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MECHANISMS OF BLOOD FLOW RESTRICTION EXERCISE IN SKELETAL MUSCLE ADAPTATIONS

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MECHANISMS OF BLOOD FLOW RESTRICTION EXERCISE IN SKELETAL MUSCLE ADAPTATIONS

by

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Dedication

To Beverley Ellis, who has shown me unbridled love and support throughout my education, also Earl Ellis, who has been my role model since my early years.

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The maintenance of skeletal muscle mass is crucial for human health and long term survival. However, the aging process is associated with an involuntary loss of muscle mass and the inability to maximally stimulate muscle growth ultimately leading to sarcopenia. Extensive research has been conducted to determine the precise mechanisms by which nutrients, hormones and exercise regulate cell signaling events that convert the anabolic stimulus to a response that enhances cell size. Unfortunately, the research is incomplete considering the insufficient explanation for the impaired muscle synthetic response associated with aging. Interestingly, a novel style of exercise utilizing low-intensity resistance coupled with local vascular occlusion called "blood flow restriction" (BFR) exercise has emerged as an exercise that stimulates muscle growth and muscle protein synthesis to a similar extent for all adults. Research in the past two decades on BFR exercise has been primarily descriptive whereas a mechanistic explanation is lacking as to how a low intensity resistance stimulus is sufficient to promote an increase

in muscle mass. Research in this dissertation is focused on investigating potential mechanisms that stimulate a muscle anabolic response following BFR exercise. Immediately following BFR exercise as the restriction cuffs are removed, reactive hyperemia occurs, and the increase in nutritive delivery to the muscle is believed to be one of the driving factors that stimulate muscle protein synthesis. Although, mimicking the effect of reactive hyperemia using a pharmacological vasodilator after low-intensity resistance exercise was insufficient to reproduce a similar increase in muscle protein synthesis or anabolic cell signaling. Moreover, mTORC1 is thought to be necessary and required for all cell growth signals; however, this mechanism has yet to be tested with BFR exercise. Use of the competitive mTORC1 inhibitor, rapamycin, determined that BFR exercise does in fact stimulate muscle protein synthesis through the activation of mTORC1. Lastly, metabolic stress such as the accumulation of lactate and G6P have been shown to play a role in the activation of mTORC1 in all muscle fibers contrary to high-intensity exercise that only stimulates fast twitch muscle fibers. Collectively, these studies further our understanding of the underlying mechanisms of BFR exercise.

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CHAPTER 1

Introduction

Skeletal muscle is a multifunctional tissue of great importance that is commonly underestimated. Besides its obvious roles in posture, locomotion, protection, and physical activities, skeletal muscle has a more fundamental role as it serves as a constant amino acid source for the whole body's protein needs and backup energy supply. On a continuous level, every tissue in the body is under constant remodeling; that is to say proteins are regularly being degraded and resynthesized from an amino acid pool maintained in the blood stream. Considering that human beings do not continuously ingest the necessary amino acids to adequately maintain plasma amino acid concentrations for this purpose, the pool is maintained primarily by the tight regulation of skeletal muscle degradation in the post-absorptive state. Therefore, the maintenance of skeletal muscle is crucial to the survival of an individual.

The term muscle metabolism is the combination of muscle anabolism (muscle building) and catabolism (muscle breakdown); a process that is under tight regulation to maintain muscle mass while still maintaining the plasma amino acid concentration. Skeletal muscle is typically maintained through the ingestion of a diet containing high quality protein that ultimately causes a transient increase in muscle protein synthesis. Thus, skeletal muscle is in dynamic equilibrium as a result of anabolism after a meal and catabolism during the post-absorptive state. In order to maintain muscle mass, the amount of muscle anabolism must on average equal the amount of muscle catabolism; a concept referred as net balance. Positive net balance occurs when muscle anabolism on

average exceeds muscle catabolism over a given period of time thereby increasing muscle mass. The opposite condition is considered negative net balance and results in muscle loss over time. This concept is important when considering a variety of conditions such as infections, diseases, burns, injuries and cancer. In such cases, the demand of amino to produce immunoglobulins, and other proteins involved with wound healing, significantly increase. Likewise, muscle catabolism concomitantly rises to provide the additional amino acids, resulting in a negative muscle net balance. For example, burns can increase muscle protein breakdown at such a rate to cause significant and sometimes lethal levels of muscle loss (233). Alternatively, conditions such as malnutrition, starvation, immobilization, bed rest or generalized inactivity, decrease muscle anabolism and also results in a negative muscle net balance. Depending on the severity and duration, all of these conditions can potentially be fatal as the catabolic condition continues to degrade muscle until insufficient mass is left to maintain the cellular functions of the other tissues. Therefore muscle loss can be viewed as the most life threatening condition when combined with another illness. Additionally, muscle loss can have other negative effects such as impaired hormone production, glucose regulation, cellular communication, and wound healing along with immune dysfunction leading to severe infections (33, 39). Countermeasures to preserve, maintain and increase muscle mass have gained significant attention in the literature for all populations.

High-intensity resistance exercise training is a well-established stimulus to enhance muscle protein synthesis to an extent as to enhance skeletal muscle mass and strength (23, 41, 142, 239). Moreover, in combination with high quality protein and carbohydrates, the muscle protein synthetic response to exercise becomes augmented (55). Unfortunately, the muscle protein synthetic responses to various anabolic stimuli

are not equal among all populations. In particular the elderly appear to have a diminished response which may play a role in the development of sarcopenia.

SARCOPENIA

As humans age, there is an inevitable, involuntary loss of muscle mass reportedly at a rate of 1-2% per year after the 50th year of age (33). Although specific criteria for the definition of sarcopenia are not well established, approximately 30-50% over the age of 80 have a muscle mass index (muscle mass/height) more than two standard deviations below the average young population (20, 100). As alluded to above, the ability to withstand an acute illness is associated with muscle mass to the extent where a 40% loss in lean mass can be fatal (120). Moreover, a strong correlation has been established between the loss of muscle mass and the incidence of falls, fractures, nursing home admissions and loss of independence (80, 212).

Although the mechanism(s) for sarcopenia are not yet clear, investigators have explored whether metabolic differences exist between young and older individuals. To this end, at basal conditions, anabolic and catabolic rates do not appear to be the differential factor between young and older individuals (47, 113, 114, 217-219). Instead, blunted responses to anabolic stimuli such as feeding (87, 113, 155, 217), resistance exercise (71, 125) and insulin (163) have been observed in the elderly population as compared to younger controls. Resistance exercise and nutrient supplementation are typically used to stimulate muscle protein synthesis and induce hypertrophy to overcome causes of muscle atrophy. However, these interventions may not be as influential if the resulting anabolic response is blunted with age.

Welle *et al*. first showed that older individuals have a blunted anabolic response to typical resistance exercise, whereby the same relative intensity of exercise, performed by both young and old individuals did not elicit an equivalent response to increase muscle protein synthesis (225). Since then, a number of other investigators observed a similar blunted anabolic response associated with age, not only with resistance exercise, but nutrition and insulin as well (47, 125, 202). In these studies, exercise intensity is standardized to each individual's maximum strength. It is important to note that the older population exhibits a significant diminishment in muscle strength, even when normalized to lean mass (73), indicating not only a loss of muscle quantity but quality as well. Therefore, when comparing a young group to an older group, the absolute exercise intensity is not equally matched. This design was thought to be of concern since the absolute intensity was different between groups. However, considering that older subjects have less muscle fibers within a given area of muscle, it is logical to assume at each muscle fiber needs to work harder to compensate. Fry *et al*. demonstrated that old and young subjects exercising at 70% of their 1-repatition maximum (1RM) elicit similar lactate responses, indicating that the muscles in both groups were experiencing the same intensity during the exercise yet muscle protein synthesis rates were lower in the older individuals (71).

Countermeasures to prevent sarcopenia are needed due to the escalating economic burden it places on the United States government. In the year 2000, it was estimated that the direct health care costs of sarcopenia were $18.5 billion (106). However, more daunting is the estimate that the population over the age of 60 will triple by the year 2050 compared to the year 2000 (118). Therefore, the discovery of specific cellular mechanisms relating to how skeletal muscle mass is regulated through nutrition, exercise

and other factors is essential to our understanding of sarcopenia. The research in this dissertation is focused on elucidating the cellular mechanisms that must exist in which cells communicate the link between an anabolic stimulus, and the resulting increase in muscle protein synthesis called cell signaling. The cell signaling process is an elaborate network of cellular enzymes that signals from one another in a fashion that can be controlled and manipulated at many checkpoints.

METABOLIC CELL SIGNALING PATHWAYS

Nutrients such as amino acids and carbohydrates play multiple roles in increasing protein synthesis leading to enhanced cell size. This section provides the technical details of how each stimulus can enter multiple anabolic or catabolic pathways with cross talk between the pathways which inevitably leads to increased cell size from many different mechanisms.

Carbohydrate regulation of cell size

The primary mechanism of carbohydrate-induced cell growth is an indirect mechanism involving the increase in postprandial insulin. Insulin binds the extracellular domain of a transmembrane receptor tyrosine kinase. These insulin receptors are heterotetramers and have kinase activity on two catalytic domains on the intracellular side of the receptor. Insulin binding induces a conformational change such that the catalytic domains move closer together and phosphorylate tyrosine residues on each other; a process called autophosphorylation. The phosphotyrosine residues attracts an SH2 domain found in insulin receptor substrate 1 (IRS1) which also contains tyrosine

phosphorylation sites and itself becomes phosphorylated by the kinase activity of the insulin receptor. IRS1 acts as a docking site since its newly acquired phosphotyrosines attract other SH2 domain containing proteins such as Phosphatidyl inositol 3 kinase (PI3K), and Grb2 (231). These pathways will be discussed separately.

Mitogen Activated Protein Kinase (MAPK) Pathway

The Grb2-IRS1 complex mentioned above binds to a Ras-guanyl nucleotide exchange factor (GEF) called mSOS which functions to exchange a GDP for GTP on a Ras protein which initiates the MAPK pathway (180).

Ras is the founding member of a very large family of small GTPases (209). The activity of Ras is significantly enhanced 100-1000 fold with the binding to GTP compared to when the nucleotide is hydrolyzed to GDP (213). One of the effectors of Ras is another GEF called Ral which acts in a positive feedback loop to enhance the activity of Ras (150). Although Ras has a secondary role in binding PI3K and activating a separate signaling cascade described below (150), the primary activity of Ras-GTP is to recruit Raf from its cytosolic location to the membrane where Ras is bound and physically associate to potentiate the MAPK pathway.

Raf is a serine/threonine kinase with its catalytic domain located at the carboxyl-terminal. The amino-terminal half of Raf contains a zinc finger structure which in its inactive state functions to obstruct its own carboxyl-terminal catalytic site (13). Furthermore the amino-terminal contains two binding sites for Ras-GTP to bind. Dual binding of Raf (140) along with the binding of the dimeric 14-3-3 proteins at the zinc finger (15, 66) is required for kinase activity to proceed. The function of Raf is to

phosphorylate and activate the MAP kinase kinase, named MEK, at serine residues 218 and 297 located in subdomain VIII (6, 127).

Once activated, MEK subsequently phosphorylates the p44 and p42 kinases, erk1 and erk2 respectively. These kinases have the diversity to phosphorylate and activate multiple other proteins including p90 ribosomal protein S6 kinase (RSK) (98, 188, 193), MAP kinase interacting kinase 1(Mnk1) (223) and tuberous sclerosis complex 2 (TSC2), (141) each having their own functions as discussed below.

RSK is a 90kDa enzyme with two catalytic domains and several sites for phosphorylation. Erk1 and 2 are known to phosphorylate threonines 360, 364 and 574. The latter is a site within the carboxy-terminal catalytic domain which when phosphorylated, leads to the autohphosphorylation of a serine residue at position 381. Together, the serine 381 and the threonine 364 phosphorylation are required for the activation of the amino-terminal catalytic domain used to continue the pathway (48). As the name implies, the primary function of RSK is to phosphorylate ribosomal protein S6 (rpS6), although it has other targets including TSC2, eukaryotic elongation factor 2 kinase (eEF2K) and eukaryotic initiation factor 4B (eIF4B) (210).

The molecular structure of Mnk1 contains an Erk interacting domain, Erk phosphorylation sites and, a carboxy-terminal catalytic domain. Unstimulated Mnk1 is exclusively bound to unphosphorylated Erk. Upon Erk phosphorylation and subsequent activation, Mnk1 becomes phosphorylated at 3 sites: threonine 197, 202 and 332. Phosphorylation of Mnk1 leads to its dissociation from Erk to bind and phosphorylate its target eukaryotic initiation factor 4E (eIF4E) at Serine 209; a step required for CAP dependent translation (223).

The fates of the targets of the MAPK pathway including TSC2, rpS6, eEF2K, eIF4B and eIF4E ultimately lead to enhancing protein synthesis each in a unique manor, although these signaling proteins are also influenced by the PI3K and mTOR pathways and thus will be described in greater detail below.

Phosphatidylinositol 3 kinase (PI3K) pathway

PI3K exists as a heterodimer consisting of an 85kDa regulating subunit with SH2 domains allowing for the physical association with phosphotyrosines on IRS1, and a 110kDa catalytic subunit capable of binding and phosphorylating phosphatidylinositols (PI). Specifically, PI3K binds and phosphorylates PI 3,4-bisphosphates (PIP_2) producing PI 3,4,5-trisphosphates (PIP_3). Recall from above that Ras also has the ability to activate PI3K. PIP_3 specifically attracts proteins that contain pleckstrin homology domains such as phosphoinositide dependent protein kinase 1 (PDK1) and protein kinase B (PKB), also known as AKT. Both of these proteins have pleckstrin homology and dock on membrane bound PIP_3.

As AKT binds to PIP_3 a conformational shape change occurs to increase the accessibility of its threonine 308 (7). The role of PDK1 in conjunction with the conformational change in AKT is to phosphorylate its threonine 308 (8). Moreover, PDK1 has a secondary role in the elaborate regulation of S6K1 as discussed in a latter section. AKT is a major hub for signal transduction as it functions to phosphorylate multiple effectors. Phosphorylation at threonine 308 although directly in the catalytic domain of AKT only partially activates AKT which is further enhanced by phosphorylation at serine 473, a site outside the catalytic domain close to the carboxy-terminal (8). Partial activation of AKT is sufficient for phosphorylating certain effectors

but not others. Details of each AKT target such as motor, TSC2, PRAS40, GSK3 and FOXO will be described in detail below as these targets are not solely regulated by AKT but also converge with other pathways.

Amino acid regulation of cell size

Although the exact mechanism has not been fully delineated, essential amino acids specifically leucine has an ability to activate the mTOR pathway via a direct or indirect mechanism (16, 21).

The essential amino acids enter the cell through a tertiary active transport system. The process starts with a system A, sodium dependent neutral amino acid transporter (SNAT2) which employs secondary active transport to import glutamine into the cell driven by the co-import of a sodium ion down its concentration gradient (112). The intracellular concentration gradient of glutamine then becomes the driving force for the anti-porter system L amino acid transporter (LAT1). LAT1 is a heterodimeric protein with a light subunit and glycosylated heavy subunit that functions by exchanging intracellular glutamine for extracellular leucine and other essential amino acid like isoleucine, valine, phenylalanine and tryptophan (99).

It is believed that SNAT2 can independently modulate cell size through a few separate mechanisms. Preliminary evidence suggests that SNAT2 may double as an amino acid sensor and directly trigger the activation of mTOR (99) or PI3K (62) pathways. Secondly, with every flux of the SNAT2, the membrane becomes more depolarized. It is believed that this slight depolarization may lead to an increased intracellular calcium ion concentration through voltage gated calcium channels (88). Intracellular calcium binds to the calcium binding protein, calmodulin (CaM) and

consequently induces a conformational change that increases its affinity to a class III PI 3-kinase, vacuolar protein sorting 34 (Vps34). Activated Vps34 forms a heterodimer with a kinase-like partner, Vps15 (17, 133), leading to the phosphorylation of PI creating PI-3-P (not to be confused with PIP_3). PI-3-P interacts with mTOR inducing a conformational change to increase its activity. Lastly, symport transporting leads to an osmotic gradient causing cell swelling. Heterodimer transmembrane proteins capable of detecting changes in cell volume called integrins can recruit a tyrosine kinase, focal adhesion kinase (FAK) to the cytosolic tail of one of the integrin subunits. Multiple FAK bound to integrins can cross phosphorylate and creating phosphotyrosine docking sites for Src kinase. Src kinase phosphorylate src at tyrosine 418 which subsequently phosphorylates and activates p38, erk1 and erk2 leading to an inhibition of proteolysis (220).

Independently, two other amino acid sensing mechanisms have surfaced in the literature linking to mTOR activation. Intracellular amino acids can activate a Ste20 family kinase, mitogen activated protein kinase kinase kinase kinase 3 (MAP4K3) using an unclear mechanism to phosphorylate its serine 170 (238). This phosphorylation site is required for amino acid induced activation of the mTOR pathway. Additionally, the proton assisted amino acid transporter, PAT1, which resides on endosomal and lysosomal membranes, is also critical for amino acid dependent mTOR activation. Again, although the exact mechanism linking to mTOR has yet to be uncovered, PAT1 is believed to export recycled amino acids from the lysosomes into the cytosol using the hydrogen ion gradient as the driving force (94).

The strongest link between amino acid availability and the regulation of cell size is through the Rag family of small GTPases which form heterodimers of subunit A or B

with subunit C or D. Each subunit binds its own guanyl nucleotide however subunits A and B are inactive when bound to GDP while subunits C and D are inactive when bound to GTP. Intracellular amino acids stimulate the reversal of the Rag-guanyl nucleotides to activate them (117). A trimeric protein complex consisting of p14, p18, and MAPK scaffold protein 1 (MP1) is collectively called the ragulater, and functions to tether the Rag-GTPases to late endosomes and lysosomes (176) to recruit and indirectly interact with mTOR through its regulatory-associated protein, RAPTOR (177). Translocation of mTOR to the endosome allows for enhanced activation by the small GTPase Ras homologue enriched in brain (Rheb) while in its active GTP state (177).

mTOR – The central hub for anabolic pathways

The mechanistic target of rapamycin (mTOR) is a large protein that exists in two complexes to serve distinct functions. mTOR is influenced by many sources including MAPK and PI3K pathways and amino acid availability as discussed earlier. In result, the regulation of mTOR is convoluted through elaborate protein signaling discussed in this section.

mTOR complex 1 (mTORC1) consists of mTOR, the regulatory-associated protein of mTOR (RAPTOR), proline-rich AKT substrate of 40kDa (PRAS40) and the G-protein β-like subunit protein (GβL). RAPTOR is a 150kDa protein consisting of a carboxy-terminal domain with seven WD repeats that binds mTOR at its FRB domain, the site competitively inhibited by rapamycin. It functions as a scaffold, by binding the mTOR substrates, ribosomal S6 protein kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) through an mTOR signaling (TOS) motif, in proximity to the catalytic domain allowing for phosphorylation of S6K1 on threonine 389 (36, 104)

and 4E-BP1 on threonine 37 and 46 (31, 36, 81). Without RAPTOR, mTOR activity is abolished (92). Likewise, GβL is also required for maximum mTOR activity, although it makes this contribution by binding directly to and stabilizing the mTOR catalytic domain (116). Lastly, PRAS40, while unstimulated, binds to the mTOR catalytic domain inhibiting mTOR kinase activity (211). As the name implies, the immediate upstream activator of PRAS40 is AKT which phosphorylates at threonine 246, inducing its dissociation from mTOR to bind 14-3-3 proteins thereby releasing its inhibitory effect on mTOR (121). Altogether, mTORC1, once activated has the ability to phosphorylate two main downstream effectors, S6K1 and 4E-BP1 (31, 36, 104) each of which independently contributes to an increase in protein synthesis as described below.

mTORC2 consists of mTOR, rapamycin-insensitive companion of mTOR (RICTOR), stress-activated map kinase-interacting protein 1 (SIN1), protein observed with RICTOR (PROTOR) and also GβL. The effects of mTORC2 are less known although it is activated by phosphorylation via AKT at serine 2448 and this activation positively feeds back by phosphorylating AKT on serine 473 which is required for full AKT activity as mentioned above (63, 179). mTORC2 dependent phosphorylation of AKT also serves to as a primer to enhance AKT susceptibility for phosphorylation on threonine 308. Additionally, mTORC2 dependent phosphorylation of AKT is also required for activation of forkhead box proteins O1 and 3 (FOXO1 and FOXO3) leading to decreased proteolysis thereby contributing to enhancing cell size (86).

While mTOR can be directly activated by phosphorylation at serine 2448 by AKT, this is not the only mechanism to activate mTORC1. As previously mentioned, the immediate upstream activator of mTORC1 is primarily Rheb, a farnesylated GTPase protein localized primarily to intracellular membranes (194). Although the natural

binding affinity is relatively low, the association of mTOR with endomembranes facilitates the probability of Rheb-TORC1 interactions (54, 194, 224, 237). In this case, Rheb has been verified to directly bind to the mTORC1 catalytic domain, GβL and Raptor's carboxy-terminal WD domain (14), all of which is necessary for mTORC1 activation (38, 200, 244). Surprisingly, in contrast to other GTPases, Rheb's affinity to binding is independent of its bound guanyl-nucleotide. However, only in Rheb's GTP state does this binding lead to the optimal configuration of the complex to promote m mTOR activity (132, 208).

Additionally, Rheb employs a separate mechanism to activate mTOR through an indirect mechanism involving phospholipase D1 (PLD1). It has been shown that Rheb directly binds and activates PLD1 in a GTP-dependent manor (192). Activated PLD1 hydrolyzes phsphatidylcholine into phosphatidic acid (PA) and choline where PA acts as a second messenger to directly bind and activate mTOR through its FRB domain (64, 65, 110). Additionally, it has also been shown that Rheb binds an endogenous mTOR inhibitor FKBP38 (19). Taken together, Rheb plays a dual indirect role in mTOR activation by binding and removing the inhibitory effects of FKBP38 while inducing PLD1 derived PA to directly bind and activate mTOR.

The activity of Rheb is dependent on its upstream regulator, TSC2 a GTPase activating protein that is also bound to mTOR although without direct mTOR regulation (79). As mentioned earlier as being activated by Erk and RSK, TSC2 is a major point of convergence for the activation of mTOR from other sources such as the direct influence from AKT and AMP activated protein kinase (AMPK). TSC2, also known as tuberin, forms an obligatory heterodimer with tuberous sclerosis complex 1 (TSC1), also known

as hemeratin (123, 126). This complex functions to hydrolyze Rheb-GTP into Rheb-GDP thereby inactivating Rheb and consequently mTOR.

Specifically, TSC2 is phosphorylated by AKT on serine 939 and threonine 1462 leading the dissociation of TSC1/2 and its inactivation, consequently, allowing Rheb to maintain its GTP ligand thereby activating mTOR (102, 149, 160). Conversely, AMPK, an enzyme activated in low energy states, directly phosphorylates TSC2 on threonine 1227 and serine 1345, also has further influences on phosphorylation on serine 1337 and 1341, and in contrast, leads to enhanced TSC1/2 activity (103). To this aspect, nutrients delivered to the cell should help reduce AMP levels and therefore prevent AMPK activation and its phosphorylation of TSC2. An additional site of regulation occurs at serine 1798 via phosphorylation by RSK which similar to AKT serves to inactivate TSC2 (173). Lastly, in a similar mechanism Erk also phosphorylates TSC2, although at serine 664, leading to TSC2 inactivation (141).

S6K1 pathway

There are two isoforms of S6K1, p70 and p85, arising from the same gene but alternative splicing. Both kinases have a catalytic domain and autoinhibitory domains separated by a linker domain, however the p85 also has an amino terminal nuclear localizing sequence (107). As result, p85 resides in the nucleus while p70 resides in the cytosol but have identical modes of action. In the basal state, the autoinhibitor domain interacts with the catalytic domain preventing any kinase activity. Activation of SK61 via mTOR requires an initial calcium dependent release of the autoinhibitory and catalytic domains, followed by phosphorylations at six locations. Phosphorylation of serine 424, 411, 418 and threonine 421 at the autoinhibitory domain are initiated by

mTOR inducing a conformational change exposing the linker domain. mTOR then has access to phosphorylate serine 371 and threonine 389 (104). This latter phosphorylation is required for the PDK1 dependent phosphorylation of S6K1 at threonine 229 and 252 in the catalytic domain to achieve maximal S6K1 activity (9, 162). S6K1 has many downstream targets namely, 40S ribosomal protein S6 (rpS6) (109), eEF2K (222) eIF4B (166), and eukaryotic initiation factor 4A (eIF4A) through an indirect approach (53). Each of these targets regulates protein translation as described in a later section. Furthermore, S6K1 also plays a role in transcription and splicing, by phosphorylating and activating the transcription factor CRE modulator (CREM) at serine 117 (49), phosphorylating the 80kDa-subunit of the CBC (CBP80) to enhance splicing (235), and S6K Aly/REF-like target (SKAR) at serine 383 and 385 used to couple transcription, splicing and RNA export (170).

rpS6 binds to the 40S subunit of the ribosome and is believed to stabilize the ribosome-mRNA interaction during translation. It can be phosphorylated by S6K1 and RSK at serine 235, 236, 240, and 244 (67). However, the role of phosphorylated rpS6 is under debate.

Eukaryotic elongation factor 2 (eEF2) mediates the transfer of a peptyl-tRNA from the ribosomal addition site, to the peptidyl site leaving the addition site vacant. This process also pushes the attached mRNA exactly 3 nucleotides exposing a new codon for tRNA binding. The activity of eEF2 is inhibited by its upstream kinase, eEF2K by phosphorylating eEF2 at threonine 56 (167). As previously mentioned the immediate upstream kinases of eEF2K are S6K1 and RSK which inhibits eEF2K activity by phosphorylating at serine 366 (222). Therefore, S6K1 and RSK enhance translation elongation by inactivating the inhibitor of eEF2.

Translation initiation complex regulation via mTOR

The regulation of eIF4E is the rate limiting step of CAP dependent translation initiation (83). A methylguanosine cap is constructed at the 5' end on the majority of mRNA transcripts about to be translated, and functions to be the site at which the translation initiation complex assembles (82). The role of eIF4E is to bind the cap and concomitantly bind another member of the translation initiation complex eIF4G. In the basal state, the activity of eIF4E is inhibited by its binding partner, 4E-BP1 which competes for the eIF4G binding site on eIF4E. Therefore, when 4E-BP1 is bound, eIF4E is unable to assemble the translation initiation complex (83). In addition to being bound to eIF4E, 4E-BP1, as discussed earlier, is tethered to mTORC1 through RAPTOR and is under the control of mTORC1. Upon appropriate stimulus, mTORC1 phosphorylates 4E-BP1 at threonine 37 and 46 (31) and appears to have indirect control of the phosphorylation of serine 65 and threonine 70, possibly through inhibition of the phosphatase PP2A (83, 105). The combination of phosphorylations changes the conformational shape of 4E-BP1 such that it dissociates from eIF4E. To further enhance the binding affinity for eukaryotic initiation factor 4G (eIF4G), eIF4E is also phosphorylated by Mnk1 at serine 209 (223).

eIF4G acts as a scaffold to bind other members of the initiation complex including the polyadenlyated binding protein (PABP), eIF4A, eIF4B and eukaryotic initiation factor 3 (eIF3). eIF4B is regulated by phosphorylation by AKT directly at serine 422 and by RSK and S6K1 at serine 406 (166, 210). eIF4B has slight helicase activity but has a role in stimulating eIF4A which has stronger helicase activity to linearize any tertiary structures in the untranslated region before the start codon (174).

eIF4B also aids with mRNA annealing with the ribosome (10). eIF4A is also under regulation through its inhibitor, tumor suppressor programmed cell death protein 4 (PDCD4) (53). Upon mTOR activation, S6K1 phosphorylates PDCD4 at serine 67 and becomes a target for degradation through its ubiquitin ligase βTRCP. When PDCD4 is degraded, eIF4A is free to bind eIF4G in the translation initiation complex. eIF3 recruits the 40S ribosome to the translation initiation complex as it also binds with eIF4G. Altogether, these components make up the full translation initiation complex called eukaryotic initiation factor 4F (eIF4F) and once assembled is ready to initiate translation (185).

That last level of regulation within translation initiation involves transporting the methionine charged tRNA to the ribosome and annealing with the mRNA. This process requires the GTPase eIF2 which binds to the charged tRNA in a GTP dependent manor. eIF2 is recharged with a GTP through its GEF, eIF2b; which is a point of regulation. eIF2b is a heteropentamer that can be phosphorylated on its ε-subunit by multiple different kinases at different phosphorylation sites (161). In response to nutrition, glycogen synthase kinase 3 (GSK3) is responsible to phosphorylate eukaryotic initiation factor 2B (eIF2B) at serine 540 (226). At this particular site, phosphorylation decreases it's activity and therefore is a negative regulator. Activation of the PI3K pathway stimulates AKT to phosphorylate GSK3 at serine 535 and inactivate its activity (46, 108, 227). To recapitulate, insulin enhances translation initiation through eIF2 activation via a PI3K induced inactivation of GSK3, which then relieves its negative regulation on eIF2B, which then regains the ability to recharge eIF2 with GTP, and thus increases eIF2 activity.

Proteolysis

In addition to enhancing anabolism, AKT also plays a role in the down-regulation of proteolysis through the FOXO family of transcription factors. Unstimulated FOXO naturally resides in the nucleus where it can bind DNA using its DNA binding domain and activate gene transcription at promoter regions. Activated AKT phosphorylates FOXO at serine 256, 319, and threonine 24 (22, 29, 119, 169, 198). These phosphorylations attract 14-3-3 proteins to bind and chaperone FOXO out of the nucleus into the cytosol where it is incapable of inducing transcription (30). There are multiple isoforms of FOXO, all with similar regulation, although specifically FOXO3 is known to act directly on the atrogin1 promoter (178). Atrogin1 is considered an ubiquitin E3 ligase that searches for and binds proteins specific proteins for degradation. In conjunction with E1 and E2 ligases, a polyubiquitin peptide is assembled on such a protein which is recognized by the 26S proteasome which subsequently degrades the protein into small peptides and free amino acids. Atrogin1 is known to target MyoD, a positive regulator of growth, and structural proteins myosin heavy chains, myosin light chains and myosin-binding protein C (43, 44, 128)

Finally, another nutrition dependent regulation of proteolysis is macroautophagy; protein degradation mediated by vesicular transport of cargo to the lysosomes to be broken down into individual amino acids. This process is essential during nutrient deprivation in order to free up essential amino acids. However, in environments of high nutrition, this process is inhibited in an mTOR dependent manor. The development of an early autophagosome, the vesicle that engulfs cargo, is triggered by an intact complex of three proteins, Atg13, ULK1 and FIP200, although mTOR is known to either directly hyperphosphorylate Atg13 and ULK1 causing its disruption from the rest of the complex

(97, 111). Moreover, phosphorylated ULK1 can also phosphorylate Atg13 to help further inhibit autophagosome development. Inhibition of autophagosomes development prevents the lysosomal degradation of protein and therefore inhibits proteolysis.

A comprehensive depiction of these pathways were published in the Journal of Cell Science and is included in the illustration below (130).

Mechanical stimulation and the regulation of cell size

In comparison to nutrient and hormone mechanisms, much less is understood about how mechanical stimuli can ultimately activate the cascade of cell signaling that results in cell growth. It was originally discovered that mechanical stimulation promotes an increase in insulin-like growth factor 1 (IGF-1), which can enhance cell size via the PI3K pathway similar to insulin (24, 25). Another proposed mechanism includes stretch activated ion channels (SACs) that can potentially generate an influx of calcium and activate the CaM/Vps34 pathway (186). Additionally, amino acid uptake has been shown to be increased during mechanical stimulation and thus may play a role in the activation of mTOR as described above (143). However recent studies have demonstrated that the major contributor of exercise induced cell signaling occurs through the production of PA with its known ability to activate mTOR as describe above (154). Although the precise mechanism is still unknown it is believed that mechanosensors can detect mechanical stimuli and relay the signal to enzymes involved with the production of PA from phosphotidylcholine, lysophosphatidic acid and/or diacylglycerol (69, 96).

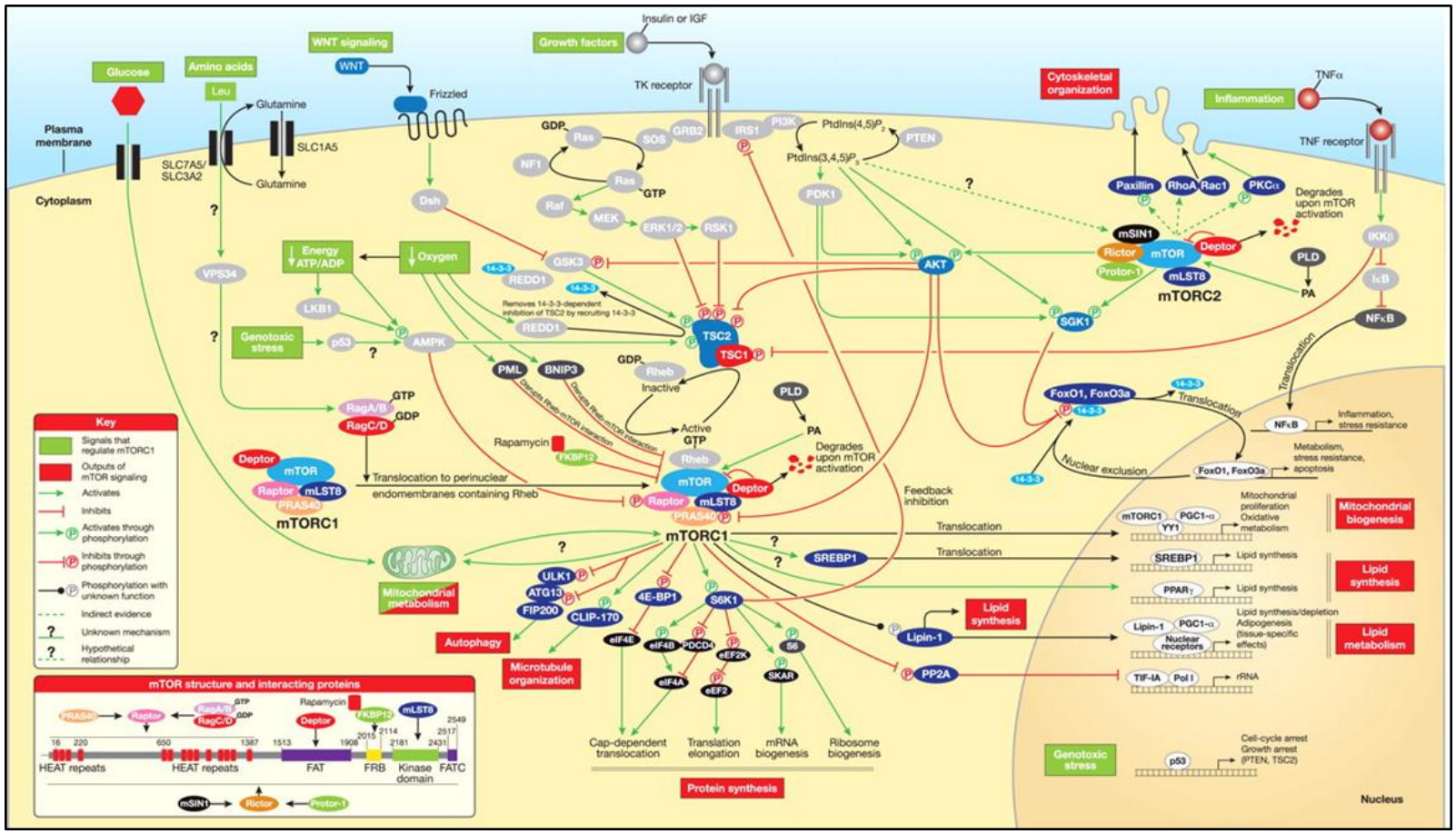


Illustration 1: Illustration of how various stimuli can signal within a cell to regulate growth. Reproduced with permission (130).

While a lot of research has been conducted in the past couple decades to determine the mechanisms of nutrient, hormonal or exercise induced muscle hypertrophy, a novel style of resistance called blood flow restriction exercise doesn't appear to fit any of the molds

BLOOD FLOW RESTRICTION EXERCISE

It was originally postulated in the 1990's that metabolic stress and muscle fatigue may play a large role in hypertrophy induced by resistance exercise (171, 181). By the turn of the millennium, a new exercise technique emerged called blood flow restriction (BFR) exercise, where human subjects would exercise with a device that applies pressure around the most proximal region of the exercising limb, restricting arterial blood flow to the active muscle and occluding venous return (183, 197). This exercise procedure quickly fatigues all fibers of the active muscle to a great extent in a short period of time, even with a relatively low resistance. Since its innovation, multiple studies have demonstrated that blood flow restriction during low intensity exercise (20% one-repetition maximum [1RM]) is sufficient to induce an increase in muscle strength (2, 4, 35, 61, 145, 156, 197, 240), muscle hypertrophy (2, 4, 145, 197), muscle function (3, 242) and muscle protein synthesis (73, 75) similar to traditional exercise training. This is a unique phenomenon because it was previously well accepted that muscle hypertrophy and strength improvements are achieved by lifting loads greater than 70% of their 1RM (122, 165). The minimum level of resistance needed using BFR has not been determined but have observed hypertrophic effects using BFR in conjunction with walking (2, 3) or simple body weight exercises (242). Additionally, tourniquet type, thickness and restriction pressures have been inconsistent among the literature, yet these variables

appear to have minimal influences on the results (136, 137). Lastly, BFR exercise training has also been tested in both men women, and shows no sex-based differences with regards to strength and hypertrophy (156, 175).

While BFR exercise has clear hypertrophic effects on skeletal muscle, there is little evidence to suggest that it is a superior method to traditional resistance exercise with heavy loads in healthy young adults. However, given that not all populations are capable of lifting heavy loads, BFR exercise may have applications in patients recovering from injuries or operations, or those who have osteoporosis or arthritis. Interestingly, BFR exercise has been evaluated in the young; 18-35 years of age; (75) and the old; 65-75 years of age (73) with similar increases in muscle protein synthesis indicating that BFR exercise uses a mechanism that overcomes the unexplained impaired anabolic response to resistance exercise in older individuals. Therefore, BFR exercise may be a useful tool to combat sarcopenia and other causes of muscle atrophy. In fact, training studies in elderly populations have already determined benefits of BFR exercise in the enhancement of muscle strength and size, as well as functional outcomes relating to quality of life (3, 242).

Safety

Considering the unusual practice of blood flow restriction exercise, some concern about its safety has been raised. Research in the literature has investigated some of these concerns and has yet to observe any adverse changes in the vascular system or the peripheral nervous system. Clark *et al.* analyzed the acute and chronic effects of BFR exercise compared to high-intensity exercise to determine if there was any added risks of performing BFR exercise (42). The authors of this study indicated that after 4 weeks of

training there are no changes in arterial stiffness, as measured by the pulse wave velocity; no change in the ankle brachial index, which measures a drop in blood pressures in the arteries of the legs; no change in plasma clotting time, and no changes in nerve conduction velocity in the sensory or motor neurons. Additionally, on an acute scale, there are no changes in the coagulation response as measured by fibrinogen or its degradation product, D-dimer content, and no changes in acute inflammation as measured by high-sensitivity C-reactive protein. These results support similar finding as encountered by other groups (73, 138, 144).

Potential Mechanisms

Although BFR exercise has been proven useful, the mechanism remains ultimately unknown. Unfortunately, BFR exercise is still in the early phases of implementation and the practicality of the exercise has not been determined. Testing a specific mechanism of BFR exercise could prove to be beneficial to seek an explanation for the differential age related responses to exercise training. In addition, elucidation of the BFR mechanism inducing positive muscle adaptations can be used to develop new, therapeutic interventions to overcome muscle atrophy in all populations.

Several correlations have surfaced in the literature including increased motor unit recruitment (197, 229), significant muscle damage and delayed onset muscle soreness (207), all of which are believed to be precursors to the muscle adaptation. However, the most commonly reported correlation in the literature is the involvement of the endocrine system. Several studies report a post-BFR exercise surge in growth hormone (GH) along with cortisol, epinephrine and norepinephrine (73, 75, 101, 146, 157, 159, 195). It is believed that the GH response is caused by the local accumulation of metabolites during

exercise stimulating intramuscular metaboreceptors that activate the hypothalamic-pituitary axis; a process called muscle metaboreflex (85). While this correlation has been repeatedly confirmed, no causality has been established nor has it been refuted. A physiological increase in GH does not have significant implications in muscle adaptations post-exercise (230, 232), however the robust increase in GH post-BFR exercise has been reported to be as high as 290 fold above baseline (196); far above a normal GH response and has not been investigated on any mechanistic level.

More recently, researchers have been investigating the effects of BFR exercise at the level of the myocyte. Nielsen *et al* discovered that both fast and slow twitch muscle fibers hypertrophy to an equal extent with a concomitant increase in myogenic satellite cells, leading to the increase in myonuclei per muscle fiber and a maintenance of the myonuclear domain (153). This data supports prior research demonstrating an increase in myogenic genes and a decrease in myostatin gene expression, each of which influence the activation of myogenic satellite cells (59, 131). Researchers have also made associations with the mTORC1 (73, 75) and MAPK (73) signaling cascades which, as aforementioned, regulates translation initiation leading to an increase in protein synthesis.

Despite our knowledge of such cellular processes, the upstream stimulus activated by BFR exercise to induce its effects remains unknown. One highly debated idea is the concept of metabolic stress. In addition to the common observation that BFR exercise generates and accumulates large amounts of lactic acid (73, 75, 197), Suga *et al.* showed that BFR exercise significantly increases the intramuscular accumulation of inorganic phosphate and deprotonated phosphate, the depletion of phosphocreatine (PCr) and a drop in pH (189-191). This conglomerate of metabolites is known to impact muscle on several levels including the interference calcium binding troponin thereby interfering

with muscle contraction, inhibition of glycolysis and the activation of class III and IV muscle afferents which all contribute to muscle fiber fatigue (28). As muscle fibers fatigue, higher threshold motor units need to be activated in order to continue the exercise, leading to the recruitment of fast twitch muscle fibers. Fast twitch fiber activation has been confirmed to occur during BFR exercise with the observation of inorganic phosphate peak splitting measured by magnetic resonance spectroscopy (189-191). This is noteworthy because typically, hypertrophy occurs when all muscle fibers have been recruited during training. On the other hand, a systemic effect has been noted on a few occasions where a muscle not affected by limb restriction, such as the gluteus maximus during squats (5) or the pectoralis major during bench press (240, 241), can hypertrophy from BFR exercise. Interestingly, Madarame *et al.* conducted a study where subjects performed BFR exercise with their legs following non-occluded low-intensity exercise of the elbow flexors which resulted in improvements in elbow flexor size and strength. These examples of a crossover effect have yet to be explained or mechanistically investigated.

Along with human experimentation, animals have been used to develop additional ideas to uncover the mechanism behind BFR exercise. A rodent model of BFR exercise has been associated with increases in heat shock proteins, proteins known to inhibit proteolysis (115). The same study also observed an increase in nitric oxide synthase-1, which has been linked to satellite cell activation through hepatocyte growth factor and its c-MET receptor (12, 199). Nitric oxide also has a role to regulate the expression of cyclooxygenase 2 (COX-2) in skeletal muscle which has been shown to enhance protein synthesis through prostaglandin production (184, 206). However, neither heat shock proteins nor nitric oxide synthesis has been shown to be up-regulated in humans after

BFR exercise. Lastly, ischemia-reperfusion has been linked with reactive oxygen species (ROS) production. While counterintuitive considering its role in atrophy, ROS is also linked to mTOR signaling in cells (18, 90, 134), which has not been ruled out as a potential mechanism of BFR exercise. In short, more mechanistic human research is needed to determine or eliminate potential mechanisms for BFR exercise.

CONCLUSIONS

In summary, skeletal muscle maintenance is important for all individuals, considering the strong correlation with muscle mass and the ability to overcome an illness or an injury. Maintenance of skeletal muscle mass is particularly important for the elderly population, who are more prone to disease and disability. Unfortunately, the aging process is associated with the gradual loss of skeletal muscle along with a decrease in the potential to rebuild muscle through nutrition or exercise interventions. In contrast, for reasons unknown at this point, BFR exercise seems to employ a unique mechanism that has the ability to overcome this phenomenon. Research into the mechanism(s) underlying the effects of BFR exercise may provide a clearer understanding of exercise-induced regulation of muscle mass. Additionally, this knowledge would provide insight as to why aging is associated with a blunted muscle anabolic response. Therefore, the purpose of the following studies is to mechanistically investigate the most prominent theories of how BFR exercise elicits the muscle anabolic response in this unconventional manor.

CHAPTER 2

Reactive hyperemia is not responsible for stimulating muscle protein synthesis following blood flow restriction exercise[1]

INTRODUCTION

It has been recently shown that increased blood flow and nutritive delivery are critical for an anabolic stimulus such as insulin to increase muscle protein synthesis (MPS) (201, 203). The anabolic effects of insulin have been shown to increase muscle protein synthesis in young adults but a blunted response is observed in older adults (201, 217), a pattern consistent with traditional resistance exercise. Insulin can activate signaling protein AKT which is known to be a regulator of growth. Also, insulin increases blood flow by inducing a nitric oxide-dependent vasodilation of the precapillary arterioles in skeletal muscle. Timmerman *et al*. has recently shown that pharmacological vasodilation in the old restores insulin's ability to induce muscle protein synthesis (202), while preventing vasodilation in the young also blocked the anabolic response to insulin (201). Together, this indicates that a hyperemic response is required to respond to the anabolic effects of insulin. One can speculate that this concept may be true for other anabolic stimuli such as low intensity resistance exercise.

The aim of this experiment was to determine whether reactive hyperemia following BFR exercise plays a major role in stimulating anabolic and catabolic cell signaling and MPS. To test this, the current study compares the effect of low-intensity

[1] Excerpts from Gundermann DM, Fry SC, Dickinson JM, Walker DK, Timmerman, KL, Drummond MJ, Volpi E, Rasmussen BB. Reactive hyperemia is not responsible for stimulating muscle protein synthesis following blood flow restriction exercise. J Appl Physiol. 112: 1520-1528, 2012. ©American Physiological Society, reproduced with permission

exercise with BFR, and low-intensity exercise with pharmacologically-induced hyperemia, on MPS and mTORC1, MAPK signaling and the ubiquitin proteasome pathway using a randomized, cross-over design in young healthy men. It was hypothesized that pharmacologically-induced hyperemia following low-intensity exercise would mimic the muscle protein anabolic effects of BFR exercise.

EXPERIMENTAL PROCEDURES

Subjects

Six young, healthy, recreationally active males (24 ± 2y; 181 ± 1cm; 82 ± 5kg) volunteered for this study. Each subject gave written informed consent before participating in the study, which was approved by the Institutional Review Board of the University of Texas Medical Branch. Screening was performed with clinical history and physical exams, electrocardiogram, and laboratory tests including complete blood count with differential liver, kidney and thyroid function tests, coagulation profile, fasting blood glucose, oral glucose tolerance test, hepatitis B and C screening, HIV test, urinalysis and drug screening. A variety of anthropometric measurements were taken along with a Dual-Energy X-Ray Absorptiometry (DEXA) scan (Hologic QDR 4500W, Bedford, MA) and a 1RM leg extension strength test. The strength test was performed on two occasions (>5 days apart) on a bilateral leg extension machine (Cybex-VR2, Medway, MA) located in the Institute for Translational Sciences Clinical Research Center (ITS-CRC) Exercise Laboratory. The strength test consisted of an initial warm-up phase with the subject performing 10 repetitions at a moderate weight to get acclimated with the machine. Following the warm-up, subjects attempted to complete 1 repetition of

the leg extension exercise at progressively greater resistance until a full repetition could no longer be performed. The weight of the last complete unassisted repetition was recorded as the 1RM. The highest weight achieved from the two strength tests was deemed their 1RM. Any subjects who lifted the entire weight stack during the strength test would continue repetitions to complete failure. In this case, the 1RM would be calculated by the formula: $1RM = \frac{W}{1.0138-(0.0267123)(R)}$ where "W" represents the weight lifted in kilograms and "R" represents the number of successive repetitions (129). The average 1RM was 135 ± 16 kg.

Study Design

Each subject was randomized to initially undergo one of two treatments: 1) low-intensity exercise with BFR; or 2) low-intensity exercise followed by pharmacologically-induced hyperemia using the vasodilator, sodium nitroprusside (SNP). At least three weeks were allowed between trials.

On both occasions, the subjects were admitted to the ITS-CRC the evening before the exercise study. They were fed a standard research dinner (10 kcal/kg of body weight; 60% carbohydrate, 20% fat and 20% protein) and snack before 2200. They were fasted overnight under basal conditions and were given ad libitum access to water. The morning of the study at 0600, a polyethylene catheter was inserted into an antecubital vein for tracer infusion and another polyethylene catheter was inserted retrogradely into a hand vein of the opposite arm and kept under a heating pad for arterialized blood sampling. After drawing a background blood sample, a primed continuous infusion of L-[*ring*-$^{13}C_6$] phenylalanine (Isotec Inc., Sigma-Aldrich, Miamisburg, OH) was begun and maintained at a constant rate until the end of the experiment (Illustration 2). The priming

dose for the labeled phenylalanine was 2µmol/kg, and the infusion rate was 0.05µmol/kg/min.

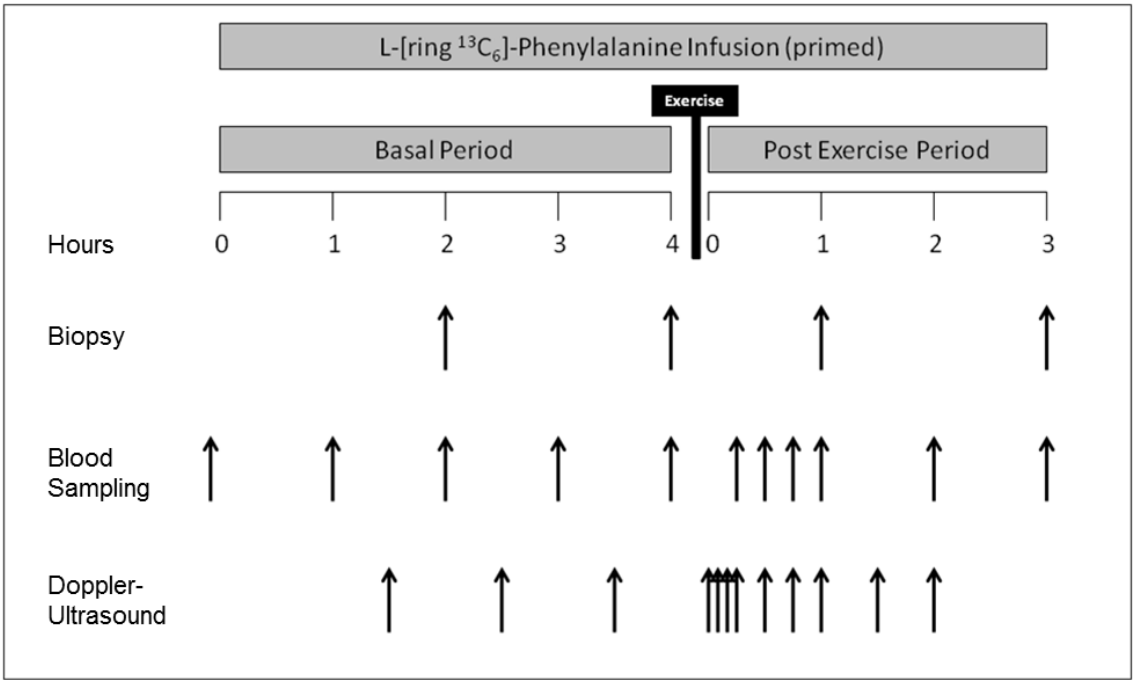


Illustration 2: Infusion study protocol. Doppler-ultrasound measurements, blood and muscle samples are indicted by arrows. Study design was identical for both groups.

Exercise

After a 4 hour basal period subjects were transported from the bed to the leg extension exercise machine where they performed 4 sets of bilateral leg extensions at 20% of their 1RM. The 4 sets consisted of 30, 15, 15 and 15 repetitions respectively with 30 seconds of rest between sets. Verbal encouragement was used to ensure each subject completed the full exercise protocol. Blood pressure and heart rate were measured and recorded immediately before and after exercise.

BFR trial

Immediately prior to the commencement of exercise, subjects performing the BFR trial were fitted with 11cm wide pressure cuffs (Hokanson SC10, Bellevue, WA, USA) placed on the most proximal portion of the upper thighs and attached to a Hokanson E20 Rapid Cuff Inflater and AG101 Air Source (Bellevue, WA). Subjects were gradually acclimated to the pressure cuff in increments of 20mmHg starting at 120mmHg to a final pressure of 200mmHg. During the acclimation phase each increment consisted of 30 seconds of pressure followed by 10 seconds of no pressure. Exercise commenced once the final pressure of 200mmHg was reached. The pressure was not released until the completion of the final set of exercise. Reactive hyperemia occurs immediately after the cuff pressure is released, therefore the subjects were promptly moved back into bed to capture the post-exercise blood flow response using Doppler-Ultrasound.

SNP trial

This trial was designed to mimic the reactive hyperemia induced following BFR exercise by pharmacologically vasodilating the femoral artery after low-intensity resistance exercise performed without blood flow restriction. During this trial only, a polyethylene catheter was placed in the common femoral artery of the same leg designated for muscle sampling and blood flow measurements. Prior to exercise, SNP was prepared with a concentration of 4mg/60mL in a syringe and connected to the femoral artery catheter. Following completion of the identical exercise protocol without blood flow restriction, SNP was infused into the femoral artery for one hour, initially at a rate of 0.14μg/min/100mL of leg volume then modified based on blood flow measurements to closely simulate the average reactive hyperemic response from BFR

exercise. As a precaution Heart rate and blood pressure were closely monitored from the femoral artery catheter and at the brachial artery for the duration of the one-hour SNP infusion.

Muscle sampling

The muscle biopsies were performed with a 5-mm Bergström biopsy needle utilizing sterile procedures and local anesthesia (1% lidocaine). Prior to exercise two muscle biopsies were obtained from the lateral portion of the *vastus lateralis* muscle between 15 and 25 cm superior to the mid-patella (Fig 1). The first biopsy was collected 2 hours after the initiation of the tracer infusion, to allow for steady state enrichments, and again 2 hours later to determine basal mixed-muscle fractional synthetic rate (FSR). Both biopsies were sampled from a common incision although the orientation of the needle was angled so that samples were taken ~5cm from each other. The third and fourth biopsies were taken 1 and 3 hours post-exercise from a second incision site proximal to the first incision on the same leg. It has been demonstrated that sequential biopsies from the same incision does not interfere with MPS measurements (214). Muscle tissue was immediately blotted, frozen in liquid nitrogen, and stored at -80°C until analysis. Right and left legs were randomly assigned to either trial; the opposing leg was used when crossing over to the other trial.

Blood flow measurements

Three blood flow measurements were conducted during the basal period and averaged together to establish a pre-exercise basal blood flow value. Flow measurements

were taken 3 minutes after the completion of exercise to allow time to remove the BFR apparatus and allow the subject to return to bed. Subsequent measurements were made every minute during the first 15 minute period to capture the immediate vascular response to BFR exercise. Thereafter, measurements were taken every 15 minutes for the remainder of the first hour post-exercise and additional measurements were made at 90 and 120 minutes post exercise to verify blood flow had returned to baseline. Blood flow measurements were performed on the same leg where the muscle biopsies were taken.

Femoral artery blood flow was determined using a two-dimensional Doppler ultrasound machine (HDI-500 ultrasound system, Philips ATL Ultrasound, Andover, MA). Online calipers were used to measure the diameter (d) of the longitudinal view of the femoral artery in quadruplicate with a high definition zoom image. Additionally, the time-averaged mean velocity (TAM) was determined using pulsed-wave Doppler with angle correction and analysis software. Femoral artery blood flow (Q) was calculated by the formula: $Q = TAM \cdot \pi(^{d}/_{2})^{2}$.

Mixed Muscle Protein FSR

Muscle intracellular free amino acids and muscle proteins were extracted as previously described (215, 216). Muscle intracellular free concentration, and enrichment, of phenylalanine was determined by gas chromatography-mass spectrometry (GCMS; 6890 Plus GC, 5973N MSD, 7683 autosampler Agilent Technologies, Palo Alto, CA) using an appropriate internal standard (L-[^{15}N]phenylalanine) (215, 216). Mixed muscle protein-bound phenylalanine enrichment was analyzed by GCMS after protein hydrolysis and amino acid extraction with the external standard curve approach (37). The FSR of mixed muscle proteins was calculated by measuring the incorporation rate of the

phenylalanine tracer into the proteins ($\Delta E_p/t$) and using the precursor-product model to calculate the synthesis rate:

$$FSR = (\frac{\Delta E_p}{t})/(\frac{E_{M1} + E_{M2}}{2}) \cdot 60 \cdot 100$$

where ΔE_p is the increment in protein-bound phenylalanine enrichment between two biopsies, t is the time between the two biopsies, and E_{M1} and E_{M2} are the phenylalanine enrichments in the free intracellular pool of the two biopsies. Data are expressed as percent per hour.

Blood sampling

Arterialized blood was collected from the retrograde hand catheter twelve times during the study (Illustration 2). Each time, nine milliliters were aliquoted for analysis of plasma glucose and lactate (Yellow Springs Instruments Co., Yellow springs, OH); and for the determination of the enrichment of labeled phenylalanine in blood using GCMS. Phenylalanine concentration was calculated using an internal standard approach as described by Wolfe and Chinkes (236) and nutrient delivery was calculated by: $(blood\ concentration)\ x\ (femoral\ arterial\ blood\ flow)$.

SDS PAGE and Western Blot Analysis

Details of the immunoblotting procedures have been published previously (56). Briefly, ~30-50mg of frozen tissue was homogenized (1:9 wt/vol) and centrifuged at 6000 RPM for 10 min at 4°C, followed by the removal of the supernatant. Total protein concentrations were determined in supernatant by using a Bradford assay (Bio-Rad, Hercules, CA, Smartspec Plus spectrophotometer). The supernatant was diluted (1:1) in

a sample buffer mixture containing 125mM Tris (pH 6.8), 25% glycerol, 2.5% SDS, 2.5% β-mercaptoethanol, and 0.002% bromophenol blue and then boiled for 3 min at 100°C. 50μg of total protein was loaded into each lane of a 7.5% or 15% polyacrylamide gel (Bio-Rad, Criterion) along with a molecular weight marker (Bio-Rad, Precision Plus protein standard) and separated by electrophoresis at 150V for 60 min. Protein was then transferred to a polyvinylidenedifluoride membrane (Bio-Rad) at 50V for 60 min. Blots were blocked in Tris-Buffered Saline with Tween 20 and 5% non-fat dairy milk then incubated in a single primary antibody overnight at 4°C (antibody concentrations are described below). The next day, blots were incubated in anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham BioSciences, Piscataway, NJ) for one hour at room temperature. Chemiluminescent solution (ECL plus, Amersham BioSciences, Piscataway, NJ) was applied to each blot for five minutes and optical density measurements were obtained with a phosphoimager (Bio-Rad) and densitometric analysis was performed with Quantity One software (Bio-Rad, version 4.5.2). Data are expressed as the fold change from baseline.

Antibodies

The following primary antibodies were purchased from Cell Signaling (Danvers, MA) and diluted in 5% bovine serum albumin: phospho-mTOR (Ser^{2448}, 1:500), phospho-p70 S6K1 (Thr^{389}, 1:250), phospho-rpS6 ($Ser^{240/244}$, 1:250), phospho-eukaryotic initiation factor 4E binding protein 1 (4E-BP1) ($Thr^{37/46}$, 1:1000), phospho-mitogen-activated protein kinase-interacting kinase 1 (Mnk1) ($Thr^{197/202}$, 1:500), phospho-extracellular signal-related kinase 1/2 (ERK1/2) (Thr^{202}/Tyr^{204}, 1:1000), phospho-eukaryotic elongation factor 2 (eEF2) (Thr^{56}, 1:2000), phospho-Akt (Thr^{308}, 1:1000). The following primary antibodies were purchased from ECM Biosciences (Versailles, KY)

and diluted in 1% non-fat dairy milk in Tris-Buffered Saline with Tween 20: muscle atrophy F-box protein-1 (Atrogin1) (1:1000), and muscle RING finger protein-1 (MuRF1) (1:1000).

Gene Expression

Details of RNA isolation, cDNA synthesis and Real-Time quantitative PCR (qPCR) have been describe previously (60). Briefly, 20-30mg of muscle was homogenized in 1mL of TriReagent (Molecular Research Center, Inc., Cincinnati), separated using 0.2mL of Chloroform, precipitated using 0.5mL of Isopropanol and washed with 75% ethanol. RNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wailmington, DE). To eliminate any potential DNA contamination, all samples were treated with DNase, using a commercially available kit (DNA-free, Ambion, Life Technologies, Grandisland, NY), according to the manufacturer's directions. RNA templates were reverse transcribed into cDNA and all isolated RNA and cDNA samples were stored at -80°C until analysis. Primer sequences used in this experiment have been previously published by Drummond *et al* (59). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene as it remained stable across time and after exercise for both trials. Real-time RT-PCR was performed using an iQ5 Multicolor Real-Time PCR cycler (BioRad) using SYBR Green fluorescence (iQ SYBR Green Supermix, BioRad). Relative fold changes were determined using the $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen (135) using the following equation: $Fold = 2^{(Ct_{TG}-Ct_{HG})_{t_x}-(Ct_{MTG}-Ct_{MHG})_{baseline}}$ where Ct is the cycle threshold, TG is the target gene, HG is the housekeeping gene and t is

time. One subject was dropped from analysis of both trials due to unstable GAPDH expression between samples.

Statistical Analysis

All values are expressed as means ± SE. Comparisons were performed using a 2-way ANOVA with repeated measures, with the effects being trial (BFR, SNP) and time. Post hoc analysis was performed with Bonferroni when appropriate. Natural log transformations were performed to establish normality where necessary. Significance was set at $P \leq 0.05$. All analyses were done with SigmaStat 11.0 (Systat Software, San Jose, CA).

RESULTS

Cardiovascular Response

There were no significant differences in exercise heart rate (96±9.8 vs 94±13), systolic pressure (143±5.6 vs 125±8.3), or diastolic pressure (71.5±4.5 vs 71±5.1) between BFR and SNP trials respectively ($P>0.05$). Heart rate significantly increased from rest only after BFR trial ($P<0.05$). Blood pressure and heart rate were continuously monitored during the SNP infusion with no significant fluctuations observed with any subjects.

Blood flow response

Femoral artery blood flow was significantly elevated post exercise during both trials ($P<0.05$). Blood flow remained significantly elevated above basal values in both

groups at 5, 10 and 15 minutes post exercise (P<0.05, Figure 1A). SNP infusion increased blood flow immediately post-exercise, however, the initial response in the BFR trial was greater than in the SNP trial for the first 10 minutes post-exercise (P <0.05). On the other hand, the total blood flow was not different (P >0.05) between trials as calculated by the area under the blood flow response curve for 1 hour (Figure 1B) and 2 hours post exercise (data not shown).

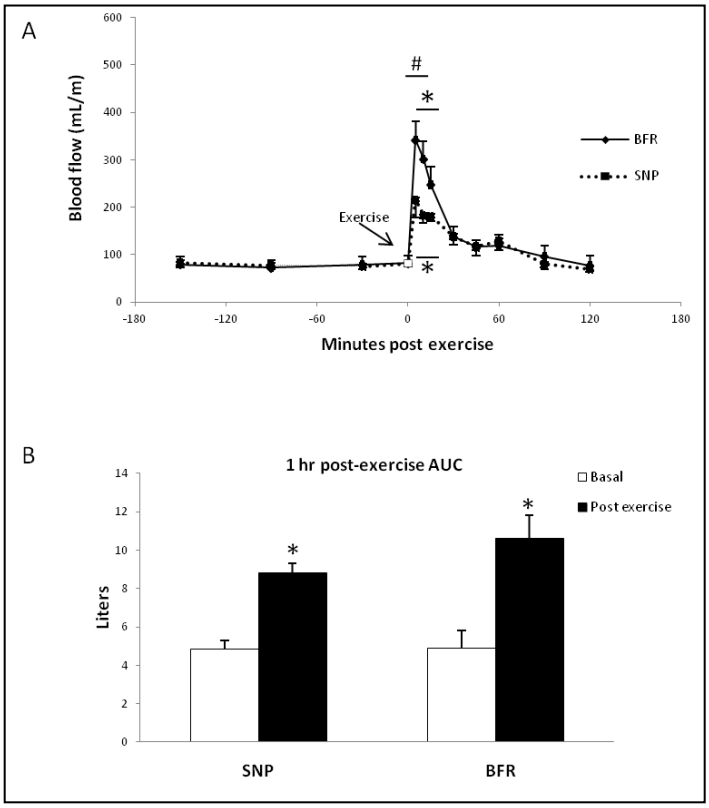


Figure 1: A. Femoral artery blood flow measured by Doppler-ultrasound. Data are presented as mL/min. Error bars represent SE. *P<0.05 vs baseline. #P<0.05 vs SNP. Open symbol represents the cumulative average basal blood flow as a reference point prior to exercise. B. Total femoral arterial blood flow within the first hour following exercise as calculated by the area under the blood flow rate curve (AUC). Presented as liters of blood through the femoral artery over the hour. Error bars represent SE. *P<0.05 vs baseline.

Arterialized Plasma Concentrations

Glucose (Figure 2A) and phenylalanine (Figure 2B) delivery (substrate concentration x blood flow) increased significantly during the first hour following exercise in both trials as determined by the area under each delivery curve ($P<0.05$). There were no differences between trials for either glucose or phenylalanine delivery.

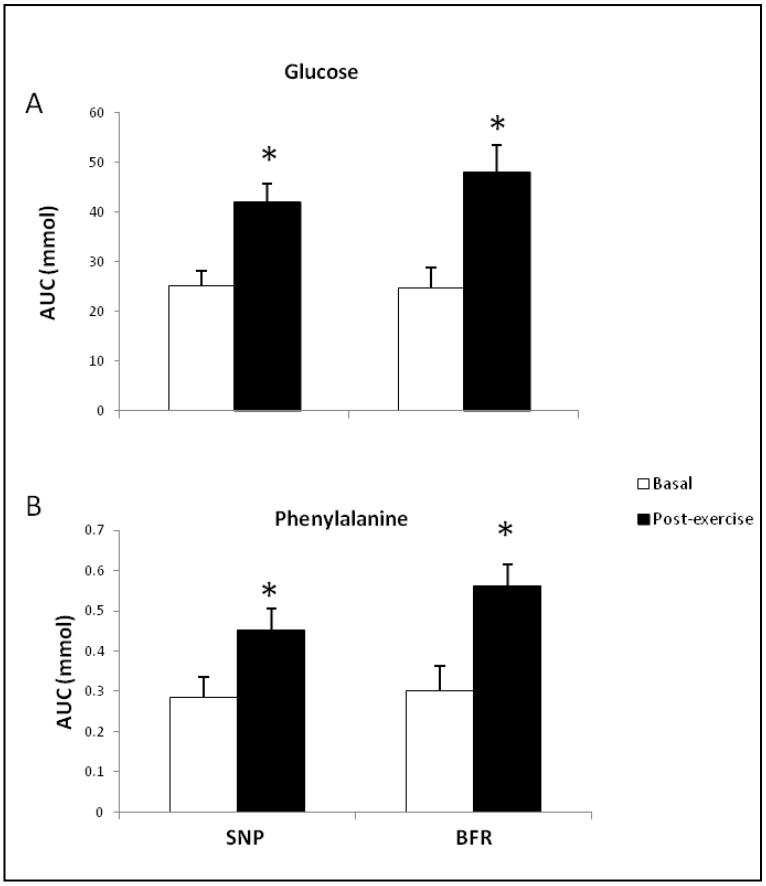


Figure 2: A. Total glucose delivery (blood glucose x blood flow) within the first hour post-exercise as calculated by the area under the glucose delivery curve. Presented as mmol of glucose through the femoral artery over the hour. Error bars represent SE. *$P<0.05$ vs baseline. B. Total phenylalanine delivery (blood phenylalanine x blood flow) within the first hour post-exercise as calculated by the area under the phenylalanine delivery curve. Presented as mmol of phenylalanine through the femoral artery over the hour. Error bars represent SE. *$P<0.05$ vs baseline.

The plasma lactate concentration increased by 3.3 fold following exercise relative to basal values during the SNP trial and remained elevated for 45 minutes ($P<0.05$). Plasma lactate during the BFR trial increased by 7.3 fold following exercise relative to basal values and remained elevated above baseline for 1 hour post exercise ($P <0.05$). Plasma lactate concentrations during the BFR trial were significantly greater as compared to the SNP trial for 90 minutes post exercise (Figure 3; $P<0.05$).

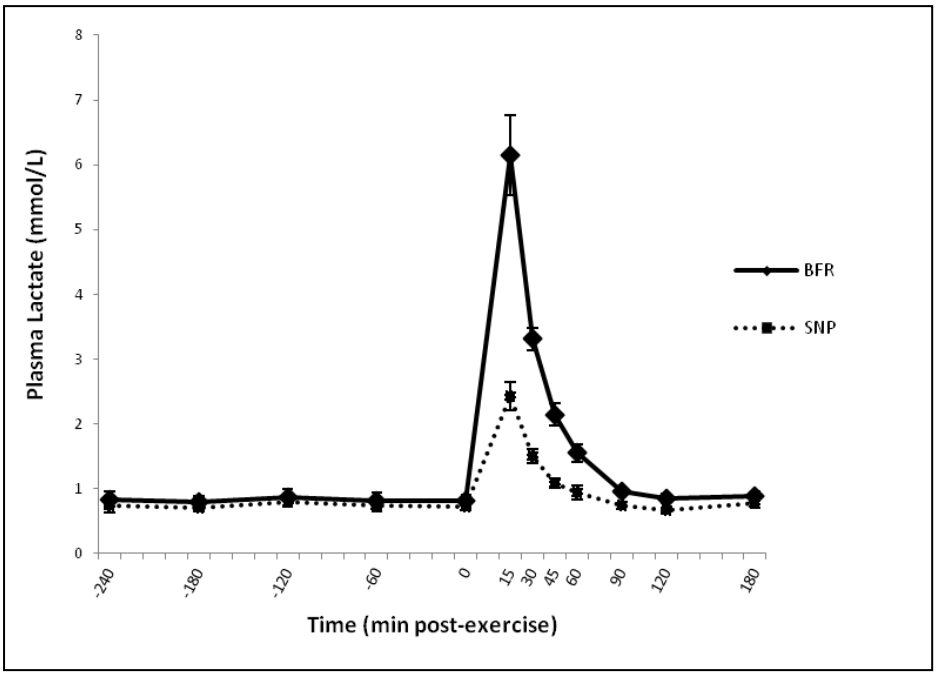


Figure 3: Plasma lactate concentrations. Data are presented as mmol/L. Error bars represent SE. At 15 min, 30 min and 45 min post exercise, lactate values were significantly greater than basal and between trials ($P<0.05$). At 1h post exercise plasma lactate was still elevated above basal during the BFR trial and was higher during the BFR trial than the SNP trial ($P<0.05$). At 1.5 h post exercise, plasma lactate was not different from basal ($P>0.05$) although lactate concentrations during the BFR trial were higher than the SNP trial ($P<0.05$). No other time or group differences were detected ($P>0.05$).

Muscle Protein Synthesis

Mixed muscle protein FSR was not different between trials at basal (P>0.05). FSR during the three hours post exercise was elevated above basal only during the BFR trial (Figure 4; P<0.05).

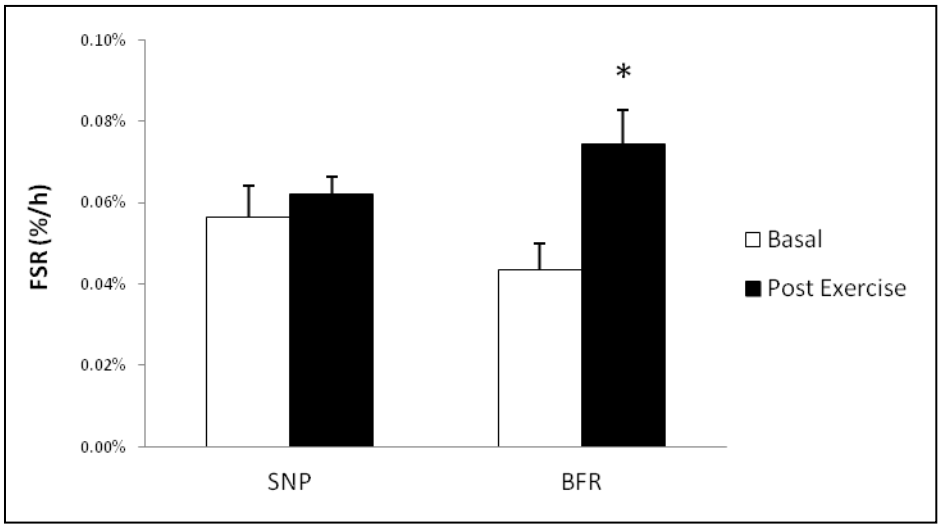


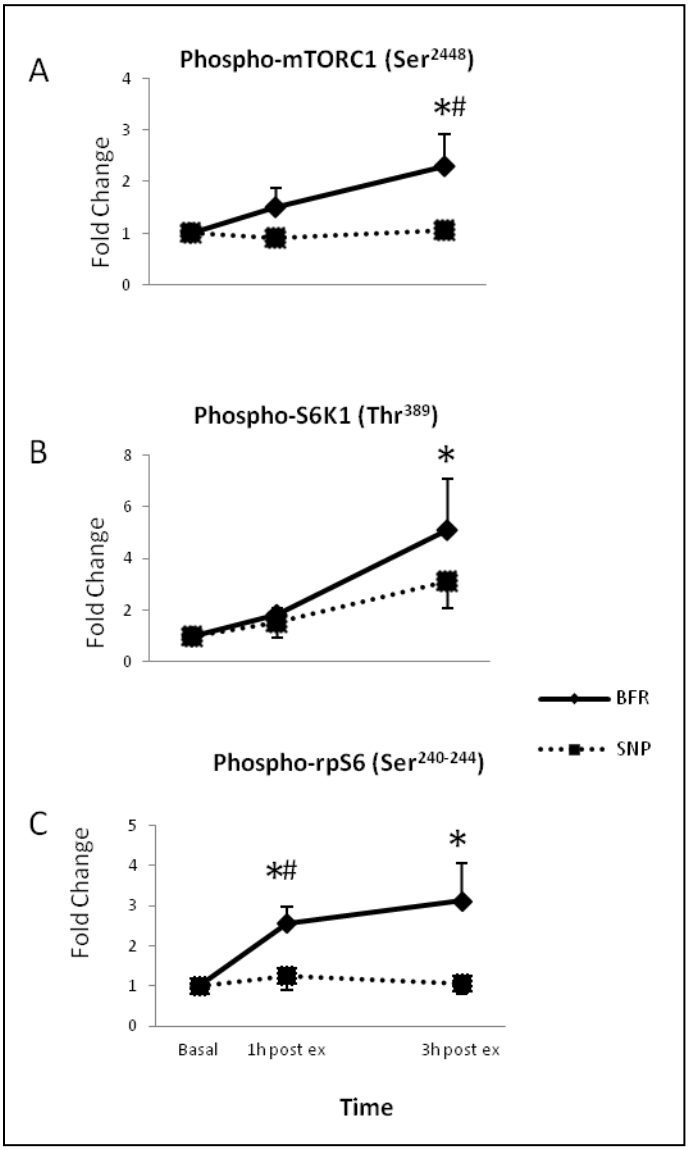
Figure 4: Mixed muscle fractional synthetic rate at baseline and 3 hours post exercise. Presented in percent per hour (%/h). Error bars represent SE. *P<0.05 vs baseline. #P<0.05 vs SNP.

Anabolic and Catabolic Cell Signaling

mTORC1 signaling

Phosphorylation of Akt at Thr^{308} was higher at 1 hour post exercise during the BFR trial compared to baseline (P <0.05; Table 1). Akt phosphorylation did not increase during the SNP trial at any time point (P>0.05). Phosphorylation of mTOR at Ser^{2448} was greater at 3 hours post exercise in the BFR trial as compared to the SNP trial and basal values (Figure 5A; P <0.05). Phosphorylation of mTOR did not increase at any time

point during the SNP trial (P>0.05). Phosphorylation of S6K1 at Thr^{389} was elevated above basal at 3 hours post exercise during the BFR trial (Figure 5B; P<0.05). S6K1 phosphorylation did not increase at any time point during the SNP trial (P>0.05).



Phosphorylation of rpS6 at $Ser^{235/236}$ was elevated above basal at 1 hour post exercise during the BFR trial and was greater compared to the SNP trial (P <0.05). rpS6 phosphorylation remained elevated above basal at 3 hours post exercise during the BFR trial (Figure 5C; P<0.05). Phosphorylation of rpS6 at 1 and 3 hours post-exercise did not change from basal during the SNP trial (P>0.05). Phosphorylation of 4EBP1 at $Thr^{37/46}$ did not change during either trial 1 hour post-exercise (P>0.05), however phosphorylation of 4E-BP1 was slightly reduced at 3 hours post-

Figure 5: Protein phosphorylation during post exercise recovery of the mTORC1 pathway from western blot analysis. Presented as a fold change from baseline. *p<0.05 vs baseline. #P<0.05 vs SNP.

exercise during the SNP trial (Table 1; $P<0.05$). Phosphorylation of eEF2 at Thr^{56} remained unchanged during both trials for the duration of the study (Table 1).

Table 1: Protein phosphorylation and expression during post exercise recovery

	1h Post	3h Post
Akt / mTORC1 signaling		
Akt (Thr^{308})		
BFR	1.29 ± 0.05 *	1.12 ± 0.11
SNP	1.20 ± 0.06	0.79 ± 0.08
4E-BP1 ($Thr^{37/46}$)		
BFR	0.87 ± 0.15	0.83 ± 0.13
SNP	1.02 ± 0.15	0.66 ± 0.10 *
eEF2 (Thr^{56})		
BFR	1.33 ± 0.44	0.90 ± 0.10
SNP	1.01 ± 0.24	0.98 ± 0.12

Data are from western blot analyses. Presented as fold change from baseline. *P<0.05 vs baseline.

MAPK signaling

There was a tendency for an increase in phosphorylation of ERK1/2 at Thr^{202}/Tyr^{204} 3 hours post-exercise during the BFR trial as compared to basal (Figure 6A; $P=0.088$). Phosphorylation of Mnk1 at $Thr^{197/202}$ was higher at 3 hours post exercise during the BFR trial compared to basal (Figure 6B; $P<0.05$). Phosphorylation of ERK1/2 or Mnk1 was not different at any time point during the SNP trial ($P>0.05$).

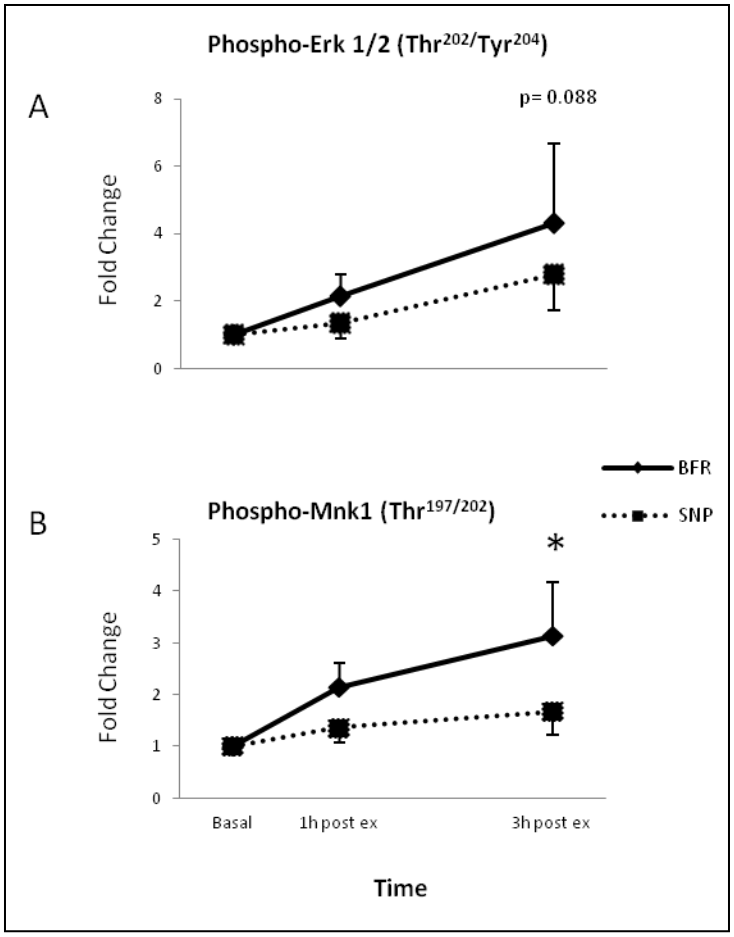


Figure 6: Protein phosphorylation during post exercise recovery of the MAPK pathway from western blot analysis. Presented as a fold change from baseline. *P<0.05 vs baseline. #P<0.05 vs SNP.

E3-Ligases

Atrogin1 and MuRF1 protein expression did not change in either trial at any time point during the study, although the mRNA expression of MuRF1 was increased at 3 hours post exercise during the BFR trial (Figure 7A; P<0.05). Atrogin1 mRNA expression was not different at any time point during either trial (Figure 7B; P>0.05).

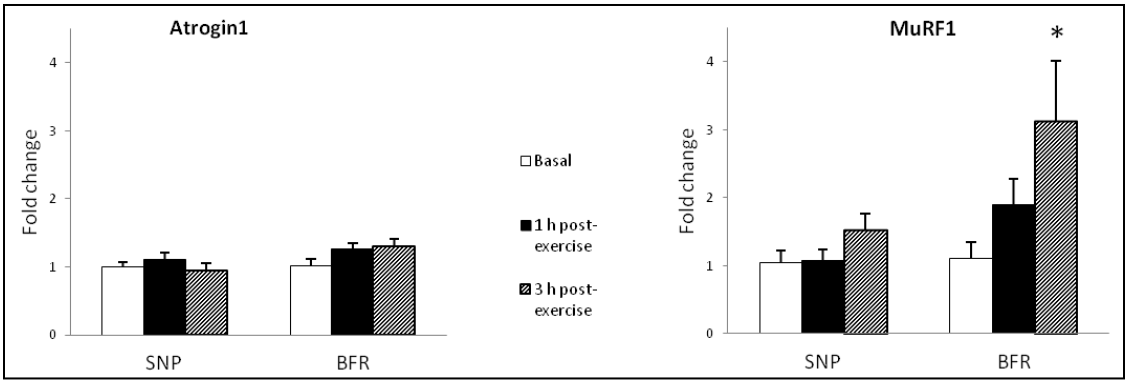


Figure 7: mRNA expression of the E3 ligases during post exercise recovery. Presented as fold change from baseline using the 2-ΔΔCt method. *P<0.05 vs baseline.

Representative blots for each protein are displayed in Figure 8.

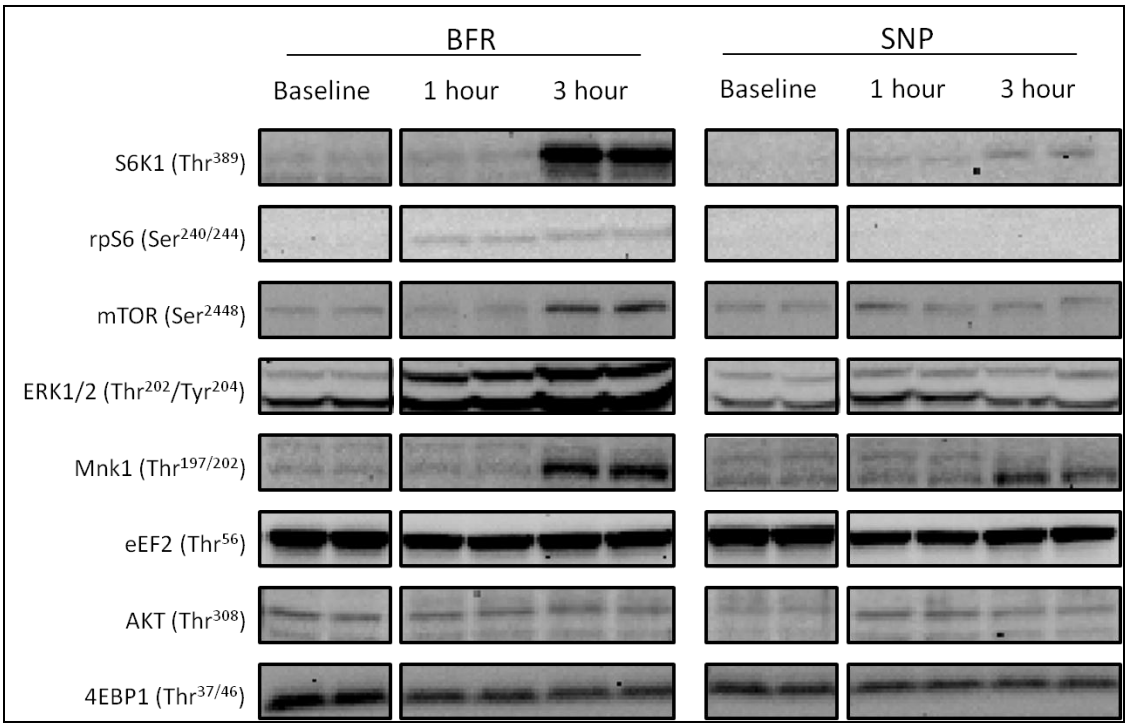


Figure 8: Representative western blots for all proteins measured.

DISCUSSION

The purpose of the study was to ascertain if reactive hyperemia is a primary mechanism to induce muscle growth. The reactive hyperemic response was simulated from BFR exercise using a pharmacological vasodilator, SNP, following non-restricted low-intensity exercise and compared the acute metabolic responses to traditional BFR exercise. This study is the first investigation designed to test a proposed mechanistic hypothesis of the anabolic response to BFR exercise. Using this approach it was demonstrated that while BFR exercise elicits an increase in MPS and anabolic signaling, a hyperemic response is not sufficient to induce these stimulatory responses when coupled with low-intensity exercise.

Specifically, the current study successfully simulated the overall reactive hyperemic response of BFR exercise using SNP, although it is important to note that while the overall 1-hour elevation in leg blood flow was accurately reproduced, the immediate post-BFR exercise response (the first 10 min) was more difficult to precisely reproduce. Thus, a limitation to the study is the inability to ascertain if the initial peak blood flow response (first 10 min) is responsible for the BFR induced MPS. However, even though the BFR trial elicited a higher initial peak in blood flow, the total blood flow and nutrient delivery during the first hour was not different between trials. Thus, this study design is capable in determining whether or not the total post-exercise increase in blood flow and the concomitant increase in nutritive delivery are responsible for the anabolic effects of BFR exercise. Despite the equivalent increase in blood flow, BFR exercise elicited a 49% increase in the 3 hour post-exercise FSR response, whereas the SNP-induced hyperemia following low intensity exercise did not change the post-exercise FSR response from basal values. The increase in FSR due to BFR exercise is

comparable to previous research with BFR exercise (73, 75) and traditional high-intensity resistance exercise (71). Likewise, the increase in FSR was associated with an increase in mTORC1 and MAPK pathway activation. Both of these pathways are widely accepted to be regulators of the rate limiting steps of protein translation following resistance exercise (57, 221, 234), however the relative contributions of each are unknown. An increase in extracellular amino acid availability is known to stimulate mTORC1 pathway activation (16, 21), a process required for an increase in MPS induced via amino acid supplementation (51). Furthermore, extensive research has shown that increased amino acid availability (216), in conjunction with carbohydrates (76), or coupled with resistance exercise (26, 27, 152, 164, 204, 205) increases MPS to a greater extent compared to resistance exercise alone. However, a major finding of this study is that an increased amino acid delivery through hyperemia is not sufficient to stimulate an anabolic response or increase mTORC1 activation. While the mTORC1 pathway has been shown to be indispensible in stimulating MPS following high-intensity resistance exercise (58) and nutritional supplementation (51), it is still unclear if BFR exercise follows the same pattern and requires mTORC1 activation or if a unique pathway is utilized, possibly relying heavier on MAPK pathway activation.

Although emphasis typically is placed on protein anabolic processes, overall muscle growth occurs when protein synthesis rates over time are higher as compared to the average rate of protein breakdown. Thus understanding muscle degradation is important from not only a growth standpoint, but also a therapeutic standpoint. Of the catabolic process, the proteasomal system plays a key role and involves the E3 ligases: Atrogin1 and MuRF1. Muscle proteins are targeted for degradation by these ligases and directed to the 26S proteasome through polyubiquitination (168). A study analyzing the

time-course of proteolytic gene expression demonstrated that the mRNA levels of MuRF1 but not Atrogin1 are elevated following high-intensity resistance exercise and endurance exercise between 1 and 4 h post-exercise, after which values are returned to basal conditions (139). A coinciding increase in protein synthesis and proteolysis is thought to be beneficial to enhance muscle quality via an overall increase in muscle protein turnover, as opposed to a futile counteraction (168). Accordingly, in a previous study Drummond *et al.* demonstrated that the mRNA transcript of MuRF1 increases 3 hours post BFR exercise (59). Therefore, a secondary aim of this research was to investigate if hyperemia exerts any influence on the transcription of the E3 ligases. Data in the current study indicates that hyperemia was insufficient to induce any changes in MuRF1 and Atrogin1. Recently Manini et al. showed a decrease in Atrogin1 and MuRF1 mRNA expression 8 h after BFR exercise when fed a standard meal (148). This does not necessarily contradict the current study considering the differences between protocols. Variations in results are mostly likely due to the differing nutritional status of subjects and the time points analyzed. To date, no research has analyzed the muscle protein fractional breakdown rate post-BFR exercise. Therefore, these results are only an initial look into the catabolic events following BFR exercise.

Although reactive hyperemia is not the primary mechanism involved in the acute anabolic or catabolic responses from BFR exercise, specifically in the young adult population, it certainly does not rule out the possibility that enhanced blood flow plays a permissive role as a supplementary variable. Evidence shows that there is an age related decline in anabolic sensitivity to traditional high-intensity resistance exercise (71, 125). Most recently, it was shown that older subjects have an impaired ability to activate MPS and mTORC1 signaling after traditional high-intensity resistance exercise in comparison

to younger subjects (71). Interestingly, BFR exercise has been shown to elicit similar increases in MPS in both young (75) and older subjects (73), indicating that BFR exercise is capable of restoring the normal muscle protein anabolic response following exercise in older adults. Previous research has found a positive correlation between an increase in blood flow and the response to an anabolic stimulus (201, 203), which could potentially explain the age related difference in responses since older adults naturally have a compromised endothelial responsiveness (77, 78, 163). Recently, it was demonstrated that the restoration of blood flow in older adults rescued the age related decline in the muscle protein anabolic response to insulin (203). The same authors also reported that increased blood flow was required for young subjects to elicit a full muscle protein anabolic response to insulin (201). Together, data in these previous studies suggests that hyperemia is a crucial component to a maximal response in MPS when coupled to an anabolic stimulus. This evidence encourages the speculation that reactive hyperemia from BFR exercise supersedes the compromised endothelial responsiveness in the elderly and thereby eliminates the age related differences in anabolic responses to resistance exercise. Future research is needed to further explore the necessity of hyperemia to induce an anabolic response from BFR exercise.

As suggested in a review by Wernbom *et al*. it is likely that BFR exercise responses are caused by multiple mechanisms (228). In the current study, BFR exercise triggered a pronounced 7.3 fold increase in lactic acid production which may be the cause and/or the effect of early muscle fatigue leading to enhanced fiber recruitment (147, 148, 197). Particularly, hydrogen ion accumulation via lactic acid may potentially act as an allosteric regulator in other pathways that alter MPS. Research by Burd *et al*. has demonstrated that exercise to volitional fatigue at any intensity is sufficient to stimulate

an increase in MPS (34). Indeed, BFR exercise may simply be an accelerated method to induce muscle fatigue and therefore produce similar results as suggested by Burd *et al*. However, unlike BFR exercise, low-intensity exercise until fatigue has not been tested to increase muscle hypertrophy and strength with training. Clearly, muscle fatigue is associated with evidence of muscle growth, although the role it plays is not clearly understood. It should not be overlooked however, that despite an increase in MPS following these unique training regimes, the physiological adaptation on a functional level may still be different. For instance, In a 12 week training study, Holm *et al*. discovered that 10 sets of 36 repetitions at 15.5% 1RM was sufficient to increase muscle cross sectional area but also resulted in a loss of myosin heavy chain type IIx (95). Furthermore, although EMG data suggest that all motor units are recruited prior to fatigue (229), it is unknown what physiological differences still exist within fiber types when fatiguing exercises occurs at different intensities.

In summary, the study demonstrated that BFR exercise elicits a dramatic increase in blood flow post-exercise that enhances nutrient delivery to the working muscles. Furthermore, it established that enhanced nutrient delivery does not appear to be the primary mechanism responsible for the stimulation of mTORC1 signaling and muscle protein synthesis following BFR exercise. Future studies are needed to determine the precise mechanisms responsible for stimulating muscle protein synthesis and promoting growth following BFR exercise and whether or not mTORC1 activation is required.

CHAPTER 3

Mechanistic Target of Rapamycin Complex 1 is Required for the Stimulation of Human Skeletal Muscle Protein Synthesis by Blood Flow Restriction Exercise

INTRODUCTION

The mechanistic target of rapamycin complex 1 (mTORC1) is believed to be a necessary enzyme in the regulation of translation initiation leading to protein accretion and ultimately muscle hypertrophy, a concept that has been confirmed in animal models (24, 84, 124) and humans (51, 58). Use of the competitive mTORC1 inhibitor, rapamycin, has demonstrated that elevations in human skeletal MPS in response to either high-intensity resistance exercise or essential amino acid ingestion requires mTORC1 activation (51, 58). Given the unconventional protein synthetic response of low-load exercise when coupled with BFR, the underlying mechanism may be unique. Previous studies have identified a correlation between muscle protein synthesis and both the mTORC1 and Erk/Mnk MAPK pathways (73, 89), yet it is unknown whether mTORC1 activation is essential to the muscle protein synthetic response.

Muscle protein accretion in humans occurs largely as a result of an increase in muscle protein synthesis which can remain elevated for up to 24 hours while changes in muscle protein breakdown play a minimal role (158). However, it is unknown whether the same responses of muscle protein synthesis and breakdown occur following BFR exercise. Therefore the purpose of this study was to determine if mTORC1 is required for the increase in muscle protein synthesis following BFR exercise. Additional aims

included determining if the muscle protein synthetic response persists up to 24 hours and whether muscle protein breakdown plays a role in the muscle metabolic response following BFR exercise. It is hypothesized that mTORC1 is required for the increase in muscle protein synthesis and 24 hour time-course closely match the pattern observed with high-intensity exercise.

EXPERIMENTAL PROCEDURES

Subjects

Sixteen young, healthy, recreationally active males (25.5 ± 0.8 yrs, 179.4 ± 1.7cm, and 24.8 ± 1.6% body fat) volunteered for this study. Each of the subjects was screened identically as the subjects described in Chapter 2.

Study Design

Each subject was randomized into one of two treatments groups. Both treatments included an identical BFR exercise protocol (Illustration 3) with the exception that one group ingested 16mg (1mg tablets) of rapamycin (Rapamune/Sirolimus; Wyeth) (RAP), whereas the other group did not ingest rapamycin (CON).

The exercise study comprised of two days of evaluation and lasted 11.5 hours on the first day and 4.5 hours in the morning of the second day. The subjects were admitted to the ITS-CRC the evening before the exercise study and were fed a standard research dinner (10 kcal/kg of body weight; 60% carbohydrate, 20% fat and 20% protein) and snack before 2200 the evening before each study day. Each day they were fasted overnight under basal conditions and were given ad libitum access to water. The morning of each day, a polyethylene catheter was inserted into an antecubital vein for

tracer infusion and another polyethylene catheter was inserted retrogradely into a hand vein of the opposite arm and kept under a heating pad for arterialized blood sampling. After drawing a background blood sample, a primed continuous infusion of L-[ring-13C6] phenylalanine and 15N phenylalanine (Isotec Inc., Sigma-Aldrich, Miamisburg, OH) was begun and maintained at a constant rate. The L-[ring-13C6] phenylalanine was maintained throughout for the measurement of mixed muscle fractional synthetic rate, while the 15N phenylalanine infusion was arrested at specific periods where the plasma decay was used to measure the fractional breakdown rates at specific periods. The priming dose for each labeled phenylalanine was 2μmol/kg, and the infusion rate was 0.05μmol/kg/min.

After a 4.5 hour basal period, subjects randomly assigned to the RAP group ingested 16mg of rapamycin and remained at rest for another hour prior to exercise (Illustration 3). This dosage has been previously validated to appear at peak concentrations in the bloodstream after an hour and to blunt the increase in mTORC1 activation in humans (51, 58), while not affecting basal muscle metabolism or basal mTORC1 conditions (50). Subjects randomly assigned to the CON group did not ingest rapamycin yet still remained at rest for an additional hour.

Exercise

After the 5.5 hour basal period subjects were transported from the bed to the leg extension exercise machine (Illustration 3). Immediately prior to the commencement of exercise, subjects were fitted with 11cm wide pressure cuffs (Hokanson SC10, Bellevue, WA, USA) placed on the most proximal portion of the upper thighs and attached to a Hokanson E20 Rapid Cuff Inflater and AG101 Air Source (Bellevue, WA). Subjects were gradually acclimated to the pressure cuff in increments of 20mmHg starting at

120mmHg to a final pressure of 200mmHg. During the acclimation phase each increment consisted of 30 seconds of pressure followed by 10 seconds of no pressure. Exercise commenced once the final pressure of 200mmHg was reached. The exercise consisted of 4 sets of bilateral leg extensions at 20% of their 1RM performing 30, 15, 15 and 15 repetitions respectively with 30 seconds of rest between sets. Verbal encouragement was used to ensure each subject completed the full exercise protocol. The pressure was not released until the completion of the final set of exercise.

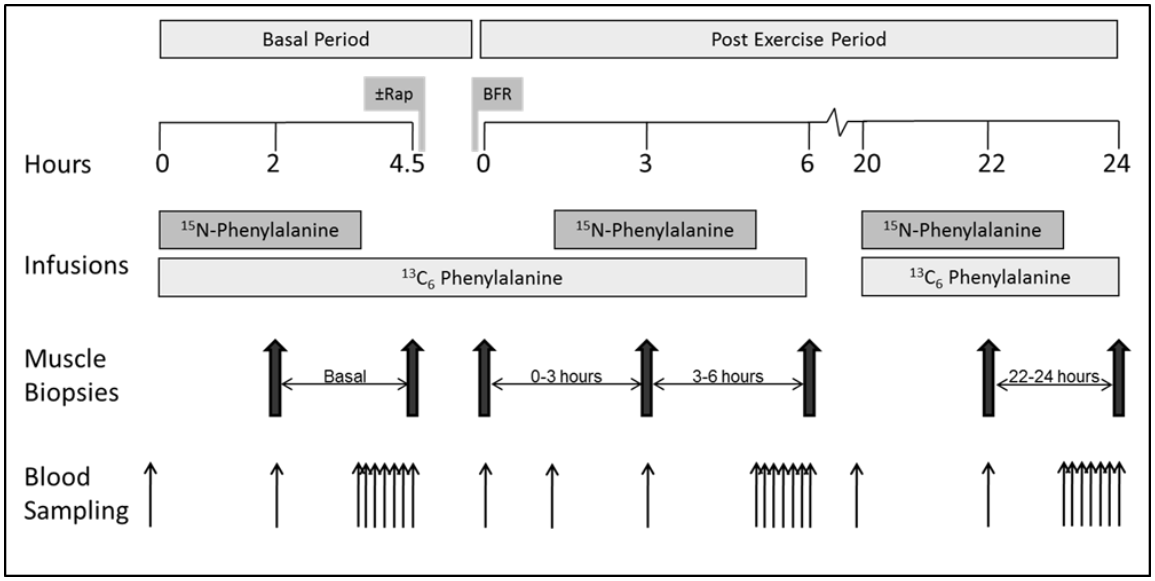


Illustration 3: Infusion study protocol. Study design was identical for both groups. ±Rap indicates the time point where the RAP group ingested 16mg of rapamycin whereas the CON group did not. Timing of blood draws and biopsies are represented by upward facing arrows. Between hours 6 and 20, subjects were fed, and then re-fasted overnight.

Muscle sampling

Seven muscle biopsies were performed over the two day trial with a 5-mm Bergström biopsy needle utilizing sterile procedures and local anesthesia (1% lidocaine).

Prior to exercise on the first day, two muscle biopsies were obtained from the lateral portion of the *vastus lateralis* muscle between 15 and 25 cm superior to the mid-patella. The first biopsy was collected 2 hours after the initiation of the tracer infusion, to allow for steady state enrichments, and again 2.5 hours later for the measurement of basal muscle metabolism. Both biopsies were sampled from a common incision on the left leg although the orientation of the needle was angled so that samples were taken ~5cm from each other. It has been demonstrated that sequential biopsies from the same incision does not interfere with MPS measurements (214). The third and fourth biopsies were taken at 0 and 3 hours post-exercise from a second incision site proximal to the first incision on the right leg. A fifth muscle biopsy was taken at 6 hours post-exercise in a new incision on the right leg. Finally, the sixth and seventh muscle biopsies were taken at 22 and 24 hours post exercise the following morning respectively from a common incision on the left leg. All muscle tissue was immediately blotted, frozen in liquid nitrogen, and stored at -80°C until analysis.

Blood sampling

Arterialized blood was collected from the retrograde hand catheter 26 times during the study (Illustration 3). Each time, two milliliters were aliquoted for the determination of the enrichment of labeled phenylalanine in blood using GCMS. Phenylalanine concentration was calculated using an internal standard approach as described by Wolfe and Chinkes (236).

Mixed Muscle Protein Fractional Synthetic and Breakdown Rates

The FSR of mixed muscle proteins was calculated using the same method and formula as described in chapter 2.

The fractional breakdown rate (FBR) of mixed muscle proteins was calculated with the L-[^{15}N] phenylalanine tracer using the tracee release method (243). This method requires the intracellular free phenylalanine enrichment to reach steady a steady-state followed by halting the L-[^{15}N] phenylalanine tracer. The muscle intracellular enrichment decay can be determined by the arterial 1 hour plasma tracer decay with frequent arterialized blood sampling as well as the free and bound muscle intracellular phenylalanine content. FBR was calculated using the formula:

$$FBR = \frac{E_M(t_2) - E_M(t_1)}{p \cdot \int_{t_1}^{t_2} E_A(t) \cdot dt - (1+p) \int_{t_1}^{t_2} E_M(t) \cdot dt} \cdot \frac{Q_M}{T}$$

where $E_A(t)$ and $E_M(t)$ are the arterialized and muscle free enrichments at time t, t1 and t2 are two time points. $P = E_M/(E_A-E_M)$ at plateau, E_A and E_M are enrichments in the arterial pool and muscle intracellular pool respectively, and Q_M/T is the ratio of free to bound phenylalanine in muscle. Data are expressed as percent per hour.

SDS PAGE and Western Blot Analysis

Details of the immunoblotting procedures are the same as described in chapter 2. Data are expressed as the fold change from baseline. The following primary antibodies were purchased from Cell Signaling (Danvers, MA) and diluted in 5% non-fat dairy milk: phospho-mTOR (Ser2448, 1:500), phospho-p70 S6K1 (Thr389, 1:250), phospho-rpS6 (Ser240/244, 1:250).

Statistical Analysis

Data are reported as mean ± standard error. Between and within group differences were tested using a two-way repeated-measures ANOVA with a random subject effect. Post hoc analysis was performed with Bonferonni when appropriate.

Significance was set at $P \leq 0.05$. All analyses were done with SigmaStat 11.0 (Systat Software, San Jose, CA).

RESULTS

Basal values of mixed muscle protein FSR were similar between groups (P>0.05). FSR significantly increased by 41.5% (P<0.05) in the CON group at 3 h post exercise and returned to baseline at 6 h post exercise. However FSR measured through the full 6 hour post exercise period was significantly elevated by 30% compared to baseline (P<0.05; data not shown). At 24 h post exercise the FSR was again elevated by 69.4% (P<0.05) compared to basal values. The FSR in the RAP group was unchanged at all time points (P>0.05; Figure 9).

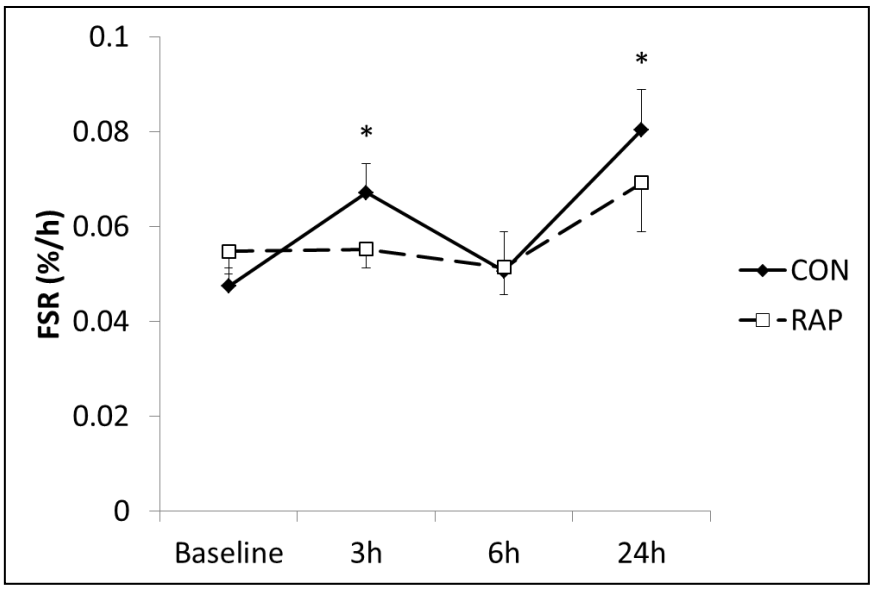


Figure 9: Mixed muscle fractional synthetic rate at baseline and 3 hours, 6 hours and 24 hours post exercise. Presented in percent per hour (%/h). Error bars represent SE. *P<0.05 vs baseline.

Phosphorylation of mTOR at Ser^{2448} increased in the CON group at all post-exercise time points, and was elevated above the RAP group at 6 and 24 hours post exercise ($P<0.05$). Conversely, mTOR phosphorylation remained as basal levels in the RAP group at all time points post exercise ($P>0.05$). Phosphorylation of S6K1 at Thr^{389} increased at all time points in the CON group post exercise ($P<0.05$). In contrast S6K1 phosphorylation did not increase at any time point after exercise ($P>0.05$) and in fact slightly decreased at 6 and 24 hours post exercise ($P<0.05$; Figure 10).

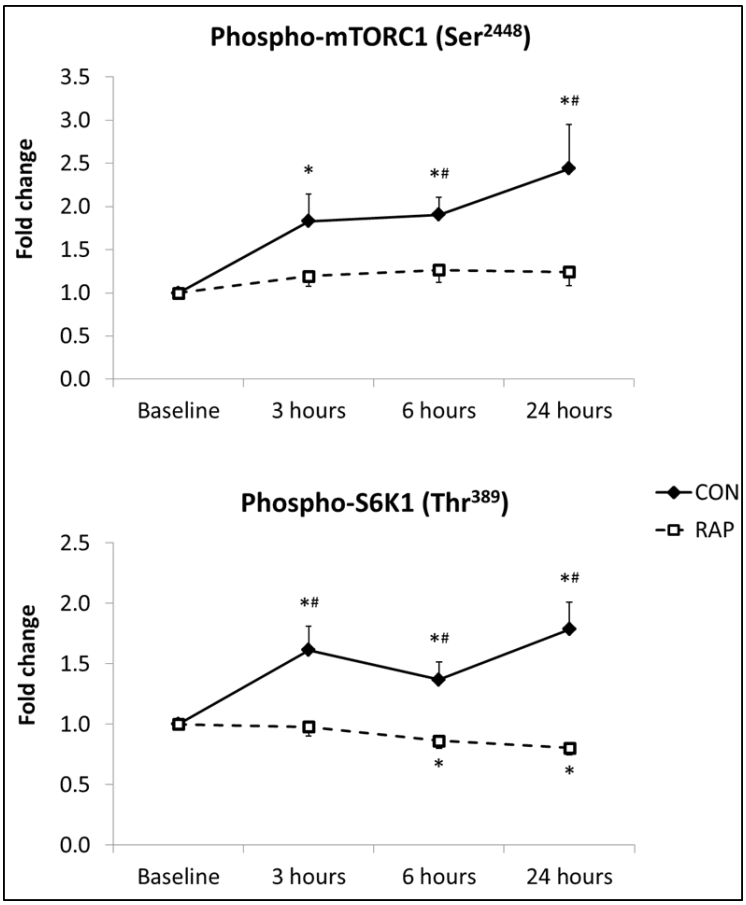


Figure 10: Protein phosphorylation during post exercise recovery of the mTORC1 pathway from western blot analysis. Presented as a fold change from baseline. *p<0.05 vs baseline. #P<0.05 vs CON.

The fractional breakdown rate remained unchanged throughout the study with no significant differences between groups or time (P>0.05; Figure 11).

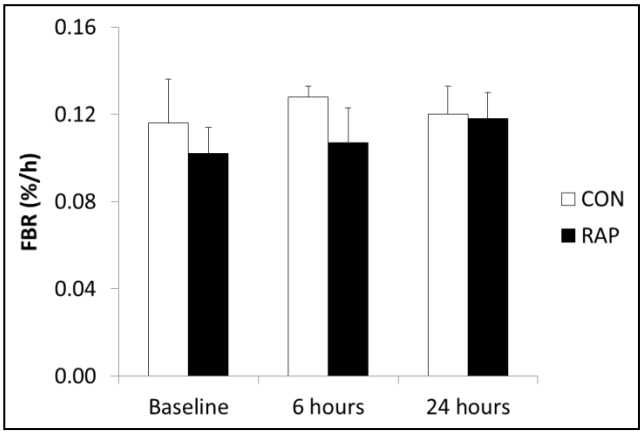


Figure 11: Muscle fractional breakdown rate at baseline, 6 hours and 24 hours post exercise. Presented in percent per hour (%/h). Error bars represent SE.

Net balance (FSR – FBR) is significantly improved in the CON trial, indicating a less catabolic environment (P<0.005; Figure 12). No net balance changes occurred in the RAP trial (P>0.05; Figure 12).

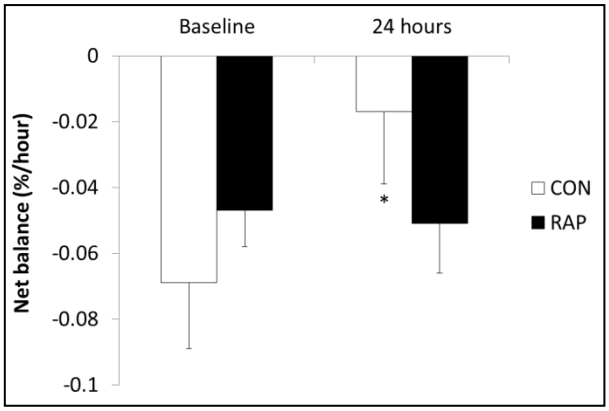


Figure 12: Net balance at baseline and 24 hours post exercise. Presented in percent per hour (%/h). Error bars represent SE. *P<0.05 vs Baseline

DISCUSSION

The primary purpose of this study was to determine the necessity of mTORC1 for the muscle anabolic response following BFR exercise. Using the mTOR inhibitor, rapamycin, it is shown for the first time that mTORC1 pathway activation is required to stimulate muscle protein synthesis following BFR exercise. To verify the effectiveness of rapamycin, the phosphorylation of mTOR and its primary downstream target, S6K1 was measured and did not increase at any time point within the RAP trial. The use of rapamycin in human clinical trials has been employed previously to investigate the necessity of mTORC1 to stimulate muscle protein synthesis following high-intensity resistance exercise (58), or after the ingestion of essential amino acids (51) with similar outcomes. Thus, despite the idea that BFR exercise functions within a unique mechanism, it appears the role that mTORC1 plays is consistent between modes of exercise and the differences must lie upstream.

The time-course of muscle synthetic effects from a single bout of BFR exercise appears to follow a similar pattern as observed with other resistance exercise studies. Fry *et al*. and Phillips *et al.* both observe a sustained elevation in mixed muscle protein synthesis up to 24 hours following a bout of resistance exercise (71, 158). The increase in FSR at 3 hours post exercise is consistent with data from previous BFR exercise studies conducted in our laboratory (73, 75, 89). However, muscle protein synthesis returned to basal levels 6 hours post exercise unlike with high-intensity exercise where it remains elevated (71). The phosphorylation of S6K1 followed a similar pattern as the FSR, though did not return to baseline at 6 hours post exercise. This response pattern may suggest that synthetic response occurs in phases such as an immediate acute response and a delayed longer lasting response, which may also suggest responses from

multiple stimuli. An alternative explanation is that BFR exercise increases skeletal muscle sensitization to ingested nutrients, and the 24h increase in MPS persists from the last meal prior to the overnight fast.

Mechanotransduction is believed to be the primary stimulus that ultimately leads to the activation of mTORC1 following resistance exercise (69). However, when considering BFR exercise, significantly less tension is applied to the muscle along with less overall time under tension, and thus, other molecular mechanisms are likely to be involved. Few studies have investigated the role of muscle protein breakdown (MPB) following resistance exercise with conflicting results. Research by Phillips *et al.* indicate that the rate of MPB increases 3 hours post exercise and remains elevated for 24 hours thereafter in a similar pattern with the rise in FSR (158). On the contrary, Fry *et al.* did not observe any differences in the rates of MPB 24 hours post resistance exercise but concluded that the rise in breakdown rates may have occurred during time points not measured and then reached baseline values after 24 hours (72). Results in the current study indicate that the rates in MPB after BFR exercise do not change at 6 or 24 hours post exercise compared to basal values. However, if the rate of protein breakdown truly follows the time course for the initial rise in FSR then it is possible that this study also missed the increase in MPB, which may have been elevated at 3 hours post exercise and returned to baseline at 6 hours. In fact, markers of proteolysis also appear to follow an unusual pattern. The gene expression of the proteolytic E3 ligase MuFR1 has been shown to be increased 3 hours post BFR exercise (59, 89) and then decreased 8 hours post BFR exercise (148). On the other hand, it is possible that MPB is not associated with FSR following BFR exercise as opposed to traditional resistance exercise, and instead follows a pattern unique to BFR exercise. The mechanisms of exercise-induced

increases in MPB are less understood than for muscle protein synthesis. With that in mind, MPB may play a partial role in the hypertrophic response with BFR training if the low intensity nature of BFR exercise is not sufficient to stimulate as robust of an increase or duration of MPB compared to traditional resistance exercise.

Previous studies have shown that MAPK signaling is increased with BFR exercise including the phosphorylation of Erk1/2 and Mnk1 (73, 89), and activation of this pathway is known to enhance protein synthesis through an mTORC1-independent mechanism as described in Chapter 1. However, data from the current study indicate that mTORC1-independent mechanisms have a minimal, or possibly only a permissive, role in the increase in muscle protein synthesis following BFR exercise.

In summary, this research demonstrates that the muscle protein synthetic response following BFR exercise is dependent on the activation of mTORC1, similar to other anabolic stimuli. Furthermore, muscle protein breakdown appears to play a minimal role, if any, in the muscle metabolic response to BFR exercise. Lastly, the rise in MPS synthesis appears to be biphasic and is elevated 24 hours post exercise suggesting the possibility of multiple stimuli or enhanced anabolic sensitivity. Further mechanistic research is needed to investigate other proposed mechanisms of BFR exercise such as metabolic stress that occurs within the muscle during BFR exercise.

CHAPTER 4

Metabolic Stress as a Mechanism behind Blood Flow Restriction Exercise

INTRODUCTION

BFR exercise is a unique method that stimulates MPS to a similar degree as high-intensity resistance exercise. However, given that the absolute amount of work performed during BFR exercise is substantially less, there must be some unique element in BFR exercise that when combined with low-intensity exercise, produces an equivocal response as high-intensity exercise.

Metabolic stress has been proposed to have some involvement as a co-anabolic stimulus. Suga *et al.* demonstrated that BFR exercise is associated with the accumulation of metabolites such as lactate, inorganic phosphate and deprotonated phosphate, and a decrease in PCr and pH (189-191). While this observation has only been associated with BFR exercise, it is believed that this conglomerate of metabolites have several roles in muscle fatigue during exercise. For example, metabolite-induced muscle fiber fatigue is known to occur via the activation of group III and IV muscle afferents at the level of the peripheral nervous system (91, 172). Additionally, inorganic phosphate has been shown to inhibit muscle force by interfering with actin-myosin binding and reducing the number of cross-bridges (68). Moreover, hydrogen ion accumulation through lactic acidosis is known to reduce force via inhibiting calcium ion (Ca^{++}) release from the sarcoplasmic reticulum, interference with Ca^{++} binding troponin, and also directly reducing the force per actin myosin cross-bridge (68, 74).

As low threshold, slow-twitch fibers become fatigued, higher threshold, fast-twitch fibers are required to be recruited to maintain muscle force, which is speculated by many to be required for muscle hypertrophy. Certainly, this theory has been the accepted argument for high-resistance exercise training (70), but this relationship has not been determined for low-intensity resistance exercise with BFR. While high-intensity exercises require immediate recruitment of fast twitch fibers to perform the exercise, the relative contribution of fast twitch fibers during BFR exercise is unknown.

Not only can these metabolites affect fatigue, but bioenergetics as well. Although pH changes per se are not thought to be a regulator of glycolysis in skeletal muscle (187), lactate has been shown to directly inhibit the glycolytic enzyme, phosphofructokinase (PFK) and down-regulate glycolysis (45). During glycolysis impedance, glycolytic intermediates upstream of PFK may accumulate. Of particular interest, glucose-6-phosphate (G6P), a glycolytic intermediate, has been recognized as a stimulant to mTORC1 (182).

The purpose of this research was to compare BFR exercise to its hypertrophic equivalent counterpart, traditional high-intensity exercise, on two fronts: 1) To determine if the pattern of mTORC1 activation in each fiber type is similar between the exercises; and 2) To determine if metabolites such as lactate and G6P follow a distinct pattern between exercises and are associated with the activation of mTORC1 signaling and protein synthesis. The hypothesis is that BFR exercise induces mTORC1 activation in a similar pattern as high-intensity exercise, though a mechanism induced by G6P accumulation.

EXPERIMENTAL PROCEDURES

Subjects

Fourteen young, healthy, recreationally active males volunteered for this study. Demographic information is provided in Table 2. Each of the subjects was screened identically as the subjects described in Chapter 2.

Table2: Subject Characteristics

	HI	BFR	P
Subjects, n	8	6	
Characteristics			
Age, years	27.1 ± 2.6	23.6 ± 1.7	0.32
Height, cm	177 ± 2.6	181 ± 1.5	0.24
Weight, kg	78.7 ± 3.7	81.5 ± 5.5	0.66
Body fat, %	21.8 ± 2.6	20.1 ± 2.3	0.66
BMI, kg/m^2	25.1 ± 1.2	24.9 ± 1.7	0.92
1RM	274 ± 14	297 ± 35	0.51

Values are means ± SE. No significant differences between groups (P>0.05)

Study Design

Subjects participated in one of two trials, low-intensity exercise with BFR (BFR) or high-intensity exercise (HI). In either trial, subjects were admitted to the ITS-CRC the evening before the exercise study. They were fed a standard research dinner (10 kcal/kg of body weight; 60% carbohydrate, 20% fat and 20% protein) and snack before 2200. They were fasted overnight under basal conditions and were given ad libitum access to water. The morning of the study at, a polyethylene catheter was inserted into an antecubital vein for tracer infusion and another polyethylene catheter was inserted retrogradely into a hand vein of the opposite arm and kept under a heating pad for arterialized blood sampling. After drawing a background blood sample, a primed

continuous infusion of L-[*ring*-$^{13}C_6$] phenylalanine (Isotec Inc., Sigma-Aldrich, Miamisburg, OH) was begun and maintained at a constant rate until the end of the experiment. The priming dose for the labeled phenylalanine was 2μmol/kg, and the infusion rate was 0.05μmol/kg/min.

BFR trial – After a 4 hour basal period, subjects were transported from the bed to the leg extension exercise machine and were fitted with 11cm wide pressure cuffs (Hokanson SC10, Bellevue, WA, USA) placed on the most proximal portion of the upper thighs and attached to a Hokanson E20 Rapid Cuff Inflater and AG101 Air Source (Bellevue, WA). Subjects were gradually acclimated to the pressure cuff in increments of 20mmHg starting at 120mmHg to a final pressure of 200mmHg. During the acclimation phase each increment consisted of 30 seconds of pressure followed by 10 seconds of no pressure. Exercise commenced once the final pressure of 200mmHg was reached. They performed 4 sets of bilateral leg extensions at 20% of their 1RM. The 4 sets consisted of 30, 15, 15 and 15 repetitions respectively with 30 seconds between sets. The pressure was not released until the completion of the final set of exercise. Verbal encouragement was used to ensure each subject completed the full exercise protocol.

HI trial – After a 4 hour basal period, subjects were transported from the bed to the leg extension exercise machine and completed 1 warm-up set at of 10 repetitions at 45% 1RM followed by 8 sets of 10 repetitions at 70%1RM with 3 minutes rest between each set.

Muscle sampling

Three muscle biopsies were collected with a 5mm Bergström biopsy needle utilizing sterile procedures and local anesthesia (1% lidocaine): before exercise (basal), shortly after exercise within 15 minutes post exercise (0h) and 3 hours post exercise (3h).

All three biopsies were sampled from a common incision although the orientation of the needle was angled so that samples were taken ~5cm from each other.

Blood sampling

Arterialized blood was collected from a retrograde hand catheter. Blood was aliquoted for analysis of plasma lactate (Yellow Springs Instruments Co., Yellow springs, OH); and for the determination of the enrichment of labeled phenylalanine in blood using GCMS.

FSR and mTORC1 signaling

The FSR of mixed muscle proteins was calculated using the same method and formula as described in chapter 2. Data are expressed as percent per hour. Details of the immunoblotting procedures are also the same as described in chapter 2. Data are expressed as the fold change from baseline. The following primary antibodies were purchased from Cell Signaling (Danvers, MA) and diluted in 5% non-fat dairy milk: phospho-mTOR (Ser2448, 1:500), phospho-p70 S6K1 (Thr389, 1:250).

Histology

Muscle tissue was immediately placed on cork with optimal cutting temperature (OCT) compound, frozen in cold 2-methyl-butyrate and stored in -80°C until analysis. Samples were cut perpendicular to the direction of the muscle fibers and molded in OCT compound with the muscle sectional face exposed. Samples were then sliced 10µm thick at -20°C using a cryostat (Jencons, Bridgeville, PA) and applied to a microscope slide. Basal and post exercise samples for each subject were placed on the same slide to minimize any variation of treatments in order to adequately visualize changes between time points. Basal and 3h samples were placed on a slide for the determination of

mTORC1 signaling via the phosphorylation of S6K1. Basal and 0h samples were placed on a separate slide for glycogen staining. Prepared slides were stored at -20°C until further analysis

Epifluorescence immunohistochemistry

Visual illumination of phosphorylated S6K1 at Thr^{421}/Ser^{424} was used as a proxy for enhanced mTORC1 activity known to be associated with an increase in muscle protein synthesis. In coordination with the illumination of myosin heavy chain type 1 (MHC1), mTORC1 activity can be visualized separately in each fiber type 3 hours after each exercise trial. Samples were fixed in cold 4% paraformaldehyde for 10 minutes, washed 3 times in phosphate buffered saline (PBS) and blocked with a 10% donkey serum and 0.5% Triton X-100 cocktail. A dilution of 1:25 rabbit anti P-p70 S6 Kinase (Thr^{421}/Ser^{242}; Cell Signaling, Danvers, MA) with 1:10 blocking solution in PBS was applied overnight at 4°C. The next day samples were incubated in a 1:2000 anti-rabbit Alexa Fluor 555 with 1:10 blocking solution in PBS for 30 minutes in the dark at room temperature. The samples were then incubated in mouse anti MHC1 (DSHB, A4.951; 1:200), and rabbit anti-laminin (Sigma, 1:200) for two hours in the dark. Afterward, a final incubation with the appropriate anti-mouse Alexa Fluor 547 and anti-rabbit Alexa Fluor 488 was applied for 30 minutes. The samples were then washed twice in PBS followed by two washes in water and left to dry. Images were captured with a fluorescence microscope (Axio Imager.M1m, Carl Zeiss, Toronto, Ontario, Canada) and AxioCam Mrm camera (Carl Zeiss, Toronto, Ontario, Canada).

Glycogen

Relative glycogen content was visualized using a Periodic Acid Schiff staining system (Sigma-aldrich-395B) following the manufacturer's instruction. Briefly, samples were fixed in cold 4% paraformaldehyde, washed 3 times in PBS and incubated in Periodic Acid Solution for 5 minutes. With adequate rinses between each step, the samples were incubated in Schiff's reagent for 15 minutes then Hematoxylin for 90 seconds. Images were captured under a microscope with a color camera (AxioCam MRc5, Carl Zeiss, Toronto, Ontario, Canada) with 20X magnification where the darker stain represents more glycogen.

Separate pieces of muscle at basal and 3h were used for a quantitative analysis of glycogen content. 5-15mg of muscle was submerged in 0.5mL of 2N hydrochloric acid and boiled at 100ºC for 2 hours then neutralized with 1.5mL of 0.67N sodium hydroxide and shaken until dissolved resulting in glycogen hydrolyzed into glucose units. The resulting hydrosylate was used for the measurement of glucose representing the glycogen content from the tissue using a commercially available glucose assay kit according to manufacturer's instructions (abcam – ab65333).

ATP, PCr and G6P Analysis

3-5mg pieces of basal and 0h muscle samples were freeze dried for 24 hours and powdered homogenously. 300µL of cold 0.5M perchloric acid with 1mM EDTA was added, vortex then centrifuged at 13,000RPM for 5 minutes at 4ºC. 250µL of the supernatant was extracted and neutralized with 62.5µL of potassium bicarbonate and centrifuged again at 13,000RPM for 5 minutes at 4ºC. The supernatant was extracted again and used for the analysis of PCr, ATP and glucose G6P by enzymatic spectrophotometric assays as described by Harris *et al.* (93)

Cell Culture

Murine C_2C_{12} myoblasts were cultured on 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) coated tissue cultureware in growth media (high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50U of penicillin/mL, 50µg of streptomycin/mL; Invitrogen, Carlsbad, CA) at 37°C in an atmosphere of 5% CO2/95% air. At ~90% confluency, differentiation media (low-glucose Dulbecco's modified Eagle medium supplemented with 2% horse serum, 50U of penicillin/mL, 50µg of streptomycin/mL; Invitrogen, Carlsbad, CA) was added to cultures for 4 days to allow formation of multinucleated myotubes. Prior to experiments on day 4, cells were serum starved for 4 hours followed by nutrient deprivation for 30 minutes in Hepes buffered saline (HBS, 20mM Hepes/Na, 140mM NaCl, 2.5mM MgSO4, 5mM KCl, and 1mM CaCl2; pH 7.4; Sigma-Aldrich).

The first experiment comprised of two treatments: control versus lactic acid treatment. Cells randomly assigned to control were left in fresh HBS for an additional 30 minutes. Cells randomly assigned a lactic acid treatment were incubated in 0.5mM lactic acid solution (Sigma-Aldrich) dissolved in HBS (Illustration 4A).

To verify the involvement of mTORC1, a separate experiment was conducted with pre-treatment of the mTORC1 inhibitor, rapamycin. In this experiment control and lactic acid groups were nutrient deprived for 60 minutes while a third subset (rapamycin) was nutrient deprived for 30 minutes and pre-treated with 50nM rapamycin dissolved in HBS for an additional 30 minutes. The control subset were then left in fresh HBS, the lactic acid subset was incubated in 0.5mM lactic acid solution dissolved in HBS and the rapamycin subset was incubated in 0.5mM lactic acid and 50nM rapamycin both dissolved in HBS (Illustration 4B).

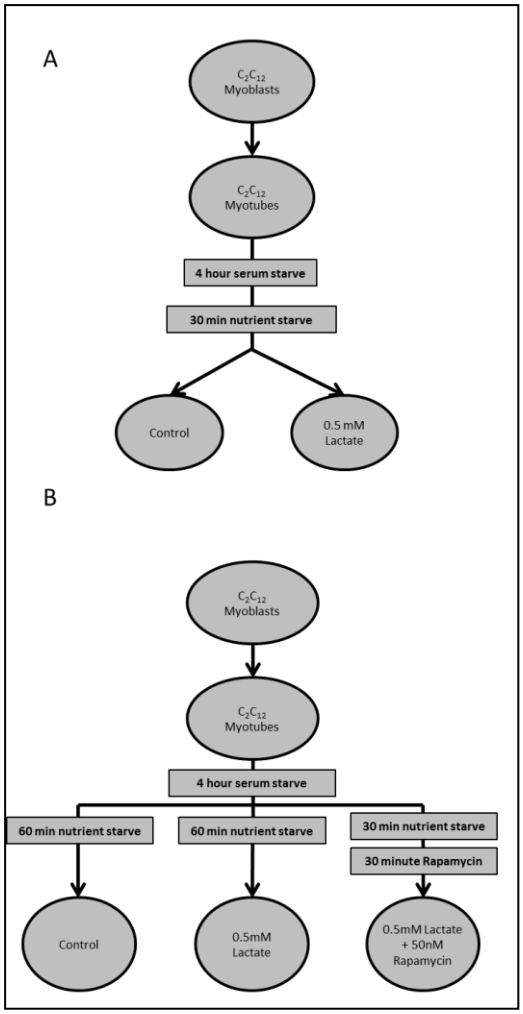
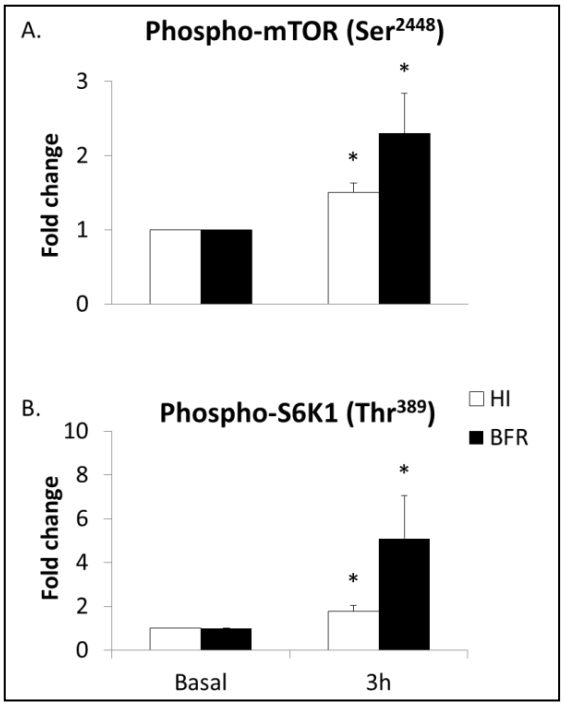


Illustration 4: Flowchart of each cell culture experiment

Following treatments, myotubes were rinsed with PBS three times. Cells were scrapped in ice-cold extraction buffer (50mM Tris-HCl, 250mM mannitol, 50mM NaF, 5mM Na pyrophosphate, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 1mM DTT, 1mM benzamidine, 0.1mM PMSF, 5µg/mL soybean trypsin inhibitor, pH 7.4) then snap frozen in liquid nitrogen and thawed to facilitate cell lyses. Cell lysates were vortexed three times and sonicated for 15 seconds. Protein concentrations were determined using a Bradford Protein Assay (Smartspec Plus, Bio-Rad, Hercules, CA). Cell lysates were diluted (1:1) in a 2X sample buffer mixture (125mM Tris, pH 6.8, 25% glycerol, 2.5% SDS, 2.5% b-mercaptoethanol, and 0.002% bromophenol blue) and then boiled for 3 minutes at 100°C. Equal amounts of total protein (7µg) were loaded into each lane on a 7.5 or 15% polyacrylamide gel (Criterion, Bio-Rad). Details of the immunoblotting procedures are the same as described in chapter 2. Data are expressed as the fold change from baseline. The following primary antibodies were purchased from Cell Signaling (Danvers, MA) and diluted in 5% non-fat dairy milk: phospho-mTOR (Ser2448, 1:500), phospho-p70 S6K1 (Thr389, 1:250), phospho-rpS6 (Ser240/244, 1:250), 4E-BP1 (Thr37/46, 1:1000).

RESULTS

Mixed Muscle Anabolic Response

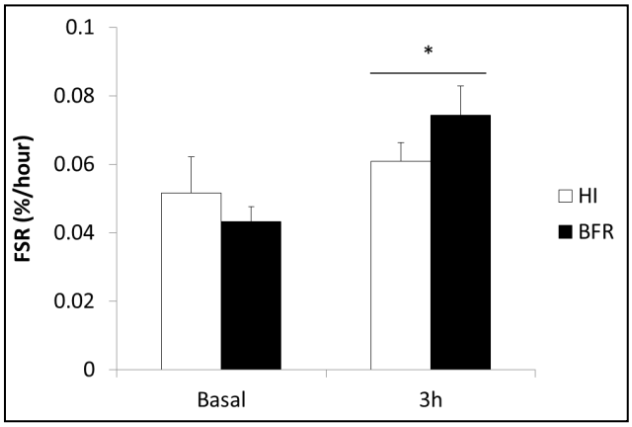


Phosphorylation of mTOR at Ser^{2448} was significantly increased compared to basal values by 50% and 230% after HI exercise and BFR exercise respectively ($P<0.05$; Figure 13A). Likewise, the primary downstream target of mTOR, S6K1 was also significantly elevated compared to baseline values in both groups ($P<0.05$; Figure 13B).

Figure 13: Protein phosphorylation during post exercise recovery of the mTORC1 pathway from western blot analysis. Bars represent SE. Presented as fold change from baseline *$P<0.05$ vs baseline.

There was a significant time effect for an increase in FSR at 3 hours post exercise in both exercise groups ($P<0.05$; Figure 14).

Figure 14: Mixed muscle fractional synthetic rate at baseline and 3 hours post exercise. Presented in percent per hour (%/h). Error bars represent SE. *$P<0.05$ vs baseline.



Fiber type-specific mTORC1 signaling

Qualitative analysis of muscle fibers using epifluorescence immunohistochemistry with stained laminin, MHC-I and S6K1 phosphorylated at Thr^{242} Ser^{244} revealed minimal S6K1 phosphorylation in any fiber type during basal conditions (Figure 15 A-C). Three hours after high-intensity resistance exercise, phosphorylation of S6K1 at Thr^{242} Ser^{244} increased primarily in fast twitch muscle fibers (Figure 15 D-F). In contrast, 3 hours after BFR exercise, phosphorylation of S6K occurs in all muscle fiber types with particular emphasis in slow twitch muscle fibers (Figure 15 G-I).

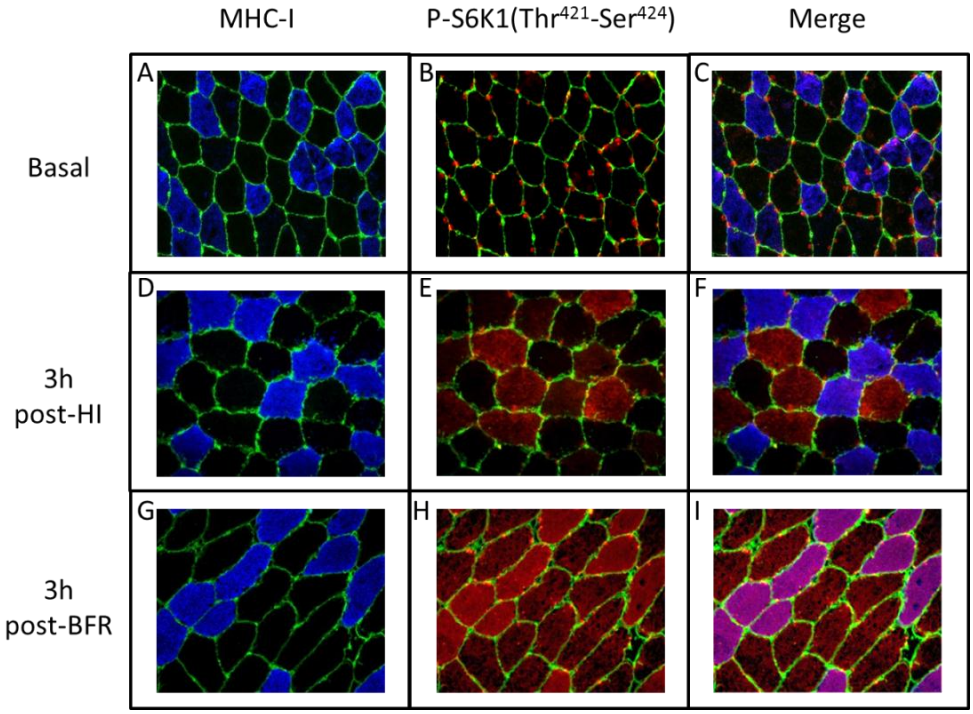


Figure 15: Epifluorescence immunohistochemistry representative images of biopsied muscle in cross section. Sarcolemma is demarcated by laminin proteins stained green. Slow twitch muscle fibers are demarcated by MHC1 proteins stained blue. mTORC1 pathway activation is indicated via red staining of S6K1 phosphorylated at Thr421 and Ser424. Purple represents an overlap of S6K phosphorylation and MHC1, indicating mTORC1 activity in slow twitch muscle fibers.

Plasma and Intramuscular Metabolites

The concentration of plasma lactate increased by 812% and 1039% in HI and BFR groups, respectively, as measured from a blood sample taken within 15 minutes after exercise and compared to a basal blood sample ($P<0.05$; Figure 16A). Intramuscular concentrations of ATP and PCr significantly decreased to a similar extent post-exercise in both groups ($P<0.05$; Figure 16B-C). Intramuscular concentrations of glycolytic intermediate G6P remained stable after HI exercise ($P>0.05$l; Figure 16D) whereas it increased by 294% after BFR exercise ($P<0.05$; Figure 16D).

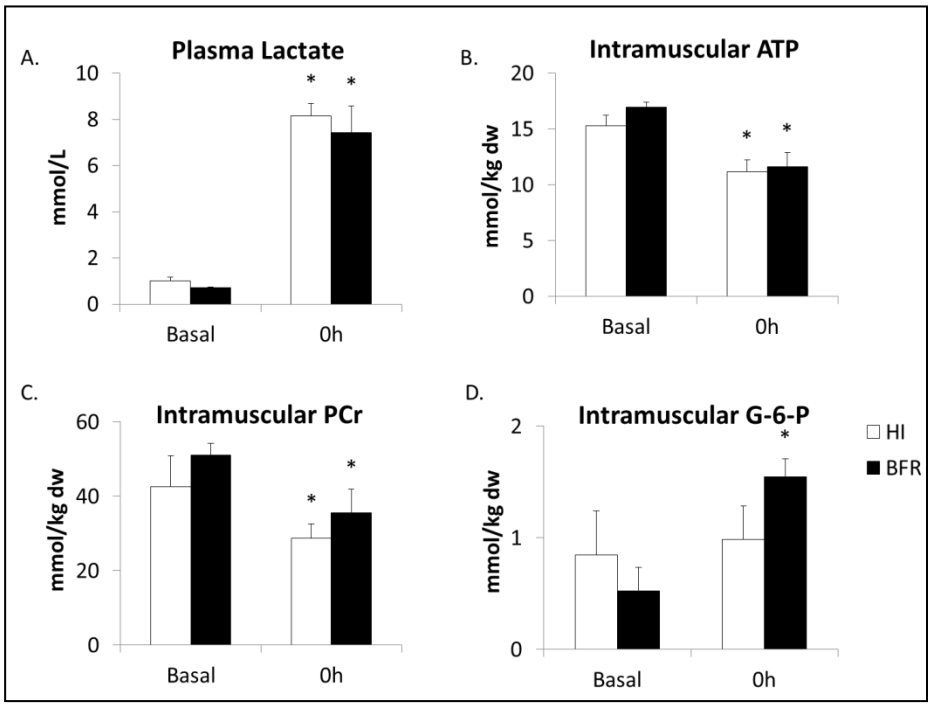


Figure 16: Metabolite analysis comparing basal conditions to tissue samples collected within 15 minutes post exercise. Bars represent SE. *$P<0.05$ vs basal.

Glycogen

Mixed muscle glycogen content at baseline was not different between groups (P<0.05; Figure 17A,C,E). Glycogen content appears to decrease during HI exercise (Figure 17B) while no noticeable changes in glycogen content are apparent after BFR exercise (Figure 17D). Quantitative analysis of mixed muscle glycogen content reveals a significant decrease in glycogen lasts 3 hours post HI exercise (P<0.05; Figure 17E) while no change in in glycogen from BFR exercise (P>0.05; Figure 17E).

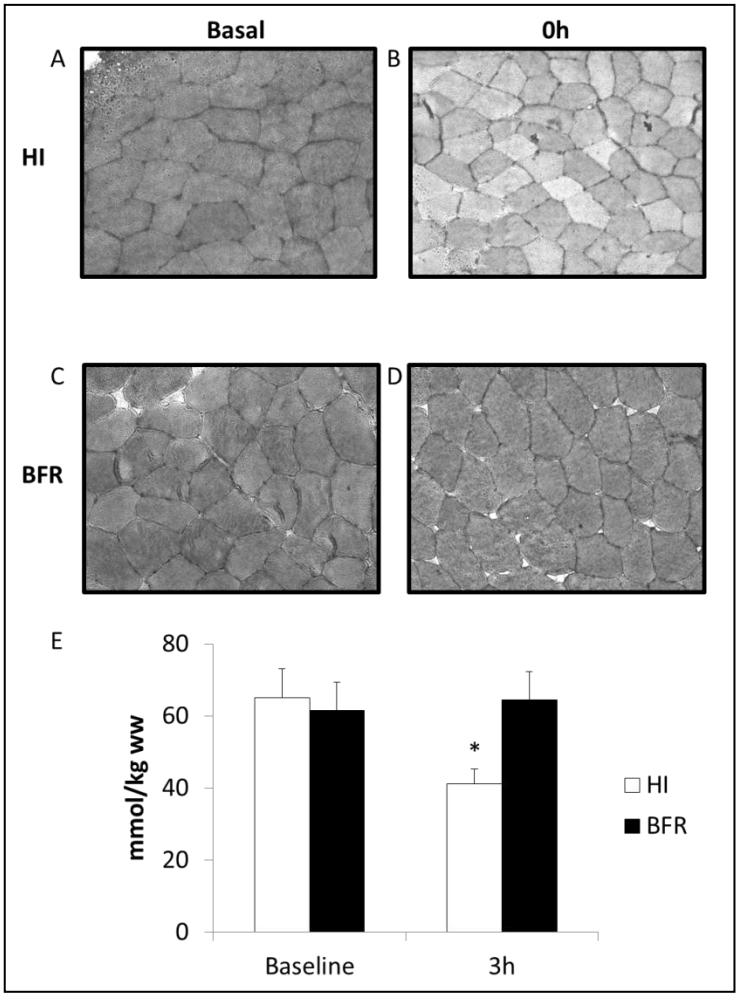


Figure 17: Glycogen content. A-D. Histological staining of glycogen using periodic acid Schiff reaction. Darker staining pattern represents higher glycogen content. Post exercise muscle sample collected within 15 minutes after exercise. E. Quantitative analysis of mixed muscle glycogen content. Presented as mmol/kg wet weight (ww). Bars represent SE. *P<0.05 vs baseline.

Cell Culture

C_2C_{12} myotubes exposed to 30 minutes of 0.5mM lactate solution increased mTORC1 activity as determined by phosphorylation of S6K1 at Thr^{389}, rpS6 at $Ser^{240/244}$, and 4E-BP1 at $Thr^{36/47}$ by 89%, 70%, and 28% respectively ($P<0.05$; Figure 18A). Pre-treatment with 50nM Rapamycin along with 0.5mM of lactate completely abolishes the increase in phosphorylated S6K1 (Figure 18B).

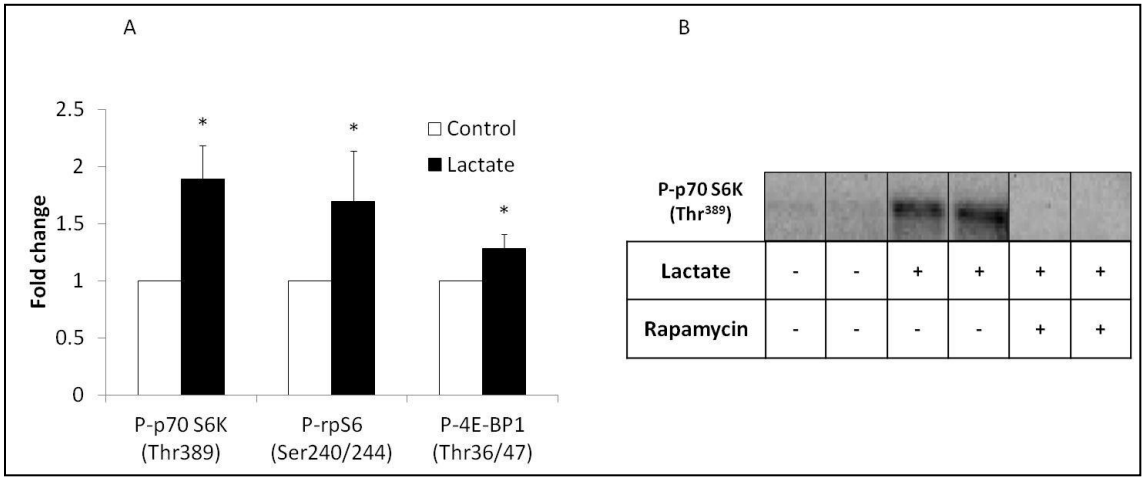


Figure 18: A. Protein phosphorylation of the mTORC1 pathway in C2C12 myoblasts after 30 minute treatment with 0.5mM lactate. Presented as fold change compared to control. Bars represent SE *$P<0.05$ vs control. B. Representative blot of Phospho-S6K1 ± 0.5mM lactate ± 50nM rapamycin.

DISCUSSION

The current research hypothesized that BFR and HI exercise both stimulated mTORC1 signaling and protein synthesis through different mechanisms. In fact, the current data support previous studies that suggest both exercise regimes stimulate protein synthesis and mTORC1 signaling (58, 75). Indeed, novel differences between the two

exercises were elucidated from this study. First, S6K1 phosphorylation appears to occur to a greater extent in fast twitch muscle fibers following HI exercise, whereas this response is more uniform across fibers following BFR exercise. Secondly, as hypothesized, G6P accumulation occurs in skeletal muscle as a result of BFR exercise.

HI exercise training results in significant muscle hypertrophy. However, a recent review concludes that the majority of hypertrophy from this type of exercise arises primarily from fast twitch muscle fibers due to a two-fold greater hypertrophy in fast twitch fibers compared to slow (11). As a matter of fact, Aagaard *et al.* demonstrated that after 14 weeks of HI resistance training, fast twitch fibers were significant hypertrophied whereas slow twitch fibers were not different from pre-training (1). The current results are in line with these studies, indicating that HI exercise increases mTORC1 primarily in fast twitch fibers. In contrast, BFR exercise has been reported to induce hypertrophy in all fiber types according to a recent study (153). This also agrees with the current data that mTORC1 activity is more distributed among fiber types following BFR exercise. Fast twitch fiber activation has been confirmed to occur during BFR exercise with the observation of inorganic phosphate peak splitting measured by magnetic resonance spectroscopy (189-191). However, a possible explanation responsible for this phenomenon involves two elements of BFR exercise.

First, despite the well-accepted idea that fast twitch muscle fibers are recruited due to early muscle fatigue, fast twitch fibers are also more sensitive to acidosis, leading to diminished muscle tension (40, 52, 68, 151). Considering the lack of venous return and general blood flow around the active muscles, lactate clearance is minimal. Thus, constant lactate production along with drastically reduced clearance would lead to a continuous rise in intracellular lactate and H^+ concentrations, decreasing pH. Therefore,

although, fast twitch muscle fibers are recruited, they would produce progressively less force as BFR exercise continues. As a result, slow twitch fibers are left with an increased load. The major difference with HI exercise is that while lactate production is high, lactate clearance mechanisms are also available to extend the duration of fast twitch muscle involvement.

The second element responsible for the distinct response to BFR exercise is the exercise intensity. Typically with HI exercise, whole muscle failure occurs in parallel with fast twitch fiber fatigue. Naturally, this is attributable to slow twitch fibers being physiologically incapable of contributing a great degree of power to lift heavy loads, and thus may be a potential limitation as to why they do not typically hypertrophy from HI exercise. On the other hand, if fast twitch muscle fibers begin to fail during BFR exercise due to acidosis, the low-intensity nature of BFR exercise may allow the fatigue resistant, slow twitch fibers to endure a higher than normal load. This rare scenario of slow twitch fibers taking over for fast twitch fibers may be the mechanism responsible for slow twitch mTORC1 activity and fiber hypertrophy.

Interestingly, this observation may also provide an explanation as to why BFR exercise appears to be equally anabolic in the elderly and younger populations (73, 75), in contrast to HI exercise which exhibits a well-documented impaired anabolic response in the elderly (71, 125). It is well accepted that aging coincides with a loss of fast twitch fibers and a decrease in size, leaving slow twitch fibers occupying a greater fraction of whole muscle as Brunner *et al.* summarized in a systematic review (32). Provided that HI exercise stimulates growth of primarily fast twitch muscle fibers, as discussed above, the mixed muscle protein synthetic response following HI exercise would be dependent on the muscle fiber composition. Therefore, HI exercise coupled with reduced fast twitch

fiber content, such as in the aging population, would result in less muscle protein synthesis, even if synthesis rates were identical at the fast twitch fiber level. Conversely, the added contribution and anabolic responses of slow twitch fibers during BFR exercise permits elderly muscle to synthesize all fibers types including the predominantly slow twitch fibers.

Lactate and the resulting acidosis play several roles in muscle fatigue at the level of the sarcomere cross-bridges, the sarcoplasmic reticulum affecting force production (68, 74) and the glycolytic enzyme PFK affecting energy production (45). The results in the current study did not demonstrate any significant differences in post exercise plasma lactate concentration between exercise trials. However, the noteworthy difference is that the 10.4 fold increase in lactate during BFR exercise was generated and contained within the muscle for the duration of the exercise. Conversely, the 8.1 fold increase in lactate from HI exercise was generated over 30-45 minutes and continuously dispersed away from the active muscles. Therefore it can be deduced that the muscle intracellular lactate concentration during BFR exercise is considerably greater than during HI exercise.

Considering the direct inhibitory role lactate has on PFK activity (45), together with data that suggests G6P is involved with mTORC1 activation (182), the association of elevated G6P after BFR exercise and the sparing of glycogen compared to HI exercise encourages the speculation that glycolysis was inhibited during BFR exercise and therefore this may be a possible additional metabolic pathway that links BFR exercise to muscle protein synthesis (Illustration 5).

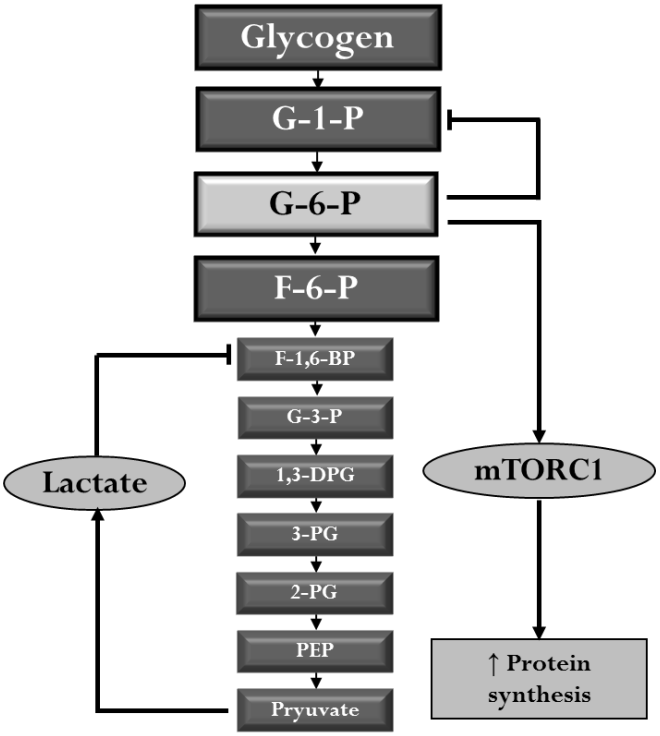


Illustration 5: Proposed mechanism of BFR exercise inducing protein synthesis: Insufficient oxygen supply for energy demands rapidly up-regulates anaerobic glycolysis producing lactate. Insufficient lactate clearance feeds back and inhibits PFK causing the increase in glycolytic intermediates upstream. G6P is a known inhibitor of glycogen phosphorylase thereby sparing glycogen. G6P also stimulates mTORC1 which enhances protein synthesis.

Human studies are often limited to associative results. However, the mechanistic investigation isolating lactate as an independent variable and removing any other confounders such as growth factors and nutrients using C_2C_{12} myotubes supports the proposed mechanism of metabolite-induced mTORC1 signaling. The current data suggest that lactate alone is sufficient to induce mTORC1 activation and is completely abolished with rapamycin treatment.

In summary, this research demonstrates that BFR exercise is unique compared to traditional HI exercise as related to metabolic stress. This research demonstrated that metabolic stress may play a direct role in inhibiting glycolytic flux, sparing glycogen breakdown and inducing the accumulation of G6P, which can activate mTORC1. Additionally, while HI exercise stimulates mTORC1 in primarily fast twitch muscle fibers, accumulating metabolites may be involved in the resulting muscle anabolic response following BFR exercise, which showed enhanced involvement in the slow twitch muscle fibers compared to HI exercise. The proposed explanation may also explain how BFR exercise can adequately stimulate protein synthesis in the elderly population. Additional studies should examine the mechanistic necessity of G6P in response to BFR exercise.

CHAPTER 5

Conclusions

Skeletal muscle has several critical roles in human physiology not limited to the common uses in physical activities, but also including hormone production, glucose regulation, wound healing, immune function and disease prevention. Moreover, in the state of severe disease, an adequate level of muscle mass is crucial and a determinant for survival. Unfortunately, during the aging process, muscle loss is inevitable leading to sarcopenia for reasons that are inconclusive. Resistance exercise is commonly used to stimulate muscle growth considering that a single bout is known to stimulate MPS within 1 hour and remain elevated up to 24-48 hours (71, 158). However, the older population has a diminished ability to stimulate muscle growth after a similar bout of resistance exercise (71, 125).

The preceding research investigated BFR exercise, a unique exercise protocol that, despite numerous differences in comparison to traditional resistance exercise training, is still capable of stimulating muscle hypertrophy to a similar degree. Interestingly, BFR exercise appears to affect older adults similarly to younger adults, thereby overcoming the age-dependent impairment of the muscle anabolic response to resistance exercise. Heretofore, research on BFR exercise has been predominantly descriptive with minimal advances as to the mechanisms involved in the induction of MPS and ultimately hypertrophy. The difficulty is that skeletal muscle protein metabolism is an elaborate network of cellular signaling pathways with several different points of entry as described in detail in Chapter 1. Understanding the mechanism of how BFR exercise can stimulate MPS could improve our understanding of how the muscle

anabolic response is an impaired with age. Furthermore, while several theories exist regarding the cellular mechanisms, research in human clinical research has a very limited ability to investigate such mechanisms. Despite this, the preceding investigations were designed to mechanistically examine BFR exercise in humans and related cell culture experiments. Ultimately, the precise mechanism(s) remain unclear though significant advances have been made.

The use of rapamycin in human studies is a well-established method to inhibit the mTORC1 pathway in order to determine the pathway's necessity in the hypertrophic response from a variety of anabolic stimuli. Previously published research using rapamycin has determined that mTORC1 activation is required for the increase in muscle protein synthesis following high-intensity resistance exercise and essential amino acid ingestion. Accordingly, the current research demonstrates that BFR exercise is no exception, and mTORC1 is indeed the master growth regulator of the cell.

Prior research has indicated that increased blood flow plays a permissive role in the regulation of MPS (201, 203). It is believed that endothelial dysfunction preventing hyperemia may be a mechanism that explains the blunted muscle anabolism in the elderly population. Fitting to this theory, BFR release causes a dramatic reactive hyperemic effect thought to possibly be a co-stimulant with low intensity exercise that together may overcome the age dependent muscle anabolic impairment. Although, infusing the pharmacological vasodilator, SNP, directly into the femoral artery immediately following low-intensity resistance exercise of the quadriceps was insufficient to reproduce the anabolic response of BFR exercise.

Metabolic stress has also been suggested to play a role in BFR exercise induced hypertrophy. Certain metabolites such as lactate, H^+, and inorganic phosphate are known

stimulants of muscle fatigue via interference with muscle contraction and inhibiting anaerobic energy production (68, 74). Of particular interest, lactate is known to specifically inhibit PFK, an enzyme involved in the regulation of glycolysis (45). Coincidently, it was determined that BFR exercise generates a substantial quantity of lactate that also accumulates in the muscle fibers due to the occlusion of venous return. Associated with the increase in lactate, was a concomitant increase in G6P, a glycolytic metabolite upstream of PFK and a known activator of mTORC1 (182). This research speculates that BFR exercise metabolic stress may in fact be a stimulus for hypertrophy via G6P, induced by intramuscular lactate accumulation. This proposed mechanism is supported by supplemental cell culture experimentation that finds lactate alone is sufficient to induce mTORC1 activity in C_2C_{12} myoblasts.

Metabolic stress inducing muscle fatigue leading to enhanced muscle fiber recruitment has also been proposed as a mechanism to stimulate hypertrophy following BFR exercise. Studies have reported EMG activity during BFR exercise to be similar to traditional HI exercise (197, 229). In addition, studies have also demonstrated that fast twitch muscle fibers are recruited during BFR exercise as measured by inorganic phosphate splitting via magnetic resonance spectroscopy (189-191). Consequently, it is believed that activation of fast twitch muscle fibers are required to stimulate hypertrophic responses. Epifluorescence immunohistochemistry analysis of muscle after HI exercise revealed that mTOR activation occurs primarily in the fast twitch muscle fibers. Interestingly, current data demonstrate BFR exercise activates mTORC1 in all muscle fibers with particular emphasis on slow twitch muscle fibers suggesting that slow fibers have a higher involvement in BFR exercise compared to HI exercise. An explanation for this response may possibly be related to enhanced fatigability in fast twitch muscle fibers.

As a result, slow twitch fibers may endure the remaining tension provided the load is low enough to be within the physiological power range of slow twitch fibers.

Due to the diminished fraction of whole muscle occupied by fast twitch muscle fibers in older individuals, activation of anabolic cell signaling and MPS in slow twitch muscle fibers may be a potential mechanism that overcomes the age dependent impairment in muscle protein synthesis following HI exercise.

Collectively, these studies further our understanding of the mechanisms underlying BFR exercise, which will contribute to our knowledge of age-related differences in the anabolic response to exercise.

Glossary

0h	Shortly post-exercise
1RM	One-repetition maximum
3h	3 hours post-exercise
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
AMPK	AMP activated protein kinase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BFR	Blood flow restriction
Ca^{++}	Calcium ion
CaM	Calmodulin
CBP80	80kDa-subunit of the CBC
CON	Control
COX-2	Cyclooxygenase 2
CREM	CRE modulator
Ct	Cycle threshold
DEXA	Dual-Energy X-Ray Absorptiometry
E_A	Enrichment in the artery
eEF2	Eukaryotic elongation factor 2
eEF2K	Eukaryotic elongation factor 2 kinase
eIF2B	Eukaryotic initiation factor 2B

eIF3	Eukaryotic initiation factor 3
eIF4A	Eukaryotic initiation factor 4A
eIF4B	Eukaryotic initiation factor 4B
eIF4E	Eukaryotic initiation factor 4E
eIF4F	Eukaryotic initiation factor 4F
E_M	Enrichment of muscle intracellular protein
EMG	Electromyography
E_P	Enrichment of bound muscle protein
FAK	Focal adhesion kinase
FBR	Fractional breakdown rate
FOXO1	Forkhead box protein O1
FOXO3	Forkhead box protein O3
FSR	Fractional synthesis rate
G6P	Glucose-6-Phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCMS	Gas chromatography mass spectrometer
GEF	Guanyl nucleotide exchange factor
GH	Growth hormone
GSK3	Glycogen synthase kinase 3
GβL	G-protein β-like subunit protein
HBS	Hepes buffered saline
HG	Housekeeping gene
HI	High intensity

ITS-CRC	Institute for Translational Sciences Clinical Research Center
IRS1	Insulin receptor substrate 1
LAT1	System L amino acid transporter
MAP4K3	Mitogen activated protein kinase kinase kinase kinase 3
MAPK	Mitogen Activated Protein Kinase
Mnk1	MAP kinase interacting kinase 1
MP1	MAPK scaffold protein 1
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
MuRF1	Muscle specific RING finger 1
OCT	Optimal cutting temperature
PA	Phosphatidic acid
PABP	Polyadenlyated binding protein
PAT1	Proton assisted amino acid transporter 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCr	Phosphocreatine
PDCD4	Tumor suppressor programmed cell death protein 4
PDK1	Phosphoinositide dependent protein kinase 1
PFK	Phosphofructokinase

PI	Phosphatidylinositol
PI3K	Phosphatidyl inositol 3 kinase
PIP2	PI 3,4-bisphosphate
PIP3	PI 3,4,5-trisphosphate
PKB	Protein kinase B
PLD1	Phospholipase D1
PRAS40	Proline-rich AKT substrate of 40kDa
PROTOR	Protein observed with RICTOR
Q	Blood flow
Q_M	Free phenylalanine content
RAP	Rapamycin
RAPTOR	Regulatory-associated protein of mTOR
Rheb	Ras homologue enriched in brain
RICTOR	Rapamycin-insensitive companion of mTOR
ROS	Reactive oxygen species
rpS6	40S ribosomal protein S6
RSK	p90 ribosomal protein S6 kinase
S6K1	Ribosomal S6 protein kinase 1
SAC	Stretch activated ion channel
SE	Standard error
Ser	Serine
SIN1	Stress-activated map kinase-interacting protein 1
SKAR	S6K Aly/REF-like target

SNAT2	System A, sodium dependent neutral amino acid transporter 2
SNP	Sodium nitroprusside
T	Bound phenylalanine content
t	Time
TAM	Time-averaged mean velocity
TG	Target gene
Thr	Threonine
Try	Tyrosine
TSC1	Tuberous sclerosis complex 1
TSC2	Tuberous sclerosis complex 2
Vps34	Vacuolar protein sorting 34

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Vita

David Miles Gundermann was born on January 30, 1983 in Kitchener, Ontario, Canada to Glenn Gundermann and Beverley Ellis. David grew up in Burlington, Ontario attending C.H.Norton public school and Lester B. Pearson high school. Following high school, David moved to Guelph, Ontario where he attended the University of Guelph and enrolled in the Honors Bio-Medical Science program. It was during his time at the University of Guelph where he gained a keen interest in exercise physiology and skeletal muscle metabolism. After graduating with a bachelor of science from the University of Guelph, David moved to Gainesville, FL to pursue a Master of Science degree at the University of Florida. At UF his research consisted of skeletal muscle catabolic cell signaling in response to disuse atrophy and peripheral arterial disease in rodent models. David also collaborated with the Aging & Rehabilitation Research Center working on a pilot project involving blood flow restriction (BFR) exercise that led him to pursue more advanced research with BFR exercise. Thus after graduating from the University of Florida, David enrolled in the Ph.D. program at the University of Texas Medical Branch in Galveston, TX to continue his research in BFR exercise.

Education

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