will be given either a forskolin supplement or a placebo in a randomized order. Two muscle biopsies will be taken from your thigh muscle at each treatment visit; one prior to ingestion of the supplement or placebo, and a second 45 minutes following the supplement or placebo. Approximately seven to fourteen days after the first treatment you will report to the laboratory for the second treatment. Prior to each testing visit, you will be asked to fast for at least 8 hours and abstain from caffeine consumption for at least 24 hours. We approximate that the first treatment visit will take 1.5 to 2 hours and the second treatment 1.5 hours. Approximately 24 hours following each treatment visit, you will return to the laboratory

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for follow up care for the biopsy site. Each follow up visit will take approximately 15 minutes. A table detailing the procedures to be performed at each visit is listed below.

Visit 1 (Testing Visit)	Visit 2 (Follow up Care)	Visit 3 (Testing Visit)	Visit 4 (Follow up Care)
1. Pre-Supp. Biopsy 2. Take Supp. or Placebo 3. 45 min Post-Supp. Biopsy	1. Biopsy site inspection 2. Application of new bandage	1. Pre-Supp. Biopsy 2. Take Supp. or Placebo 3. 45 min Post-Supp. Biopsy	1. Biopsy site inspection 2. Application of new bandage

Payment: $50		Payment: $50	

Anthropometrics – Your anthropometric data, including height and weight will be recorded at the first treatment visit. Height will be measured using a Seca 216 stadiometer (Hanover, MD). Weight will be measured with a Tanita scale, with shoes and excess clothing removed.

Blood Pressure – Your blood pressure will be measured at the each treatment visit to ensure that you do not have low blood pressure.

Pregnancy Test – Upon arriving at each acute testing visit, you will be asked to complete a home pregnancy test, to ensure that you are not pregnant. If you become pregnant between testing visits, please inform the researchers via the contact information listed below.

Supplement – The supplement used in the investigation is Futurebiotics 100% Vegetarian Forskolin with an active ingredient of 62.5 mg Coleus Forskohlii root extract (standardized for a minimum of 40% forskolin). Other ingredients include microcrystalline cellulose, modified cellulose, magnesium stearate, and silica. You will be given two capsules at the acute visit, supplying a total of 50mg of forskolin.

Placebo – The placebo supplement is veggie capsules containing maltodextrin. Each placebo pill will contain between 408 and 816 mg of maltodextrin. The ingredients of the veggie capsule are Hydroxypropyl methylcellulose (HPMC), vegetable source and water. You will be given two capsules at the acute visit. Maltodextrin is a carbohydrate containing 3.8 calories per gram.

Muscle Biopsies – You have been informed that one of the purposes of this study is to measure the effect of forskolin on a signaling molecule (cAMP) in the muscle tissue we collect. By obtaining a small sample of your muscle tissue (size of a pencil eraser or small finger nail), we will be able to measure the concentration of cAMP in your muscle. All muscle tissue samples (biopsies) will be taken from the outer portion of the front of the thigh using a needle biopsy technique by Andy Fry, PhD. Andy Fry, PhD, Professor of HSES, has performed over 100 muscle biopsies over the past year and has assisted on over 500 biopsies over the past six years on various populations (athletes, sedentary people, elderly etc.) with no significant complications and nothing more than minimal adverse reactions. The procedure is being overseen by Dr.

Jeffrey Burns M.D., who is a medical doctor in Neurology at the KU Medical Center in Kansas City, Kansas supervises the procedure, but will not be physically present for the biopsies. The total size of the muscle biopsy will be approximately the size of a pea. You will be placed on an examination table lying down on your back (supine) so that the muscles of the leg are relaxed. The skin will be thoroughly cleaned with antiseptic solution (Betadine) using sterile cotton swabs after which a surgical cover will be placed around the sampling site. If you are allergic to Betadine, an alternative antiseptic solution will be used to clean the skin. A small amount (2ml or 2cc) of a local aesthetic (2% Lidocaine) will be injected into the tissue under the skin around the site to be sampled. During this injection you may feel a slight burning sensation. If you are allergic to the local anesthetic, or have had allergic reactions to other anesthetics (i.e.: Novocain) then you will be disqualified from the study. Following the injection of the numbing agent into your thigh, a minimum of five (5) minutes will be allowed to pass to ensure adequate time for the agent to take effect in the area where the incision will be made. A small incision (1 cm) will then be made in the skin overlying the muscle and the biopsy needle inserted into the middle of the muscle (muscle belly) at a depth of 3 cm (about 1 inch). During the time that the sample is being taken (about 5 seconds) you may feel some deep pressure and cramping that will be moderately painful. You have been informed that if you have been previously diagnosed as having a bleeding disorder, a blood clotting problem, take blood thinning medication or have sensitivity to scarring, you will tell the researcher and not participate in the biopsy procedure.

Sterile disposable instruments and sterile gloves will be used for the preparation of the site and the reusable biopsy needle will be thoroughly sterilized (via steam and heat autoclave) after each biopsy. Approximately 100 mg (size of a pencil eraser or small finger nail) of skeletal muscle tissue will be removed. With the invasive skeletal muscle biopsy technique there is the possibility of a blood related infection (HIV, Hepatitis B), but the reusable needles will be cleaned and sterilized using an autoclave, a generally accepted method of cleaning surgical instruments.
A total of **four (4)** biopsies will be performed over the course of the study.

RISKS

Muscle biopsies – The use of local anesthetics will result in a slight burning sensation, lasting approximately 5 seconds. There is a risk of allergic reactions to the local injection (1 in 1 million). There is a small chance of bleeding from the biopsy site. The principle concern would be prolonged bleeding which would produce a bruise in the area. This would extend the muscle soreness, but is adequately treated with rest, ice, a compression bandage, and keeping the leg elevated as much as possible. Nausea, dizziness, and fainting can occur (1 in 100) during the biopsy process. As a result, the subjects will be in a supine position during the biopsy procedure. There is a risk of infection (1 in 1000) and irritation associated with the biopsy procedure. The use of aseptic techniques, careful cleaning of the skin and keeping the area dry will minimize the risk of infection. There is a risk of bruising (1 in 100) from the biopsy procedure. This will be minimized by placing ice over the site following the procedure and by applying a compression bandage on the site for the 24 hours following the biopsy. In some instances (1 in 200), some motor nerves may be damaged which may cause local numbness. There is likely to be a small scar where the biopsy is performed. This scar usually dissipates over a period of 6-12 months at which time the scaring is very modest.

Supplement - As a nutritional supplement, forskolin has not been evaluated and approved by the Federal Food and Drug Administration. Previous clinical studies, have demonstrated that forskolin supplementation is well tolerated and without adverse side effects in healthy individuals. Studies have demonstrated that forskolin supplementation of 50 mg/day for 12 weeks had no significant detrimental biochemical, hematological or blood pressure effects. Forskolin should not be taken by individuals with asthma, stomach ulcers, low blood pressure.

Potential side effects associated with forskolin supplementation include: decreased blood pressure, decreased intraocular pressure (observed during long term supplementation), increased stomach acid levels, and increased effectiveness of prescription drugs (listed below).

Potential drug interactions:

- Antihypertensives (Calcium Channel Blockers):
    - Nifedipine (Adalat, Afeditab, Nifediac, Procardia etc.)
    - Verapamil (Calan, Isoptin, Verlan, Covera-HS etc.)
    - Diltiazem (Cardizem, Cartia XT, Dilacor XR, Diltia, Diltzac, Matzim LA, Taztia XT, Tiazac etc.)
    - Isradipine (Dynacirc)
    - Felodipine (Plendil)
    - Amlodipine (Norvasc)
- Nitrates:
    - Nitroglycerin
    - Isosorbide (Imdur, Monoket, ISMO, Dilatrate-SR, Isochron, Sorbitrate, etc.)
- Anticoagulant/Antiplatelet Medication:
    - Aspirin
    - Clopidogrel (Plavix)
    - Diclofenac (Cambia, Cataflam, Voltaren-XR, Zipsor, Zorvolex etc.)
    - Ibuprofen (Advil, Motrin etc.)
    - Naproxen (Aleve, Naprosyn etc.)
    - Dalteparin (Fragmin)
    - Enoxaparin (Lovenox, Clexane etc.)
    - Heparin (Heparin Sodium ADD-Vantage)
    - Warfarin (Coumadin, Jantoven)

Forskolin should not be taken by pregnant women. To date, there is no scientific evidence that forskolin supplementation is detrimental to pregnancy. Given the wide range of processes that cAMP (the chemical which forskolin may increase) affects, forskolin is contraindicated for pregnant women as a precaution.

FOLLOW UP CARE

Following the procedure you will be provided with a biopsy care sheet, extra bandages and contact information for Andy Fry, Ph.D. After 24-hrs you must report back to the testing coordinator to check the wound. At this time the bandage will be removed and properly disposed of and a new sterile dressing placed over the wound. You will again be asked to report to the test

coordinator after 3-days and be contacted via phone one week after the biopsy or whenever necessary to ensure normal recovery. The biopsy procedure often results in a small fine scar at the site of the incision, however, all care post-treatment will aid in reducing the potential for scarring. All care will be taken to aid in the healing of the wound. The entire biopsy procedure will be performed under sterile conditions. All testing staff and associated personnel will be trained in first aid and will be familiar with emergency procedures.

There have been no other major complications reported in the scientific literature as a result of taking small tissue samples from the skeletal muscle using the percutaneous needle biopsy technique described above. This procedure has been performed on numerous subjects by qualified personnel in many institutions worldwide with only slight discomfort being reported. During the muscle biopsy it is common to feel a strong cramping sensation in the muscle while the biopsy is being performed. However, muscle function is not impaired. In fact, subjects have been reported to continue participation in sporting events immediately following a muscle biopsy. It is common for subjects to experience mild soreness, moderate pain and bruising near the biopsy site, similar to a "Charlie-horse" the day after the procedure. In order to allow the incisions to heal properly and minimize the risk of infection, you should not get the biopsy site wet for 24-hours and avoid prolonged exposure to water for 4-days. Daily showers are acceptable (after the first 24-hours), but baths, swimming, sauna's etc. should be avoided for 4-days following the biopsy procedure.

BENEFITS

You may not directly benefit from participating in this investigation. Information obtained from your participation will be used to determine if forskolin supplementation can affect cAMP levels in skeletal muscle. You will receive financial compensation for your participation.

PAYMENT TO PARTICIPANTS

You will receive $100 for completing this investigation. If you complete only one of the two testing visits, you will receive $50. Investigators may ask for your social security number in order to comply with federal and state tax and accounting regulations.

Visit 1 (Testing Visit)	Visit 2 (Follow up Care)	Visit 3 (Testing Visit)	Visit 4 (Follow up Care)
Payment: $50	No Payment	Payment: $50	No Payment

PARTICIPANT CONFIDENTIALITY

Your name will not be associated in any publication or presentation with the information collected about you or with the research findings from this study. Instead, the researcher(s) will use subject numbers. Your identifiable information will not be shared unless (a) it is required by law or university policy, or (b) you give written permission. Permission granted on this date to use and disclose your information remains in effect indefinitely. By signing this form you give

permission for the use and disclosure of your information for purposes of this study at any time in the future."

INSTITUTIONAL DISCLAIMER STATEMENT

In the event of injury, the Kansas Tort Claims Act provides for compensation if it can be demonstrated that the injury was caused by the negligent or wrongful act or omission of a state employee acting within the scope of his/her employment.

REFUSAL TO SIGN CONSENT AND AUTHORIZATION

You are not required to sign this Consent and Authorization form and you may refuse to do so without affecting your right to any services you are receiving or may receive from the University of Kansas or to participate in any programs or events of the University of Kansas. However, if you refuse to sign, you cannot participate in this study.

CANCELLING THIS CONSENT AND AUTHORIZATION

You may withdraw your consent to participate in this study at any time. You also have the right to cancel your permission to use and disclose further information collected about you, in writing, at any time, by sending your written request to: Andrew Fry, Ph.D., University of Kansas, 1301 Sunnyside Avenue, Robinson Center Room 101, Lawrence, Kansas 66045. If you cancel permission to use your information, the researchers will stop collecting additional information about you. However, the research team may use and disclose information that was gathered before they received your cancellation, as described above.

QUESTIONS ABOUT PARTICIPATION

Questions about procedures should be directed to the researcher(s) listed at the end of this consent form.

PARTICIPANT CERTIFICATION:

I have read this Consent and Authorization form. I have had the opportunity to ask, and I have received answers to, any questions I had regarding the study. I understand that if I have any additional questions about my rights as a research participant, I may call (785) 864-7429 or (785) 864-7385, write the Human Subjects Committee Lawrence Campus (HSCL), University of Kansas, 2385 Irving Hill Road, Lawrence, Kansas 66045-7568, or email irb@ku.edu.

I agree to take part in this study as a research participant. By my signature I affirm that I am at least 18 years old and that I have received a copy of this Consent and Authorization form.

_________________________ _________________
Type/Print Participant's Name Date

____
Participant's Signature

Researcher Contact Information

Adam Sterczala, MS
Principle Investigator
Applied Physiology Lab
101 Robinson Hall
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Lawrence, KS 66045
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University of Kansas
Lawrence, KS 66045
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APPENDIX I

PRE-EXERCISE TESTING HEALTH & EXERCISE STATUS QUESTIONNAIRE

Name ______________________________ Date__________

Home Address____

Phone Number ____________________ Email ____________________

Person to contact in case of emergency________________________________

Emergency Contact Phone ____________________

Personal Physician ______________________ Physician's Phone__________

Gender_______ Age _____(yrs) Height _____(ft)_____(in) Weight_____(lbs)

Does the above weight indicate: a gain____ a loss____ no change____ in the past year?
If a change, how many pounds?_________(lbs)

A. JOINT-MUSCLE STATUS (✓Check areas where you currently have problems)

Joint Areas
- ( ) Wrists
- ( ) Elbows
- ( ) Shoulders
- ( ) Upper Spine & Neck
- ( ) Lower Spine
- ( ) Hips
- ( ) Knees
- ( ) Ankles
- ( ) Feet
- ( ) Other________________

Muscle Areas
- ( ) Arms
- ( ) Shoulders
- ( ) Chest
- ( ) Upper Back & Neck
- ( ) Abdominal Regions
- ( ) Lower Back
- ( ) Buttocks
- ( ) Thighs
- ( ) Lower Leg
- ( ) Feet
- ( ) Other________________

B. HEALTH STATUS (✓Check if you currently have any of the following conditions)

- ( ) High Blood Pressure
- ( ) Low Blood Pressure
- ( ) Heart Disease or Dysfunction
- ( ) Peripheral Circulatory Disorder
- ( ) Lung Disease or Dysfunction
- ( ) Arthritis or Gout
- ( ) Edema
- ( ) Epilepsy
- ( ) Multiply Sclerosis
- ( ) High Blood Cholesterol or Triglyceride Levels
- ( ) Allergic reactions to rubbing alcohol
- ( ) Asthma
- ( ) Acute Infection
- ( ) Stomach Ulcers
- ( ) Diabetes or Blood Sugar Level Abnormality
- ( ) Anemia
- ( ) Hernias
- ( ) Thyroid Dysfunction
- ( ) Pancreas Dysfunction
- ( ) Liver Dysfunction
- ( ) Kidney Dysfunction
- ( ) Phenylketonuria (PKU)
- ( ) Loss of Consciousness
- ( ) Glaucoma

* *NOTE: If any of these conditions are checked, then a physician's health clearance will required.*

C. PHYSICAL EXAMINATION HISTORY

Approximate date of your last physical examination______________________________

Physical problems noted at that time____

Has a physician ever made any recommendations relative to limiting your level of physical exertion? _________YES __________NO

If YES, what limitations were recommended?___________________________________

____

D. FEMALE REPRODUCTIVE HISTORY

If you are male, skip to Section E.

Did you begin menses within the past year? _________YES _________NO

Have you had consistent menstrual periods for the last 3 months?
YES_________ NO_________

Date of onset of last menstrual period____

Have you used a hormonal contraceptive within the last 3 months?
YES__________ NO_________

Are you currently pregnant? YES_________ NO_________

E. CURRENT MEDICATION USAGE (List the drug name, the condition being managed, the length of time used)

MEDICATION	CONDITION	LENGTH OF USAGE
____________________	______________________________	_________________
____________________	______________________________	_________________

F. PHYSICAL PERCEPTIONS (Indicate any unusual sensations or perceptions. ✓Check have recently experienced any of the following during or soon after *physical activity* (PA) during *sedentary periods* (SED))

PA	SED		PA	SED	
( )	( )	Chest Pain	( )	( )	Nausea
( )	( )	Heart Palpitations	( )	( )	Light Headedness
( )	( )	Unusually Rapid Breathing	( )	( )	Loss of Consciousness
( )	( )	Overheating	( )	( )	Loss of Balance
( )	( )	Muscle Cramping	( )	( )	Loss of Coordination
( )	( )	Muscle Pain	( )	( )	Extreme Weakness
( )	( )	Joint Pain	( )	( )	Numbness
( )	( )	Other______________________	( )	( )	Mental Confusion

G. FAMILY HISTORY (✓Check if any of your blood relatives . . . parents, brothers, sisters aunts, uncles, and/or grandparents . . . have or had any of the following)

( ) Heart Disease
( ) Heart Attacks or Strokes (prior to age 50)
( ) Elevated Blood Cholesterol or Triglyceride Levels
( ) High Blood Pressure
( ) Diabetes
( ) Sudden Death (other than accidental)

H. EXERCISE STATUS

Do you regularly engage in aerobic forms of exercise (i.e., jogging, cycling, walking, etc.)? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

What is your fastest 5 km time? ______________

What is your fasted 10 km time? _____________

What is your fasted mile time? _______________

What is your fasted times at other distances not listed? ___________________________________

Do you regularly lift weights? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

What is your back squat 1 repetition maximum (RM)? _____________

What is your deadlift 1 RM? ____________

What is your power clean 1 RM? ____________

What are your other 1 RMs that are not listed? ____

Do you regularly play recreational sports (i.e., basketball, racquetball, volleyball, etc.)? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

APPENDIX J

SUBJECTS WANTED!!

SUPPLEMENT STUDY

Eligibility: Healthy females between the ages of 18 and 35.

The University of Kansas is an equal opportunity institution.

If you are eligible and interested, please contact:

Adam Sterczala, MS
101 Robinson Center
KUCAMPStudy@gmail.com

Your total time commitment will be approximately 3.5 - 4 hours.

Purpose: To determine if forskolin supplementation affects cAMP concentrations in skeletal muscle

APPENDIX K

Does overtraining affect force via a β2-adrenoceptor-Na+/K+ mechanism?

INTRODUCTION

The Department of Health, Sport and Exercise Sciences at the University of Kansas supports the practice of protection for human subjects participating in research. The following information is provided for you to decide whether you wish to participate in the present study. You may refuse to sign this form and not participate in this study. You should be aware that even if you agree to participate, you are free to withdraw at any time. If you do withdraw from this study, it will not affect your relationship with this unit, the services it may provide to you, or the University of Kansas.

PURPOSE OF THE STUDY

Insufficient recovery from repeated bouts of stressful exercise can induce overreaching or overtraining, states characterized by training specific decreases in performance. Additionally, several detrimental physiological changes can occur including changes in stress hormone levels and skeletal muscle protein levels. The purpose of this investigation is to determine whether the changes in stress hormone and skeletal muscle protein levels are the cause of overtraining induced performance decreases.

BASIS FOR SUBJECT SELECTION

In order to participate in this study you must be male, between the ages of 18 and 30, healthy, non-obese (BMI <28 kg/m2), non-smoking, and free of any cardiovascular, metabolic or musculoskeletal conditions that may affect the results of the investigation. Only male subjects will be recruited for participation due to previously observed gender differences in the sympathetic response to exercise. Additionally you must be free of any bleeding or blood clotting disorders as these conditions represent an increased risk for adverse events due to the muscle biopsy procedure. You will be screened for participation using a health-history questionnaire for contraindications to exercise by American College of Sports Medicine (ACSM) guidelines.

Only resistance trained individuals will be recruited for this study. To qualify as resistance trained, you must be capable of performing a 1.5x body weight back squat and a 1x body weight bench press. Additionally, you must have at least two years of resistance training experience, and have been actively training for at least six months prior to the onset of the investigation.

PROCEDURES

You will be asked to perform to fatiguing exercise bouts, before and after 8 days of supervised resistance training in the KU applied physiology laboratory. Muscle biopsies from your thigh muscle and blood draws will be taken before an after each of the two fatiguing exercise bouts. A

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general timeline of the study and of the fatiguing exercise bout is provided below. Overall the study will take approximately 2.5 weeks to complete.

Figure 1. Overall Investigation Timeline (times presented in relation to 4 week training period)

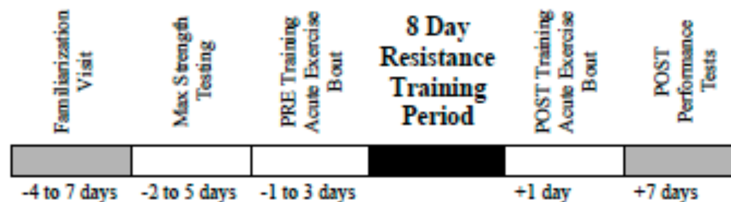
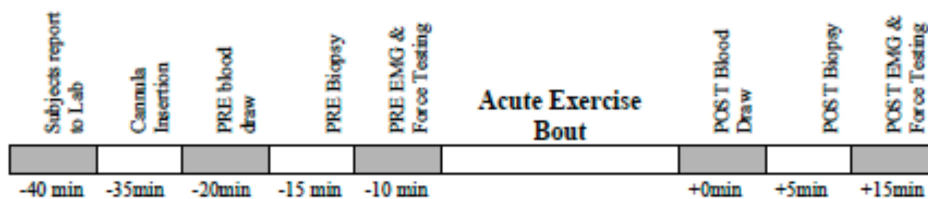


Figure 2. Acute Exercise Bout Timeline (times presented in relation to acute exercise bout)



Familiarization – Prior to participating in the investigation, you will complete a familiarization visit. At this visit you will be introduced to the various procedures and protocols that will occur during the investigation. Proper form on the resistance exercises employed by the training protocol will be demonstrated. You will practice the resistance exercise movements as well as the EMG and isokinetic force tests. Additionally, your anthropometric data (height, weight, body composition) will be collected at this visit. This visit will take approximately 1 hour.

Anthropometrics – Your anthropometric data, including height, weight and body composition will be collected at the familiarization visit. Height will be measured using a Seca 216 stadiometer (Hanover, MD). Weight will be measured with a Tanita scale, with shoes and excess clothing removed. Body composition will be measured using a three site skinfold (chest, abdomen, thigh) protocol to determine body density. An appropriate formula will then be applied to determine percent body fat.

Resistance training program design – You will be asked to perform 8 days of resistance training. If assigned to the control group you will be training three times during the 8 day period with approximately 48 hours rest between training sessions. Therefore, the control group will not train on successive days. If you are assigned to the overtraining group, you will be training twice

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daily (sessions at least 4 hours apart), each of the 8 days. You will be asked not to perform any additional resistance training for the duration of the investigation. All resistance training sessions will be performed in the University of Kansas Applied Physiology Laboratory facilities under the supervision of NSCA Certified Strength and Conditioning Specialists. Each training session will take approximately 45 minutes.

Strength Testing – To determine the loading used during the training period, max strength testing will be completed a few days before the training period. Back squat and knee extension one repetition maximum will be determined at this visit. The max strength testing visit should take approximately 30 – 40 minutes. On the eighth day of the training period, a second back squat 1RM will be collected. Seven days following the last day of the training period you will be asked to perform a single set of five back squats at 60% 1RM followed by a third back squat 1RM test.

Acute Exercise Bout – The acute exercise bout will consist of back squats, five sets of five repetitions at 60% 1RM, and leg extensions, three sets of 10 repetitions at 70% 1RM. Before and after the acute exercise bout, we will be taking muscle biopsies from the thigh muscles of your left leg. We will also be inserting an indwelling cannula in your forearm so that we may take blood draws before and after the acute exercise bout. Muscle function will be measured via electromyography (EMG) sensors attached to the muscles of your right leg. The muscle function tests include three maximum knee extension isometric contractions followed by several knee extension submaximal isometric contractions. We will also ask you to perform three dynamic knee extension contractions at two different speeds. Knee extensions contractions will be separated by one to two minute rest periods. After these trials, we will ask you to perform a 30 second sustained contraction of your leg muscles. The force and torque measurements will be performed before and after the acute exercise bout. The acute exercise bout, including all of the tests will take approximately 1.5 hours.

Training Questionnaires - You will be asked to complete a 15-question survey prior to each training session. Questions include measures of readiness to train, eagerness to train, and feelings of soreness. The survey should take approximately 5 minutes to complete.

Muscle Biopsies – You have been informed that one of the purposes of this study is to measure the cellular markers of muscle growth from the muscle samples that we collect. By obtaining a small sample of your muscle tissue (size of a pencil eraser or small finger nail), the different types of proteins in your muscle may be determined which will be helpful in the evaluation of health and exercise performance. All muscle tissue samples (biopsies) will be taken from the outer portion of the front of the thigh using a needle biopsy technique by Andy Fry, PhD. Andy Fry, PhD, Professor of HSES, has performed over 100 muscle biopsies over the past year and has assisted on over 500 biopsies over the past six years on various populations (athletes, sedentary people, elderly etc.) with no significant complications and nothing more than minimal adverse reactions. The procedure is being overseen by Dr. Jeffrey Burns M.D., who is a medical doctor in Neurology at the KU Medical Center in Kansas City, Kansas supervises the procedure, but will not be physically present for the biopsies. The total size of the muscle biopsy will be approximately the size of a pea. You will be placed on an examination table lying down on your back (supine) so that the muscles of the leg are relaxed. The skin will be thoroughly cleaned with

antiseptic solution (Betadine) using sterile cotton swabs after which a surgical cover will be placed around the sampling site. If you are allergic to Betadine, an alternative antiseptic solution will be used to clean the skin. A small amount (2ml or 2cc) of a local aesthetic (2% Lidocaine) will be injected into the tissue under the skin around the site to be sampled. During this injection you may feel a slight burning sensation. If you are allergic to the local anesthetic, or have had allergic reactions to other anesthetics (i.e.: Novocain) then you will be disqualified from the study. Following the injection of the numbing agent into your thigh, a minimum of five (5) minutes will be allowed to pass to ensure adequate time for the agent to take effect in the area where the incision will be made. A small incision (1 cm) will then be made in the skin overlying the muscle and the biopsy needle inserted into the middle of the muscle (muscle belly) at a depth of 3 cm (about 1 inch). During the time that the sample is being taken (about 5 seconds) you may feel some deep pressure and cramping that will be moderately painful. You have been informed that if you have been previously diagnosed as having a bleeding disorder, a blood clotting problem, take blood thinning medication or have sensitivity to scarring, you will tell the researcher and not participate in the biopsy procedure.

Sterile disposable instruments and sterile gloves will be used for the preparation of the site and the reusable biopsy needle will be thoroughly sterilized (via steam and heat autoclave) after each biopsy. Approximately 100 mg (size of a pencil eraser or small finger nail) of skeletal muscle tissue will be removed. With the invasive skeletal muscle biopsy technique there is the possibility of a blood related infection (HIV, Hepatitis B), but the reusable needles will be cleaned and sterilized using an autoclave, a generally accepted method of cleaning surgical instruments.

A total of **four (4)** biopsies will be performed over the course of the study (see timeline, above).

Blood Draws – We will measure hormonal markers found in your blood prior to supplementation, after supplementation but prior to exercise, and at the end of exercise. Four blood draws will be performed during this study (see timeline, above). In this procedure, a small amount of blood will be taken from a forearm vein for each blood draw by a trained phlebotomist using an indwelling cannula. Approximately 10 cc of blood (~ 2 teaspoons) will be drawn for each time-point. The blood draw will take about 5 minutes.

Urine Collection – To analyze nocturnal urinary catecholamines, urine will be collected twice throughout the investigation. You will be provided with collection containers collect your urine immediately upon waking on the morning of the PRE and POST Acute Exercise Bout visits. If your acute bout is scheduled for the morning, you will bring the sample with you to the acute bout. If your acute bout is schedule for the afternoon, arrangements will be made to obtain your sample in the morning. The estimated time commitment is about two minutes per collection.

RISKS

Muscle biopsies – The use of local anesthetics will result in a slight burning sensation, lasting approximately 5 seconds. There is a risk of allergic reactions to the local injection (1 in 1 million). There is a small chance of bleeding from the biopsy site. The principle concern would

be prolonged bleeding which would produce a bruise in the area. This would extend the muscle soreness, but is adequately treated with rest, ice, a compression bandage, and keeping the leg elevated as much as possible. Nausea, dizziness, and fainting can occur (1 in 100) during the biopsy process. As a result, the subjects will be in a supine position during the biopsy procedure. There is a risk of infection (1 in 1000) and irritation associated with the biopsy procedure. The use of aseptic techniques, careful cleaning of the skin and keeping the area dry will minimize the risk of infection. There is a risk of bruising (1 in 100) from the biopsy procedure. This will be minimized by placing ice over the site following the procedure and by applying a compression bandage on the site for the 24 hours following the biopsy. In some instances (1 in 200), some motor nerves may be damaged which may cause local numbness. There is likely to be a small scar where the biopsy is performed. This scar usually dissipates over a period of 6-12 months at which time the scaring is very modest.

Blood Draws – The blood sample has a small risk of infection and bruising of the area. The needle stick for the blood draw will be mildly painful and only lasts a couple of seconds.

Exercise Testing and Training – During the strength testing sessions test there is a risk potential even though no health problems exist. The risk and discomforts that are associated with this type of test include muscle fatigue, lightheadedness, chest discomfort, and very rarely death. The potential for death during or immediately following the test (or any vigorous exercise) is approximately 0.5 per 10,000 tests, according to the American College of Sports Medicine. Following these tests however you will experience muscle soreness due to the eccentric component of the resistance exercise. This soreness generally referred to as delayed on-set muscle soreness, as it is most likely to occur in the days following the exercise bout. All exercise training will be prescribed relative to each subject and the principles of proper exercise progression and overload will be applied so as to reduce the risk of injury to the subject. Laboratory personnel will stay in contact with you after a testing/training session to ensure that you are comfortable. You will be given a 24-hour contact number for the Applied Physiology Laboratory personnel to convey any type of unusual discomfort.

Overtraining – Overtraining is associated with muscle soreness, fatigue and training specific performance decreases. Soreness and fatigue subside quickly after the training period has ended. Performance decreases in maximal strength may take several weeks to recover. Due to the strenuous and fatiguing nature of the overtraining protocol, there is a slightly increased risk of musculoskeletal injury when compared to normal resistance training.

FOLLOW UP CARE

Following the procedure you will be provided with a biopsy care sheet, extra bandages and contact information for Andy Fry, Ph.D. After 24-hrs you must report back to the testing coordinator to check the wound. At this time the bandage will be removed and properly disposed of and a new sterile dressing placed over the wound. You will again be asked to report to the test coordinator after 3-days and be contacted via phone one week after the biopsy or whenever necessary to ensure normal recovery. The biopsy procedure often results in a small fine scar at the site of the incision, however, all care post-treatment will aid in reducing the potential for scarring. All care will be taken to aid in the healing of the wound. The entire biopsy procedure

will be performed under sterile conditions. All testing staff and associated personnel will be trained in first aid and will be familiar with emergency procedures.

There have been no other major complications reported in the scientific literature as a result of taking small tissue samples from the skeletal muscle using the percutaneous needle biopsy technique described above. This procedure has been performed on numerous subjects by qualified personnel in many institutions worldwide with only slight discomfort being reported. During the muscle biopsy it is common to feel a strong cramping sensation in the muscle while the biopsy is being performed. However, muscle function is not impaired. In fact, subjects have been reported to continue participation in sporting events immediately following a muscle biopsy. It is common for subjects to experience mild soreness, moderate pain and bruising near the biopsy site, similar to a "Charlie-horse" the day after the procedure. In order to allow the incisions to heal properly and minimize the risk of infection, you should not get the biopsy site wet for 24-hours and avoid prolonged exposure to water for 4-days. Daily showers are acceptable (after the first 24-hours), but baths, swimming, sauna's etc. should be avoided for 4-days following the biopsy procedure.

BENEFITS

If interested, you will be provided with all of your personal results, including your muscle fiber type composition.

The results of this investigation will promote a greater understanding of the mechanism through which overtraining attenuates performance. As such, findings from this investigation could influence resistance training program design to help prevent overtraining. Additionally, these findings may provide the basis for improved markers for the detection of overtraining and treatments for overtraining symptoms.

PAYMENT TO PARTICIPANTS

If you complete all phases of the study, you will be compensated in the form of cash payment of $200. Payment will be prorated as follows: $50 for completing the first acute exercise bout only, $100 for completing the first acute exercise bout and the 8 day training period, and $200 for completing the entire investigation. If you withdraw from the study, you will be allowed access to the results of your respective data. Investigators may ask for your social security number in order to comply with federal and state tax and accounting regulations.

PARTICIPANT CONFIDENTIALITY

Your name will not be associated in any publication or presentation with the information collected about you or with the research findings from this study. Instead, the researcher(s) will use subject numbers. Your identifiable information will not be shared unless (a) it is required by law or university policy, or (b) you give written permission. Permission granted on this date to use and disclose your information remains in effect indefinitely. By signing this form you give permission for the use and disclosure of your information for purposes of this study at any time in the future."

INSTITUTIONAL DISCLAIMER STATEMENT

In the event of injury, the Kansas Tort Claims Act provides for compensation if it can be demonstrated that the injury was caused by the negligent or wrongful act or omission of a state employee acting within the scope of his/her employment.

REFUSAL TO SIGN CONSENT AND AUTHORIZATION

You are not required to sign this Consent and Authorization form and you may refuse to do so without affecting your right to any services you are receiving or may receive from the University of Kansas or to participate in any programs or events of the University of Kansas. However, if you refuse to sign, you cannot participate in this study.

CANCELLING THIS CONSENT AND AUTHORIZATION

You may withdraw your consent to participate in this study at any time. You also have the right to cancel your permission to use and disclose further information collected about you, in writing, at any time, by sending your written request to: Andrew Fry, Ph.D., University of Kansas, 1301 Sunnyside Avenue, Robinson Center Room 101, Lawrence, Kansas 66045. If you cancel permission to use your information, the researchers will stop collecting additional information about you. However, the research team may use and disclose information that was gathered before they received your cancellation, as described above.

QUESTIONS ABOUT PARTICIPATION

Questions about procedures should be directed to the researcher(s) listed at the end of this consent form.

PARTICIPANT CERTIFICATION:

I have read this Consent and Authorization form. I have had the opportunity to ask, and I have received answers to, any questions I had regarding the study. I understand that if I have any additional questions about my rights as a research participant, I may call (785) 864-7429 or (785) 864-7385, write the Human Subjects Committee Lawrence Campus (HSCL), University of Kansas, 2385 Irving Hill Road, Lawrence, Kansas 66045-7568, or email irb@ku.edu.

I agree to take part in this study as a research participant. By my signature I affirm that I am at least 18 years old and that I have received a copy of this Consent and Authorization form.

_________________________ _______________
Type/Print Participant's Name Date

____

Participant's Signature

Researcher Contact Information

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APPENDIX L

PRE-EXERCISE TESTING HEALTH & EXERCISE STATUS QUESTIONNAIRE

Name ______________________________ Date ____________

Home Address ______________________________________

Phone Number ____________________ Email ____________________

Person to contact in case of emergency ______________________________

Emergency Contact Phone ____________________

Personal Physician ____________________ Physician's Phone ____________

Gender ________ Age ______(yrs) Height ______(ft)______(in) Weight______(lbs)

Does the above weight indicate: a gain____ a loss____ no change____ in the past year?
If a change, how many pounds?__________(lbs)

A. JOINT-MUSCLE STATUS (✓Check areas where you currently have problems)

Joint Areas
( ) Wrists
( ) Elbows
( ) Shoulders
( ) Upper Spine & Neck
( ) Lower Spine
( ) Hips
( ) Knees
( ) Ankles
( ) Feet
( ) Other____________________

Muscle Areas
( ) Arms
( ) Shoulders
( ) Chest
( ) Upper Back & Neck
( ) Abdominal Regions
( ) Lower Back
( ) Buttocks
( ) Thighs
( ) Lower Leg
( ) Feet
( ) Other____________________

B. HEALTH STATUS (✓Check if you currently have any of the following conditions)

( ) High Blood Pressure
( ) Heart Disease or Dysfunction
( ) Peripheral Circulatory Disorder
( ) Lung Disease or Dysfunction
( ) Arthritis or Gout
( ) Edema
( ) Epilepsy
( ) Multiply Sclerosis
( ) High Blood Cholesterol or Triglyceride Levels
( ) Allergic reactions to rubbing alcohol

( ) Acute Infection
( ) Diabetes or Blood Sugar Level Abnormality
( ) Anemia
( ) Hernias
( ) Thyroid Dysfunction
( ) Pancreas Dysfunction
( ) Liver Dysfunction
( ) Kidney Dysfunction
( ) Phenylketonuria (PKU)
( ) Loss of Consciousness

* *NOTE: If any of these conditions are checked, then a physician's health clearance will required.*

C. PHYSICAL EXAMINATION HISTORY

Approximate date of your last physical examination______________________________

Physical problems noted at that time____

Has a physician ever made any recommendations relative to limiting your level of physical exertion? _________YES __________NO

If YES, what limitations were recommended?___________________________________

____

D. FEMALE REPRODUCTIVE HISTORY

If you are male, skip to Section E.

Did you begin menses within the past year? _________YES _________NO

Have you had consistent menstrual periods for the last 3 months?

YES_________ NO_________

Date of onset of last menstrual period____

Have you used a hormonal contraceptive within the last 3 months?

YES_________ NO_________

E. CURRENT MEDICATION USAGE (List the drug name, the condition being managed, the length of time used)

MEDICATION	CONDITION	LENGTH OF USAGE
_____________________	______________________________	_________________
_____________________	______________________________	_________________

F. PHYSICAL PERCEPTIONS (Indicate any unusual sensations or perceptions. ✓Check have recently experienced any of the following during or soon after *physical activity* (PA) during *sedentary periods* (SED))

PA	SED		PA	SED	
( )	( )	Chest Pain	( )	( )	Nausea
( )	( )	Heart Palpitations	( )	( )	Light Headedness
( )	( )	Unusually Rapid Breathing	( )	( )	Loss of Consciousness
( )	( )	Overheating	( )	( )	Loss of Balance
( )	( )	Muscle Cramping	( )	( )	Loss of Coordination
( )	( )	Muscle Pain	( )	( )	Extreme Weakness
( )	( )	Joint Pain	( )	( )	Numbness
( )	( )	Other______________________	( )	( )	Mental Confusion

G. FAMILY HISTORY (✓Check if any of your blood relatives . . . parents, brothers, sisters aunts, uncles, and/or grandparents . . . have or had any of the following)

( ) Heart Disease

( ) Heart Attacks or Strokes (prior to age 50)

( ) Elevated Blood Cholesterol or Triglyceride Levels

( ) High Blood Pressure

( ) Diabetes

( ) Sudden Death (other than accidental)

H. EXERCISE STATUS

Do you regularly engage in aerobic forms of exercise (i.e., jogging, cycling, walking, etc.)? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

What is your fastest 5 km time? ______________

What is your fasted 10 km time? _____________

What is your fasted mile time? _______________

What is your fasted times at other distances not listed? ___________________________________

Do you regularly lift weights? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

What is your back squat 1 repetition maximum (RM)? _____________

What is your deadlift 1 RM? ____________

What is your power clean 1 RM? ____________

What are your other 1 RMs that are not listed? ____

Do you regularly play recreational sports (i.e., basketball, racquetball, volleyball, etc.)? YES

How long have you engaged in this form of exercise? ______ years ______ months

How many hours per week do you spend for this type of exercise? _______ hours

APPENDIX M

SUBJECTS WANTED!!

RESISTANCE TRAINING STUDY

Eligibility: Healthy, resistance trained males between the ages of 18-30. To qualify as resistance trained, you must be capable of performing a 1.5x body weight back squat and a 1x body weight bench press. Additionally, you must have at least two years of resistance training experience, and have been actively training for at least six months prior to the onset of the investigation.

The University of Kansas is an equal opportunity institution.

If you are eligible and interested, please contact:

Adam Sterczala, MS
Andrew C. Fry, PhD
101 Robinson Center
KUOvertraining@gmail.com

Subjects will perform 3 – 16 training bouts of approximately 45 minutes over an 8-day period. The estimated time commitment outside of training is approximately 8 hours. Training and testing will be conducted in the Robinson Fitness Center (Robinson 102/103) and the Applied Physiology Laboratories (Robinson 101 and 207).

Benefits: Subjects will be compensated for their time.

Purpose: To determine if changes in stress hormone and skeletal muscle protein levels are the cause of overtraining induced performance decreases.

APPENDIX N

Subject:__________ Session:________ AM or PM Date:___________

Fill out this questionnaire prior to your workout. Circle the number corresponding to

"HOW DID YOU FEEL TODAY?" (Questions 1-6)

1. How anxious did you feel?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

2. How sad or depressed did you feel?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

3. How confused did you feel?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

4. How angry did you feel?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

5. How energetic did you feel?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

6. How fatigued did you feel?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

"HOW DO YOU FEEL NOW" (Questions 7-12)

7. Are you looking forward to this workout?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

8. Do you feel strong?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

9. Do you feel recovered from your last workout?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

10. Are your leg muscle sore?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

11. Do your knees hurt?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

12. Is your lower back sore?

0--------------------1--------------------2--------------------3--------------------4
Not at all A Little Moderately Quite A Bit Extremely

COMMENTS:____