<u>Thesis Proposal</u>

Thesis Title: **An Acute Bout of Concurrent Exercise on Mechanotransducing Properties of the Skeletal Muscle Fiber**

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An Acute Bout of Concurrent Exercise on

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Abstract

Aim It was the aim of this study to extrapolate the affect which an acute bout of concurrent training, aerobic exercise coupled with resistance exercise, as compared to resistance exercise alone, has on the mechanically sensitive signaling proteins of the muscle fiber and their downstream signaling proteins as to give insight into the intracellular metabolic milieu. **Method** Eleven healthy males performed unilateral tricep extension exercise with both arms. One randomly designated arm took part in the concurrent training bout comprising an acute aerobic intervention shortly followed by resistance exercise. Biopsies were obtained from the M. Triceps Brachii at three time-points; Pre-RE, Post+15 RE, and post+3-hr RE. Tissue was then analyzed for mechanically sensitive proteins A7B1 Integrin, Yes-Associated Protein, Focal Adhesion Kinase, and downstream signaling proteins AMP-Kinase, p70s6k, and 4ebp1. **Results** AMPK showed a significant arm X time interaction ($p < 0.05$). P70s6k showed no condition X time interaction. 4E-BP-1 showed a significant condition X time interaction ($p < 0.05$). Beta-1 Integrin showed a main effect of time increasing from time-point 1 to time-point 3 across both arms. FAK showed a main effect of time decreasing from time-point 1 to time-point 3 across both arms. YAP showed no condition X time interaction. **Conclusions** Here we demonstrate the ability of the triceps brachii to respond in a similar manner between concurrent exercise and resistance-only exercise concerning the skeletal muscle specific integrin isoform, alpha7 beta1. Moreover, we demonstrate the large decrease of 4E-BP1 and increase of AMPK immediately following the AE protocol along with possible augmentation of FAK through AMPK inhibitory mechanisms. This denotes the possibility that, as compared to past studies, the triceps brachii uniquely responds and regulates intracellular remodeling mechanisms following an acute bout of concurrent exercise as compared to the lower body.

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LIST OF ABBREVIATIONS

YAP = Yes-Associated Protein

FAK = Focal Adhesion Kinase

mTORC1 = Mechanistic Target of Rapamycin Complex One

mTORC2 = Mechanistic Target of Rapamycin Complex Two

PI3K = Phosphoinositide 3-Kinase

IGF-1 = Insulin-Like Growth Factor One

IGFR = Insulin-Like Growth Factor Receptor

IRS1 = Insulin Receptor Substrate One

TSC1/2 = Tuberous Sclerosis Complex

S6K1 = Ribosomal S6 Kinase

UBF = Upstream Binding Factor

ERK = Extracellular Signal-Regulated Kinase

MTJ = Myotendinous Tendon Junction

NMJ = Neuromuscular Junction

TEAD = TEA Domain

Rheb = Rhas Homolog Enriched in Brain

AMPK = AMP-activated serine/threonine protein kinase

ILK = Integrin Linked Kinase

PA = Phosphatidic Acid

PLD = Phospholipase D

SAC = Stretch-Activated Channels

DGC = Dystrophin-Glycoprotein Complex

Chapter 1: Introduction

Mechanical stimuli exerted by, and upon, the individual skeletal muscle fiber serve as a unique medium to alter the intracellular milieu consequently influencing protein synthesis. The intrinsic process constituting a mechanical stimulus being transduced into a biochemical response is termed mechanotransduction. Upon the seminal work of Goldberg with his observation of hypophysectomized mice reaching the same level of muscle mass relative to their wild type counterparts (1), it soon thereafter began to be questioned as to whether the increase in muscle size and protein synthesis was an intrinsic process rather than reliance upon circulating growth compounds. This was the necessary revolutionizing force behind the paradigmatic shift concerning the unique role with which mechanical stimuli may play on the intracellular protein synthetic machinery.

The regulation of skeletal muscle mass is directly influenced by the balance between protein synthesis and protein degradation (2). For example, when the synthesis:degradation ratio is in favor of synthesis, muscle growth occurs. However, when the ratio is in favor of degradation, a decrease in muscle mass takes place and atrophy occurs. Concerning the intracellular anabolic environment, it has become readily apparent that a signaling protein playing a significant role in transducing external environmental cues into an increase in downstream signaling proteins and subsequent protein synthesis is present; this protein is called the mechanistic target of rapamycin (mTOR) (3). The mTOR complex of proteins is found in two distinct states within the skeletal muscle fiber; the rapamycin-sensitive complex 1 (mTORC1) and the rapamycin-insensitive complex 2 (mTORC2) (4). Multiple studies have found mTORC1 to be the key complex which regulates cell growth and will thus be the complex focused on throughout this paper.

There are a multitude of mechanically-sensitive upstream effectors which are able to respond to the external mechanical stimuli and transduce it into a biochemical response subsequently increasing mTORC1 activity. Of specific interest to this paper is the muscle-specific transmembrane protein alpha7 Beta1 Integrin, Focal Adhesion Kinase, and Yes-Associated Protein. These will be reviewed below.

Integrin Signaling

Integrins are a family of heterodimeric cell surface transmembrane proteins consisting of an alpha and beta subunit. Currently, 18 alpha and 8 beta subunits have been identified totaling 24 specific integrin types dependent upon cellular location and function (5). These integrins span the cellular membrane binding to, in the skeletal muscles case, laminin protein in the extracellular matrix and, indirectly through a complex of proteins found within the costamere, the actin-cytoskeleton (6). Thus, integrins structurally connect the extracellular environment to the intracellular cytoskeleton. This proves important as a myriad of protein synthetic machinery have been found to be immobilized upon the cytoskeleton of which may become active following mechanical perturbation (7).

Specifically concerning the skeletal muscle fiber, the primary integrin-type is the alpha7 beta1 integrin. These integrins can be found at the myotendinous junction (MTJ), neuromuscular junction (NMJ), and the costamere (6). The integrin can be activated in both an inside-out and outside-in manner (8). Specifically, in a rested state, the extracellular head of the integrin is in a bent, low-affinity, conformational state. Upon mechanical activity, the signaling protein Talin is recruited to the beta subunit of the cytoplasmic region of the integrin (9). This event allows for the two integrin subunits to separate resulting in a high-affinity conformational change of the extracellular head of the integrin as to allow for its interaction with its specific extracellular

ligand. Upon this change in structure of the integrin, a cascade of regulatory proteins, both structural and signaling, are recruited to the integrins cytoplasmic tail. This is paramount due to the integrin lacking intrinsic kinase capabilities. One of the key signaling proteins which becomes active following integrin stimulation is the focal adhesion kinase (FAK).

FAK is a mechanically sensitive cytoplasmic protein tyrosine kinase with a predominant resting location on the myosin protein within the sarcomere (10). Upon muscle activity, it has been demonstrated that FAK disperses to a myriad of sites such as the nucleus, Z-line, and the costamere. We will specifically consider FAK's role at the costamere. Upon FAK's costomeric translocation, it binds to the tail of the beta integrin through its N-terminal FERM domain (10). This allows for FAK phosphorylation and subsequent ability to act as a signaling protein. Concerning its impact on the overall hypertrophic effect, FAK has been demonstrated to indirectly increase mTORC1 activity through two unique events; first, through the increase in the PI3K/AKT signaling pathway of which the end result, AKT, has been demonstrated to directly increase mTORC1 activity by-way of raptor phosphorylation increasing mTORC1 activity (11), along with FAK phosphorylating the mTORC1-inhibitor TSC2 (12). Thus, upon integrin stimulation and downstream FAK activation, mTORC1 activity may increase as to induce protein synthesis. Moreover, FAK has been demonstrated to increase cytoskeletal actin polymerization as to increase cytoskeletal tension. This increased cytoskeletal tension has been shown to increase YAP activity (13). This signaling protein will be discussed below.

YAP Signaling

The Yes-Associated Protein (YAP) is a transcriptional co-activator which has been demonstrated to be regulated by a number of stimuli; however, most noticeably, the Hippo Pathway (14). In its active state, YAP is dephosphorylated, translocates from the cytoplasm, and localizes within the

nucleus binding TEAD (TEA Domain) transcription factors (15). Following the co-localization with TEAD, YAP has been shown to increase various ribosome biogenesis transcription factors such as UBF and c-Myc (16), both of which have been shown to take place in a rapamycin independent manner denoting an mTORC1-independent signaling cascade. McCarthy et al have demonstrated the necessity for adequate ribosome biogenesis and translation capacity to take place for robust and long-term hypertrophy (17). Thus, further analysis into YAP dynamics upon translational capacity may prove advantageous.

Theoretical Basis

The underlying theme of this study is to better understand the exercise-specific effects on skeletal muscle fiber mechanotransducing pathways following two divergent styles of exercise. Specifically, it is the authors goal to delineate between an aerobic stimulus alone with an aerobic stimulus coupled with a resistance stimulus as to determine the specific mechanotransducing pathways which the cell uses. This will give insight into proper exercise prescription to those populations which suffer from the inability to provide proper activity (elderly/bed-ridden/injured) to the muscle subsequently altering the mechanically sensitive proteins listed above leading to an atrophic environment.

Statement of the Problem

Delineating the signaling apparatus concerning the above-mentioned mechanically sensitive proteins has been scarce when considering the skeletal muscle fiber. Further, even more scarce is the use of in-vivo studies within human subjects. This scarcity coupled with rarely utilizing invasive investigations of the upper body musculature denotes this study as highly favorable as to have broad impact upon the exercising and sedentary community alike. Thus, the purpose of this study is to analyze the impact of aerobic-only training and concurrent training

(aerobic+resistance) on the mechanically-sensitive proteins alpha7 beta1 integrin, focal adhesion kinase, and the Yes-Associated Protein, along with their downstream signaling proteins.

Hypothesis and Specific Aims

Hypothesis

It is hypothesized that the concurrent training intervention will augment YAP and FAK, however, will have minimal impact on the alpha7 beta1 integrin. Further, the aerobic intervention will augment FAK but will have minimal impact on the alpha7 beta1 integrin and YAP.

Specific Aim 1:

Determine as to whether the concurrent training intervention uniquely alters the functional activity of the three specific proteins as compared to the aerobic-only intervention. Along with this, analyze key downstream signaling proteins which will further give insight into the intracellular anabolic, or lack-there-of, environment. These proteins will consist of mTOR, p70s6k, 4EBP1, and AMPK. These will all be analyzed from total to phosphorylated.

Rationale

Here we strive to give further insight into the intracellular mechanically-sensitive apparatus and, specifically, how its alteration in function can dictate downstream transcription and translation properties as to augment or attenuate protein synthesis. Integrins are at the bedrock of mechanotrasduction and have proved necessary for proper muscle formation. Further, downstream signaling proteins, such as FAK, which directly interact with the integrin, have been studied rigorously with the results demonstrating their necessity in functional tissue formation. Lastly, more and more bodies of literature are being published concerning the role of YAP in functional cell growth. The key area lacking in the majority of these studies is their role in

human skeletal muscle. Thus, this lack in study concerning the various mechanotransducing pathways within human skeletal muscle warrants further investigation and is what this project wishes to shed light on.

Chapter 2: Review of Literature

Regulation of Skeletal Muscle

The regulation of skeletal muscle is a dynamic, ever-changing, continuous flux of properties which both synthesize and degrade structural and functional proteins within the muscle fiber. It has often been propagated that the predominant source of increase in muscle mass is that of an increase in protein synthesis. However, it has been demonstrated that the continuous degradation is required for adequate protein synthesis. Thus, the overall increase in skeletal muscle mass should be seen as the harmonious integration of both catabolic and anabolic intracellular properties as to adequately meet the external demands placed upon the muscle.

There are a multitude of factors which play into the overall protein synthetic and hypertrophic effect; these being amino acid content, intracellular energy environment, circulating growth factors, and mechanical stimuli (4). While the initial factor acting upon the intracellular milieu may be different, the large majority of these pathways act through the protein synthesis 'hub' termed the mechanistic target of rapamycin complex one (mTORC1).

There are two functionally distinct mTOR complexes, mTORC1 and mTORC2, which have thus far been identified; both of which are comprised of similar structural and functional characteristics. Recently, however, it has been demonstrated that mTORC1 comprises a rapamycin-sensitive domain termed raptor, which mTORC2 does not possess, and has been demonstrated to be the key complex which increases important downstream proteins concerning translation machinery such as 4EBP1 and S6K1 (4).

As mentioned above, p70 S6Kinase1 (S6K1) and eIF4E Binding Protein (4EBP), are crucial signaling proteins concerning translation initiation. S6K1, upon direct phosphorylation from mTORC1, can phosphorylate eIF4B which is considered to be a positive regulator of the

5'- cap binding eIF4F complex (4). The large majority of proteins necessary for protein synthesis to take place consist of a 5' TOP complex mRNA sequences thus requiring S6K1 activity to allow this to take place. 4EBP also plays an important role in allowing protein synthesis. Specifically, 4EBP is inhibitory in nature concerning protein synthesis, through its ability to bind and sequester eIF4E attenuating eIF4E's ability to form the necessary eIF4F complex (4). Thus, through mTORC1 activation and subsequent 4EBP phosphorylation, 4EBP disassociates from eIF4E permitting efficient translation initiation to take place. This being so, an increase in mTORC1 activity is paramount for proper translation initiation and protein synthesis.

mTORC1 can also play other roles concerning protein synthesis such as its ability to act as a direct promotor of ribosome biogenesis through its nuclear translocation (18), its ability to augment mitochondrial biogenesis through increasing pgc1alpha transcription (18), altering the cellular framework through feedback increase of focal adhesion kinase (FAK) which effects cytoskeletal dynamics further influencing the cellular tension-state and subsequent intracellular and extracellular force transmission, among many others. This latter finding may prove important as a large number of the intracellular proteins, specifically those concerning the translation apparatus consisting of ribosomes and mRNA, are found to be immobilized upon the cytoskeletal struts (7).

The predominant upstream signaling cascade which has been studied in the greatest depth concerning the activity of mTORC1 is that of the IGF1-PI3K-AKT signaling axis. Briefly, circulating growth factors, such as insulin-like growth factor, bind to its receptor Insulin-like Growth Factor 1 (IGFR). Upon binding, intracellular Insulin Receptor Substrate 1 (IRS1) is activated. Through a number of steps, IRS1 can then activate PI3K which then increases activity of AKT. AKT has been demonstrated to increase mTORC1 activity both directly through

phosphorylation and inhibition of the mTORC1-inhibitory protein PRAS40 and indirectly through phosphorylation of TSC1/2. This latter step reduces TSC2's ability to keep rheb in a GDP-state (11). It has been shown that the activity state of rheb is a direct effector of mTORC1. Concerning the latter mechanism, AKT phosphorylates TSC1/2 subsequently leading to its translocation away from lysosome, allowing rheb to take on a GTP-state and subsequent increase in mTORC1 (4). A large number of mTORC1 and rheb subpopulations have been demonstrated to bind directly to the lysosome in the presence of sufficient intracellular amino acids (11). Thus, the lysosome appears to be an important signaling hub concerning the intracellular metabolic milieu.

With the above taken into consideration, a number of past investigators have demonstrated incongruences and the indispensability of the IGF-1-mediated, PI3K-inducing, and thus AKT activity on the muscle fiber hypertrophic effect following exercise. For example, Spangenburg (19) utilized a transgenic mouse model which possessed a dominant-negative IGF-1 receptor of the skeletal muscle as to inhibit its ability to bind IGF-1 and subsequently inhibit the IGF1-PI3k-AKT signaling axis. However, when the IGFR dominant-negative mutant mice population were exposed to mechanical stimulation through the implementation of a functional overload model, the skeletal muscle hypertrophy was identical to their wild-type counterparts of whom were also exposed to the functional overload. Thus, this study demonstrates, along with others (20,21), that the IGF1-PI3K-AKT axis is not required for skeletal muscle hypertrophy following mechanical overload.

This being so, it is widely accepted that mechanical tension is the key driver of the hypertrophic response (2); more specifically, mechanotransduction, the ability of the muscle fiber to transmit mechanical perturbation into a biochemical response. There are a multitude of

factors by which this mechanotransucing effect may increase protein synthesis. Presently, stretch activated channels, cell-membrane spanning proteins termed integrins, phosphatidic acid, and yes-associated proteins, are those which have garnered the most attention. Concerning this study, we will be investigating the alpha7 beta1 integrin, yes-associated protein, and focal adhesion kinase along with their downstream effectors as to determine their role in the intracellular protein synthesis environment following exercise.

Alpha7 Beta1 Integrin

The Alpha7 Beta1 Integrins are a family of transmembrane proteins physically connecting the intracellular environment, specifically the alpha-cytoskeleton through costameric or focal adhesion proteins, with the extracellular matrix, specifically the laminin 211. Integrins are formed from two distinct subunit types comprising alpha and beta subunits. Currently, 18 alpha subunits and 8 beta subunits have been identified totaling 28 unique integrin combinations dependent upon cell-type. Within the mature human skeletal muscle, the alpha7 beta1 integrin has been identified as the predominant integrin type (6).

The integrin comprises two distinct roles; that of structural and functional characteristics. Dr. Donald Ingber of Harvard University was the first to describe the structural characteristics of the integrin, regardless of cell type, and how their structural characteristics uniquely alter the intracellular environment through direct tension alteration and force transmission (7). Dr. Ingber, while a graduate student at Yale University, noticed that the cell structure acts in a similar manner as a tensegrity model. Tensegrity is a model which consists of discontinuous compression elements being upheld by a continuous web of tension. A model of the architectural structure can be found below in figure one.

This type of architecture can similarly be found within the muscle fiber. This can be seen through the binding of the transmembrane alpha7 beta1 integrin with the extracellular matrix. The cytoplasmic head of the integrin then binds indirectly to the alpha-cytoskeleton. The cytoskeleton can then be found to connect indirectly to the nuclear matrix and chromosome through other transmembrane proteins located at the nuclear membrane (23). Thus, there is a continuous structural link from the extracellular matrix to the nucleus by which force can be propagated. The cytoskeleton is the primary site of this tensegrity architecture, specifically byway of the cytoskeletal microfilaments, intermediate filaments, and microtubules. The microfilaments and intermediate filaments take on the role of continuous tension whereas the microtubules comprise the compression elements (24). The tensegrity model is based upon the idea of a continuous state of pre-stress. This being so, if there is any alteration or mechanical perturbation on one aspect of the muscle fiber, due to the continuity of the structural properties of the cell, all other proteins within this tensegrity architecture will also be affected. This is paramount for force to be transmitted properly throughout the muscle fiber along with it to be transmitted to the extracellular matrix and subsequently to the tendon to initiate movement. Concerning the integrin, this can specifically be found within the costamere.

Costamere's are rib-like structures found along the muscle fiber membrane which align in parallel with the z-disk of peripheral sarcomeres allowing for physical coupling of the myofibril to the sarcolemma (25). One of the key cytoskeletal proteins physically connecting the z-disk to the costameric-cytoskeleton is the protein desmin (32). While the costamere is formed by more than 100 proteins, one of the key and most important concerning mechanotransduction, is the alpha7 beta1 integrin. As has been previously touched upon, integrins contain a short cytoplasmic tail 20-70 amino acids in length and a large extracellular head consisting of several

hundred residues (26). In its inactive state, integrins rest with their extracellular head in an approximately 45-degree conformational state causing inactivity with its extracellular binding substrate. However, upon activation, the integrin goes through a conformational transformation causing its head to swing out and bind to its specific extracellular protein. The integrin quantity and activity-state is necessary for the transmission of force to be transmitted properly along with its impact on a number of signaling proteins altering the anabolic environment as has been demonstrated in a number of studies; most recently is that of Boppart's laboratory.

Boppart and colleagues conducted a study investigating the impact that an increased quantity of muscle-specific integrins have on efficient force transmission, muscle injury, and intracellular signaling cascades (27). This consisted of analyzing two distinct litters of mice, specifically, the wild type mouse population with physiologically normal levels of the integrin, and the transgenic mice overexpressing the alpha7BX2 gene which concomitantly induces increased levels of alpha7beta1 integrin. The exercise intervention for these mice consisted of downhill running on a 20% decline as to impose elevated eccentric muscle actions. This exercise intervention led to significant muscle fiber damage in the wild-type mice demonstrated by increased intracellular Evans blue-dye indicating muscle fiber membrane damage. However, the transgenic mice overexpressing the alpha7beta1 integrin found decreased level of Evans blue-dye. These findings demonstrate that increased integrin quantity resulted in greater force propagation efficiency and subsequent decreased membrane damage.

With the above taken into consideration, it was also demonstrated that the increased quantity of the alpha7 beta1 integrin of transgenic mice reduced the intracellular signaling cascade. This, however, should not come as a surprise. The supraphysiological increase in the integrin quantity will, as previously described, improve efficiency of force transmission

throughout the muscle fiber. This, then, would result in a reduced intracellular mechanical perturbation subsequently reducing the intracellular signaling cascade. It must be noted that mechanical perturbation is a stressor within the myofiber and, if this stressor is dampened through increased adaptations such as an increase in integrin signaling, the relative response to this stressor will also be dampened which should be viewed as a positive adaption.

Boppart further demonstrated the necessity of adequate integrin dynamics for efficient muscle fiber force transmission by introducing alpha7 null mice to acute and chronic exposure consisting of downhill treadmill running (28). The alpha7 null mice were observed to have high levels of damage preferentially localized at the myotendinous junction and the costamere. Further, in the wild type mouse population, the transcribing process of the alpha7 gene increased following exercise denoting the importance of increased expression of the alpha7 beta1 properties following a strenuous mechanical stimulus.

More recently, the work of Wang et al. further gives insight into the muscle damage-resisting effects of functional integrin activity (29). Wang's team required two distinct populations of mice to exercise on a treadmill with a 10% incline. The mouse population were divided into the wild type group and the Integrin-Linked Kinase (ILK) null mice. ILK is a direct binder to the beta1 tail of the integrin cytoplasmic tail and, upon stimulation, increases in phosphorylation and activates a number of downstream proteins. Within this study, ILK was demonstrated to be necessary for adequate integrin function. Following three weeks of the above-mentioned exercise intervention, the tissue was analyzed and the investigators found that significant damage of the skeletal muscle fiber was present through the observation of increased intracellular methylene blue staining. It was postulated that, due to the decrease in ILK quantity,

integrins were expressed randomly at the membrane resulting in inefficient muscle force transmission and stress-induced damages.

Thus, it is made readily apparent that the transmembrane protein alpha7 beta1 integrin is not only important but required for efficient myofiber force transmission. This increase in quantity and/or activity reduces membranous damage at both the sarcolemma and the site of the myotendinous junction. Further, the alpha7 beta1 integrin is required for the harmonious integration of force transmitted signals to be dissipated throughout the cytoskeletal web through its tensegrity communication characteristics. Along with these structural and force transmission properties, integrins have been demonstrated to not only indirectly influence but, amazingly, directly localize with growth factor receptors as to influence their biochemical signal transducing properties (30). This has been demonstrated to form a direct complex with the IGF-1R and Insulin Receptor Substrate-1 (IRS-1) properties; both of which are key activators of the PI3K-AKT-mTOR signaling cascade and thus gives insight into the unique aspect of integrin force propagation directly influencing growth factor receptors as a means to induce downstream anabolic signaling cascades in lieu of direct extracellular growth factor stimuli. This coordinated crosstalk between the structural characteristics of the muscle fiber and the circulating growth factor/hormonal muscle fiber influencers demonstrates the complex and deeply integrated extra- and intracellular environment of the myofiber.

Integrins also play a significant role in transducing extracellular mechanical signals into an intracellular biochemical response termed outside-in signaling. The integrin lacks intrinsic enzymatic properties and thus requires their binding to accessory proteins such as laminin or collagen that, upon direct or indirect localization with the integrin, can become phosphorylated and initiate a downstream signaling cascade. This takes place due to proteins containing a

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phospho-tyrosine-binding (PTB) property which then can recognize the NXXY motifs of the subunit-specific beta integrin tail (31). Of importance to this paper is the effect that this phosphorylation process has on the increase in protein synthesis. Thus, specifically, how it impacts the activity-state of FAK and mTORC1.

FAK is a key mechanically sensitive, 125 kilo dalton, cytoplasmic, non-receptor tyrosine kinase with predominant intracellular resting locations on the myosin protein within the sarcomere (33). Structurally, FAK consists of three major domains such as the N-Terminal 4.1 ezrin, radixin, moesin (FERM) domain, a catalytic tyrosine kinase domain, and a C-Terminal focal adhesion-targeting (FAT) domain, (33). Within skeletal muscle, FAK has been found to directly bind to the B1-integrin through its C-Terminal FAT domain within the muscle-like focal adhesions, the costamere. Due to FAKs resting location within the sarcomere and direct localization upon the myosin, its ability to rapidly respond to mechanical stimuli is made readily apparent.

Upon mechanical activity of the skeletal muscle, various sites on FAK can become phosphorylated, such as tyr397, 407, 576/577, 861, and 925 (34). While each of these unique phosphorylation sites are important and allow FAK to play diverse roles, this paper is specifically interested in the phosphorylation at residue tyrosine 397. The phosphorylation of this site, upon its translocation to the costamere and subsequent B1 integrin binding, causes a shift in FERM domain position leading to rapid auto-phosphorylation of this specific residue. This has been demonstrated to take place within the first thirty seconds of perturbation (35).

Following a complex signaling cascade, FAK can regulate the intracellular anabolic environment through direct and indirect modulators of the mTORC1 protein complex. This can

be seen through two distinct steps; namely, phosphorylation of TSC1/2 and phosphorylation of AKT. Each of these will be described in detail below.

TSC1/2 are a key set of proteins which negatively regulate cell growth through their relationship with the AMP kinase (AMPK) energy-sensing protein and thus is increased in activity-state during decreased intracellular energy levels and decreased during high energy levels. Specifically, in nutrient deprived states or alteration in ATP:AMP ratio as to favor AMP, AMPK is activated and directly phosphorylates TSC2. TSC2, when active, acts as a suppressor of rheb. Rheb can be in a GTP (active) or GDP (inactive) state. When TSC2 is activated, it sequesters rheb to remain in a GDP-bound state and subsequently does not permit mTORC1 activity (4). Thus, the increase in TSC2 activity indirectly decreases mTORC1 activity through impacting rheb's GDP/GTP state. It has been demonstrated that the C-terminal FAT domain of FAK is that which binds to TSC2 between residues 609-1080 allowing the direct interaction between FAK and TSC2 (12). However, it has also been demonstrated that FAK can phosphorylate TSC2 in an Src-independent manner subsequently giving rise to the possibility that FAK, upon mechanical perturbation and myosin-sarcomere migration, may impact TSC2 without previous translocation to the costamere and integrin binding; or, rather, that integrin-induced FAK phosphorylation and subsequent TSC2 inhibition occurs without further phosphorylation from Src. This TSC2 binding and phosphorylation has shown to augment S6K1 which is a key downstream mTORC1 signaling protein impacting protein synthesis through upregulation of translation-initiation (12).

Along with TSC2 phosphorylation, FAK has been demonstrated to positively impact the PI3K-AKT-mTOR pathway (36). This can be seen through, upon B1 integrin activation and FAK phosphorylation, FAK can bind with the SH2 domain of the p85 subunit of PI3K (37). This

can then activate the p110 catalytic subunit of PI3K allowing for increased AKT activity through phosphorylation of residue serine 473. Increases in AKT and PI3K were found to be attenuated when B1 integrin was inactivated along with FAK-null cells thus demonstrating a B1-integrin – FAK dependent increase in PI3K-AKT. This is important in that AKT impacts a myriad of signaling cascades in relation to protein synthesis. For example, similar to FAKs role in phosphorylating TSC2, AKT also phosphorylates TSC2 causing its translocation away from the lysosome which then allows rheb to take on its GTP activity and activate mTORC1.

With the above taken into consideration, the Hornberger lab, among others, have shown that through the induction of the PI3K-inhibiting factor Wortmanin, mechanically-induced mTORC1 remained elevated following mechanical perturbation (38). Moreover, Spagenburg et al, as previously described, used a IGF-1R dominant negative mouse model resulting in the inhibition of the IRS1-PI3K-AKT pathway. However, upon mechanical stimulation, mTORC1 was not altered. Thus, it would appear that while FAK contributes in the increase of PI3K, this is not required for the downstream anabolic effect of mechanical stimuli. This, however, may only be the case in the acute timeframe whereas, if investigated in a chronic manner, PI3K-AKT may be necessary for full hypertrophic adaptations.

While FAK has predominantly been described to reside at the costamere following mechanical activity, recent investigations have demonstrated FAK to translocate to the Z-Disc and to the nucleus (39). Specifically concerning the nucleus, FAK has been demonstrated to act as an epigenetic factor through structural chromatin remodeling along with acting as a transcription factor to the muscle regulatory gene myogenin (40). This was found to be the case through the introduction of a hypoxic stimulus consequently causing FAK to interact with MBD2 reducing its interaction with HDAC1 and methyl-CpG binding sites of the myogenin

promotor. DNA methylation has been widely demonstrated to reduce gene expression, thus, the inhibition of methyl binding is paramount for enhanced protein synthesis. However, as previously mentioned, this was seen in a hypoxic state which may not give insight as to whether mechanical stimuli can cause this nuclear translocation. AMPK, too, has demonstrated to alter HDAC activity through the HDAC nuclear exclusion. Thus, the energy status of the cell may be a required stimulus as to influence intranuclear HDAC activity and subsequent protein synthetic response following training.

However, it must be understood that, while FAK plays a central role within the mechanotransducing complex, it can also respond to a myriad of circulating growth factors such as Insulin and IGF-1. FAK has been demonstrated to be required for the downstream anabolic signaling cascade following the increase in IGF-1 and/or Insulin in an FAK-dependent manner (41). While this may be the case, as previously mentioned, the functional characteristics of the integrin may be the sole influencer of circulating growth factors due to the physical relationship it has with the IGF-1/Insulin receptor.

In conclusion, FAK is an incredibly dynamic protein which, upon mechanical stimulation, translocates to multiple sites within the muscle fiber and attenuates catabolic signaling processes through an increase in PI3K activity, inhibition of TSC2, and upregulation of myogenin.

YAP

The Yes-Associated Protein (YAP) is a transcriptional co-activator with its role consisting of the regulation of cell growth and size (42). It can take on this role through nuclear translocation and binding to transcription factors with the TEA Domain (TEAD) 1-4 transcription factors having been demonstrated as the important and necessary concerning cellular growth. TEAD has been

demonstrated to be necessary for YAP transcriptional activity due to YAP not having DNA-binding activity (43). Thus, YAP works with and through TEAD. YAP is able to shuttle back and forth from the nucleus to the cytoplasm dependent upon phospho- or de-phospho status. If phosphorylated, YAP remains in the cytoplasm in an inactive state. If not phosphorylated, YAP translocates to the nucleus and, as previously mentioned, impacts gene expression through the TEAD transcription factors.

The tumor suppressor pathway, the Hippo Pathway, is the predominant pathway which regulates YAP activity. As the above description alludes, the hippo pathway is antithetic toward cellular growth through a myriad of intracellular stimuli. Energy status, nutrition status, and mechanical stimuli can differentially alter hippo signaling. In intracellular states of low energy, specific signaling proteins within the hippo pathway such as NF2, the adaptor protein Salvador, and kinases MST1/2, and LATS1/2. LATS1/2 is the end stage of the pathway which, when active, phosphorylates YAP at residue S127 (43). The suppressive nature of the hippo pathway, and subsequent anabolic nature of YAP, can be seen through a number of knock-out and knock-in studies. For example, a recent study (44) demonstrated that mutant YAP lacking S127 causes nuclear YAP location and a significant increase in cellular size. Moreover, the utilization of a mouse model consisting of a knock out LATS protein, caused a significant increase in nuclear YAP location and subsequent increase in cellular growth. Moreover, the TEAD transcription factors have been demonstrated as being required for functional regeneration following muscle damage (45). For example, upon TEAD 1 and 4 silencing, various gene sequences were reduced in activity such as a number of structural protein genes, signaling protein genes, and genes comprising the hippo pathway.

While YAP has widely been demonstrated as an oncogene in a number of specific tissue-types, its role in the regulation of skeletal muscle fiber size has yet to be analyzed in-depth. Watt et al (46) showed that there was a positive regulation of muscle size through nuclear YAP location upon the utilization of short hairpin-RNA (shRNA) targeting YAP which led to the overall reduction in YAP activity. This then reduced YAP-mediating genes and subsequent decrease in overall muscle fiber size. Interestingly, this decrease in protein synthesis of the shRNA YAP was not due to a decrease in mTORC1. Thus, the YAP-TEAD complex appears to be a unique signaling mechanism of which does not necessitate interaction with the typical anabolic mTORC1.

In contrast to that mentioned above, YAP also appears to increase in overall activity through mechanisms independent of the hippo signaling pathway. Upon muscle activity and an increase in tension transmitted across the costamere and through the integrin, force is transmitted and tension is increased within the cytoskeletal environment of the individual muscle fiber. This has then been demonstrated to increase the activity of RhoA-GTPases leading to a decrease in YAP serine 127 phosphorylation and subsequent nuclear translocation. Moreover, substrate rigidity has also demonstrated to be important in YAP activity. The Piccolo Lab has given significant insight through plating cells on different substrate rigidity environments which, when placed on a soft matrix, YAP takes on a cytoplasmic position whereas when plated on a stiff matrix, YAP takes on a nuclear position. This is interesting in relation to muscle fiber as the extracellular matrix, following exercise, is in a constant protein flux with enhanced MMP-2 activity and ECM reorganization and subsequent alteration of relative stiffness wherein the muscle cell resides which would lead one to conclude that impact of which the ECM has upon YAP dynamics.

These findings prove very important in that YAP is tightly regulated by the energy-sensing and growth-controlling hippo signaling pathway, but also appears to work in an intrinsic fashion consisting of direct communication through the tension-state of the intracellular environment. This latter statement sheds light upon the direct modulation of mechanically sensitive properties due to the tensegrity model. Thus, the continuous flux of ECM properties and cytoskeleton lead to continuous alteration in tensegrity characteristics of which YAP is sensitive to leading to nuclear translocation and increased gene expression.

YAP may also play a role in the overall increase in protein synthesis through the increase in ribosome content, termed ribosome biogenesis, and subsequently an increase in the capacity of protein synthesis termed translational capacity. McCarthy (47) has demonstrated that, while the initial increase in protein synthesis maybe be driven by an increase in translation initiation and capacity in an mTORC1 dependent manner, long term hypertrophy is potentially driven in an mTORC1-independent and YAP-dependent manner (42). This can be seen from a recent study which, upon electrical stimulation and rapamycin injection, ribosome biogenesis transcription factors c-Myc and UBF were not altered. Both of these transcription factors have been found to be increased in direct relation with YAP (42). Thus, YAP may prove important concerning the temporal aspect of protein synthesis when hypertrophy is observed chronically.

Interestingly, Nader recently investigated the relative external load alterations and ribosome biogenesis. Within this model, Nader incorporated a partial synergist ablation model where greater and greater amounts of muscle were amputated as to gradually increase the external load upon the plantaris. As the overall mechanical load upon the plantaris increased and a concomitant increase in muscle mass was observed, so did that of a key mTORC1 signaling protein, p70s6k. However, this increase in p70s6k was only for the first 20% increase in muscle

mass. Following that, p70s6k levels plateaued. It is speculated that this may be the case in that p70s6k is a key protein responsible for translation-initiation and, as previously mentioned, a long-term increase in translation-capacity is required. Thus, YAP may be a key component in long-term increase in muscle mass due to its positive regulation of ribosome biogenesis factors.

Lastly, YAP has been observed to coordinate various aspects of the SMAD signaling complex (42). Specifically, SMAD2/3 have been shown to localize and increase in activity following myostatin signaling through the transforming growth factor receptor-1 (TGF1). SMAD 4 then translocates and localizes with SMAD2/3, forming a SMAD2/3-4 complex, which is then shuttled to the nucleus to take on its role as a transcription factor with specific atrogenes such as MuRF1 and Atrogin-1. Another subfamily of SMAD proteins exist, SMAD 6/7, which act as an inhibitory property concerning the SMAD2/3-4 complex. This has been demonstrated to take place through SMAD6/7 recruitment to the TGF1 receptor attenuating its ability to activate the SMAD2/3 complex following myostatin-TGF-1 binding. Strictly concerning the YAP, Ferrigno et al showed that an increase in intracellular YAP lead to the recruitment of SMAD 7 to the TGF1 receptor thereby rendering its proteolytic capabilities inactive. Having said this, it has also been widely demonstrated that YAP can bind to SMAD 2/3 negating its nuclear translocation capabilities and subsequently attenuating its transcription activity. Moreover, with either an increase of LATS1/2 or MST1/2, both key signalers within the hippo signaling pathway which, as previously mentioned, negatively regulate YAP, it was demonstrated that their increase and YAP decrease resulted in the attenuated of SMAD2/3 complex formation.

With the latter information from above being taken into consideration, it may then be postulated that the increased activity of YAP is necessary for the SMAD2/3-4 nuclear

translocation to take place which would appear to contrast that of the above-mentioned YAP-SMAD 7 inhibitory influence upon proteolytic SMAD2/3-4 function. However, TGFB1 signaling has been shown to be a crucial cytokine in relation to the enhancement of ECM properties. This may give insight as to the role with which YAP plays in the enhancement of TGFB1 downstream components. Due to YAPs high sensitivity in relation to cellular tension, the increase of the ECM or cytoskeletal stress-state leads to the activation of this specific cytokine subsequently enhancing YAP activity. Thus, it may be that YAP is influenced and impacted through a multitude of stimuli all of which uniquely impact YAP's function (48). It is clear that greater exploratory endeavors need to take place as to shed light upon the specific function of YAP properties.

In conclusion, the mechanically sensitive properties of the musculoskeletal system are a complex, integrated, ever-evolving field of study which proves promising concerning a number of disease-states. However, the harmonious integration of all external signals, both biophysical and biochemical, resulting into a coordinated response requires further intensive investigation. FAK, YAP, and the alpha7beta1 Integrin appear to be key plays within the field of mechanobiology.

Chapter 3: Methods

STUDY DESIGN

Eleven men performed unilateral consecutive bouts of arm extensor aerobic exercise (AE; ~40 min) and resistance exercise (RE; 4 sets of 7 reps) interspersed by 15 min recovery. Test subjects served as their own control. Arms, within each subject, were randomized concerning the arm which took part in the concurrent training intervention and which arm did the resistance-only intervention. Muscle biopsies were obtained from the m. triceps brachii of each arm immediately before, 15 min, and 3 h following the RE bout. Peak concentric power output was recorded for the RE intervention while Newton Meters were quantified for the aerobic exercise. Muscle samples were assessed for gene expression and protein phosphorylation of markers orchestrating the response to mechanical stress and muscle adaptations to AE and/or RE.

SUBJECTS

Eleven men (181 cm +/- 6cm, 81 +/- 8kg, 28 +/-5years), determined as recreationally active through the criteria of taking part in 2-3 days/wk of moderate RE training, volunteered to take part in the study. The study experiments and procedures were explained prior to subjects giving their written informed consent. Approval was granted through the Regional Ethical Review Board in Stockholm and the Institutional Review Board of the University of Kansas.

EXERCISE EQUIPMENT

Aerobic exercise was performed using an isokinetic dynamometer (Biodex) offering isotonic resistance. Subjects performed repeated arm extensions in an upright seated position with their upper arm strapped comfortably to a resting pad (Fig 1A). The concentric portion of the movement was assisted by a researcher as to minimize fatigue of the elbow flexors. The resistance exercise equipment consisted of flywheel ergometer contraption (Exxentric™, Sweden) with a 0.025 kg/m2 wheel to offer resistance (Fig 1B). The device was attached upside

down on the wall and the subject, standing underneath, performed tricep "pushdown" concentric-eccentric maximal muscle actions. Subjects were verbally encouraged to provide full effort while form/body posture were monitored by two researchers throughout the study. Concentric peak power was calculated using an optical sensor (Exxentric™).

Figure 1A – B (working left to right): Isokinetic Dynamometer (AE) and the Exxentric Flywheel (RE)

PRE-TESTING & FAMILIARIZATION

Subjects attended two familiarization sessions. In the first session, the subject completed a health and activity questionnaire. The study was subsequently described verbally by the researcher. If the subject was deemed eligible for participation and so chooses to sign the informed consent, he was allowed to be familiarized with the exercise protocol and equipment. In the second familiarization session, subjects performed unilateral RE (2 x 7) for both arms (same as the testing intervention as previously mentioned). The results in this bout were used to determine the subject's dominant arm. Subjects were then randomized to use either their dominant or non-dominant arm for the AE exercise. The subject then performed an incremental workload test in

the arm dynamometer to determine MWL. No upper-body RE was allowed between

familiarization days and test day.

EXERCISE PROTOCOL

Experimental trials were performed in the morning between 7:00am – 9:00am as to prevent

possible diurnal variation. One randomly designated arm was first subjected to 40 minutes of AE

in the Biodex dynamometer with an external workload set to 70% of the maximal workload

(MWL; see pre-test section) using a cadence of 30 rpm (1-second eccentric, 1-second

concentric). Ratings of perceived exertion (RPE) and heart rate (HR) were collected every 5

minutes to ensure that the exercise was moderately strenuous. After 40 minutes, resistance

increased by 2 Nm every 30 secs until an RPE of 18-20 and/or the participant could not continue

with the prescribed cadence. Fifteen minutes after completion of the AE bout, subjects

performed unilateral RE for both arms (4 x 7 for each arm) with 2 min of rest between sets.

Subjects were verbally encouraged to ensure maximal effort during each repetition. Peak
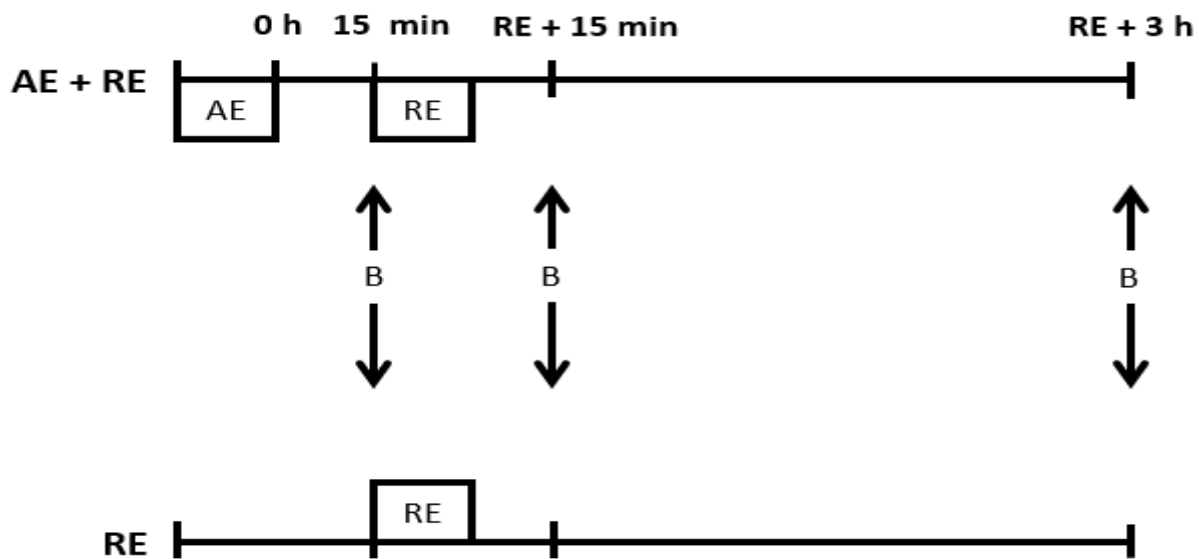

Figure 2: outline of testing intervention. Resistance Exercise (RE) Aerobic Exercise
(AE) Biopsy (B)

concentric power was recorded for each repetition during the RE. Please see figure two for a description.

MUSCLE BIOPSIES & DIET CONTROL

Muscle biopsies were obtained from the m. triceps brachii under local anesthesia immediately before, 15 min, and 3 h after the acute RE bout from both arms (Figure 1). The 3hr time-point was chosen to accommodate for changes in both protein phosphorylation and gene expression. A conchotome was used to obtain ~50 mg of tissue that was subsequently cleansed of excess blood, fat, and connective tissue before being frozen in liquid nitrogen and stored at -80°C. Subsequent biopsies were obtained from separate incisions, moving from distal to proximal. Since all tests were performed in the morning, an attempt to control the breakfast intake through providing detailed recommendations for dinner and breakfast prior to the testing intervention were given. Upon entering the testing facility, no further food intake was allowed during the experimentation.

PROTEIN EXTRACTION & WESTERN BLOTTING

Approximately 30mg of muscle tissue was manually homogenized in RIPA buffer. The resulting homogenate was placed on a rocker for 60 min at 4C. Sample was then centrifuged for 14 minutes at 11,500 rpm. The supernatant was then collected and placed into a separate microcentrifuge tube. Protein concentrations were then determined using the Bradford Protein Assay. A detailed description of the assay can be found here. Samples were then aliquoted and stored in -80C for later use.

Thirty micrograms of protein per sample were loaded on a 4-20% SDS pre-cast gel. Sample was then separated by electrophoresis alongside a protein ladder at 4C. Running time was specific to the protein being analyzed. The gel was then transferred onto PVDF membranes using wet

transfer for 60-90 minutes' dependent upon protein of interest. Following transfer, membranes were then allowed to dry for 90 minutes, activated with methanol, and subsequently blocked with blocking buffer for 60 minutes at room temperature. Membranes were then sectioned into their designated phospho-specific primary antibody and incubated overnight (4C). Target proteins and their respective phospho-residues are as followed: mTOR (ser2448, 1:500), YAP(ser127, 1:1000), FAK(Tyr397, 1:500), Beta1(Y783, 1:1,000), AMPK(Thr172, 1:1000),p70s6k(Thr 389, 1:1000), and 4EBP1(Thr 37/46, 1:500). Following overnight incubation, membranes were washed 3 times for 5 minutes with TBST (.2%). Membranes were then incubated with secondary antibody of either Rabbit (phosphor, 1:10,000) or Mouse (total, 1:10,000) for 60 minutes followed by another round of 3x5minute washes. Membranes were then rinsed with TBS and scanned. Membranes were scanned on the Licor CLx Odyssey Imageing System. Images were analyzed using ImageJ software with all phosphorylated proteins expressed relative to total protein concentration of the control protein (GAPDH, 1:5,000).

DATA ANALYSIS

Dependent variables were analyzed by a two-by-three way repeated-measures ANOVA (factors: arm and time). Pearson's product moment correlation will be used to examine the relationships between changes in acute molecular protein responses (FAK, YAP, A7B1, mTOR, 4EBP1, p70s6k, AMPK). Data skewness will be assessed through histograms and the Shapiro-Wilk test. Cohen's D effect sizes will be used to determine the magnitude of differences between the training intervention and signaling protein activity. Significance will be accepted at the 5% level ($P < 0.05$).

RESULTS

Workload test and baseline data. Workload during the incremental test amounted to an average

of 26 ± 7 N, lasting 6:14 min ± 23 sec. During resistance exercise, the RE arm produced an average of 94 ± 42 W and AE + RE 96 ± 39 W. There were no significant differences between limbs in baseline measurements (P > 0.05).

Exercise characteristics Average workload during the aerobic exercise intervention was 12 ± 5 N. Workload increased to 22 ± 7 N during the final exhaustion phase, lasting 2:37 min ± 37 sec. RPE was 15 during the 40-min bout increasing to 19 after the final exhaustion stage. Average HR across subjects during aerobic exercise was 98 ± 8 beats/min and increased to 126 ± 15 beats/min after the final exercise stage. During the resistance exercise, the AE + RE showed 12% lower (78 ± 37 W; P > 0.05) average peak concentric power than the RE arm (88 ± 40 W)

Protein Expression AMPK showed a significant condition X time interaction [$F_{(2,18)}$= 8.688; p=0.002]. AMPK was significantly higher at T1 compared to T2 (p=0.043) and T3 (p=0.001) in CT only. T2 was significantly different from T3 (p=0.05) in CT. AMPK was significantly higher at T1 in CT compared to RT. AMPK did not change at any time point in RT [$F_{(2,18)}$= 1.89; p=0.18). There was no condition X time interaction [$F_{(2,18)}$=0.305; p=0.741) for p70s6k. There was no main effect for condition [$F_{(1,9)}$=0.078; p=0.787] or time [$F_{(2,18)}$=0.857; p=0.441]. 4E-BP-1 a significant condition X time interaction [$F_{(2,16)}$= 5.175; p=0.018]. p-4E-BP-1 was significantly different at T1 compared to T3 (p=0.023). T2 tended to be significantly different from T3 (p=0.051). p-4E-BP-1 was significantly different between conditions at T1 (t(8)=-3.7; p=0.006) and T2 [t(8)= -2.89; p=0.02]. p-4E-BP-1 was different at T1 compared to T2 (p=0.015) in RT only. Beta-1 Integrin showed no condition X time interaction [$F_{(2,18)}$=1.049; p=0.37). There was no main effect for condition [$F_{(1,9)}$=0.209; p=0.659]. There was a main effect of time [$F_{(2,18)}$=6.53; p=0.007]. T1 was significantly different from T3 (p=0.003) across both interventions. T2 tended to be different from T3 (p=0.081). FAK showed no condition X time

interaction [F(2,18)=1.976; p=0.168). There was no main effect for condition [F(1,9)=0.151; p=0.707]. There was a main effect of time [F(2,18)=4.66; p=0.023]. T1 was significantly different from T3 (p=0.045). T2 tended to be different from T3 (p=0.056). YAP There was no condition X time interaction [F(2,18)=2.31; p=0.127). There was no main effect for condition [F(1,9)=1.60; p=0.27] or time [F(2,18)=2.15; p=0.145].

Chapter 4: Manuscript to be Submitted to the Journal of Strength and Conditioning Research

Introduction

The Skeletal muscle is a dynamic tissue-type with a myriad of unique intracellular signaling properties ultimately culminating into a directed response(s) dependent upon inputted stimuli. While diverse, these can often be sectioned into two distinct signaling mechanisms; that of circulating compounds binding with specific receptors upon or within the muscle fiber resulting in an intracellular signaling cascade, and mechanotransduction - the ability of a cell to convert a mechanical stimulus into an intracellular biochemical response (24). This latter pathway, while clearly prevalent in cellular function, still remains elusive. Mechanotransduction has been demonstrated to influence gene expression in lieu of any circulating growth factors (24). Moreover, it has been demonstrated that this mechanotransducing pathway can increase nuclear transcription activity at rates 45 times faster than growth factor induced signaling cascades (13). Thus, mechanotransduction appears prime and ready to sense mechanical overload during a training intervention and convert and transmit that stimulus into a functional intracellular response.

The mechanistic target of rapamycin (mTOR), specifically complex 1 (mTORC1), has been demonstrated to be a key signaling hub through which multiple external stimuli, namely circulating amino acids, growth factors, and mechanical perturbation, integrate and subsequently convert these signals into an increase in protein synthesis through the increase or decrease in multiple downstream signaling proteins impacting both gene transcription and translation (3). Two of the primary downstream proteins influenced by mTORC1 as to augment protein synthesis is the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and

ribosomal protein s6 kinase (P70s6k) (4). Both proteins work through increasing translational activity of 5-cap dependent TOP-motif mRNA which has been shown to be the key influencer in protein synthetic dynamics. Furthermore, mechanically sensitive proteins have been postulated to be essential in converting mechanical perturbation into an intracellular anabolic signal through the increased activity in the above-mentioned downstream signaling proteins; namely, focal adhesion kinase (FAK), alpha7 beta1 integrin (A7B1), and yes-associated protein (YAP) (38).

While mechanically sensitive proteins can be found throughout the entirety of the muscle fiber, there are specific locations within the fiber which are found to be highly localized with these protein aggregates. These are often sites through which force is propagated from outside or inside of the muscle fiber. These sites are termed focal adhesions, also known as costameres, within the muscle fiber (25). While costameres are found to contain more than 100 proteins, a key protein within this complex, which links the intracellular milieu with the extracellular environment, is the integrin. The alpha7 beta1 integrin is the predominant subunit found within the mature skeletal muscle fiber. Upon mechanical activity, such as that with exercise, the integrin becomes phosphorylated on its cytoplasmic tail, its extracellular head swings open allowing interaction with its specific extracellular ligand, and a subsequent direct connection is formed between the outside and inside of the cell allowing force to be transmitted and downstream signaling cascades to ensue (26). Boppart et al has demonstrated the necessity of the integrin for hypertrophy following eccentric muscle activity (28). Moreover, Wang et al. showed the requirement of adequate beta1 integrin activity and positioning for efficient muscle force transmission and minimizing membrane damage (29). However, how the downstream signaling mechanisms which drive this hypertrophic and membrane reinforcement response has been elusive and remains unclear. A prime protein to fit this role is the focal adhesion kinase.

Upon muscle activity, FAK has been demonstrated to translocate from its resting place upon the sarcomeric protein myosin to the cytoplasmic tail of the integrin as to become phosphorylated. Phosphorylated FAK can then drive mTORC1 activity through two distinct mechanisms; inhibition of the mTORC1-inhibitor TSC2 subsequently allowing Rheb to take on a GTP-state which has been demonstrated to be necessary for full mTORC1 activity, and secondly, through phosphorylation and activation of phosphoinositide 3-kinase (PI3K) which, through multiple steps, can further increase mTORC1 activity (36).

With the above taken into consideration, mechanically sensitive proteins can also drive protein synthesis in mTORC1-independent mechanisms. The yes-associated protein (YAP) has demonstrated to be capable to work in this mTORC1-independent manner through dephosphorylation, translocation to the nucleus, co-localization with a set of transcription factors termed TEAD, and subsequent enhancement of gene expression such as MyoD and Myc reporters (42). YAP can be influenced by several factors such as alteration in cytoskeletal dynamics, terminally phosphorylated and subsequent cytoplasmic retention and inhibition through the HIPPO signaling proteins MST1/2 and direct phosphorylation by the energy-sensing AMPK (46). Interestingly, while YAP can drive protein synthesis in an mTORC1-independent manner, it can also directly influence mTOR dynamics through increase the leucine transporter LATS1 and reducing mTORC1 repression through inhibition PTEN following micro RNA-29 regulation (49). Thus, YAP is poised to be a prominent mechanically sensitive signaling protein influencing the protein synthetic machinery through a myriad of mechanisms.

Concerning the present study, interest is centered upon how these mechanically sensitive proteins are influenced following diverse exercise protocols; namely, endurance- and resistance-style training. Evident in their name, mechanically sensitive proteins are often postulated to

require a mechanical threshold to be met as to stimulate their activity. Thus, a relatively insignificant level of mechanical perturbation, as is often found with an aerobic exercise intervention, would theoretically provide an insufficient stimulus to drive their activation. Lundberg et al, however, found mTORC1 to be increased following the combination of aerobic and resistance styles of training, termed concurrent training (50). This being so, it raises the question as to whether anabolic mechanotransducing pathways may be augmented following an endurance-style of stimuli leading to an increase in downstream anabolic properties.

Thus, a key question is whether an aerobic training intervention can influence, either through attenuation or augmentation, the muscle fiber integrin dynamics, subsequently altering FAK, similar to that of resistance training. Moreover, due to YAP's unique ability to sense a multitude of external stimuli, does the temporal phosphorylation, or lack-there-of, respond uniquely to either resistance training, aerobic training, or the combination of the two subsequently influencing the intracellular metabolic environment? These are the key questions that the present study wishes to address.

METHODS

General design. Eleven males, considered moderately active, performed unilateral consecutive bouts of tricep extensor movements comprised of AE (~45 min, <70% of maximal workload) and RE (4 sets of 7 reps at maximal exertion) interspersed by 15 minutes of recovery. The contralateral arm was subjected to RE-only. The arm designated for AE+RE or RE-only were randomized. Analysis of M. Tricep Brachaii muscle biopsies, obtained from both arms before RE (pre), 15 minutes following RE (post-15), and 3 hours following RE (post-3), measured the phosphorylation status of the sought-after proteins. Power output during the AE exercise were

measured via Newton Meters along with the collection of HR and RPE every five minutes. Peak concentric power was measured during the RE session.

Subjects. Eleven healthy male volunteers (181 cm ± 6 cm, 81 ± 8 kg, and 28 ± 5 years). completed the study protocol. Subjects were considered moderately active, performing recreational activities ~2-3 times a week. The study experiments and procedures including risks and discomforts were explained before subjects gave their informed written consent to participate. The study protocols were approved by the Regional Ethical Review Board in Stockholm and the Institutional Review Board at the University of Kansas.

Exercise Equipment. Resistance exercise emphasizing the M. Triceps Brachaii muscle was performed in a standing position. Non-gravity dependent devices, described elsewhere, were employed to conduct this exercise. The Exxentrix Flywheel device, using the inertia of a spinning flywheel to offer unlimited resistance during coupled concentric and eccentric actions, were used for the resistance exercise protocol. Subjects stood upright while performing the exercise. The range of motion consisted of full tricep extension 180 degrees and 90 degress of elbow flexion. Peak concentric power was gathered following each repetition. All repetitions were monitored by a trained researcher.

Exercise Protocol. Aerobic exercise was performed using the isotonic dynamometer. Subjects were comfortably seated in an upright position with their upper arm passively held in an approximately 90-degree position in relation to the torso. Test subjects, using 70% of their predetermined maximal workload, did continuous tricep extensions at a cadence of 30 rpm. Following 40 minutes, the individual was the subjected to a fatiguing period comprised of an additional 2nm increases in external resistance. An additional 2nm were added every 30 seconds until the individual could not maintain the required 30 rpm cadence or reached a RPE of 18+.

Following 15 minutes of passive rest, the test-subject then took part in the RE session comprised of 4 reps of 7 repetitions alternating arms and 2 minutes of rest between each arm. Subjects were verbally encouraged by the researcher during each repetition.

Muscle Biopsies.

Muscle biopsies were obtained from the m. triceps brachii under local anesthesia before (PRE), 15 mins (Post), and 3 hours (Post-3hr). Biopsies were taken distal to proximal with approximately 3 centimeters of separation between incisions. With the utilization of a choncotome, ~50 mg of muscle sample was collected, cleaned, and frozen in liquid nitrogen and stored at -80C for future use.

Protein Concentration. Approximately 30mg of muscle tissue was manually homogenized in RIPA buffer. The resulting homogenate was placed on a rocker for 60 min at 4C. Sample was then centrifuged for 14 minutes at 11,500 rpm. The supernatant was then collected and placed into a separate microcentrifuge tube. Protein concentrations were then determined using the Bradford Protein Assay. A detailed description of the assay can be found here. Samples were then aliquoted and stored in -80C for later use.

Western Blotting. Thirty micrograms of protein per sample were loaded on a 4-20% SDS pre-cast gel. Sample was then separated by electrophoresis alongside a protein ladder at 4C. Running time was specific to the protein being analyzed. The gel was then transferred onto PVDF membranes using wet transfer for 60-90 minutes' dependent upon protein of interest. Following transfer, membranes were then allowed to dry for 90 minutes, activated with methanol, and subsequently blocked with blocking buffer for 60 minutes at room temperature. Membranes were then sectioned into their designated phospho-specific primary antibody and incubated overnight (4°C). Target proteins and their respective phospho-residues are as followed: mTOR (ser2448,

1:500), YAP(ser127, 1:1000), FAK(Tyr397, 1:500), Beta1(Y783, 1:1,000), AMPK(Thr172, 1:1000),p70s6k(Thr 389, 1:1000), and 4EBP1(Thr 37/46, 1:500). Following overnight incubation, membranes were washed 3 times for 5 minutes with TBST (.2%). Membranes were then incubated with secondary antibody of either Rabbit (phosphor, 1:10,000) or Mouse (total, 1:10,000) for 60 minutes followed by another round of 3x5minute washes. Membranes were then rinsed with TBS and scanned. Membranes were scanned on the Licor CLx Odyssey Imaging System. Images were analyzed using ImageJ software with all phosphorylated proteins expressed relative to total protein concentration of the control protein (GAPDH, 1:5,000).

Results

Statistics: A repeated measures ANOVA was used to test for a condition X time interaction. In the case of a significant interaction, post-hoc analyses were conducted with one-way (time) repeated measures ANOVA for each condition separately. A protected Fisher's LSD was used to determine differences between time points. Paired sample t-tests were used to determine differences between conditions at T1, T2, and T3. Significance was determined as $p \leq 0.05$

Aerobic Exercise: Average workload during the aerobic exercise intervention was 12 ± 5 N. Workload increased to 22 ± 7 N during the final exhaustion phase, lasting an average of 2:37 min $\pm$ 37 sec (Figure 3A). Average RPE was 15 (Fig 3B) during the 40-min bout increasing to 19 following the final exhaustion stage. Average HR across subjects during aerobic exercise was 98 $\pm$ 8 beats/min and increased to 126 ± 15 beats/min after the final exercise stage (Figure 3C).

Resistance Exercise: The AE + RE intervention showed a 12% reduction (78 ± 37 W; $P > 0.05$) in average peak concentric power as compared to the RE-only arm (88 ± 40 W) (Figure 4).

Protein Phosphorylation. A repeated measures ANOVA was used to test for a condition X time interaction. In the case of a significant interaction, post-hoc analyses were conducted with one-

way (time) repeated measures ANOVA for each condition separately. A protected Fisher's LSD was used to determine differences between time points. Paired sample t-tests were used to determine differences between conditions at T1, T2, and T3. Significance was determined as $p \leq 0.05$. AMPK showed a significant condition X time interaction [$F_{(2,18)}$= 8.688; p=0.002]. AMPK was significantly higher at T1 compared to T2 (p=0.043) and T3 (p=0.001) in CT only. T2 was significantly different from T3 (p=0.05) in CT. AMPK was significantly higher at T1 in CT compared to RT. AMPK did not change at any time point in RT [$F_{(2,18)}$= 1.89; p=0.18]. There was no condition X time interaction [$F_{(2,18)}$=0.305; p=0.741) for p70s6k. There was no main effect for condition [$F_{(1,9)}$=0.078; p=0.787] or time [$F_{(2,18)}$=0.857; p=0.441]. 4E-BP-1 a significant condition X time interaction [$F_{(2,16)}$= 5.175; p=0.018]. p-4E-BP-1 was significantly different at T1 compared to T3 (p=0.023). T2 tended to be significantly different from T3 (p=0.051). p-4E-BP-1 was significantly different between conditions at T1 ($t_{(8)}$=-3.7; p=0.006) and T2 [$t_{(8)}$= -2.89; p=0.02]. p-4E-BP-1 was different at T1 compared to T2 (p=0.015) in RT only. Beta-1 Integrin showed no condition X time interaction [$F_{(2,18)}$=1.049; p=0.37). There was no main effect for condition [$F_{(1,9)}$=0.209; p=0.659]. There was a main effect of time [$F_{(2,18)}$=6.53; p=0.007]. T1 was significantly different from T3 (p=0.003) across both interventions. T2 tended to be different from T3 (p=0.081). FAK showed no condition X time interaction [$F_{(2,18)}$=1.976; p=0.168). There was no main effect for condition [$F_{(1,9)}$=0.151; p=0.707]. There was a main effect of time [$F_{(2,18)}$=4.66; p=0.023]. T1 was significantly different from T3 (p=0.045). T2 tended to be different from T3 (p=0.056). YAP There was no condition X time interaction [$F_{(2,18)}$=2.31; p=0.127). There was no main effect for condition [$F_{(1,9)}$=1.60; p=0.27] or time [$F_{(2,18)}$=2.15; p=0.145].
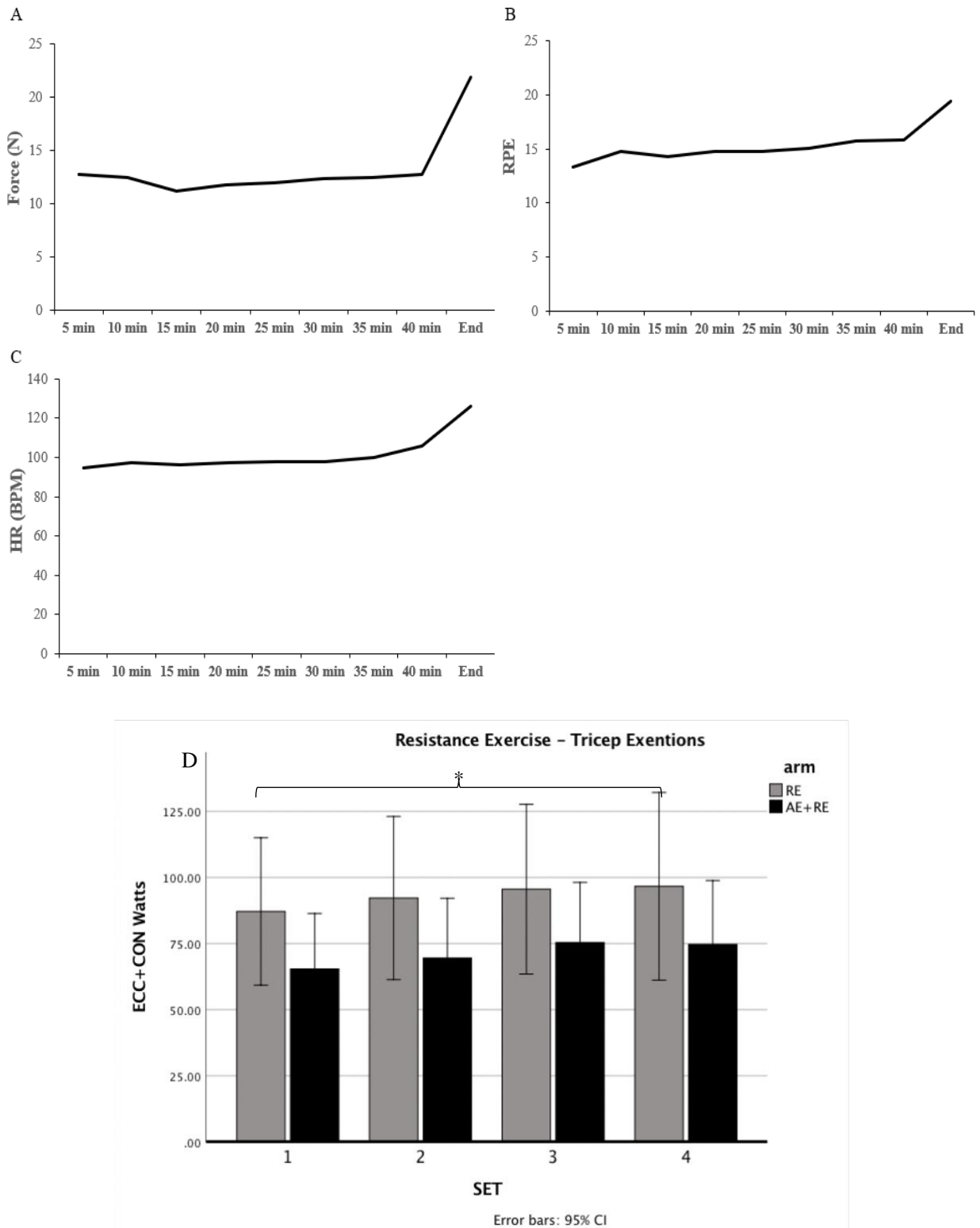
A

B

C

D

Figure 3. A-D: Mean workload, RPE, and HR during the aerobic exercise bout and mean peak concentric power of AE+RE and RE-only. * indicates significantly different from between arms at all time-points, $p < 0.05$.

Discussion

The current study set out to investigate the ability of an aerobic intervention implemented for ~45 minutes at 70% of one's maximal workload, coupled with a resistance training exercise of maximal exertion, termed concurrent training, to influence the dynamics of mechanically sensitive proteins YAP, Alpha7 Beta1 Integrin, and FAK ultimately altering their downstream anabolic or catabolic signaling properties. It was hypothesized that the implementation of an acute aerobic exercise intervention prior to resistance exercise would fatigue the muscle of interest (triceps brachii) to an extent that would reduce power output capabilities. This attenuation was readily observed during the resistance exercise of the AE+RE arm. This insufficient power production would hypothetically lead to reduced muscle mechanical output which would subsequently reduce the recruitment of mechanically sensitive proteins, of which are speculated to require heightened levels of mechanical perturbation leading to their increased activity. Here we demonstrate that, in contrast to our initial hypothesis, mechanically sensitive properties, specifically the muscle-specific beta1 integrin, was increased to the same degree between the RE and AE+RE intervention.

Mechanotransduction has been an area of much interest for a considerable period of time. Indeed, in the 1960's Goldberg demonstrated that muscle fibers possess an intrinsic mechanism allowing it to respond to external perturbation (1). Moreover, Hornberger and colleagues have demonstrated through a number of studies the ability of the muscle fiber to respond to mechanical perturbation in lieu of previously-recognized growth factor-induced anabolic signaling cascades; specifically, that of the PI3k-AKT-mTOR signaling axis (20,21,38). Spangenburg, too, found that following the implementation of the synergist ablation model with either wild type mice or those with IGF-1 mutated receptors as to render them nonfunctional, muscle size and intracellular anabolism was not attenuated (19). These studies demonstrate the

intrinsic mechanisms by which muscle can respond to a mechanical stressor through a process termed mechanotransduction – the ability of a cell to convert a mechanical stimulus into an intracellular biochemical response.

Key mechanically sensitive properties within the skeletal muscle, such as the alpha7 beta1 integrin and FAK, are postulated to require a high level of mechanical tension as to lead to their activation. This is proposed because of their role as structural proteins which provide a scaffolding framework allowing cellular force transmission in a multidirectional manner (26,31,33). Moreover, due to high levels of mechanical tension being required for their recruitment, an individual who is fatigued and unable to exert high levels of force would be unable or, rather, insufficiently, recruit these proteins. A concurrent training regimen would then be an excellent protocol to investigate these questions, specifically, does a fatiguing endurance exercise protocol prior to resistance training lead to decreased power output and thus not recruit mechanically sensitive proteins relative to resistance-only exercise? As mentioned above, we did not find this to be the case. On the contrary, we found alpha7 beta1 to be increased similarly between arms across all time-points. However, an area of interest is time-point one. Immediately following the endurance exercise, beta1 integrin was not phosphorylated to a greater degree than the "rested" arm. Thus, while AE may not impede upon a subsequent RE session, it, in-and-of itself, may not provide a sufficient stimulus as to increase integrin activity. As a result, the present study demonstrates the ability of the muscle to activate the alpha7 beta1 integrin following resistance exercise to a similar extent while fatigued and with reduced power output. This being so, the utilization of AE prior to RE does not negatively impact overall beta1 integrin phosphorylation.

This may prove important as there are a myriad of adaptations which may increase to a greater extent through the utilization of an endurance-style of training relative to resistance training alone, namely those consisting of oxidative adaptations. However, focusing on either style of exercise, whether AE or RE, at the expense of the other, may lead to overall performance deficiencies. For example, the alpha7 beta1 integrin is highly recruited with overloaded eccentric-styles of exercise (27,28). Increased integrin quantity and phosphorylation allow for improved intracellular force transmission resulting in reduced cellular damage. Moreover, this increase in integrin activity allows for rapid force propagation through the cytoskeleton to the nucleus as to influence gene expression in a matter of milliseconds (13). Thus, taking into consideration the findings of the present study, the implementation of two divergent styles of training, namely AE and RE, may be advantageous when seeking muscular adaptations concerning both structural reinforcement and intracellular oxidative improvements.

FAK has been found to drive intracellular anabolism following an external stimulus, specifically that which is mechanically strenuous, lending it as a mechanosensor (36). Its anabolic effect has been proposed to work through two key mechanisms, the phosphorylation and inhibition of TSC1/2 leading to its translocation away from the lysosome and change of GDP-bound Rheb to GTP-Rheb, along with phosphorylation of the mTORC1 binding protein called Raptor, both of which increase mTORC1 activity (51). Moreover, the alpha7 beta1 integrin has been demonstrated to partially drive its growth inducing effects in an FAK-dependent manner. This can be seen through, upon muscle activity, FAK translocation from its resting place upon the sarcomeric myosin, binding to the cytoplasmic tail of the beta 1 integrin, ultimately becoming phosphorylated (51). Here, however, we did not find this integrin-FAK link. On the contrary, it was demonstrated that FAK phosphorylation decreased over the three time-

points relative to integrin activity over the same time-points; however, this did not reach significance.

Interestingly, it has been demonstrated that AMPK negatively regulates FAK tyrosine397 phosphorylation (52). Indeed, Kline et al also found the AMPK upstream activator, Liver Kinase B (LKB), to negatively regulate FAK phosphorylation (53). Thus, it is proposed that, while integrin signaling increased across the three time-points, this on average 1.4-fold increase was not a sufficient increase to combat the inhibitory effect of AMPK upon FAK. However, with AMPK having decreased 2.5-fold by time-point three, it would be interesting to analyze FAK phosphorylation at later time-points as to see if the possible AMPK inhibition subsides leading to enhanced FAK activity.

With the above being taken into consideration, the impact that AMPK activity has on intracellular anabolism has recently been scrutinized. It has been proposed that the isoform-specific AMPK which has widely been shown to attenuate anabolic processes through AICAR stimulation, AMPKalpha2, may not be the predominant isoform activated via exercise, AMPKalpha1. Interestingly, Baar et al found high levels of AMPKalpha1 phosphorylation with a simultaneous increase of anabolic processes following one week of overload (54). Moreover, Lundberg et al utilized an identical training protocol, using the lower body, to the one implemented within this study and found AMPK increased following an endurance exercise with no attenuation of the anabolic protein 4E-BP1 (50). Here, however, we do not see this lack of 4E-BP1 attenuation following an increase of AMPK. On the contrary, we demonstrate a significant decrease in 4E-BP1 phosphorylation following AE with a simultaneous increase in AMPK. However, in the Lundberg study, a 1.5-fold increase in AMPK following AE was found as compared to the rested leg. Within this study, we found AMPK to increase 2.5-fold following

the AE intervention as compared to the unexercised arm. This large difference may drastically influence anabolic processes to a greater extent. This being so, it may be that an acute AE exercise intervention of the triceps musculature requires greater relative effort, while using a similar relative load, as compared to the much larger quadriceps. Therefore, an acute AE exercise protocol with the upper body may respond to a greater degree leading to substantially greater AMPK activity and subsequent reduction in 4E-BP1 signaling. Thus, anabolic and catabolic dynamics of the upper and lower extremities appear to differ following acute AE exercise with a similar relative load.

These dissimilarities between the previous study by Lundberg et al. and the current study may primarily be due to the functional characteristics of the muscle fibers within the muscles utilized, namely, their fiber-type distribution. Concerning the triceps brachaii, Johnson et al found it to be comprised of ~67.5% fast twitch fibers (55) while Korhonen et al. found the vastus lateralis to contain ~50% fast twitch fibers (56). Consequently, the relatively minimal oxidative capabilities of the triceps brachii renders it susceptible to easily fatigue as compared to the vastus lateralis. This being so, intracellular energy-sensing mechanisms are likely to be influenced to a greater degree, namely AMPK, within the triceps brachii, with an equally greater disruption of the anabolic signaling properties.

Interestingly, this negative influence of a preceding endurance bout upon RE-induced anabolic properties does not reflect that which was found by Kazior (57); that being an increase in mTOR and AKT following a seven-week endurance training program. Similarly, Pugh et al found anabolism to be increased following a high intensity interval style of exercise (58). Again, however, we propose this difference is driven by the catabolic properties brought about by an

endurance-style of exercise within the upper body which differs from that of the lower body and may ultimately render it more susceptible to the "interference effect".

Interestingly, p70s6k did not show similar dynamics as compared to 4E-BP1. Previous investigations have found strong correlation between p70s6k signaling and overall increase in muscle size and have thus proposed p70s6k to be an important mediator of mTORC1 signaling and 5-TOP mRNA translation (59). However, recent investigations have proposed that the family of 4E-BPs, specifically 4E-BP1, as the predominant regulator of mTORC1's dynamics influencing 5-TOP mRNA translation and subsequent protein synthesis (60). Thus, it is proposed that the lack of p70s6k signaling does not infer attenuated protein synthetic machinery. On the contrary, it is proposed that the altered dynamics of 4E-BP1 phosphorylation at the different time-points is a greater indicator of mRNA translation.

To our surprise, YAP activity did not change across the three time-points. There are a number of reasons which may be at play here, namely, unknown temporal regulation of YAP phosphorylation following exercise and muscle-specific differences in YAP activity. This being so, it is proposed that future investigations examining YAP activity following exercise include a greater number of time-points along with using the lower body as to receive greater insight into possible YAP fluctuation following an exercise intervention.

Lastly, there are a few confounding factors that may have influenced the findings; most importantly is the possible "cross-over effect" such as increased systemic inflammatory markers following the aerobic session altering intracellular signaling cascades of both the AE+RE and RE-only arms. For example, Landers-Ramos et. al, have demonstrated an increase in the inflammatory marker interleukin-6 (IL-6) immediately following an acute aerobic intervention (62). IL-6 has been demonstrated to drive catabolism the decreasing p70s6k signaling and

subsequent decrease in myofibrillar protein synthesis (61). While this possible interaction may take place, much of the literature concerning systemic effects and inflammatory markers following exercise are analyzed following lower body exercise. This being so, the possible rise in inflammatory markers following an acute aerobic exercise with the upper body may not induce a sufficient inflammatory response as to attenuate anabolism of the contralateral arm. This line of inquiry requires further investigation.

In conclusion, we demonstrate the ability of the triceps brachaii to respond in a similar manner between concurrent exercise and resistance-only exercise concerning the skeletal muscle specific integrin isoform, alpha7 beta1. Moreover, we demonstrate the large decrease of 4E-BP1 and increase of AMPK immediately following the AE protocol along with possible augmentation of FAK through AMPK inhibitory mechanisms. This denotes the possibility that, as compared to past studies, the triceps brachii uniquely respond and regulate intracellular remodeling mechanisms following an acute bout of concurrent exercise relative to the lower body.
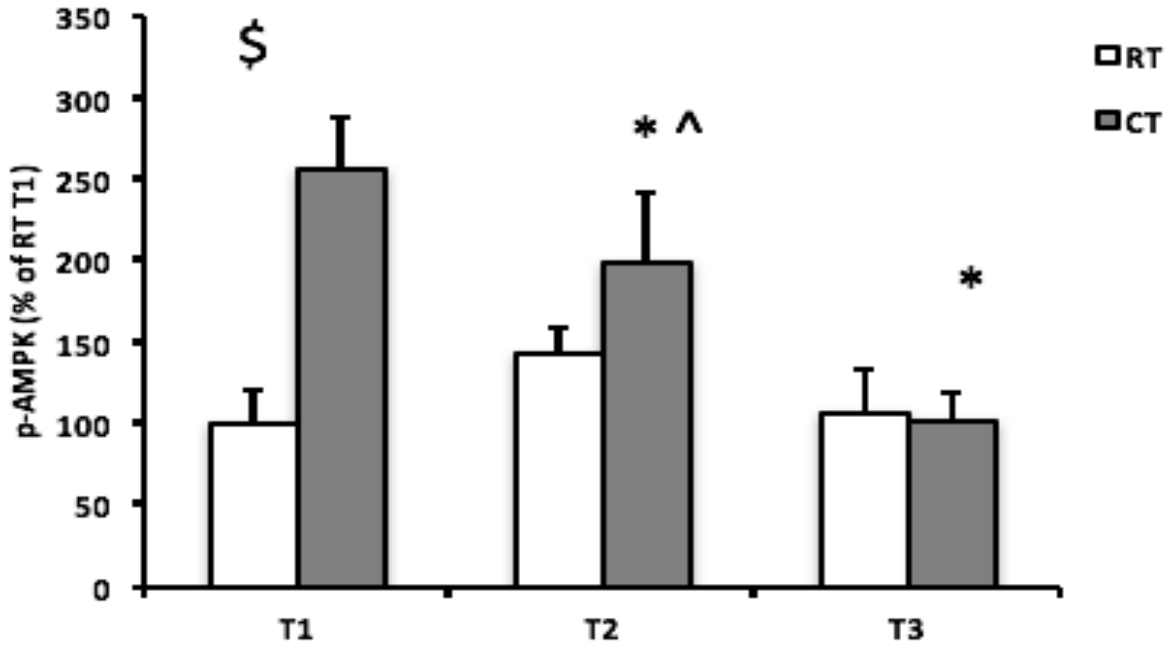
Fig.4
Phospho-AMPK before (T1), Post 15 minutes (T2), and Post 3 hours (T3) acute resistance exercise. Significant effect (P<0.05): $ indicates significantly different between RT and CT at respective time-point, * indicates significantly different from T1 in CT only, ^ indicates significantly different from T3 in CT only
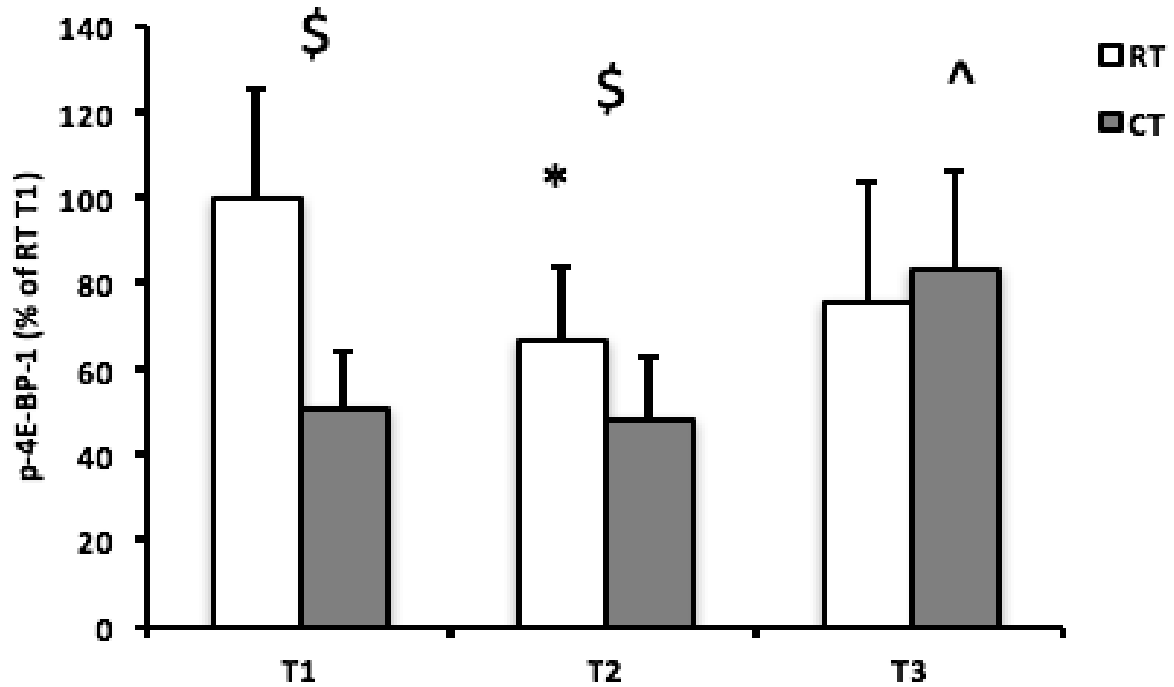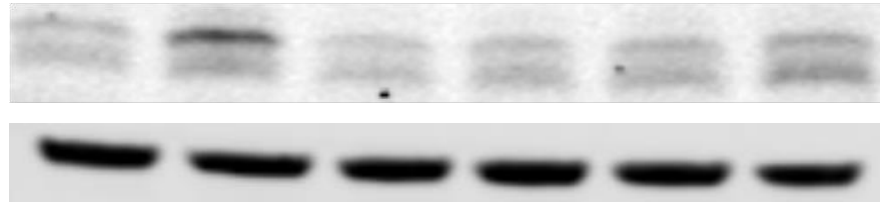
Fig. 5
Phospho-4E-BP1 before (T1), Post 15 minutes (T2), and Post 3 hours (T3) acute resistance exercise. Significant effect (P<0.05): $ indicates significantly different between RT and CT at respective time-point, ^ indicates significantly different from T1 in CT only at respective time-point * indicates significantly different from T1 in RT only at respective time-point
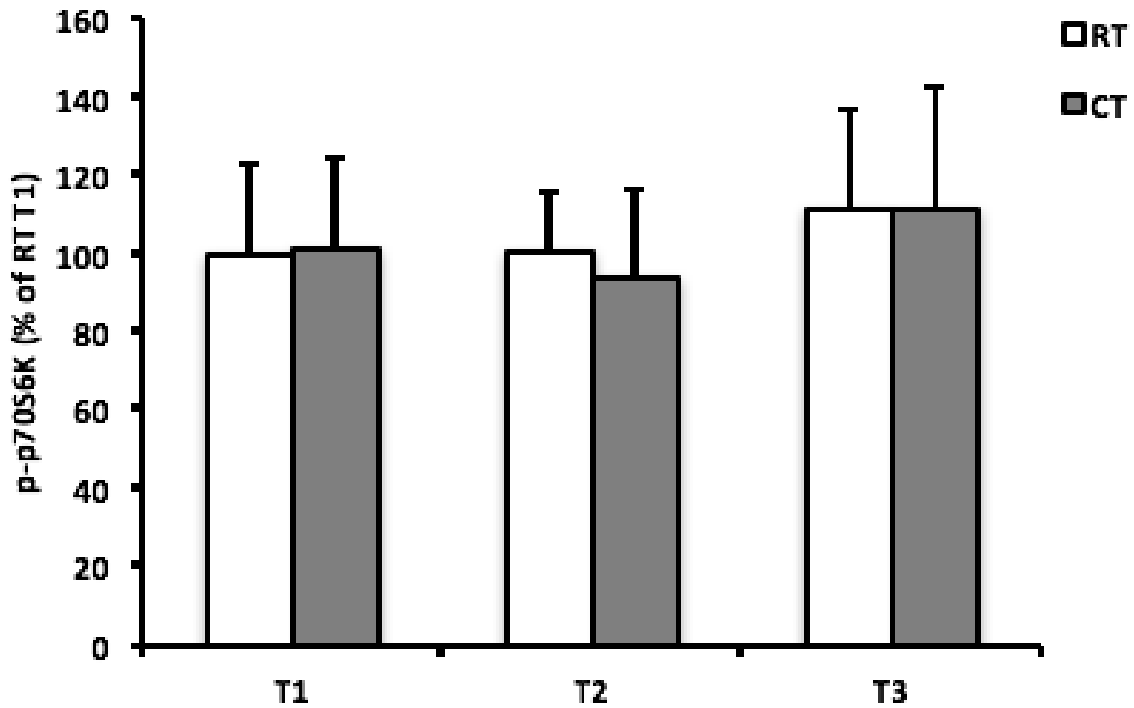
Fig. 6
Phospho-p70s6k before (T1), Post 15 minutes (T2), and Post 3 hours (T3) acute resistance exercise. Significant effect ($P<0.05$): No significant difference over time or between conditions
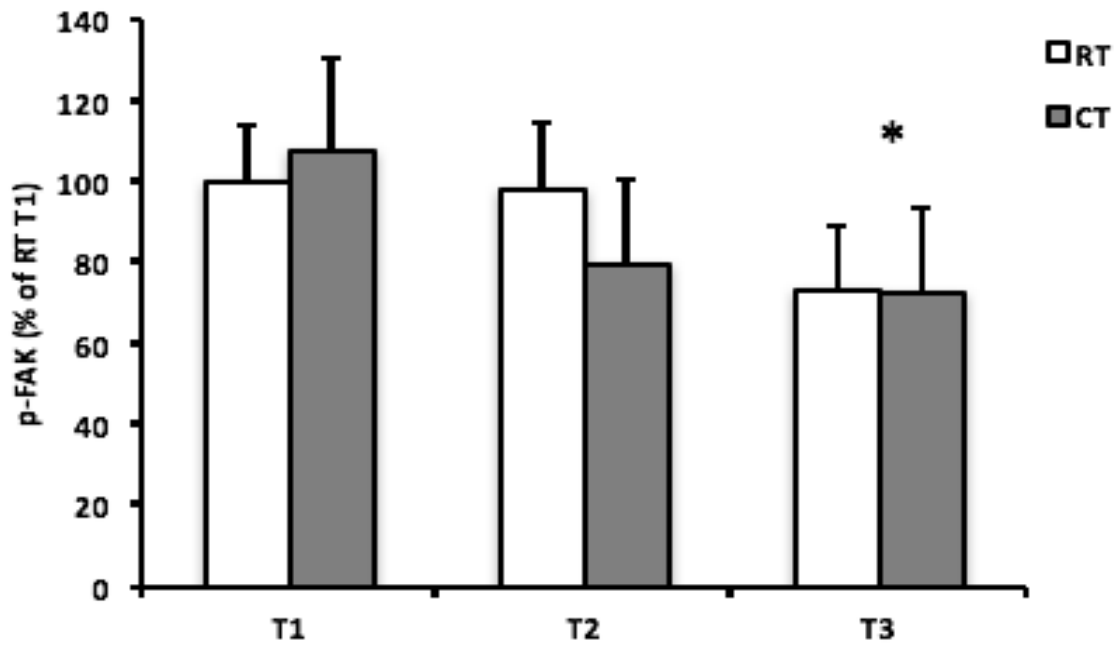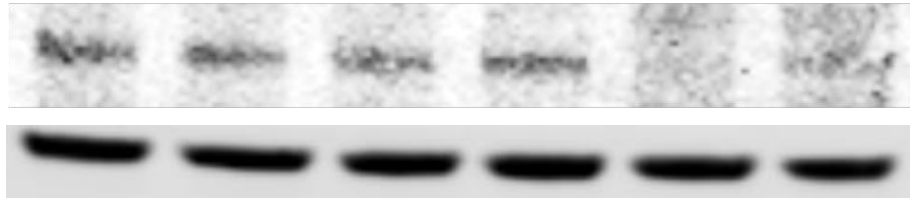
Fig. 7
Phospho-FAK before (T1), Post 15 minutes (T2), and Post 3 hours (T3) acute resistance exercise. Significant effect (P<0.05): * indicates main effect of time, T3 significantly different from T1

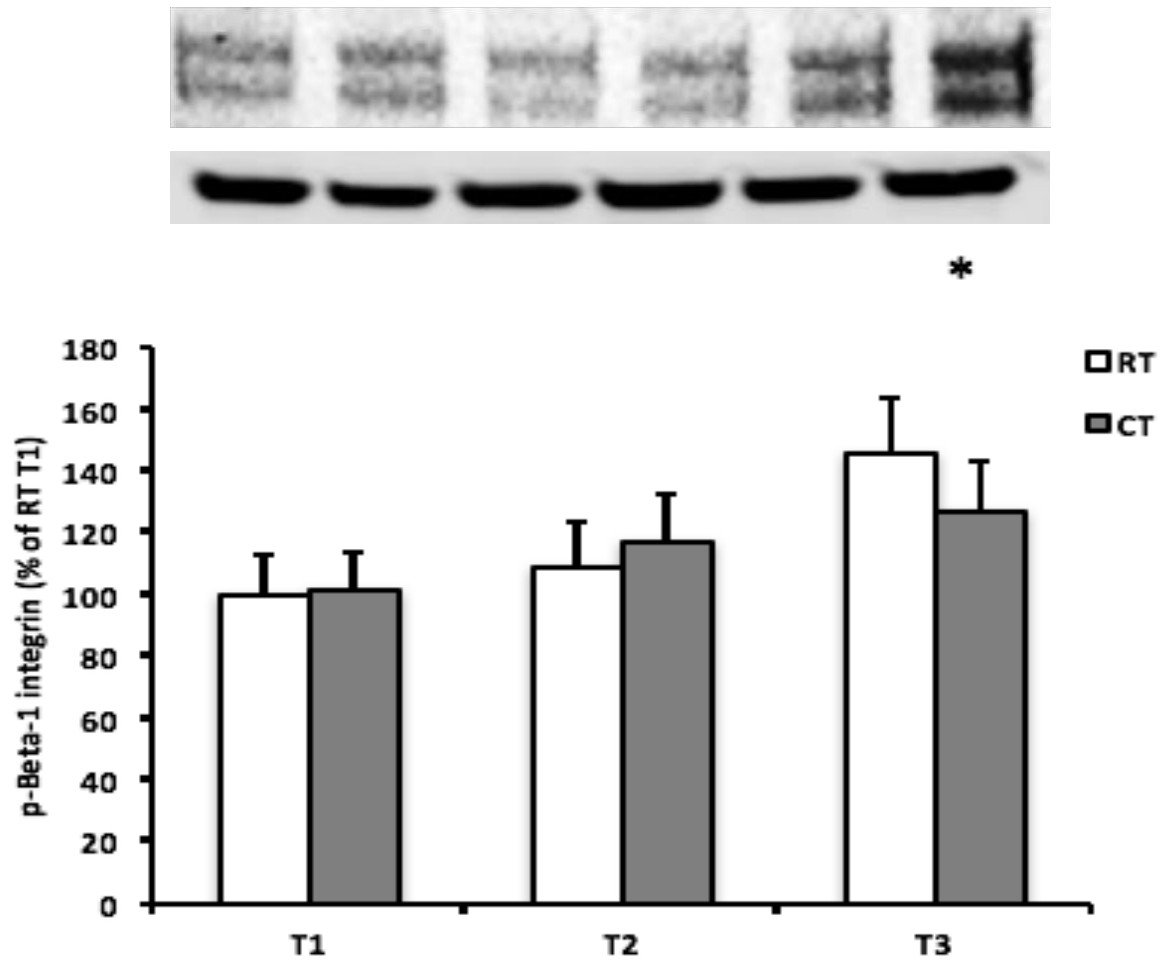Fig. 8
Phospho-Beta1Integrin before (T1), Post 15 minutes (T2), and Post 3 hours (T3) acute resistance exercise. Significant effect (P<0.05): * indicates main effect of time, T3 significantly different from T1
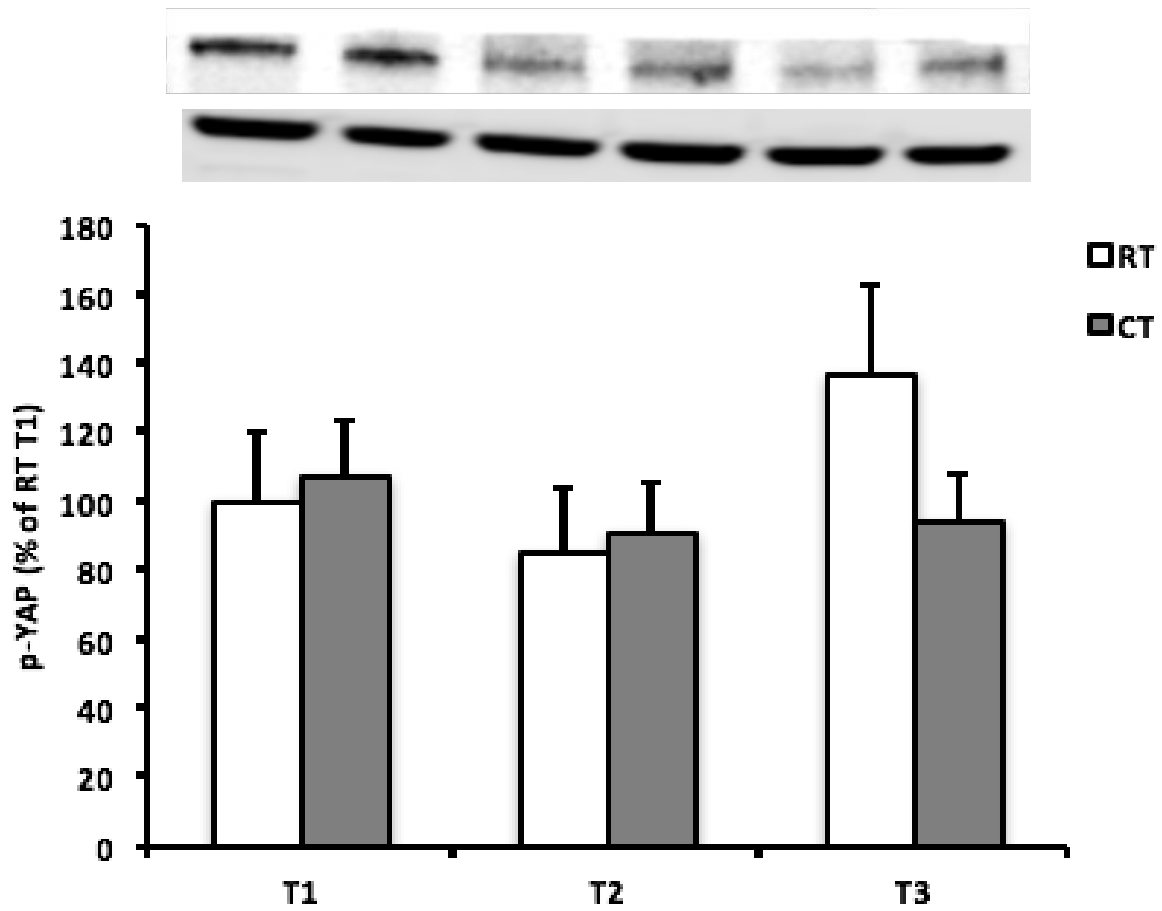
Fig. 9
Phospho-YAP before (T1), Post 15 minutes (T2), and Post 3 hours (T3) acute resistance exercise. Significant effect (P<0.05): No significant difference over time or between conditions

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