

**MECHANICAL STRETCH AND ELECTRICAL STIMULATION IN MOUSE
SKELETAL MUSCLE *IN VIVO*: INITIATION OF HYPERTROPHIC
SIGNALING**

A Thesis
Presented to
The Academic Faculty

By
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In Partial Fulfillment
Of the Requirements for the Degree
Master of Science in Bioengineering

Georgia Institute of Technology
July 2004

MECHANICAL STRETCH AND ELECTRICAL STIMULATION IN MOUSE
SKELETAL MUSCLE *IN VIVO*: INITIATION OF HYPERTROPHIC SIGNALING

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Dedicated to
The Trinity, Brathwaites, Hostens

ACKNOWLEDGEMENT

I would like to thank Dr. Burkholder for allowing me to experience a time in my life when things often seemed like there was no light at the end of the tunnel but through it all I learned how to trust more and develop a more confident and structured character.

I would like to thank Anne Claire Bellott for sharing in this experience and continuing to motivate me while we both struggled to reach this point.

A special thanks to Brenda Bondesen who worked tirelessly to help me figure out what the problems were and never said no to a request regardless of how much time it would take out of her own schedule.

Special Thanks to Dr. Grace Pavlath and Dr. Cheng Zhu for their commitment towards excellence and their oversight of the thesis process.

Thanks to Georgia Tech and Emory University for their facilities

Finally, thanks to all the people who have lent support in their various ways from the first day I started this program until the final days of its completion, again, thank you.

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LIST OF ABBREVIATIONS

4EBP1 – 4E Binding Protein 1

ANOVA – Analysis of variance

AP1 – Activator protein 1

BCA – Bicinchronic acid assay

bFGF – Basic fibroblast growth factor

BSA – Bovine serum albumin

CAN – Calcineurin

cDNA – Complementary deoxyribonucleic acid

CFW – Carworth Farm-Webster

CONT – Control

COX – Cyclooxygenase

DHPR – Dihydropyridine receptors

DNA – Deoxyribonucleic Acid

EC – Active lengthening (Eccentric)

ECM – Extracellular Matrix

EDTA – Ethylene Diamine Tetra Acetate

EI – Eccentric Injury

EIF-4E – Eukaryotic translation initiation factor 4E

ERK – Extracellular-signal related kinase

FG – Fast glycolytic

FGF – Fibroblast growth factor

FOG – Fast oxidative glycolytic

FRAP- Fluorescence recovery after photobleaching

IGF – Insulin like Growth Factor

IL- Interleukin

IP3 – Inositol tri-phosphate

IRS1 – Insulin Receptor substrate 1

Iso – Isometric activation

JNK- Jun N-terminal kinase

MAP kinase – Mitogen activated protein kinase

MEF2 – Myocyte enhancer binding factor 2

MEK – Mitogen activated protein kinase kinase

mRNA – Messenger ribonucleic acid

mTOR – Mammalian target of Rapamycin

NaCL – Sodium chloride

NFAT – Nuclear Factor of Activated T cells

NSAIDs – Non-steroidal anti-inflammatory drugs

PAS – Passive lengthening

PCR – Polymerase chain reaction

PK1 - 3-phosphoinositide-dependent protein kinase-1

PG - Prostaglandin

PI3 Kinase – Phosphatidylinositol-3-OH Kinase

PKB – Protein kinase B

PKC – Protein kinase C

PLC – Phospholipase C

PLSD – Protected least significant difference

P70 – P70 S6 Kinase

RAP – Rapamycin

RNA – Ribonucleic Acid

RyR – Ryanodine receptors

SAPK – Stress activated protein kinase

SDS – Sodium dodecyl sulphate

TA – Tibialis anterior muscle

SUMMARY

Skeletal muscle has an integral role in many activities. Although mechanical stretch and active force generation are known to be required for the maintenance of healthy muscle function, the mechanism by which those signals mediate muscle growth is unknown.

This project was based on the hypothesis that *stretch and force generation activate the Calcineurin/NFAT pathway and induce Cox-2 expression and initiate muscle hypertrophy*. The specific aims of this study were to 1) develop a minimally invasive system capable of initiating hypertrophic signaling in mice, 2) characterize the effects of isometric activation, passive lengthening, and active lengthening on signaling cascades, and 3) determine the involvement of the Calcineurin/NFAT pathway and activation of COX-2 gene expression. We propose a pathway in which stimuli increase intracellular calcium, which activates the phosphatase calcineurin. Calcineurin dephosphorylates NFAT, which is translocated into the nucleus and initiates transcription of the COX-2 gene. COX-2 mediated synthesis of PG_{G2} is the rate-limiting step in bioactive prostaglandin synthesis. Prostaglandins then stimulate known hypertrophic signals including the PI-3 Kinase and MAP Kinase signaling cascades.

Adult female mice were subjected to passive stretch, isometric activation or active stretch of the tibialis anterior muscle. Phosphorylation of p42 MAP kinase, p38 MAP kinase, p70 S6 Kinase, 4E-BP1 and NFAT was evaluated after a 3-hour recovery period. COX-2 gene expression was evaluated by quantitative PCR. There were significant, protocol dependent responses within the PI-3 Kinase and MAP Kinase signaling

cascades, but no significant dephosphorylation of NFAT and no detectable induction of COX-2 expression. These findings demonstrate that stretch and activation independently activate a range of signaling cascades, but suggest that initiation of the response does not involve dephosphorylation of NFAT or upregulation of COX2. This represents incremental understanding of the specific mechanisms of hypertrophy and suggests alternate mechanisms that may promote muscle hypertrophy and prevent atrophy.

INTRODUCTION

CLINICAL RELEVANCE

Growth and adaptation of skeletal muscle are ongoing processes. Skeletal muscle adapts to muscle loading, injury, or disease by hypertrophy or atrophy (1-6).

Muscle hypertrophy, which is an increase in muscle mass, is mediated through two mechanisms, cell growth and proliferation. Muscle overload initiates hypertrophy (1-4) by increasing protein synthesis and decreasing protein degradation (1,2), elevating amino acid synthesis and transport (3), increasing total cellular RNA content (4), and increasing satellite cell proliferation (5,6,7). Satellite cells are normally quiescent in adult muscle, but can be activated by extensive stretch, severe exercise or injury. Activated satellite cells proliferate and migrate to the injury site where they differentiate and fuse to form new fibers or enlarge existing fibers (8,9,10). Early indicators of hypertrophic signaling can be observed within one hour of the stimulus (11).

Muscle atrophy, the loss of mass and force generating capacity, can be induced by aging (12,13), inactivity (14), spaceflight (15), muscle disorders (16), and repeated muscle injury (17) (figure 1). During normal aging, the muscle cross sectional area is reduced (18), strength is reduced (19), and muscles are more susceptible to damage and degeneration (20). Sarcopenia, an age related loss of muscle mass and function has the effect of reducing force generation and mobility in the aged (21). As a person approaches 70 years of age, sarcopenia symptoms arise, and nearly half of all persons over 80 years of age suffer from sarcopenia (22). The 2000 United States census estimated the population of people older than age 65 would increase by 137% between 1999 and 2050.

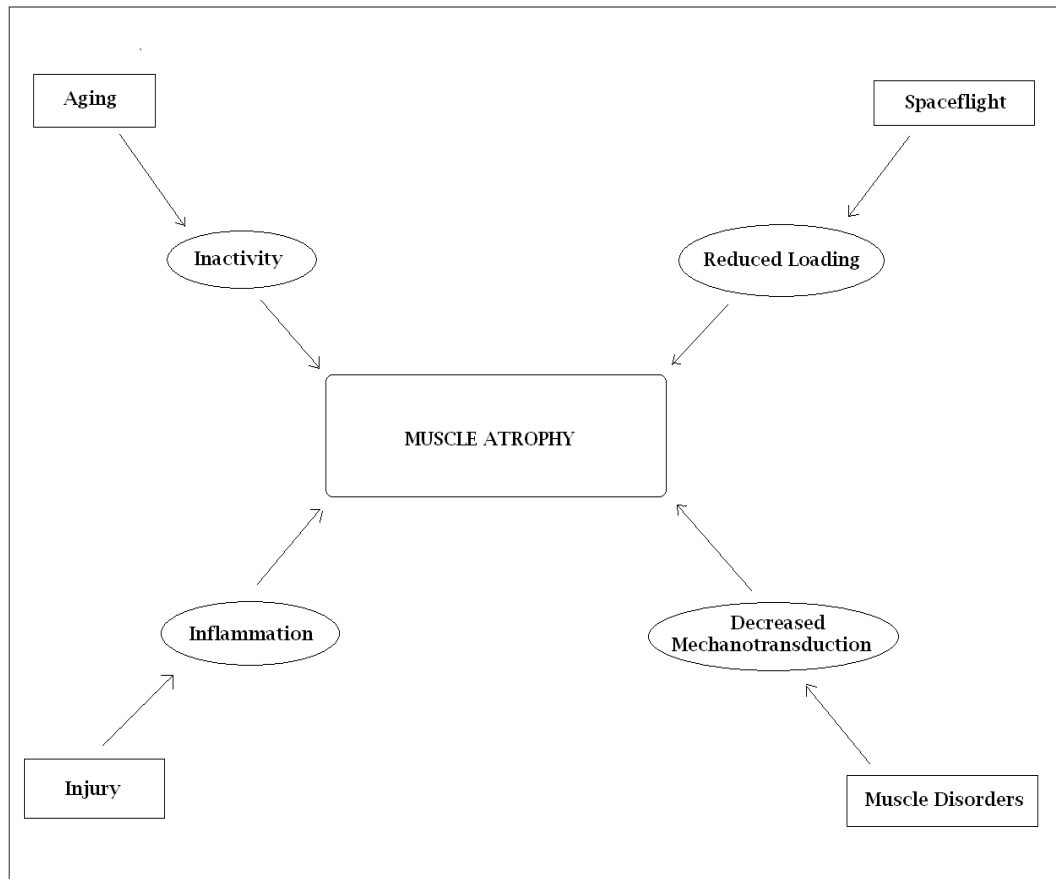


Fig. 1. Muscle atrophy occurs through aging, spaceflight, muscle disorders and injury through various mechanisms.

Several mechanisms contribute to sarcopenia, including loss of motor neurons in the spinal column, impairment of endogenous growth hormone, androgen and estrogen production, inadequate protein intake, deregulation of catabolic cytokines, and reduced physical activity (23). Key elements necessary for muscle maintenance including myogenin, MyoD, and growth hormones are reduced in aged muscle (24). Despite reductions in these factors, aged muscle has been shown to maintain the ability to hypertrophy through resistance exercise (25), demonstrating that extensive sarcopenia may be prevented. Although exercise can reduce this atrophy, aging skeletal muscle cannot respond to the extent to which younger muscle responds to the physical activity (24) and may still lose mass (26). The resulting reduction in force further limits mobility (27), promoting a downward spiral of functional degeneration. Functional limitations can promote more sedentary lifestyles for older individuals, which is another causative mechanism for atrophy.

The Centers for Disease Control and Prevention (CDC) reported that four out of ten Americans age 45-64 are sedentary, increasing to six out of ten for those 75 and older (28). These individuals are not active enough to derive health benefits from their activity (29). The fraction of adults that do not achieve the recommended activity levels continues to rise (27). Muscle inactivity reduces the load on the muscle, leading to atrophy (21), enzymatic and contractile property changes (30), reduced peak power (31), and fiber type change (32). It is not clearly understood how inactivity initiates these deficits but research into the effects of spaceflight produced valuable information on the results of decreased loading.

Spaceflight causes dramatic reduction in the loads applied to muscles, and consequently inhibits expression of genes for cell proliferation and growth factor

cascades, including cell cycle genes and signal transduction proteins (33). Reductions in force generating capacity, and increases in the proportion of fast myosin in some fibers are also seen in rats and humans during spaceflight (34,35,36). Atrophy in the extensor muscles is exaggerated in space due to their normal function to maintain extension against gravitational loads, which in these limbs is greatly reduced (37). Hindlimb suspension, which unloads the anti-gravity muscles to simulate microgravity, induces atrophy in rat muscles similar to spaceflight (38,39). Exogenous growth hormones and insulin-like growth factor I (IGF-I) along with exercise have shown to prevent this muscle wasting in suspended rats (40). These factors are normally found to be secreted from cells during force generation and act as paracrine and autocrine factors for chemical signaling.

Muscle disorders, including the muscle dystrophies, disrupt the transmission of muscle force and the associated signaling cascades. Muscle cells are anchored to the extracellular matrix (ECM) and surrounding cells through membrane receptors including integrins, cadherins, selectins and cell adhesion molecules (41,42). These receptors, clustered within focal adhesion complexes and intercellular complexes, are physically attached to the cell cytoskeleton (43). Cell-generated stresses and external mechanical forces converge on adhesion sites and the transmembrane receptors present in these sites provide molecular supports for the transfer of mechanical signals across the cell surface (44). Without the ability of the cell to sense changes in its structure and environment, it loses its ability to adapt, which in skeletal muscle is essential (16,45,46). Deficits in mechanical signaling in dystrophic muscle lead to abnormal repetitive cycles of degeneration-regeneration and compromised function (47). This

degeneration-regeneration cycle is typically associated as a result of fiber damage and not normal activity.

Muscle fiber damage requires the removal of damaged or necrotic tissue and regeneration by satellite cells (48). Chronic exposures to repeated strains result in extensive muscle fibrosis (49) and this active lengthening can induce the inflammatory response (50,51). Although it is important for healing, inflammation is associated with phagocyte infiltration to the injured muscle, elevation of protein degradation, and often aggravation of the initial insult. Inflammation subsequently leads to pain (52), swelling (53), and cellular release of cytokines. Cytokines mediate inflammation and are involved in cell proliferation, cell migration, and regeneration. Myocytes in culture respond to damage by upregulating a number of these proinflammatory cytokines (interleukin-1 (IL-1), IL-6), and also prostaglandin E2 (PGE2). Interleukin-1 enhances the expression of cyclooxygenase-2 (COX-2), a proinflammatory enzyme, which plays an important role in the synthesis of prostanoids such as PGE2 and prostaglandin F2 alpha (PGF2 α) (54,55).

Eccentric injuries (EI) are also accompanied by factor release although through a different mechanism. An EI occurs when an active muscle is forcibly lengthened (56,57). This leads to rupture of the cell plasma membrane, loss of sarcolemmal integrity (58,59), and subsequent release of chemical mediators associated with cell growth (60). Non-steroidal anti-inflammatory drugs, which target COX enzyme activity, are often prescribed to decrease the painful effects of inflammation. However, they have been shown to cause side effects of slowing the regenerative process (61). These observations have made these enzymes appear very important in mediating the hypertrophic response and have helped to form a strong basis for studying countermeasures that may activate them.

COUNTERMEASURES

The loss of muscle mass and function associated with aging, inactivity, spaceflight, and disease can be minimized by appropriate interventions. Increased physical activity provides a direct antagonistic signal. In situations in which increasing activity is not possible, direct application of growth factors associated with exercise may be a suitable alternative.

Exercise

Exercise combines mechanical stretch and muscle fiber activation and promotes muscle growth and maintenance by inducing gene expression and increasing protein synthesis (62,63). While the exact mechanism is not known, exercise increases intracellular calcium (64) and initiates many muscle signaling pathways thought to be involved in growth (65,66). Passive lengthening, isometric activation, and active lengthening, actions done during exercise, generate different intensities of muscle force and involve different structures and pathways to elicit responses.

Passive force in muscle involves the connective tissue, cellular membrane, and structures within the normal myofibrillar structure, such as titin. It is used to maintain muscle strength, length, and mass in patients who are recently injured or immobilized in the clinical setting (67). During passive stretch, mechanically induced bilayer distortions may influence the conformational change of membrane-associated proteins. These changes in the membrane local curvature or thickness could activate mechanically gated channels (68). It is also thought that this change in structure can activate cytoskeletal associated signaling molecules, thus setting off growth cascades.

Calcium, one of the predominant signaling molecules, can be released into cells independent of biochemical signals from the plasma membrane in response to physical forces (69). Voltage gated calcium channels can also participate in myogenic responses through opening by a direct effect of stretch (70). This influx is not likely due to disruptions in the membrane because stretches in relaxed fibers generally cause no detectable damage (71). An effect of repetitive passive stretch to the gastrocnemius muscle of anesthetized rats is an increase in the expression of myogenin mRNA (72). As stated previously, myogenin is a key element in maintaining muscle mass. Stretches overloading skeletal muscles in chickens have also led to an increase in α -actin, one of the muscle structure proteins (73). Stretching passive myotubes in culture induces increases in the efflux of PGE2 and PGF2 α in a time and frequency dependent manner. Passive stretching of a rabbit extensor digitorum longus also showed an increase in IGF-1. These second messengers regulate skeletal muscle protein turnover rates (74).

An isometrically activated muscle is held at a constant length during force generation. Excitation-contraction coupling that results in tension increase in the muscle produces force generation. Depolarization of the cellular membrane allows for calcium influx through voltage-gated channels and subsequent activation of the contractile apparatus (75). Isometric activation of denervated muscle reduces atrophy (76), increases force capacity (77,78), restores specific tension, and stabilizes fiber number. Electrical stimulation induced isometric activity is effective in elevating intracellular calcium, but it produces non-physiological chronic membrane depolarization and tonic elevation of intracellular calcium (79). Those conditions do not mimic cellular responses to physiological patterns of phasic neuronal activity (79). Calcium dependent pathways including the calcineurin/NFAT (Nuclear Factor of Activated T cells) pathway and

phosphatidylinositol-3-OH kinase (PI3K) pathway can be activated by the influx, and associated cytoskeletal signaling molecules can be affected by the change in tension (80).

The muscle being forcibly lengthened while activated denotes active lengthening or eccentric stretch. These components (figure 2) are widely viewed as normal during exercise. The eccentric component of movement provides the deceleration forces needed for the maintenance of balance, stability, posture, and mobility (81). Along with the results of passive lengthening, and isometric activity, active lengthening can incur damage to muscle cells, setting off inflammation and degeneration (50,51). Disruptions in the plasma membrane may mediate the increases in protein synthesis associated with this type of activity (58,59) and through increases in calcium and release of growth factors like basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (74,82,83).

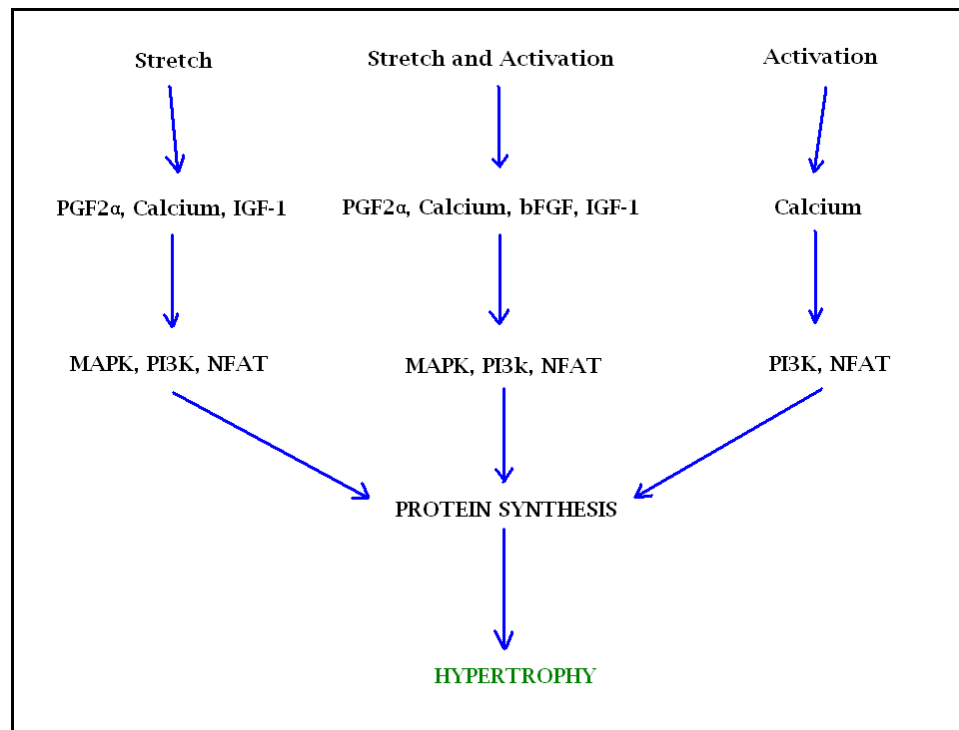


Fig. 2. The components of exercise lead to activation of signaling molecules, which initiate the various hypertrophic pathways. These events result in hypertrophy.

Second Messengers

The components of exercise are thus associated with the release and synthesis of factors including bFGF (84), IGF-1 (85), and PGF2 α (54,55). Direct use and treatment of muscles with these growth factors can mimic the effects of exercise by activating signaling molecules associated with muscle hypertrophy.

The FGF family has nine different isoforms. In addition to an increasing satellite cell proliferation, the FGF family also slows the conversion of satellite cells to myofibers (86). Basic Fibroblast growth Factor 2 (FGF-2) and bFGF are released by stretch in whole muscle and culture and stimulate proliferation, repress differentiation by suppressing MyoD and myogenin, and activate the mitogen-activated protein kinase (MAPK) pathway (87). The MAPK pathway is also important in transducing the FGF-induced increase in satellite cell proliferation but does not mediate the FGF-mediated repression of satellite cell differentiation (88).

IGF-I also utilizes the MAP kinase (89) along with the calcineurin/NFAT (90), and PI3K pathways (91). Skeletal myofibers secrete IGF-I, which is important in skeletal muscle regeneration and has been shown to enhance satellite cell proliferation and increase muscle mass in aged animals (92,93). Exercise elevates IGF-I levels in the muscle, which leads to an increase in DNA content and ensuing hypertrophy of skeletal muscle (85,94).

PGF2 α promotes the skeletal muscle growth by increasing protein synthesis efficiency at the ribosomes (95) and activating NFAT (96), which is a calcium dependent factor. Intermittent stretching of isolated rabbit muscles induces a calcium dependent increase in protein synthesis and prostaglandin-F2 alpha release (97). Repetitive mechanical stimulation of differentiated skeletal muscle in tissue culture also increases

the long-term production of $\text{PGF2}\alpha$ (98). It is thought that an initial intracellular event following $\text{PGF2}\alpha$ binding to its receptor involves the activation of a G-protein-sensitive phospholipase C (PLC). Activation of PLC generates the second messenger inositol trisphosphate (IP3) that binds to receptors in the sarcoplasmic reticulum to stimulate the release of calcium ions and an elevation in the concentration of cytoplasmic calcium (99). This suggests that $\text{PGF2}\alpha$ not only is regulated by calcium, but also plays a role in calcium release. This suggests that $\text{PGF2}\alpha$ is important in the downstream activation of calcium dependent signaling pathways like the MAP kinase and PI-3 Kinase pathways.

Prostaglandins are often produced as part of an inflammatory response and are involved in initiation of pain and swelling. Non-steroidal anti-inflammatory (NSAIDs) are widely used to alleviate these symptoms and are found in many over-the-counter drugs. NSAIDs inhibit prostaglandin synthesis, reducing inflammation and pain but also muscle regenerative capacity (100). Although NSAIDs initially protect muscle cells from inflammatory degradation, they impair functional capacity and histology in late stage regeneration (61). The histology of tibialis anterior muscle in rats that had undergone controlled uniaxial strain to induce injury and then immobilized post-injury, showed a delay in inflammatory reaction and muscle regeneration (61,103).

Molecules downstream of bFGF, IGF-1 and $\text{PGF2}\alpha$, also modulate the regenerative effect. These molecules are integral in the transduction of the signaling cascades. Responses to muscle stimulation have shown kinase activation (P70 S6, P42 (ERK2), p38) in vivo and changes in 4E binding protein phosphorylation within these signaling cascades (figure 3). These molecules all play roles in cell growth and adaptation signaling cascades (figure 4). These molecules all play roles in cell growth and adaptation

within the calcium dependent PI3 kinase pathway, MAP kinase pathway, and calcineurin/NFAT pathways (figure 3).

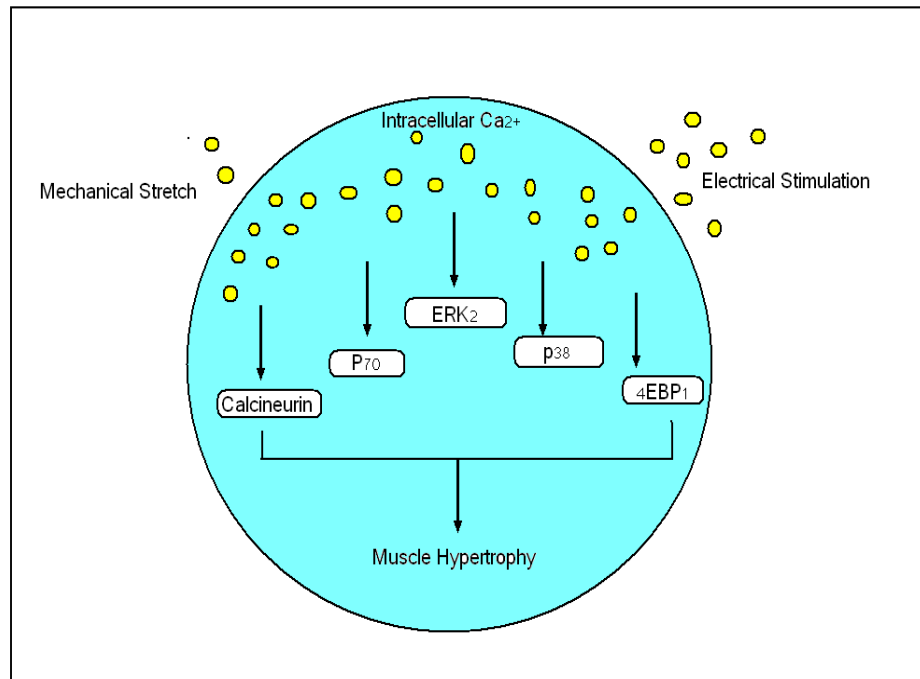


Fig. 3. Calcium plays a role in inducing skeletal muscle hypertrophy through pathways that include each of the signaling molecules: Calcineurin, P70^{S6K}, ERK, p38, and 4EBP1. Mechanical stretch and electrical stimulation induce increases in intracellular calcium.

HYPERTROPHIC PATHWAYS

Exercise and growth factors induce hypertrophy and are associated with activation of specific signaling cascades. Identification of how the cascades (figure 3) mediate the hypertrophic response would allow rational design of pharmacological interventions to most effectively counteract the deleterious effects of aging and disease.

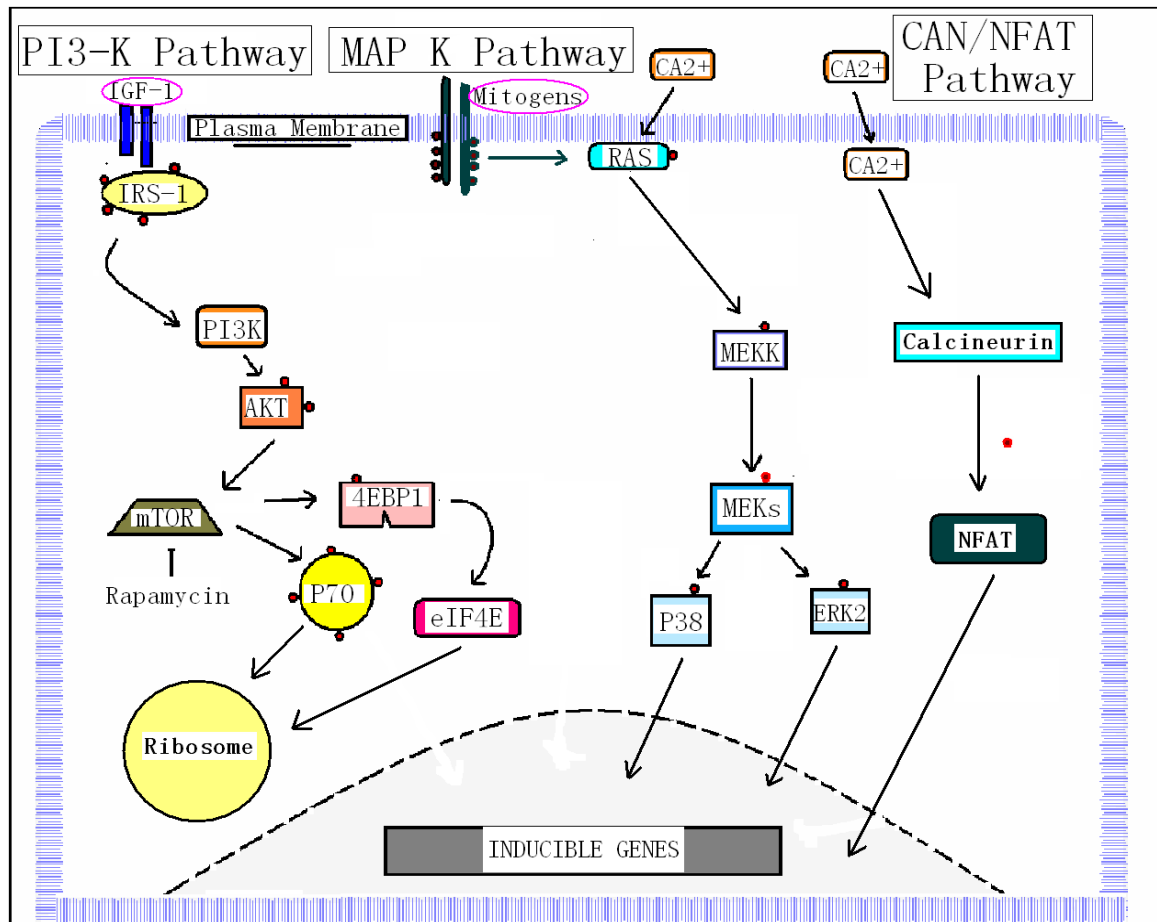


Fig.4. The PI3 Kinase, MAP Kinase and Calcineurin/NFAT pathways induce hypertrophy through activation by internal stimuli. IGF-I is the only ligand capable of activating PI3 kinase. These stimuli include increases in receptor binding, and intracellular concentration of calcium in response stress including electrical stimulation and mechanical stretch. These pathways initiate responses in transcriptional activity.

Calcium Dependent Signaling

Force development is controlled by calcium, which is rapidly released from the sarcoplasmic reticulum through the opening of ryanodine receptors (RyR) and dihydropyridine receptors (DHPR) channels following sarcolemmal depolarization (101). Elevations in intracellular calcium levels activate the calcium dependent molecules that include calpains, calmodulin, PKC and the phosphatase calcineurin (102,103).

Calcineurin is a calcium dependent phosphatase that plays an important role in the adaptive response (104,105,106). Repetitive high-amplitude calcium spikes associated with excitation-contraction coupling activates calcineurin. Calcineurin directly dephosphorylates and activates the transcription factors NFAT and myocyte enhancer binding factor 2 (MEF2) (107) which controls the transcription of, among other things, myogenin and MyoD (108). Calcineurin is also essential for induction of hypertrophy by mechanical overload (109,110).

NFAT

Five distinct isoforms of Nuclear factor of activated T cells (NFAT) have been identified (111,112). Phosphorylated NFATc1 remains in the cytoplasm when calcium channels remain inactive in unstimulated cells (113). Activation of calcineurin causes NFATc1 to be rapidly dephosphorylated and become nuclear transport ready, but actual nuclear transport proceeds slowly (114). Electrical stimulation results in elevated cytosolic calcium resulting in nuclear translocation and appearance of foci of intranuclear NFATc1 (115).

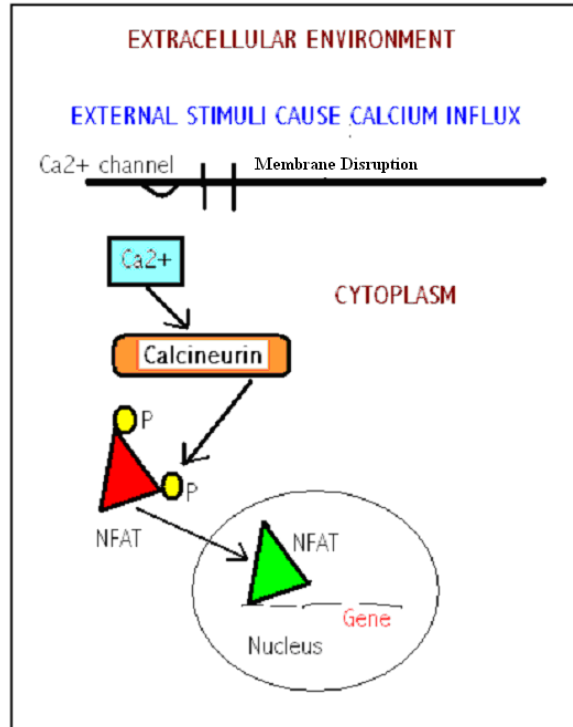


Fig. 5. External stimuli induce a change in intracellular calcium that activates the phosphatase calcineurin. Calcineurin dephosphorylates NFAT, which allows NFAT to enter the nucleus to stimulate gene expression.

Dephosphorylation of NFATc1 reveals a nuclear localization signal, which allows it to migrate into the nucleus (figure 5). Once in the nucleus, NFATc1 binds to AP-1, which is a dimer composed of Fos and Jun proteins (116,117). Induction of NFAT and AP-1 requires both calcineurin, which promotes NFAT dephosphorylation, nuclear translocation, and Ras, which promotes the synthesis, phosphorylation and activation of members of the Fos and Jun families of transcription factors (116). NFAT acts synergistically with AP-1 proteins on composite DNA elements that contain adjacent NFAT and AP-1 binding sites, where they form highly stable ternary complexes to regulate the expression of diverse inducible genes (118). These include the inflammatory and adhesion factors like cytokines IL-2, IL-3, IL-4, IL-5, granulocyte-macrophage

colony-stimulating factor, tumor necrosis factor- α , as well as several cell-surface molecules, such as CD40L and Fas Ligand (119). There is recent evidence that Cyclooxygenase-2 (COX-2) gene expression in human T lymphocytes is also specifically regulated by NFAT (120).

COX-2

COX-2 is one of three isoforms of cyclooxygenase. A wide variety of stimuli including antigens, mitogens, hormones, growth factors and inflammatory mediators induce Cox-2 (121,122,123,). The COX-2 gene has consensus sequences in its promoter region for the NFAT protein. The Cox-2 promoter includes a NFAT sequence at nucleotides -117 to -91 and an NFAT/AP-1 sequence at nucleotides -82 to -58 (120,124). Expression of COX is associated with stretch. An increase in cyclooxygenase enzyme activity was observed within 4 hours of mechanical stretch of muscle in tissue culture and that increase remained for 24 hours. This increase in activity was also accompanied by cellular hypertrophy (125) through a G-protein dependent process. Human myometrial cells grown on flexible bottom culture plates and subjected to 1 or 6 h static stretch produced increases in COX-2 mRNA expression (126). Also, cultured rat bladder smooth muscle cells subjected to continuous cycles of stretch/relaxation showed time-dependent increases in COX-2 expression after stretch, with maximal mRNA and protein levels occurring after 4 h (127).

COX is also responsible for mediating conversion of arachidonic acid to primary prostaglandins (figure 6). These primary prostaglandins all have a common metabolic origin (128). The first step is the synthesis of cyclic endoperoxide prostaglandins (PGG) from arachidonic acid by oxygenation and cyclisation catalyzed by the endoperoxide

synthase component of COX isoenzymes. The second step is catalyzed by the peroxidase component of COX isoenzymes, in which prostaglandins of G class are reduced to form prostaglandins of H class. H class prostaglandins are later acted upon by respective enzymes to form primary prostaglandin molecules including PGE₂ and PGF₂ α (128). Since COX-2 is associated with stretch and mediates prostaglandin synthesis, it appears to be associated with the cellular hypertrophic response. (125,126,127)

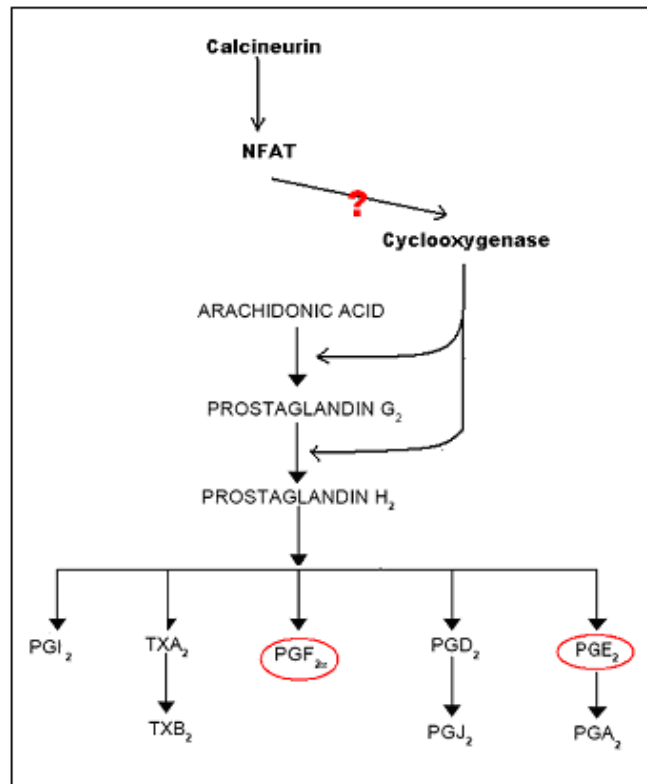


Fig. 6. Biosynthesis of prostaglandins involves cyclooxygenase enzymatic activity. Cyclooxygenase isoenzymes act to convert arachidonic acid to G class prostaglandins and reduce G class prostaglandins to H class prostaglandins. Specific enzymes then convert the H class prostaglandins into the respective prostaglandin molecules. PGE₂ and PGF₂ α both exist in skeletal muscle. H class prostaglandins are acted upon by insoluble endoperoxidase isomerase enzymes and endoperoxidase reductase.

PGF2 α is one of the factors that can activate the PI3K signaling cascade. This cascade is also affected through interactions with IGF-1 and calcium. Downstream effectors of this pathway include P70 S6 Kinase and 4E binding protein, which modulate translational efficiency and translational activity respectively.

P70 S6 Kinase

P70 S6 Kinase (P70^{S6K}) is a member of the Ser/Thr kinase family and enhances the translation of mRNAs encoding ribosomal proteins and elongation factors, integral components of the protein synthesis machinery (129). The nuclear form of P70 phosphorylates the ribosomal S6 subunit in the nucleoplasm and this modification may have a role in ribosome biogenesis in the nucleolus (130). P70^{S6K} plays a role in regulating the translation of mRNA transcripts that contain an oligopyrimidine tract at their transcriptional start site (131). Protein synthesis in response to insulin is blocked by inhibition of P70^{S6K} (132), and the mammalian target of rapamycin activates P70S6k through phosphorylation. P70^{S6K} phosphorylation increases rapidly after plantar flexor caused by strong bouts of electrostimulation exercises (133,134), and this increase in phosphorylation is closely correlated with muscle hypertrophy after repeated exercise (134).

4E Binding Protein

Like P70, 4EBP1 is phosphorylated in vivo on multiple residues and phosphorylated by FRAP/mTOR (135). In quiescent cells, dephosphorylated 4E-BP1 competes with eIF-4G for binding to eIF-4E (figure 7) and represses translation by preventing assembly of eIF4. Initiation factor 4F complexes with mRNAs through the

interaction of its eIF-4G subunit with eIF-4E, and is necessary for translation all eukaryotic mRNAs. Active mTOR phosphorylates 4E-BP1, which decreases its affinity for eIF-4E and releases the block on cap-dependent translation. Phosphorylation of 4E-BP1 increases protein synthesis and promotes muscle hypertrophy (135).

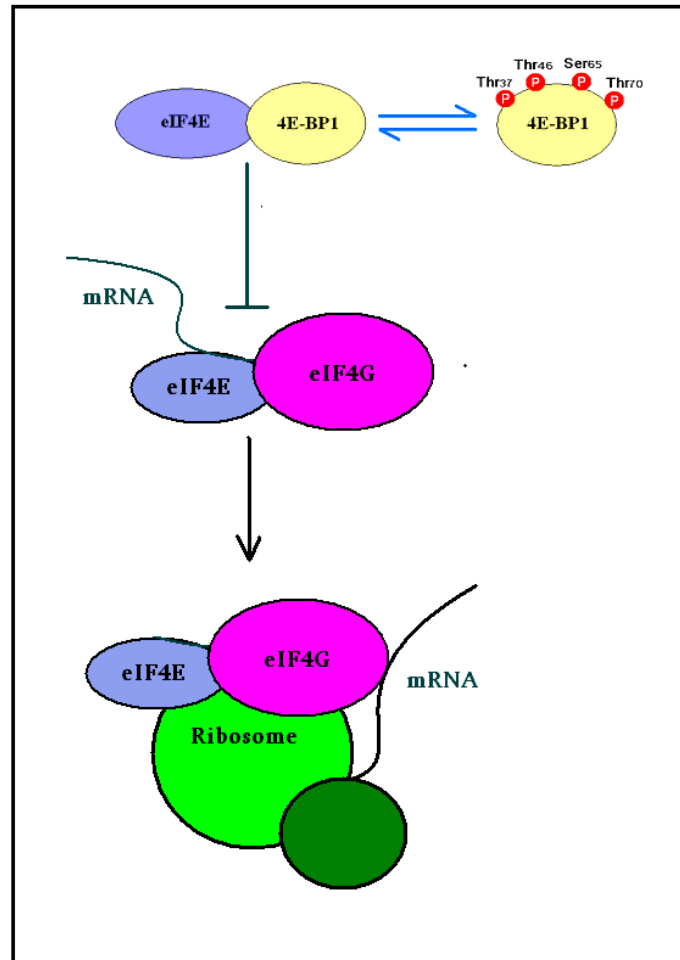


Fig. 7. Phosphorylation of 4E Binding protein results in dissociation from eIF4E. This allows eIF4E to initiate translation.

Other downstream targets that are activated during exercise are extracellular-regulated kinase (ERK) and p38 MAP kinases. Another of the MAP kinase family

includes the stress-activated protein kinases JNK (136). It was proposed that $\text{PGF}_2\alpha$ activates phorbol ester-sensitive protein kinase C isoforms, which phosphorylate and activate Raf to initiate the ERK MAP kinase signaling cascade. Activated Raf then phosphorylates MEK1, which leads to the phosphorylation and activation of ERK1 and ERK2 (137). ERK Map kinases are also activated by bFGF (138) while cytokines and metabolic stressors have an effect on P38 activation (139). PGE2 was found to activate p38 in fibroblasts (140) and may be a method of its activation in skeletal muscle.

ERK and p38

The mitogen-activated protein kinase (MAPK) signaling network (figure 8) regulates gene transcription and protein synthesis and may be a mechanism through which exercise/muscle contraction leads to increased expression of muscle proteins (141).

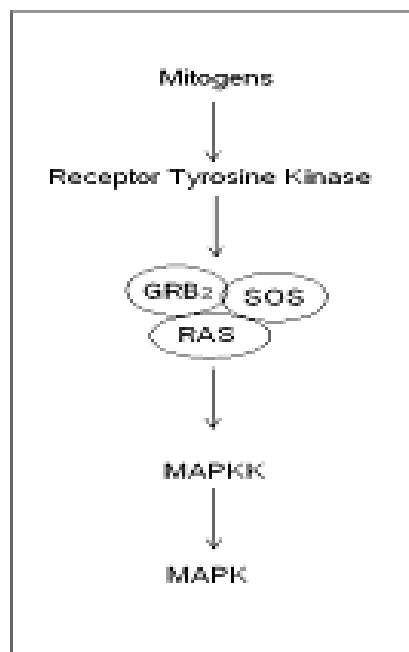


Fig. 8. The MAP kinase pathway is activated through mitogens binding to the tyrosine receptor that set off a chain of kinase activity.

MAP kinases ERK and p38 are activated in human and rat skeletal muscle by exercise (142). Muscle contraction evoked by electrical stimulation also leads to the activation of p42/44 ERK and p38 MAP kinase (143). MAP kinases have been shown to translocate to the nucleus and directly phosphorylate numerous transcription factors such as c-Jun, which is involved in the induction of structural muscle proteins including skeletal α -actin (144), c-Myc, which plays a role in apoptosis mediation (145), and myocyte enhancer binding factor 2 (MEF2) (146,147). MAP kinases can also activate other downstream substrates that can translocate to the nucleus and phosphorylate numerous transcription factors (148).

These MAP kinases are essential for hypertrophy and increased transcription. Activation of ERK1/2 and JNK by exercise and contraction is associated with the rapid induction of immediate early genes such as c-fos and c-jun. These associated proteins form the AP-1 complex through which NFAT initiates transcription in the calcineurin/NFAT pathway. The interdependencies of these three pathways led us to research them to determine the effects of stretch and activation.

This project hypothesizes that **stretch and force generation act to independently activate the calcineurin/NFAT pathway and induce Cox-2 expression.** The specific aims of this study were to 1) show the ability to initiate biochemical hypertrophic signaling through muscle force generation and lengthening 2) provide an analysis of the independent effects of passive lengthening, isometric activation, and active lengthening on known hypertrophic initiators, and 3) determine the involvement of the calcineurin/NFAT pathway and activation of COX-2 gene expression.

RESEARCH DESIGN

Research Rationale

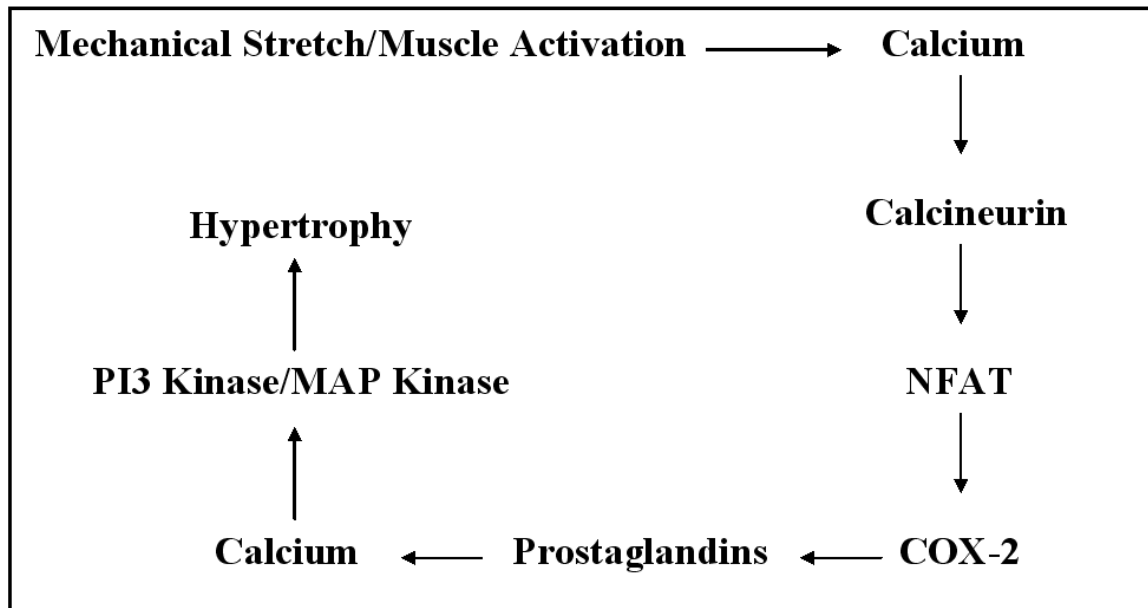


Fig. 9. The proposed pathway has a link between calcium dependent activation of NFATc1 and expression of the Cox-2 gene. Increases in calcium due to passive lengthening or isometric activation during exercise may lead to increases in prostaglandin synthesis and subsequent muscle hypertrophy.

Although stretch and activation have been shown to activate the signaling cascades described above, there is no unified signaling model describing the response. This project is based on the model illustrated in figure 9. Our model derives from the observation that passive lengthening, isometric activation, and active lengthening of the muscle stimulate the release of calcium into the cell (69,70,71). Increased calcium may activate calcineurin leading to dephosphorylating NFAT (114,115). NFAT is able to

translocate into the nucleus to upregulate COX-2 (127). This isoenzyme then plays a role in the biosynthesis of PGF2 α from arachidonic acid (128). We believe that this PGF2 α not only leads to sustained increases in intracellular calcium but also plays a role in activating the MAP kinase pathway and PI3 kinase pathways (137). These pathways are known protein synthesis pathways, which result in cellular and consequently muscle fiber hypertrophy and atrophy prevention (135,141). This research uses separate bouts of passive lengthening, isometric activation and active lengthening *in vivo* to separate the effects of stretch and force generation and to test the proposed signaling model.

Subject Selection

Mice were selected for this work because their short life span, proclivity for reproduction, known genetic background, minimal expense for purchase and maintenance, and fast metabolism. Due to their size, mouse subjects, were simple to work with within our laboratory and apparatus constraints. We selected *CFW (Carworth Farm-Webster) Mice*. They originate from the stock of non-consanguineous Swiss-Webster mice of Carworth Farm; consanguine since 1964. This model was chosen because outbred mice are less susceptible to genetic aberrations that might be associated with inbred lines. Mice represent the primary species used in research.

The selection of mice in this foundational work gives us the ability to determine baseline levels of activation as a precursor to more detailed genetic mutation studies. The existing data would allow for genuine comparison studies as the lab continues to study the effects of exercise with the purpose of defining these hypertrophic pathways.

Muscle Selection

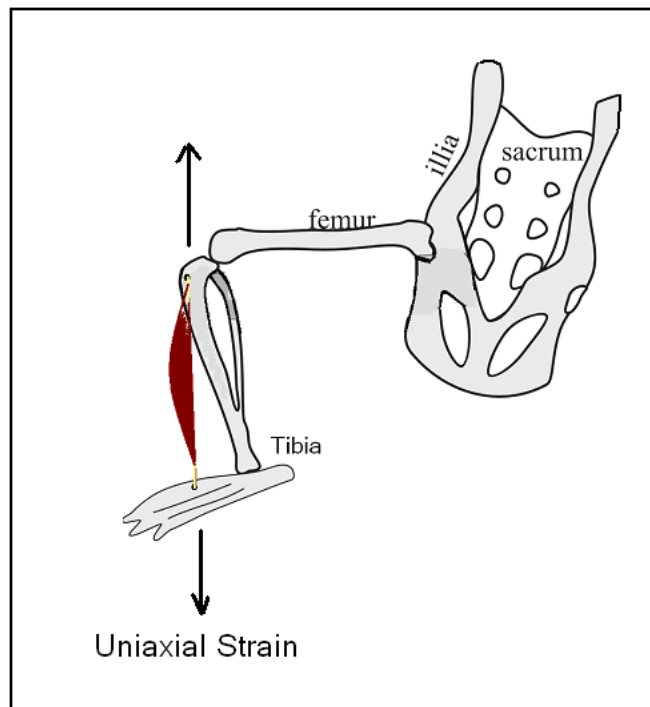


Fig. 10. Location of the Tibialis anterior and its capacity to be stretched uniaxially during flexion and extension of the ankle joint.

The Tibialis anterior muscle is located at the anterior calf. It originates on the lateral condyle of the tibia (figure 10), proximal two thirds of the lateral tibial surface and interosseus membrane and deep fascia of the leg and inserts on the medial cuneiform at the base of the first metatarsal (149). The simple architecture and anatomy of this muscle allows straightforward determination of fiber deformation from externally imposed motion of the ankle joint. The tibialis anterior muscle in mice is almost entirely made up of Type IIA (fast oxidative glycolytic - FOG) and IIB (fast glycolytic - FG). The majority of the fibers are of the FOG variety (150).

Several properties of calcineurin signaling suggest that it may participate in the control of slow skeletal muscle gene expression. A) Its selective activation by prolonged elevation of calcium and insensitivity to high amplitude calcium spikes correspond to the type of signaling thought to activate slow fiber-specific genes. B) The control regions of several slow fiber-specific genes contain adjacent binding sites for NFAT and MEF2 factors, C) transplant patients maintained on calcineurin inhibitors, develop skeletal myopathy and lose skeletal muscle oxidative capacity (151). Since fast muscle has less dependency on calcium signaling than slow muscles, it will be more sensitive to an interaction with calcium during the protocols. This may allow for observation of more pronounced reactions through less intense exercise.

Testing Apparatus

The stretch platform (figure 11) was used as a controlled stimulation environment for experimentation. A foot pedal system was chosen to limit variation in movement between animals and to focus the strain on the tibialis anterior muscle. This provides a controlled environment and movement in which we could relate the movement to the pending results.

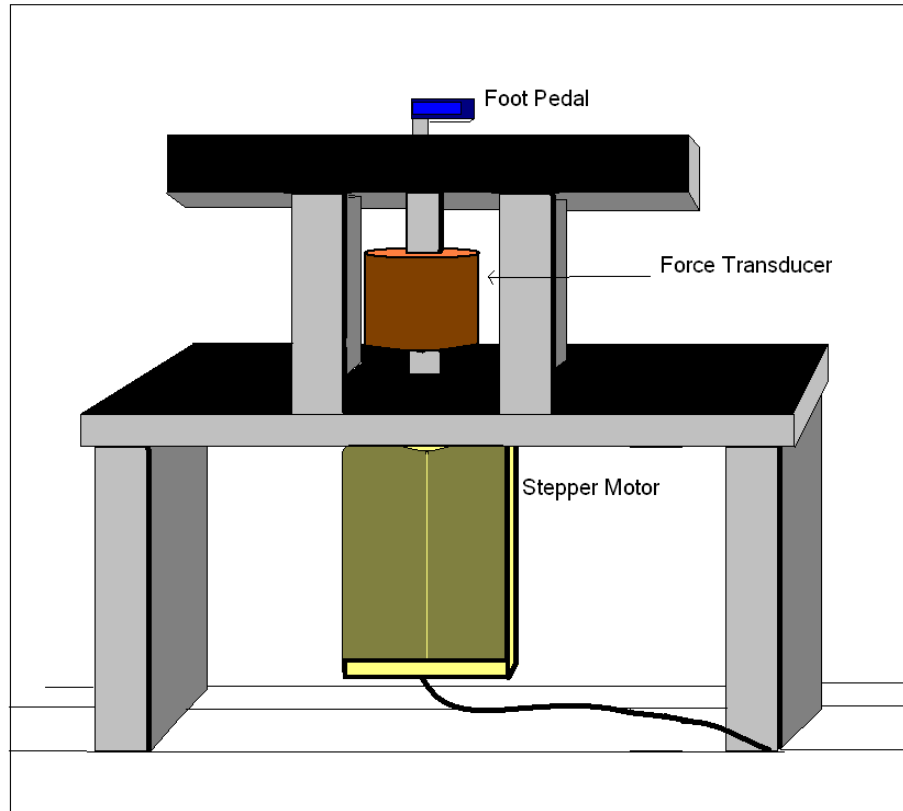


Fig. 11. The stepper platform allows for easy access to subjects and integrated force feedback. The mice were kept warm via a connected water heater, which circulated warm water through tubing that ran underneath the top plate.

Selection of signaling cascade markers

Western blotting was used for cellular proteins due to their general high specificity separation and for accepted protocols for relative quantification. We used real-time PCR as the method for analyzing COX-2 because our protocols called for early COX-2 gene expression. Using real-time PCR, we would be able to quantify our results and the primary sequences would enable high specificity and accuracy in our target analysis.

Sample size calculations

We utilized the formula, $n \geq 2 \left(\frac{\sigma}{\delta} \right)^2 \{t_{\alpha[v]} + t_{2(1-P)[v]}\}^2$ ations, σ = true standard deviation, δ = the smallest true difference that it is desired to detect, v = degrees of freedom of the sample standard deviation with the number of groups and n replications per group, α = significance level, P = desired probability that a difference will be found to be significant, and $t_{\alpha[v]} + t_{2(1-P)[v]}$ = values from a two tailed t-table with v degrees of freedom and corresponding to probabilities of α and $2(1-P)$, respectively. This formula helped us devise the sample population that we would need to study for this research. The signaling cascades examined in this work are extremely sensitive markers of biological changes, with increases of 5-10 folds being common, but the assays for activation of those cascades are relatively imprecise. For this reason, the difference desired to detect was set to 1.5 standard deviations. P was set to 0.85, which gave us $v=16$. By convention, the statistical confidence was set to 0.05. This gave us a number of replications needed to detect a given difference between means of 4.67 samples. We utilized minimum of 5 subjects per test.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Adult female CFW (n = 22 total, n = 5 (Passive, Isometric, None), n = 7 (Eccentric), mass = 27.3g +/- 3.38g) mice were used in the experiments. The mice were housed in a controlled animal housing facility (Georgia Institute of Technology). They were allowed access to rat chow and water ad libitum. The Georgia Tech Institutional Animal Care and Use Committee approved all procedures.

STRETCH PLATFORM

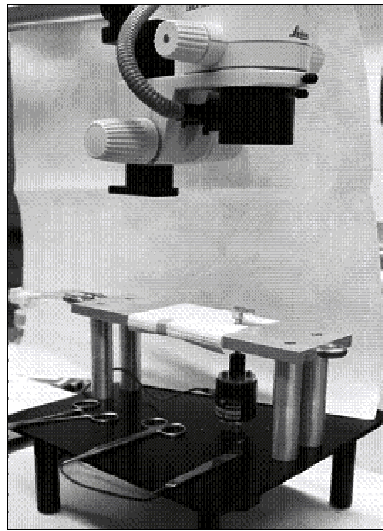


Fig. 12. Photo of Stretch Platform

The stretch apparatus is comprised of two platforms connected by standing columns, which are spaced appropriately for optimal free user movement (figure 12). The upper plate temperature is maintained by a recirculating water bath to provide thermal homeostasis. A foot pedal (152) is mounted above the upper plate to allow

consistent horizontal plane rotation of the mouse foot. Parameters of that movement and the relative timing of muscle activation can be fully adjusted through associated Lab View software.

A stepper motor mounted to the underside of the bottom plate was attached to the foot pedal by a steel shaft and provided control of ankle angle. A torque transducer was added in later experiments to give an indication of torque produced in our subjects.

Muscle Stimulation

For all experimental procedures, the animals were anesthetized through intraperitoneal injection (ketamine 90 mg/kg, acepromazine 2 mg/kg, supplemented as necessary). All animals were positioned in the testing apparatus with left heels flush with the rear of the pedal and the foot restrained to prevent sliding within the pedal. The mouse body was secured to prevent momentum shifts and the tibia was immobilized at the knee by a spring clamp to prevent movement. A stainless steel stimulating electrode was used to transcutaneously activate the left peroneal nerve. Animals were randomly assigned to one of three groups.

Isometric Stimulation Protocol. External stimulating electrodes were attached and voltage applied (1.5-4 V) to the electrode was adjusted to produce maximal palpable contractile force. Further stimuli were delivered at twice this intensity to assure maximal activation. Muscle contractions were generated by delivering 300 ms trains of 0.1ms square pulses at 100 Hz, while the ankle was held fixed at 90 degrees in the foot pedal. Activation of the gastrocnemius muscles was occasionally observed, but is not expected to affect the observations in TA. Stimuli were applied every 30 seconds for 30 minutes, for a total of 60 stimuli.

Passive Stretch Protocol. The foot was moved from an ankle angle of 40 degrees (50 degrees dorsiflexion) to 140 degrees (50 degrees of plantarflexion) in 100 ms, held for 300 ms and returned to its starting position in 1 s. This motion produces a stretch of approximately 15% to the TA and was repeated every 30 seconds for 30 minutes.

Eccentric Stretch Protocol. A combination of the stimulation protocol and the stretch protocol was conducted. The peroneal nerve was stimulated as described above. Electrical stimulation was applied as described previously, but with the foot initially at 40 degrees. After 100ms of stimulation, the foot was plantarflexed to 140 degrees under the same conditions as the passive stretch group. The 100 ms delay allowed isometric tension to fully develop, and the motion was completed 100 ms prior to the termination of electrical stimulation. The muscle was fully relaxed before the foot was returned to the starting position. This protocol was also conducted once every 30 seconds for 30 minutes.

TISSUE COLLECTION

After a recovery period of 3 hours, animals were sacrificed by CO₂ asphyxiation, and the tibialis anterior was exposed. Muscle length was measured with the ankle held at 90 degrees. The muscle was harvested by cutting the tendon at its insertion on the foot and separating the muscle from the connective tissue at its origin. Tissue was pinned to corkboard at the measured length and flash frozen in melting isopentane. The tissue was immediately placed in an -80 degree freezer until later use. Muscles were harvested from both the experimental and the contralateral limb.

ANTIBODIES

The following antibodies were used: Phospho-44/42 Map Kinase Thr202/Tyr204 Antibody (Cell Signaling Technology, Cat. No. 9101), ERK2 (BD Transduction Laboratories, Cat. No. 610104), P38 Map Kinase alpha Antibody (Cell Signaling Technology, Cat. No. 9218), Phospho-p38 MAP Kinase Thr108/Tyr182 Antibody (Cell Signaling Technology, Cat. No. 9211), 4E-BP1 Antibody (Cell Signaling Technology, Cat. No. 9452), NFAT2 Antibody (Affinity Bioreagents, Cat. No. MA3-024), NFATc1 7A6 (Santa Cruz Biotechnology, Cat. No. sc-7294), P70 S6 Kinase C-18 (Santa Cruz Biotechnology, Cat. No. sc-230), Phospho-P70 S6 Kinase, Thr 389 1A5 Monoclonal Antibody (Cell Signaling Technology, Cat. No. 9206),

PROTEIN ANALYSIS

Protein Collection. Muscle tissue was homogenized at medium speed with a mechanical homogenizer in 10µl/mg of tissue of cold non-denaturing lysis buffer (5 mM tris, 3 mM NaCl, 0.25 mM EDTA, 1% Triton X-100) containing 4mg/ml sodium fluoride (Sigma), 4µg/ml Sodium Vanadate (Sigma) and 1ul/ml protease inhibitors (Sigma). The tissue was then enzymatically digested for 30 minutes on ice. Tissue homogenate was then centrifuged at 13000g at 4 °C for 5 minutes. The supernatant was removed and placed in fresh tubes and stored at -20 °C.

Bicinchronic Acid Assay. The protein concentration was assayed using the Bicinchronic Acid assay (BCA) kit according to the manufacturer's instructions. Protein standards were diluted from 10mg/ml bovine serum albumin (BSA) stock in TNE (10 mM tris, 15 mM NaCl, 0.5 mM EDTA) to produced standards from 2mg/ml to 0.032mg/ml BSA. Aliquots of standards and protein samples were loaded and 200ul of

working reagent (50 parts reagent A with 1 part Reagent B) was subsequently added. The plate was covered and incubated at 37 degrees for 30 minutes, allowed to cool to room temperature and read with a microplate reader (Biotek: μ Quant) at 562nm using 977nm as a reference.

SDS Page and Immunoblotting. Equal masses of protein samples were diluted in SDS sample buffer (Tris, SDS, b-ME), denatured by boiling for 5 minutes and separated by SDS-PAGE (7.5%, 10%, or 12.5% polyacrylamide gel). Resolved proteins were transferred onto nitrocellulose membranes and incubated in blocking solution (5% fat-free milk) for 1 hr at room temperature. The membranes were then incubated for 1 hr with monoclonal antibodies against phospho-44/42 (1/2000), phospho-p38 (1/2000), phospho- P70^{S6K} (Thr-389, 1/1000), 4EBP1 (1/2000), or NFATc1 (1/5000). The membranes were washed three times in TBS-T and incubated with the appropriate horseradish peroxidase linked secondary antibody (1/10,000). After the secondary incubation, the membrane was washed three times and visualized with enhanced chemiluminescence (ECL, Amersham) by exposure to radiographic film (Kodak). Membranes probed with phospho-specific antibodies were subsequently stripped and reprobed with non-phospho specific antibodies.

Gel Analysis. Exposed films were digitized using a transmission flatbed scanner (Epson). A densitometry program was created in MatLAB to calculate integrated optical densities from the ECL Western blots. These raw optical density measurements were subsequently processed to produce consistently interpretable results.

Data Normalization. P42 MAP kinase and P38 MAP kinase calculated from the ratio of phosphorylation specific signal to non-phosphorylation specific signal to account for differences in loading and variation in basal expression of these MAP kinases within

each animal. Each sample ratio was then given as a percent control of the mean of the non stimulated controls limb.

Myoblast cell extracts, cultured with IGF-I, were used as positive controls for P70^{S6K} phosphorylation. Ten micrograms of these extracts were used in each western blot to normalize with experimental animal protein samples. The level of Thr389 phosphorylation in whole tissue homogenates was normalized as a percent of the IGF-I positive controls.

Phosphorylation of 4EBP1 was estimated by differential mobility. This protein migrates as α , β , and γ , depending on the extent of phosphorylation, and the γ form is known to permit translation (153). Phosphorylation of 4EBP1 is expressed as the ratio of γ to $(\alpha+\beta+\gamma)$ optical densities, which precludes the necessity to normalize to controls.

NFAT migrates as an extended smear ranging from approximately 175 kD to 91 kD. The moment of optical density about 175 kD (based on Ramos Jurkat cell lysate obtained from Affinity Bioreagents) was calculated by multiplying each pixel intensity by its distance from 175kD, then summing the intensity-distance values for all pixels in each lane. This value was then normalized to the summed intensity of the total NFAT present to account for variations in exposure conditions and total NFAT. This procedure results in a determinate value that increases with decreasing net phosphorylation of NFAT. Sure would be nice if we had a couple control samples with which to demonstrate that claim.

REAL TIME PCR

RNA. Muscle tissue was homogenized in 1ml of Trizol for 30 seconds in a microcentrifuge tube. The homogenate was then allowed to incubate for 5 minutes at

room temperature. RNA was separated by phenol-chloroform extraction and precipitated with isopropanol. The precipitated RNA was pelleted by centrifugation at 12000g for 10 min at 4 degrees. The supernatant was aspirated and the RNA pellet was washed with 75% ethanol. This was mixed gently and centrifuged at 7500g for 5 minutes at 4 degrees. The supernatant was removed and the pellet allowed to air dry for 10-15 minutes before resuspension in 30µl of diethylpyrocarbonate (DEPC) treated water and stored at -20 degrees.

Reverse Transcriptase/PCR. Reverse transcription was performed using a commercial kit (Invitrogen) according to the manufacturer's instructions.

Standards. cDNA standards were generated from COX-2 cDNA (kindly provided by Brenda Bondesen and Grace Pavlath, Emory University). The 539 bp standard was amplified by conventional PCR and resolved on a 1-1.5% agarose gel containing 50µl of ethidium bromide for 35 minutes at 100 volts. The amplicon size was confirmed and each band was cut from the gel with a scalpel and placed into pre-weighed Eppendorf tubes. The PCR product was then purified using the QIAquick gel extraction kit (Qiagen), and the resulting DNA was eluted in 50µl of buffer. DNA concentration was determined by A260 at a 1:20 dilution in the spectrophotometer. Standard stock and working dilution (1ng/µl) aliquots were stored at -80 degrees C.

Primer Sequences. Two Cox-2 reverse primers were obtained with one Cox-2 forward primer. Reverse 1 primer, short sequence (COXR1), was used in real time application while Reverse 2, long sequence (COXR2), was used to generate COX-2 standards using PCR. The forward primer was used in both applications (Table 1).

β -actin was used as a normalizing agent to account for differences in total sample cDNA. It was also used as a verification of sample integrity. It consists of a forward primer and a reverse primer (Table 1).

Table 1. Primer Sequences for COX-2 and β -actin.

| Primer | Short Name | Sequence |
|---------------------|------------|------------------------------------|
| COX-2 Standard Fwd | COXF | 5'CCTGCT GCCCGA CACCTT CA 3' |
| COX-2 Standard Rev | COXR2 | 5'CAGATG AGAGAC TGAATT GAGGCA G 3' |
| COX-2 Real Time Fwd | COXF | 5'CCTGCT GCCCGA CACCTT CA 3' |
| COX-2 Real Time Rev | COXR1 | 5'AGCAAC CCGGCC AGCAAT CT 3' |
| β -actin Fwd | BACTF | 5'TTCAAC ACCCCA GCCATG T 3' |
| β -actin Rev | BACTR | 5'TGTGGT ACGACC AGAGGC ATA C 3' |

Cycle parameters. COX2 and β -actin were amplified by 40 cycles of denaturation at 95 degrees for 30 seconds, annealing at 56 degrees for 30 seconds, and extension at 72 degrees for 30 seconds. Thermocycling was performed in a real-time PCR system (BioRad), using SYBR green as an indicator.

Purity. Melt curve. Product purity was analyzed by separating samples on a 1-1.5% agarose gel. Gels were visualized by ethidium bromide staining on a UV transilluminator.

IMMUNOHISTOLOGY

Histology. Blocks of tissue were embedded in O.C.T cryoembedding medium (Tissue-Tek) and sectioned using a Leica cryostat. Nominal section thickness was set at 8 μ m.

Immunoblotting. Sections were fixed for 20 minutes (4% paraformaldehyde in 0.1M phosphate buffer) at room temperature in a humidified slide box. They were then washed twice in PBS/1%BSA. The sections were permeabilized in 1% Triton X-100 for five minutes and then rinsed twice for 5 min with PBS/0.1% Triton X-100. All subsequent rinses and incubation were carried out in PBS/0.1% Triton X-100. The sections were then blocked in Avidin/Biotin blocking system (Vector) for 1 hr, and rinsed twice. The sections were then incubated for 1 hour in working solution of Mouse Ig blocking reagent (Vector), rinsed twice and incubated with the anti-NFATc1 antibody at a 1:100 dilution for 1 hr. The slides were then rinsed three times for 10 min and incubated with anti-mouse IgG reagent secondary antibody (Vector) for 1 hr. They were then rinsed three times for 10 min, incubated in fluorescein isothiocyanate (FITC) conjugated streptavidin at manufacturer's recommended concentration for 10 min. The sections were rinsed twice, coverslipped with glycerol for visualization. We also alternatively used DAB staining and followed the manufacturer's protocol (Vector).

STATISTICS

STATview software was used to view and analyze data. Differences among groups were determined by two-way ANOVA, factorial by Protocol with repeated measure of Intervention against contralateral. Fisher's PLSD correction was applied to post-hoc T-tests used to identify individual group differences. Log transformations were applied to MAP kinase results to correct for normalization to controls. Statistical threshold was set to $p \leq 0.05$, and trends in this study were identified using $p < 0.15$. Unless otherwise noted, results are reported as mean +/- SD.

RESULTS

We tested whether muscle activation and mechanical stretch acting on a muscle separately could produce hypertrophic signaling within the tibialis anterior muscle. Complications during experiments resulted in the death of one animal during the recovery period.

4E BINDING PROTEIN PHOSPHORYLATION

Increased 4E binding protein phosphorylation has been correlated with increased rates of protein translation (135,154). Cell culture samples were used as positive and negative control to determine the authenticity of results of our protocols. C2C12 cells treated with IGF-1 showed increased intensity of the hyperphosphorylated γ form while treatment with rapamycin to block mTOR activation increased the hypophosphorylated α form (figure 13).



Fig. 13. IGF-1 induces a maximal hyperphosphorylation state in 4EBP1.

Phosphorylation at the γ site permits translation.

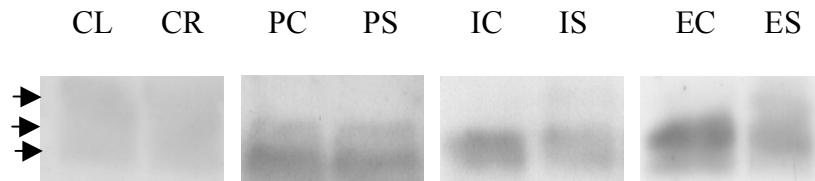


Fig. 14. Representative Western blots for 4E Binding Protein of (L-R) control animals, passively lengthened animals, isometrically activated, and actively lengthened animals.

4EBP1 is seen between 18kb and 22kb.

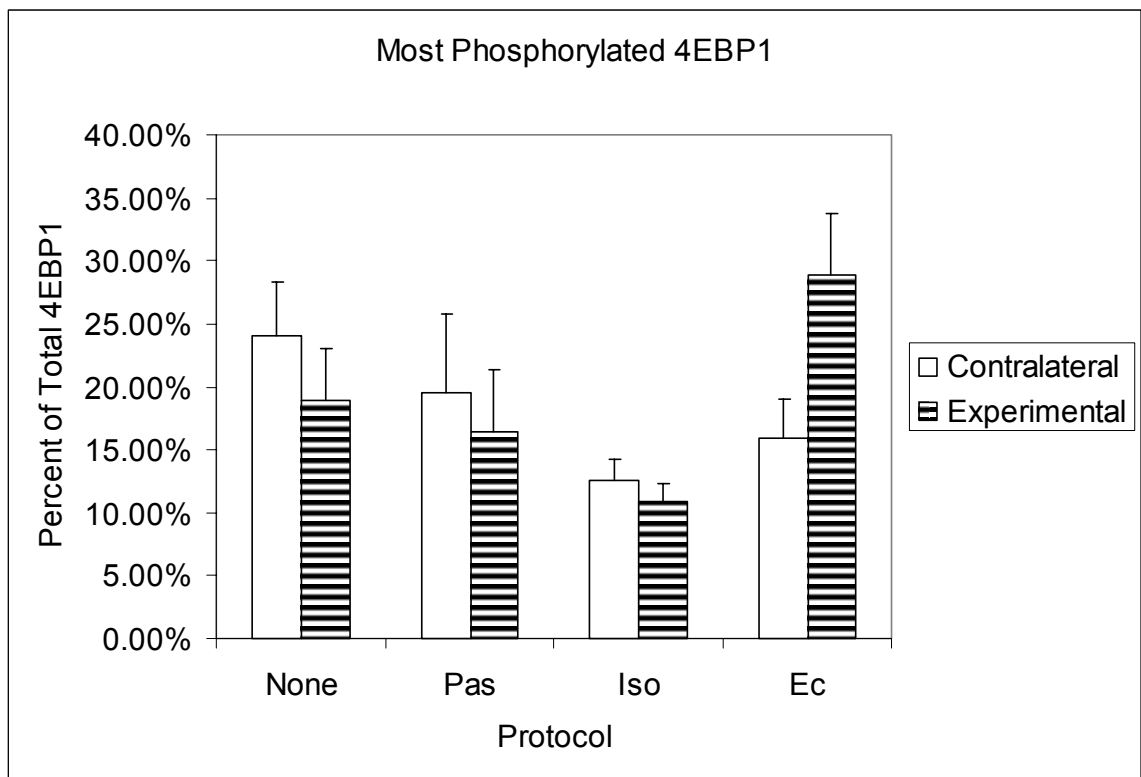


Fig. 15. Significant differences in 4EBP1 were seen based on protocol ($n \geq 5$).

A significant effect of intervention was found ($p=0.05$) in the phosphorylation of 4EBP1 (figure 14, 15). However, a wide variation was observed to occur between stimulation groups. While changes in phosphorylation in each protocol could not be statistically

resolved, the EC protocol resulted in a dramatic increase in the phosphorylation ratio of the stretch-stimulated limb versus its contralateral control limb.

P70^{S6K} PHOSPHORYLATION

The kinase activity of P70^{S6K} is correlated with its phosphorylation on Thr389; the level of phosphorylation of the molecule was investigated by Western blot (figure 17).



Fig. 16. Representative Western blots demonstrating P70^{S6K} phosphorylation observed after a 3-hour recovery period. (L-R) Control animal with IGF control, passively lengthened, electrically stimulated, and actively lengthened. P70^{S6K} is seen ~70kb.

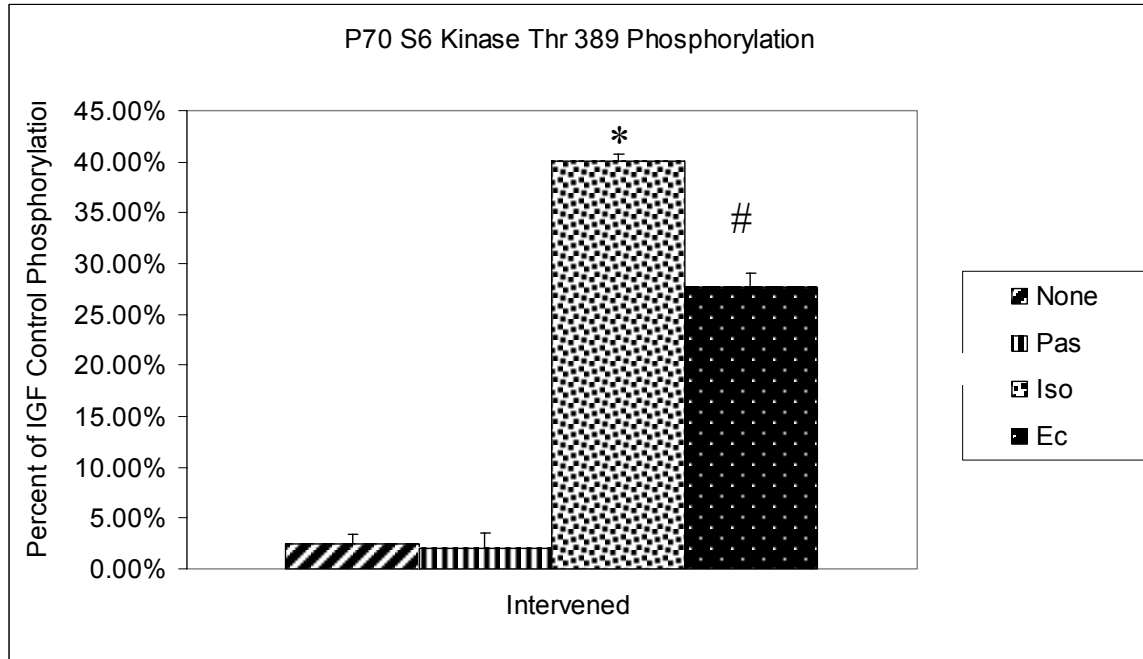


Fig. 17. P70^{S6K} phosphorylation varied with protocol (*) denotes statistical confidence $p < 0.05$, (#) trends, $p < 0.15$ in the results. We used the results of the Thr389 Western blots in comparison to the IGF-1 controls to determine differences in phosphorylation. ($n \geq 5$).

Changes in P70^{S6K} phosphorylation were more evident between animals than was seen in 4E Binding protein (figure 16, 17). Though they are both regulated by mTOR, they showed differences in phosphorylation. ANOVA revealed a significant effect of intervention and, while there was no global effect of protocol, a significant interaction ($p = 0.05$) between protocol and intervention indicates that the intervention effect is limited to the ISO and EC protocol. Muscle activation induced a 16-fold induction ($p = 0.0385$) in phosphorylation of Thr389 versus the non-stimulated subjects. We also observed significant differences in phosphorylation of actively lengthened muscles versus isometrically activated muscle ($p = 0.015$). No effects were seen by just passively lengthening the muscle on the phosphorylation of P70^{S6K}.

P42 PHOSPHORYLATION

Stimulating the animals by mechanical stretch and electrical stimulation caused a significant increase ($p=0.015$) in phosphorylation. Isometric activation increased ERK2 phosphorylation by 300% ($p=0.02$) over controls 3 hours after the stimulation (figure 18, 19). A similar 262% ($p=0.05$) increase was seen after active lengthening of the muscle. Isometric activation and active lengthening also proved to have a trend to be different than passively lengthened muscle at this time point ($p=0.053$ and $p=0.091$, respectively).

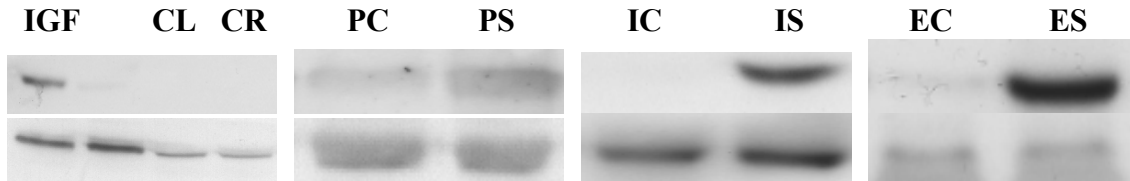


Fig. 18. Phospho Western blot ERK2 (P42) was analyzed versus non-phospho Western blot ERK2. Values of phosphorylation were then shown as percent phosphorylation of experimental muscle versus mean phosphorylation of controls.

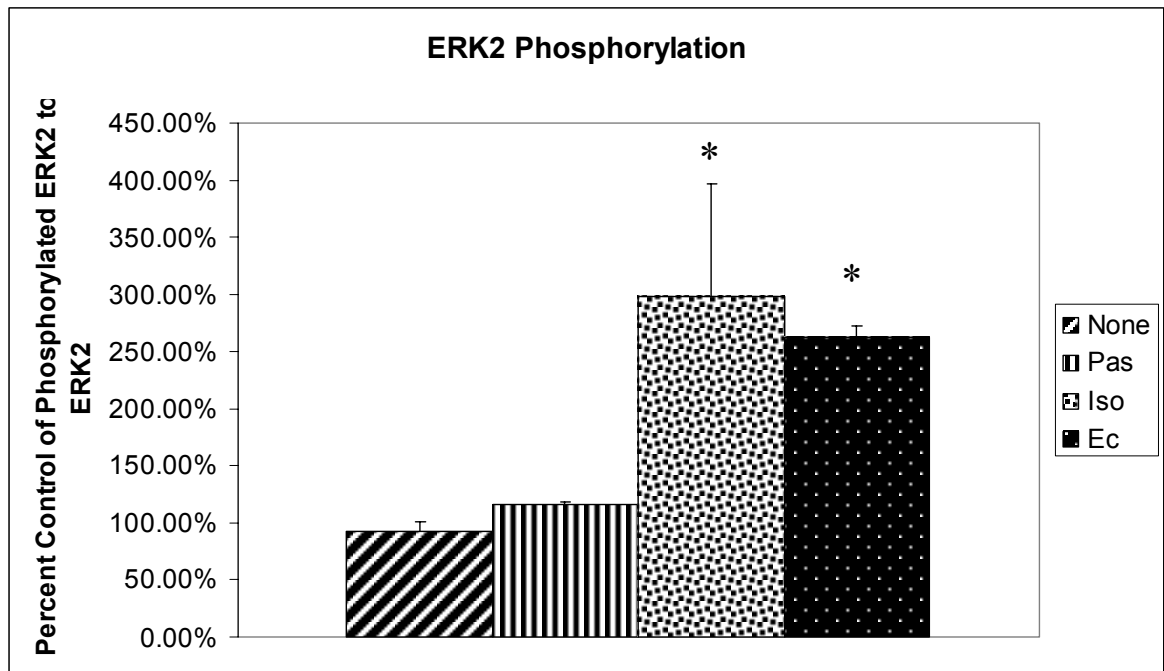


Fig. 19. Effects of stimulation seen in Western blots on p42 protein expression in mouse skeletal muscle. Phospho. ERK-2 is labeled p42 in the above figures. (*) denotes statistical significance $p < 0.05$. ($n \geq 5$).

P38 PHOSPHORYLATION

Significant effects ($p < 0.0001$) of intervention and protocol ($p = 0.03$) were found on the phosphorylation of p38MAPK (figure 20,21). A significant interaction ($p < 0.05$) indicates the response to PAS and EC is significantly different than None and ISO. We observed a 280% increased ($p = 0.038$) in P38 phosphorylation during passive lengthening (figure 20,21). We also observed that combining this passive lengthening with isometric activation produced a 270% increase ($p = 0.041$) in phosphorylation.

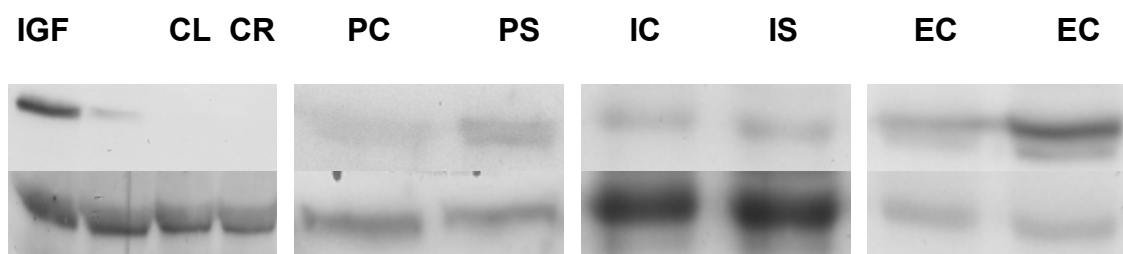


Fig. 20. Phospho Western blot P38 was analyzed versus non-phospho Western blot P38. Values of phosphorylation were then shown as percent phosphorylation of experimental muscle versus mean phosphorylation of controls.

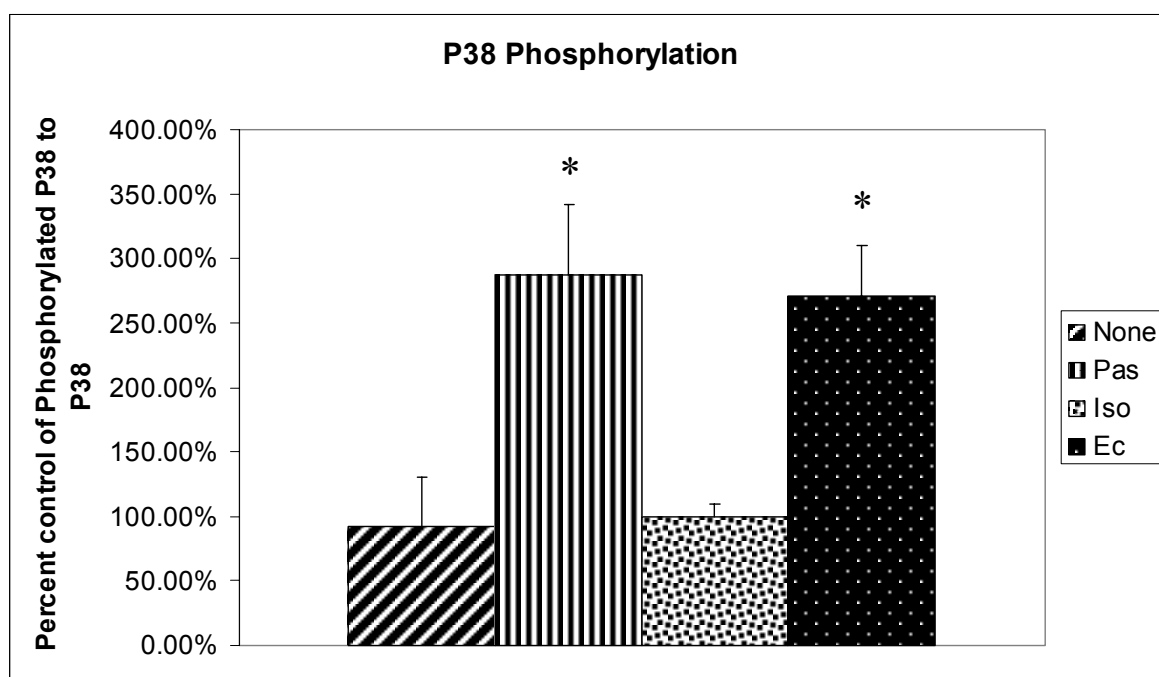


Fig. 21. Phosphorylation of p38 in mouse skeletal muscle induced by bouts of passive lengthening, isometric lengthening and active lengthening. (*) denotes statistical significance $p < 0.05$. ($n \geq 5$).

NFAT

We hypothesize that NFAT dephosphorylation is a key element in the induction of COX-2 mRNA. Through our Western blotting (figure 22) we found no significant increases in dephosphorylation of NFAT ($p=0.7$) (figure 23).

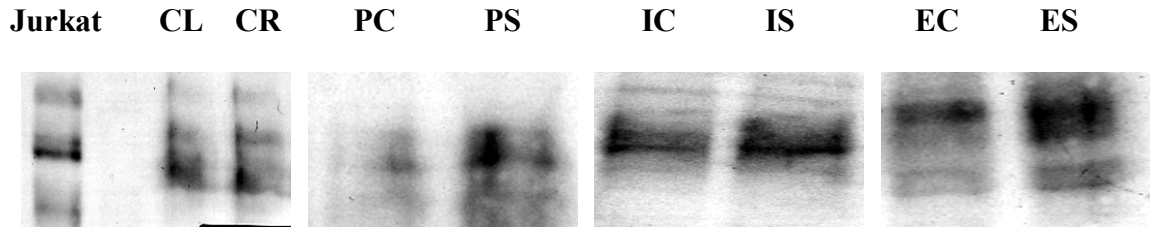


Fig. 22. These Western blots show the range of NFAT from MW 91-175. The Jurkat cell extracts were obtained from Affinity Bioreagents and used as the positive control.

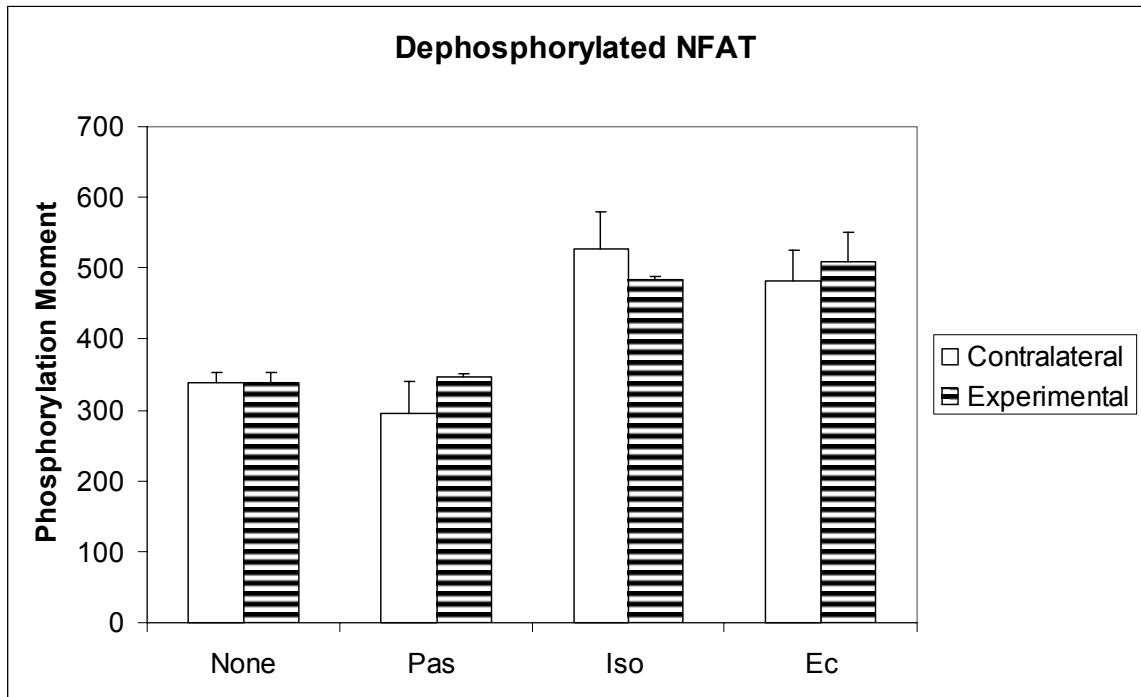


Fig. 23. NFAT showed no increase in phosphorylation under the analysis of total cellular protein.

COX-2

Quantitative real time PCR failed to detect COX2 expression following combinations of stretch and activation. The integrity of cDNA was verified by expected amplification of b-actin in parallel with COX-2. BLAST search revealed that the COX2 primers used for qPCR are specific to COX2, indicating that the 200-1000 bp smear (figure 24, 25) is an amplification artifact. Validity of these primers was verified by amplification of COX-2 from freeze injury induced muscle and COX-2 myoblast cell extract (figure 25). Amplification conditions were optimized by running samples through a temperature gradient.

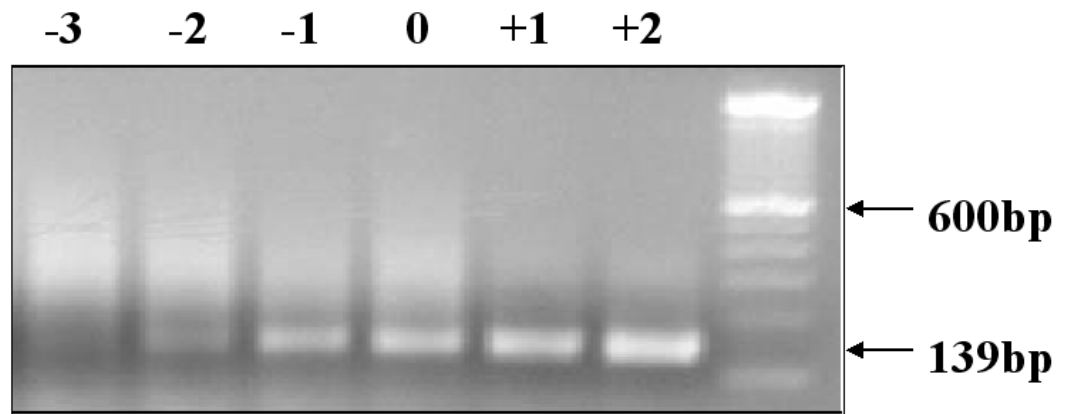


Fig. 24. COX-2 Standards declined in specificity as they decreased in concentration from 1×10^2 pg/ μ l to 1×10^{-3} pg/ μ l.

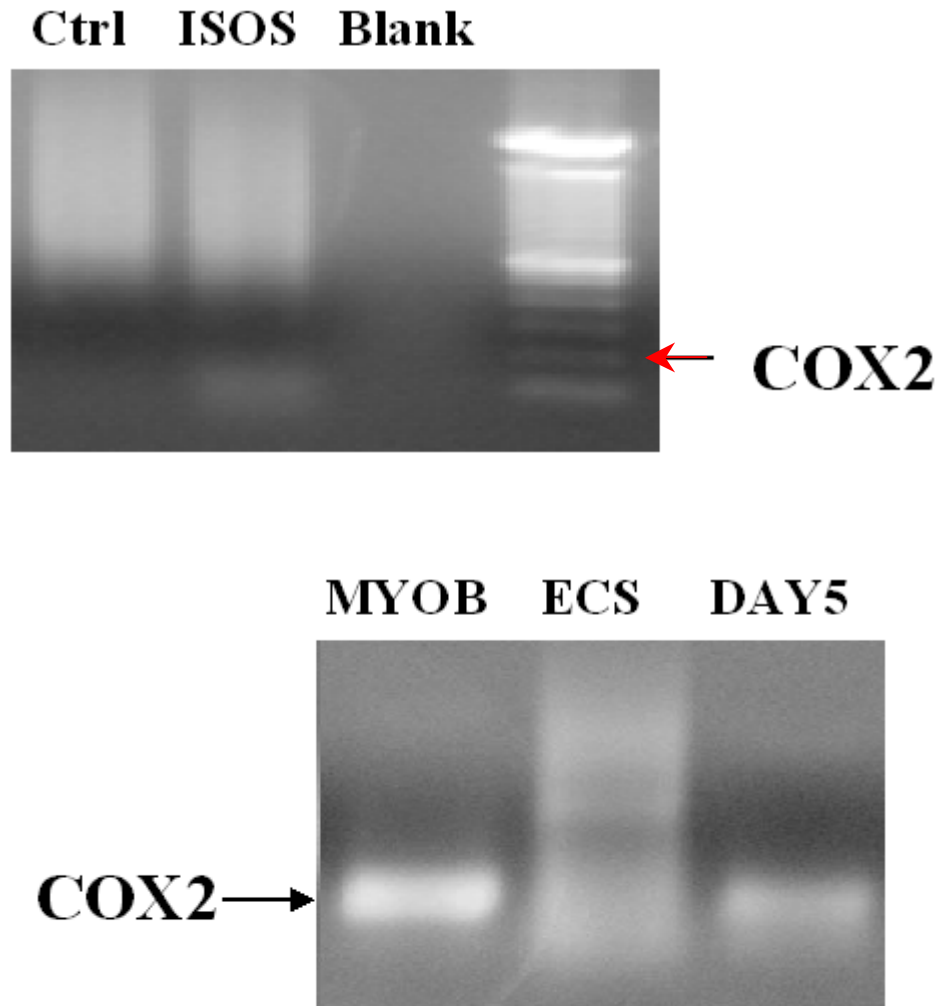


Fig. 25. Agarose gels led us to determine that no COX-2 product was being amplified at detectable levels. A representative Control and Isometric activated sample shows no product at the correct 139bp weight. When compared to a myoblast and a Day 5 injured samples, the EC sample showed no product amplification. Arrows indicate the proper location for COX-2 cDNA product.

BAND PURITY AND BETA ACTIN

Cox-2 cDNA standards were found to be pure based on acrylamide gel separation (figure 26). Band purity was determined based on singleness of bands and correct size.

The standard bands migrated to the appropriate 539bp weight and did not contain extra product. Also, experimental samples did show the ability to be amplified using Beta Actin primers (figure 27).

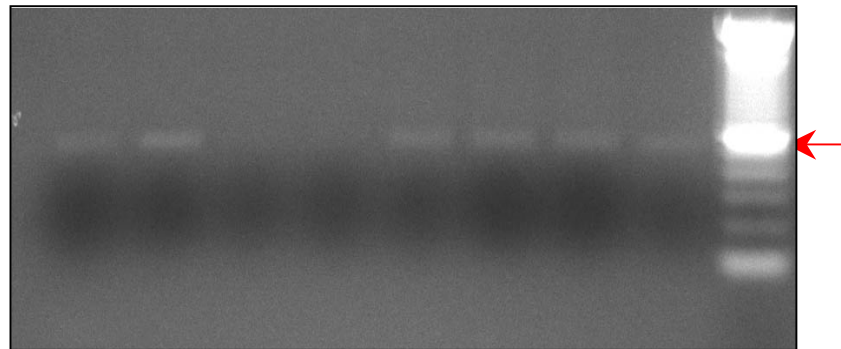


Fig. 26. Agarose gel showing COX-2 cDNA standards. Arrow denotes the 600bp marker.

COX-2 standards are 539bp in size.

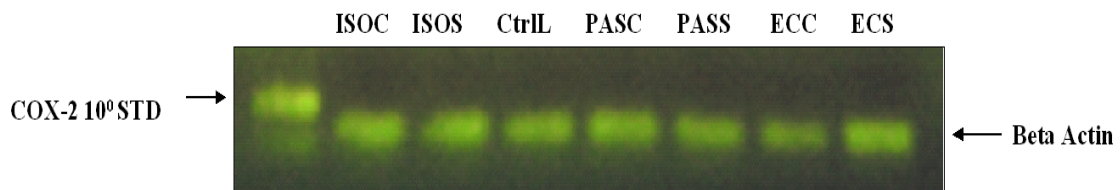


Fig. 27. Representative gel showing experimental samples amplified using Beta Actin primers.

IMMUNOHISTOCHEMISTRY

The initial process of evaluating NFATc1 dephosphorylation involved immunohistochemical staining of muscle sections. We were unable to detect the

presence of NFATc1 located within the tissue (figure 28). We therefore utilized the Western blot method to quantify NFATc1 dephosphorylation in our sample tissue.

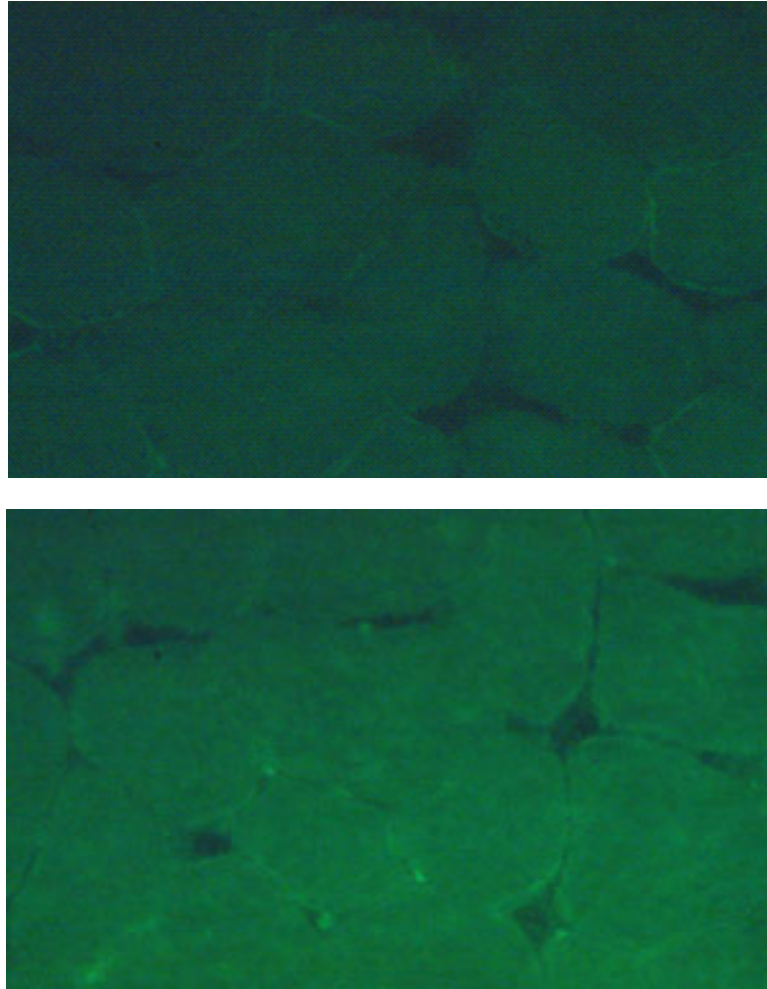


Fig. 28. Muscle sections with immunoprobed with NFATc1 antibody and fluorescein conjugated secondary failed to detect NFATc1 protein. Top - Control Bottom - Isometric Activation.

HISTOLOGY

Random tissue sections were collected and stained with hematoxylin and eosin to observe if changes in gross morphology due to electrical stimulation and/or mechanical strain were present (figure 29). This was an effort to test whether our interventions were causing damage to the tissue or inducing an inflammatory response.

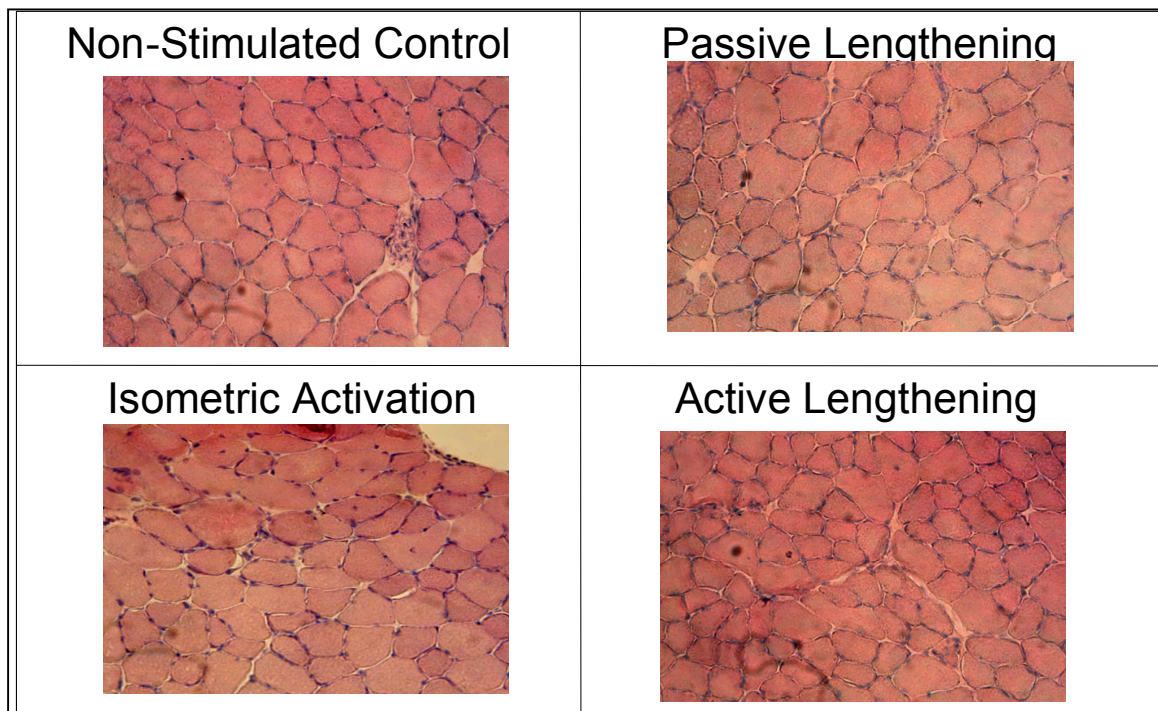


Fig. 29. Visualization with Light Microscopy showed no gross morphological damage to the muscle.

DISCUSSION

The objective of this study was to determine if *stretch and force generation activate the calcineurin/NFAT pathway and induce Cox-2 expression and initiate muscle hypertrophy.* Previous studies have shown that exercise, a combination of isometric activation and passive lengthening, acts as stimulus for increase in muscle mass and as a countermeasure for atrophy. The signaling cascades that mediate these effects are numerous and interdependent, but consistent with activation by PGF2 α (95).

THE ROLE OF NFAT

We observed no differences in NFAT dephosphorylation during the stimulations. The phosphorylation of 4EBP1, p70^{S6k}, P42 MAP kinase and P38 MAP kinase increased in the absence of detectable NFAT dephosphorylation, which suggests that NFAT does not play a role in initiating the growth response following exercise, contrary to the proposed model.

EXPRESSING COX-2

Likewise, COX-2 expression was not detected following mechanical stretch and electrical stimulation in skeletal muscle, contrary to the proposed model. Increased COX2 activity would be required for increased PGF2 α synthesis, which has been shown to be an early signal in hypertrophy (74,97,128). However, NSAIDs, which inhibit pain by specifically blocking COX-2, and have side effects of delaying muscle growth (61), demonstrate a role must exist for these isoenzymes during muscle growth and

regeneration. COX-2 expression is seen in smooth muscle cells 4-24 hours after stretch (127), so COX-2 may have a role in maintaining or enhancing the hypertrophy response rather than being the initiator. Also, COX-2 expression is elevated after injury in regenerating muscle at 24 hours; therefore this is another indication that COX-2 may have a later role muscle adaptation (155). There are other mechanisms capable of initiating stretch and activation dependent activation of the hypertrophic pathways. One of these mechanisms may be the influx of circulating growth factors to exercised muscle through increased blood flow. This increased blood flow, specifically to slow muscle, may act to increase the binding of growth factors to membrane receptors that activate the kinase pathways (156).

KINASE ACTIVATION

The activation of PI-3 kinases and MAP kinases in this study are consistent with previous results. Nader and Esser (157) used intense stimulation of the sciatic nerve in unconstrained rats to produce lengthening activations of the TA. They demonstrated that a 20 minutes of high frequency electrical stimulation resulted in a four-fold increase in phosphorylation of p70^{S6k} at 3 hr, where the present study shows a 16-fold increase after 30 minutes intervention (figure 30). A key difference between the two protocols is within the fixation of the limb and our use of specifically targeting the Thr389 site as the one producing the greatest kinase activation. Also, our system controls limb length thus limiting the lengthening of the TA.

Nader and Esser report a $280 \pm 62\%$ increase of p38 phosphorylation immediately following a single bout of high frequency electrical stimulation, where the present study shows a 270% increase following controlled active lengthening. We

found that even at 3 hours after 30 minutes of passively lengthening the muscle, we could still resolve increases in phosphorylation that were very similar to the ones determined by Nader and Esser. The increase in phosphorylation after mechanical stretch also confirms the initiation of signaling through mechanical intervention.

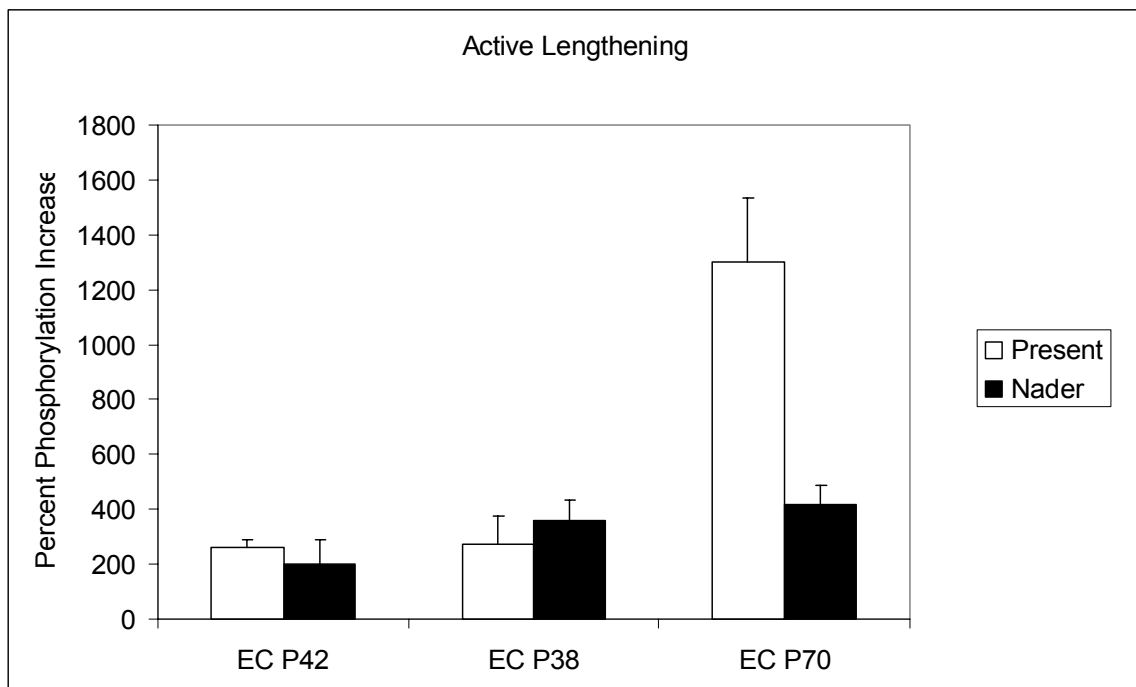


Fig. 30. Nader and Esser observed changes in phosphorylation that compared to the present study.

Martineau and Gardiner conducted a study that looked at increasing and shortening the length of the plantaris muscle during activation (158). They conducted their test by isolating the plantaris from the surrounding musculature, detaching it from its tendon, and tying it to a motor. This allows for direct extension of the muscle without dissipation of strain within the elastic tendon.

They reported a 5-fold increase in P42 phosphorylation compared to a 3-fold increase in the current study during active lengthening (figure 31). They observed increased levels of phosphorylation as tension increased as they applied stimulation to their isolated plantaris muscle. This removal of the muscle from its tendon and surrounding muscle allows for increased stretch without the structural impediments of a limb. They also observed no changes in P38 MAP kinase regardless of their protocol and conditions. We found that mechanical stretch induced a rise in P38 phosphorylation, which was absent within the Martineau and Gardiner studies. The specific increase in P38 phosphorylation during mechanical stretch showed an ability to induce muscle response in our system with muscle lengthening alone.

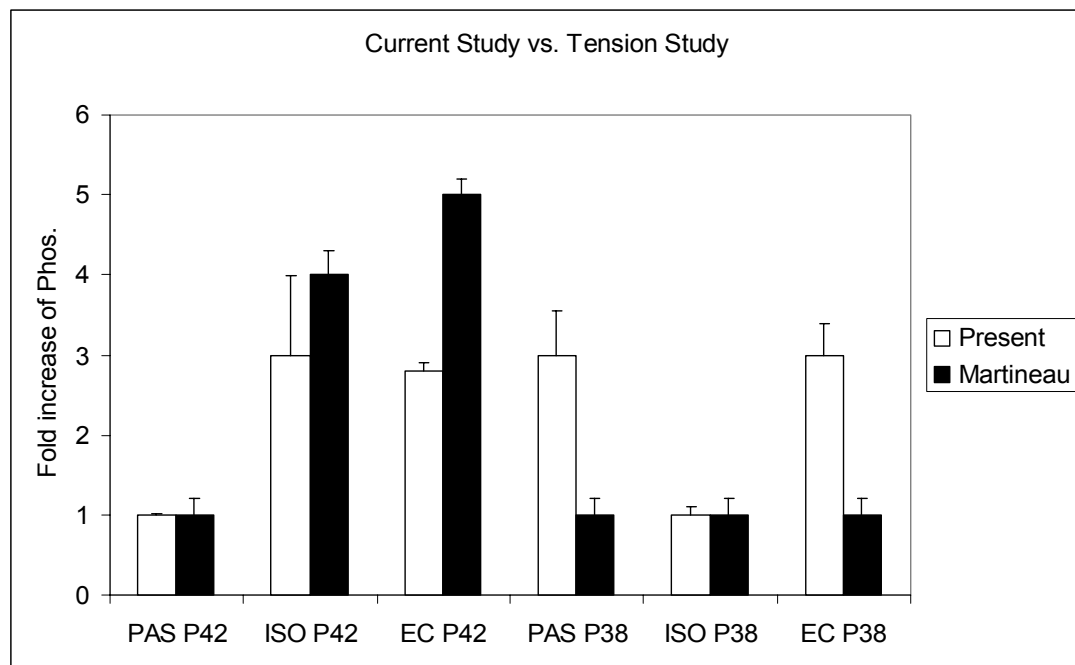


Fig. 31. Matineau and Gardiner saw no change in phosphorylation due to passive lengthening and no elevations in P38 phosphorylation.

PROTEIN PHOSPHORYLATION TRENDS: MECHANICAL STRETCH V. MUSCLE

ACTIVATION

Our second aim was to characterize activation of these markers of hypertrophy. Phosphorylation of the signaling molecules was not consistent with generic activation of classical signaling cascade groups and demonstrates the complexity of these cascades and their interactions.

4EBP1 and p70^{S6k} are classical effectors of the PI3K cascade (135), and P42 and P38 are representative of the MAP kinase family (141). Each of these proteins is linked to protein translation and transcription and might be expected to show similar changes in stimulated muscle due to common upstream regulation in each cascade (148). However the results of these experiments demonstrate the complex control of muscle hypertrophy, in that these markers showed different responses to the same stimulations.

Phosphorylation of p70^{S6k} and p42 MAP kinase was increased following ISO and EC, both protocols that include generation of active force. MAP kinases do act to phosphorylate p70^{S6k}, which may be a cause for their common response. The primary response to force generation suggests that calcium signaling plays a key role in their activation. The response of these kinases to electrical stimulation also has an implication of the effectiveness of intermittent electrical stimulation of muscle during extended bed rest for maintaining muscle mass.

In contrast, p38 MAP kinase phosphorylation was increased following PAS and EC, both protocols that included a stretch component. It is thought that passive stretch plays a role in muscle maintenance but not much is known on its mechanisms (159). Phosphorylation of p38 shows that changing the physical structure of the muscle and its underlying cytoskeletal network in some way allows it to turn on its protein synthesis

machinery. P38 is required for the expression of MyoD responsive genes and muscle differentiation (160). Inhibition of P38 prevents the differentiation program in muscle cells (161). Passive stretch showed in this study to activate only P38, possibly linking cell stretch to a switch for initiation of differentiation.

Differences between the two MAP kinases may be attributable to their function. The p42/p44-Extracellular-signal Responsive Kinases is classically activated by growth factors including PGF2 α (99,100), where p38-Stress Activated Protein Kinase is activated by cytokines, heat stress, and oxidative stress (148). ERKs have been shown to be reliably activated by stretch (158) but our data suggest that prolonged activation may be more strongly calcium dependent, through processes like calcium-dependent PKCs. In contrast, P38 phosphorylation increases following stretch, which may indicate that the stress response is due to physical stress to the cell structure, while not directly regulated by depolarization dependent processes.

Although 4EBP1 and p70^{S6K} are both members of the PI-3 Kinase cascade regulated by mTOR, they responded very differently to the applied stimuli. There are differences in their regulation that may explain these differences. P70 activation is activated in sequence in that approximately four sites have to be phosphorylated to allow it functional ability (130). Both molecules depend on multi-stage phosphorylation: only the γ form of 4E allows translation, and only the β form can be phosphorylated into the γ form (135). Though similar in sequential phosphorylation, the entire P70^{S6K} pseudo substrate domain must be phosphorylated before Thr389 is exposed (129).

It has been suggested that PDK1 might directly phosphorylate P70^{S6K} thus making its activation sites more accessible than those of 4EBP1 (132,162). It has also been suggested that MAP kinases play a role in the early parts of this sequential

phosphorylation (132). MAP kinases were activated during our protocols so if they made the P70^{S6K} more accessible for mTOR activation then this could be a reason for the difference in its activity. Since P42 was activated in the same manner as P70^{S6K} it is conceivable that P42 is more apt to play a role in the regulation of P70^{S6K} than is P38.

The complex nature of the hierarchy of phosphorylation of the other sites in 4E-BP1 suggests that they are targets for a range of proline-directed kinases but they have yet to be identified. Mitogenic stimuli induce the phosphorylation of 4EBPs and the release of eIF4E. Several kinases have been found to phosphorylate 4E-BP1 in vitro, including ERK16; these have not been studied in vivo (163). Although rapamycin has an effect on both of these, our results suggest that there are differences in the effectors that allow each of these proteins to become active. The data suggests that the calcium dependent PI3K pathway is only one of the control mechanisms for p70 and 4EBP1 phosphorylation since there is differential activation under the same stimuli. Another of the PI3 pathway controls is Insulin and IGF-I. Insulin has also been shown to increase the phosphorylation of P70^{S6K}, 4EBP1 and Protein Kinase B (PKB/Akt) in skeletal muscle, indicative signal proteins in the PI 3-kinase pathway. IGF-I increases PKB/Akt phosphorylation in skeletal muscle, but has no effect on P70^{S6K} phosphorylation (164). Exercise induces increases in calcium and sensitivity to insulin, both, which are active in mediating the PI3 kinase pathway responses. This suggests multiple activators and amplifiers of the cascade.

COMBINATION OF MECHANICAL STRETCH AND ELECTRICAL STIMULATION

Active lengthening has previously been shown to have a greater effect on some of these cascades than either lengthening or activation alone (158). This observation

highlights the ongoing controversy surrounding the relative benefits of concentric, isometric, and plyometric exercise. Within the four markers, only 4EBP1 phosphorylation was numerically increased by the combination of stretch and activation, although this increase was not significant. These results gave the first indication that there may not be one pathway that activates each of these molecules like that of the proposed NFAT/COX-2 pathway. In fact, it could be suggested that the mechanical stretch and electrical stimulation processes may be redundant or work as primary or secondary mechanisms to provide the necessary adaptation to the muscle.

COX-2 AND INITIATION OF HYPERTROPHY

This study suggests that the pattern of effector activation does not begin with NFAT dephosphorylation and COX-2 expression. It is however known that prostaglandins are involved in the hypertrophic response and the conversion of Arachidonic acid to prostaglandin by COX-2 is the rate-limiting step. Prostaglandin synthesis can be initiated by the activation of MAP kinases. Recent evidence has demonstrated that MAP kinases are able of activating cytoplasmic phospholipase A_c(PLA) that can mediate the release of arachidonic acid from the cell membrane (165). MAP kinase pathways can be activated by the binding of growth factors to membrane receptors (141). Exercise has been shown to increase growth factors in the bloodstream and during exercise blood flow is increased to the musculature (84, 85,161). While the original model suggested that increases in intracellular calcium stimulated upregulation of COX-2 and the accelerated conversion of arachidonic acid to prostaglandins, we conclude that activation of the MAP kinase and the PI3 kinase effectors are not associated with simultaneous NFAT dephosphorylation or COX-2 upregulation. Further

study could help to establish alternate time period at which these exercise protocols could induces activation and upregulation of these targets.

DISCUSSION SUMMARY

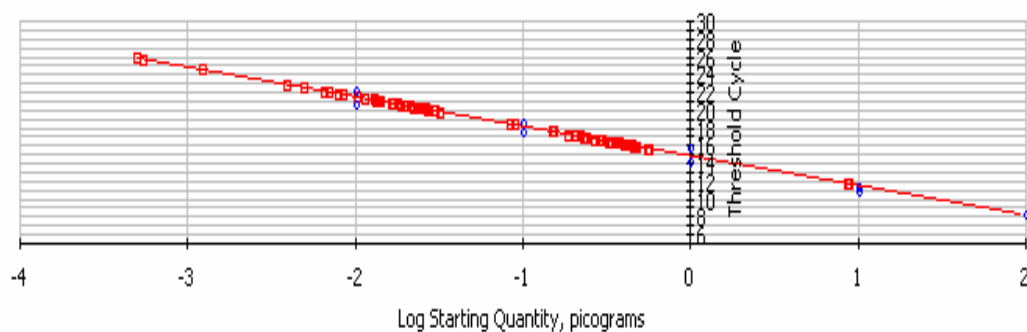
In summary, the results of this study indicate mechanical stretch and isometric activation influence the hypertrophic signaling in the tibialis anterior. The increases in activation support the hypothesis that calcium plays an important role in activating these pathways either by depolarization of the membrane or through indirect action by changes in the membrane structure. It is also suggested that at 3 hour after the recovery of exercise these pathways are not mediated through a calcineurin dependent mechanism. However, a definite conclusion cannot be made from this study that activity of NFAT and COX-2 are not present during alternate stages after exercise recovery. Stretch and activation initiate separate but overlapping signaling cascades, as evidenced by the activation the MAP kinases and PI-3 kinases targets. This type of interaction in vivo shows the complexity of controls within its native environment, and demonstrates that effective countermeasures to atrophy will need to compensate for both the chemistry of activation and the mechanics of stretch.

APPENDIX

APPENDIX A – COX-2 REAL-TIME

Correlation Coefficient: 0.992 Slope: -3.343 Intercept: 14.808 $Y = -3.343 X + 14.808$
 PCR Efficiency: 99.1 %

□ Unknowns
 ◇ Standards



PCR Standard Curve: 061504 Ricky.opd

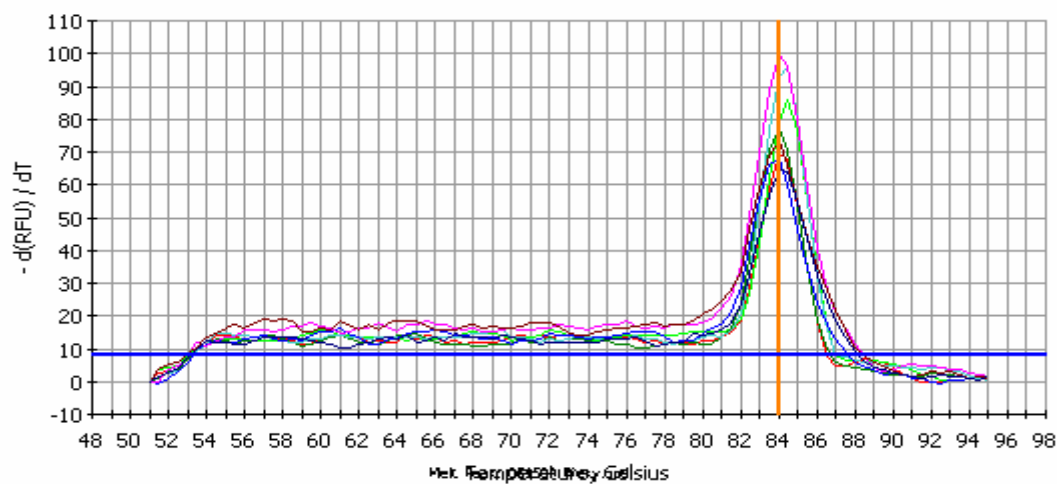


Fig. 32. Melt curves for standard maintained shape and form in the higher standards but around $1e-2$ pg/ul they began to lose shape and were therefore unresolvable.

APPENDIX A CONT.

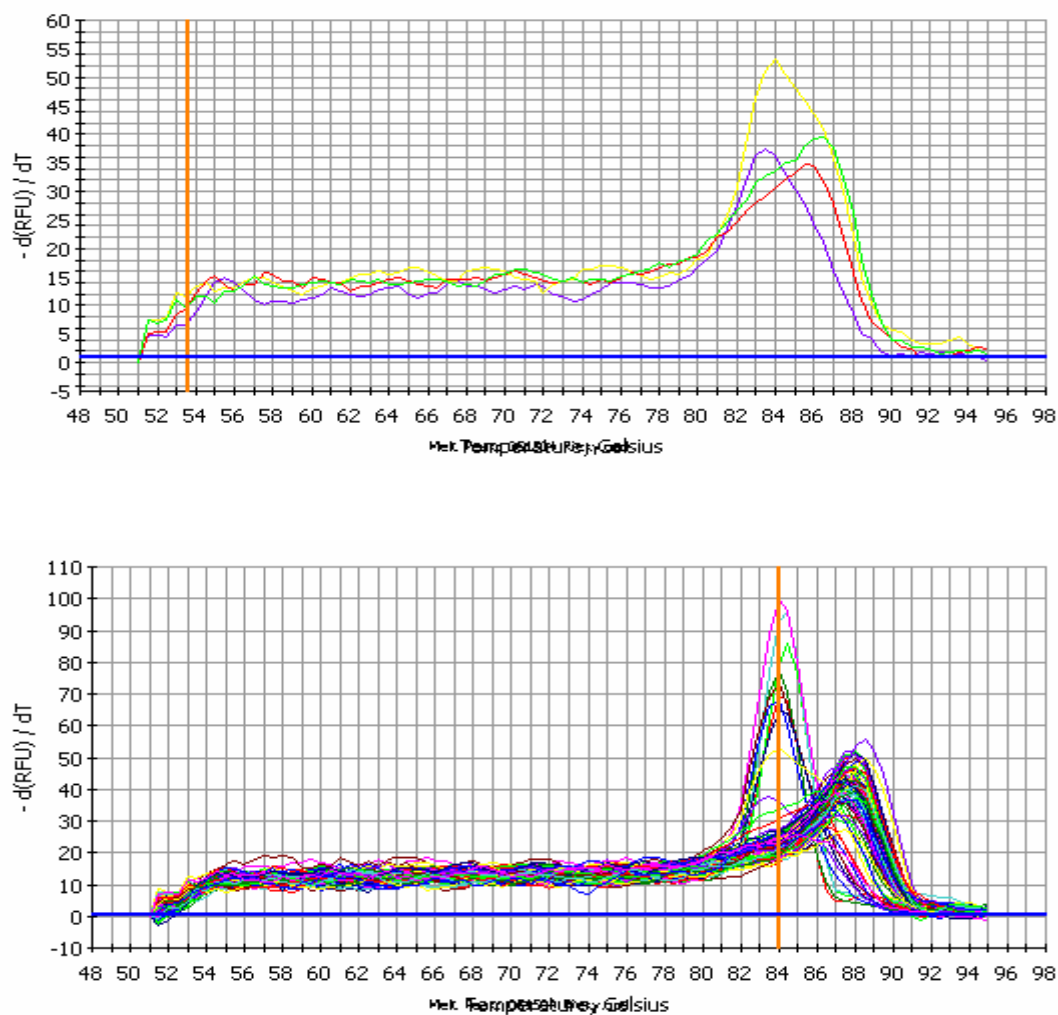


Fig. 33. This same pattern was observed in the sample which also when quantified were within the low standard range.

APPENDIX A CONT.

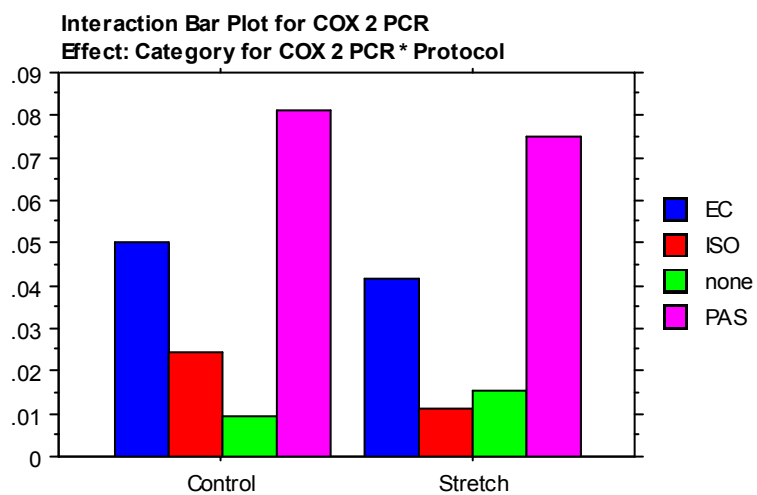


Fig. 34. Initial inspection of the unresolvable levels showed no differences between control and stretched samples.

APPENDIX B – TIME POINT

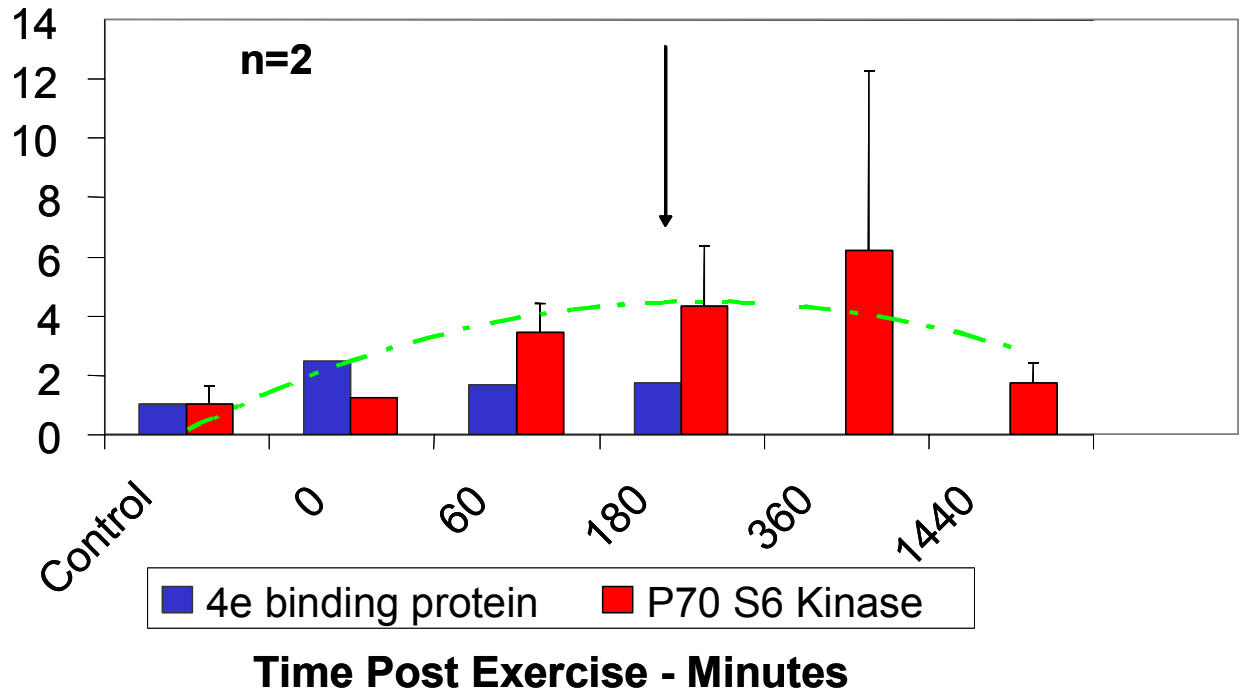


Fig. 35. Initial experiments showed that PI-3 Kinase activity was maximized by analyzing the effect at 3 hours after stimulation.

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