Université de Montréal

"Absence of a refractory period for mechanical activation of p54-JNK in rat plantaris *in situ*."

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Ce mémoire intitulé:

"Absence of a refractory period for mechanical activation of p54-JNK in rat plantaris *in situ*."

présenté par:

Petros Tzavaris

a été évalué par un jury composé des personnes suivantes:

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SOMMAIRE

Les kinases MAPK sont impliquées dans les signalisations intracellulaires incluant la mécano-transduction, le mécanisme de transformation des forces mécaniques en signaux biochimiques dans les muscles squelettiques. Ces signaux sont considérés essentiels pour l'hypertrophie musculaire. Dans cette étude, divisée en trois parties, nous avons premièrement examiné les niveaux d'activation de plusieurs kinases MAPK (p54-JNK, p44-ERK, et p42-ERK) pendant 3 heures après une séance de stimulation mécanique. Ce protocole comprend une chirurgie préparatoire *in situ* du nerf sciatique ainsi que l'isolation du muscle plantaire chez le rat qui était ensuite exposé à des étirements excentriques pendant 5 minutes.

L'analyse de l'activité des trois kinases démontre une activation maximale immédiate ainsi qu'une déactivation assez rapide, 30 minutes après la fin du protocole. Le résultat intéressant réside dans l'observation de l'oscillation de l'activation des kinases de la famille ERK (p42 et p44). Ces observations suggèrent que la régulation des kinases MAPK s'effectue selon un système dynamique et non linéaire. De plus, il est très probable que les trois MAPK possèdent des voies de contrôle ainsi que des fonctions différentes.

De plus, concernant le p54-JNK, nous avons examiné l'existence possible d'une période réfractaire suite à une deuxième séance de stimulation mécanique. Le protocole a été répété deux heures après la première séance, lorsque le niveau d'activation de p54-JNK est à environ 50% du maximum ('demie-vie'). Les résultats montrent que l'on peut encore obtenir les niveaux d'activation maximale. Ainsi, même en présence d'une régulation négative des MAPK, la relation entre la stimulation mécanique et le niveau d'activation de p54-JNK n'est pas modifiée.

Finalement, nous avons examiné la relation quantitative entre les niveaux d'activation des trois MAPK et la performance du muscle plantaire pendant le protocole. En général, l'activation des trois MAPK est fortement liée à la production de force absolue et plus encore, selon le type de MAPK, à la production de force corrigée pour les propriétés intrinsèques physiologiques et/ou le poids du muscle.

Ces résultats suggèrent fortement que chaque MAPK a des fonctions et des voies de contrôle differentes pendant la mécano-transduction. Ainsi, il est probable que chaque MAPK n'a pas la même implication dans la régulation de la synthèse protéique et dans l'hypertrophie du muscle squelettique.

MOTS CLÉS : signalisations intracellulaires ; mécano-transduction ; kinases MAPK ; étirements excentriques ; *in situ* ; période réfractaire ; oscillation de l'activation.

ABSTRACT

MAPKs are integral components of intracellular signaling pathways, including mechanotransduction in skeletal muscle. These biochemical signals are considered vital to the hypertrophy process. This study, divided in three parts, first examined the time course of activation of several MAPKs (p54-JNK, p42-ERK, and p44-ERK) for a period of three hours after a mechanical stimulation bout. The protocol involved *in situ* isolation of the rat sciatic nerve and plantaris muscle prior to a 5-minute bout of eccentric contractions.

Biochemical analysis of the MAPKs demonstrated immediate maximal activation followed by significant negative regulation within 30 minutes. The interesting finding is the presence of a biphasic response in the ERK family, which suggests that MAPKs are part of a dynamic, non-linear signaling system. Thus, it is probable that the MAPKs have independent functions and regulation during mechanotransduction.

Also, we examined the possible presence of a refractory period for p54-JNK following a second bout of mechanical stimulation. The stimulation protocol was repeated near the approximate p54-JNK half-life time, two hours following the first bout. Maximal activation was attained therefore, amidst negative regulation the relationship between mechanical stimulation and p54-JNK activation was unaltered.

Finally, we performed a quantitative analysis for the relationship between mechanical workload and MAPK activation. In general, MAPK activation is strongly related to tension. More specifically, depending on the MAPK, activation levels can either be strongly related to absolute or relative tension. Once again, this can be interpreted as evidence that the MAPKs have independent functions and regulation.

In conclusion, it is very likely that each MAPK has a different role during mechanotransduction and hypertrophy. Our findings can contribute to understanding diseases such as muscular dystrophy and in the design of training protocols and disuse atrophy countermeasures.

KEY WORDS: intracellular signaling; mitogen-activated protein kinases; *in situ*; mechanotransduction; eccentric contraction; time course; biphasic; refractory period.

TABLE OF CONTENTS

LIST of FIGURES

LIST of TABLES

LIST of DIAGRAMS

LIST of ABBREVIATIONS

Ca^{++} - calcium ion

ECM – extracellular matrix

FGF – fibroblast growth factor

GPCR – G protein-coupled receptor

HFES – high frequency electrical stimulation

HGF – hepatocyte growth factor

IGF – insulin-like growth factor

LFES – low frequency electrical stimulation

MAPK – mitogen activated protein kinase
JNK: c-Jun $NH_{2\text{-terminal}}$ kinase
ERK: extracellular signal regulated kinase

MEK – MAP/ERK kinase

MEKK – MEK kinase

MKK – MAPK kinase

MGF – mechano growth factor

MHC – myosin heavy chain

MKP – MAPK phosphatase

MTJ – myotendon junction

PKA/PKC – protein kinase A/protein kinase C

T-60 – average peak tension after 60 seconds

T-300 – average peak tension after 300 seconds

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Thank you.

Graciously yours,

Peter Tzavaris.

INTRODUCTION

A cell uses various messenger systems in order to adapt to changes in its extracellular and intracellular environment, thus achieving proper functionability. Each signaling system does not act independently of one another since there are many points of convergence and divergence. This apparent complexity and interactivity may allow for a specific cell response to a variety of stimuli. The mitogen-activated protein kinases (MAPK) are a vital component of this signaling network by acting as a point of integration (Pearson, 2001). MAPKs are a family of protein kinase cascades that exist in multiple parallel pathways. The three main pathways utilize the following subfamily as their MAPK representative: 1) c-Jun NH2-terminal kinase (JNK), 2) extracellular signal-regulated kinase (ERK), and 3) p38. In a wide variety of cell types, they have been induced by hormones (Liebmann, 2001), growth factors (Coolican, 1997), neural factors (Si, 1999), and chemical and physical stresses such as decreased pH (Xue and Lucocq, 1997), reactive oxygen species (Adler, 1999), UV light (Price, 1996), hyperosmolarity (Kultz and Burg, 1998), heat shock (Adler, 1995), and static stretch (Boppart, 2001).

Physical activity is one type of physiological stress that can elicit immediate and delayed responses. During exercise, mechanical and chemical signals are transduced in contracting skeletal muscle cells by the process of mechanotransduction. Via their cytoskeletal components, skeletal muscle cells both generate and sense mechanical forces (Chicurel, 1998a), and this is a form of stress that activates MAPK signaling. It is well documented (reviewed by Widegren 2001) that MAPK activation increases in rats (Aronson, 1997) (Ryder, 2000) and humans (Boppart, 1999) undergoing elicited

contractions or physical activity. Furthermore, factors such as fiber type (Wretman, 2000), the exercise protocol used (Nader and Esser, 2001) (Wretman, 2001) and the amount of tension generated (Martineau and Gardiner, 2001) all can have an effect on individual MAPK activation. In turn, they could target downstream nuclear and cytosolic substrates, including several transcription-activating elements (Thomson, 1999) (Pearson, 2001) (Garrington and Johnson, 1999). These elements affect gene and protein expression. Therefore, MAPK are considered to play an important role in the signal transduction process during exercise and the adaptive process after exercise. Nevertheless, nuclear and initial mechanotransduction events are unclear. For leads, we can look at the response of other cell types to mechanical tension such as smooth muscle (reviewed by Gerthoffer and Gunst, 2001) (Li and Xu, 2000) and cardiomyocytes (reviewed by Schaub, 1997). Regardless of the form or direction, many cell types are sensitive to mechanical forces (Goldspink, 1999) (Kumar, 2002) (Jo, 1997) (Lew, 1999). In muscle and other cell types, it is evident that MAPKs, especially JNK, are highly implicated with a cell's response to mechanical forces when considering its magnitude dependant response behavior (Martineau and Gardiner, 2001) (Boppart, 2001) (Hamada, 1998) (Jo, 1997).

Resistance training has been shown to produce several known adaptations in skeletal muscle, namely, fiber hypertrophy and phenotype changes (Baldwin and Haddad, 2001) (Goldspink, 1999). However, the mechanisms for these two types of changes are probably different (Murgia, 2000) with cell size governed by mechanical forces (Goldspink, 1999). Acutely, mechanical tension elicits a variety of temporal and spatial molecular responses perhaps all implicated with the hypertrophic response and probably

mediated by MAPKs. For example, increased mRNA levels of the immediate early genes *c-fos* and *c-jun* (Aronson, 1997) (Dawes 1996), increased p70 phosphorylation (Baar and Esser, 1999), and relocation of mRNA and ribosomes to focal adhesion complexes (Chicurel, 1998b).

As shown in Figure 1, each MAPK is activated by a specific kinase (MAPK2), which in turn is a point of convergence for several upstream activators (MAPK3). Scaffold proteins (Figure 2) are thought to coordinate a specific response by the MAPK system (Garrington and Johnson, 1999). This response then diverges since every MAPK may share common protein kinase and nuclear substrates in addition to their own specific high affinity substrates (Pearson, 2001). Furthermore, there is the possibility of receiving input from other signaling systems. In skeletal muscle, calcineurin, which is a calcium-calmodulin regulated phosphatase, can influence MAPK substrates (De Windt, 2000) (Wu, 2000) (Dunn, 2000) (Dunn, 2001). Therefore, at a given time, the relative amount of each MAPK represents a specific response to a given stimuli as enabled by the high level of coordination of the whole signaling system.

A family of MAPK phosphatases activated by MAPKs through a negative feedback system tightly regulates each MAPK thus, resetting the signaling system and allowing for the detection of changes in cellular environment (Figure 3). Once activated, MAPK can translocate to the nucleus thereby inducing the synthesis of various members of the MAPK phosphatase family (MKP-1, -2, -3, etc.), each possessing different MAPK substrate affinities and activity localization (Haneda, 1999).

An activated MAPK can be rapidly deactivated in the nucleus or cytosol even if the activating stimulus is still present and, moreover, inactivated MAPK in the cytosol

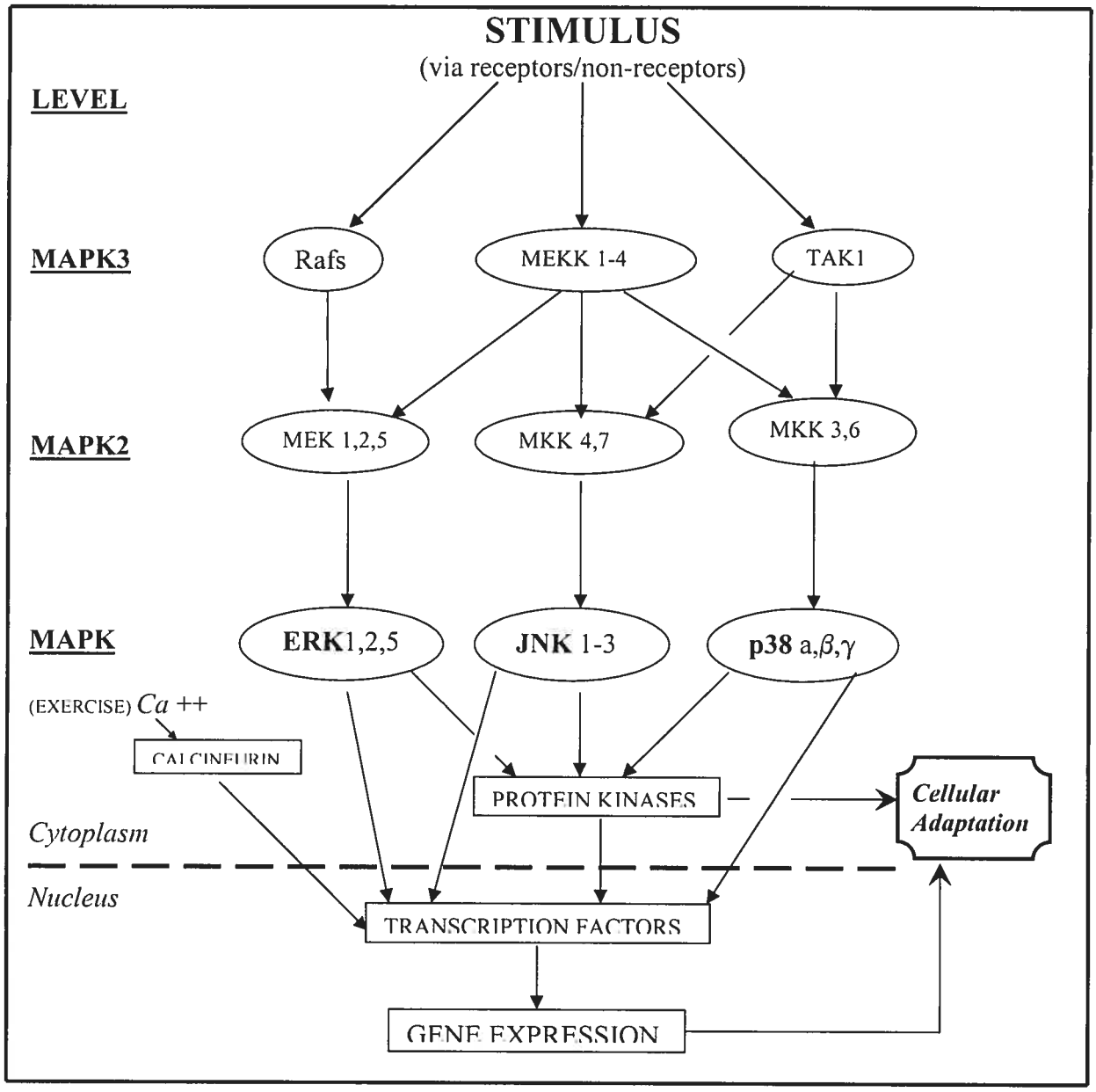

Figure 1 – MAPK pathways & exercise – upstream activators & downstream substrates.
(modified version from Garrington & Johnson, 1999)

LEGEND

LEVEL 3 – MAPK3 = MAPKKK = MAPKK kinases
LEVEL 2 – MAPK2 = MAPKK = MAPK kinases
LEVEL 1 – MAPK = mitogen-activated protein kinases

can be prevented from becoming activated (Camps, 2000). Such regulation could result in a refractory period for MAPK reactivation as demonstrated in other cell types (Fucini, 1999) (Brecht, 1999) (Meskiene, 1998) (Polakiewicz, 1998). This could have implications for the optimization of training protocols and disuse atrophy countermeasures. Constitutive expression of MKP-1 in cardiomyocytes attenuated the hypertrophic response (Bueno, 2000). This demonstrates a relationship between MAPK activation, hypertrophy, and levels of negative regulators.

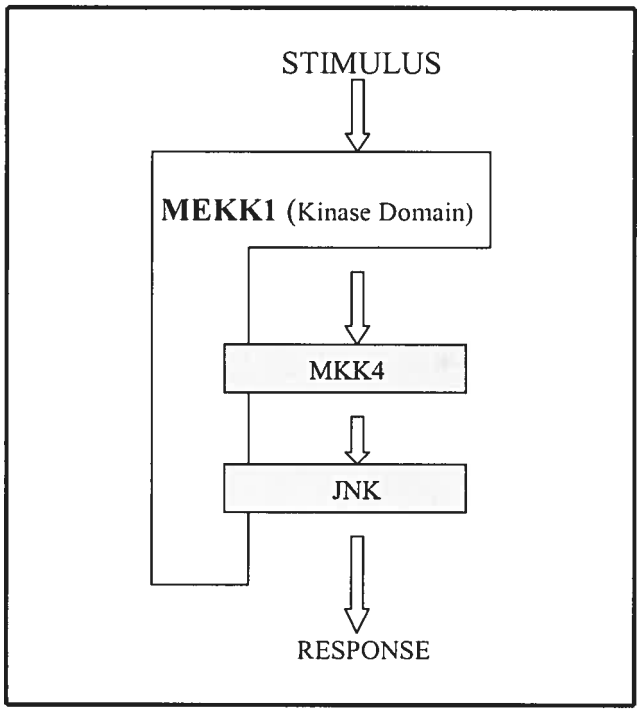

Figure 2 – MEKK1 as Scaffold Protein.
(from Garrington & Johnson, 1999).

We hypothesized that, just below its half-life time, the existence of a refractory period would prevent the reactivation of p54-JNK. Already demonstrated in our lab, we utilized a protocol associated with a high amount of tension generation and also known for its immediate activation of JNK and ERK in rat plantaris *in situ* (Martineau and

Gardiner, 2001). Initially in this study, a time course of activation and a quantitative analysis provided some characteristics of ERK and JNK. To gain some insight into MAPK regulation, we tested the hypothesis by restimulating the rat plantaris 2 hours after the initial bout of mechanical stimulation, at a time when p54-JNK activation had returned to approximately 50% of its peak as determined earlier in the study. Results indicate that near its half-life time, p54-JNK can be reactivated and attain peak levels of activation, similar to those measured immediately after the initial bout.

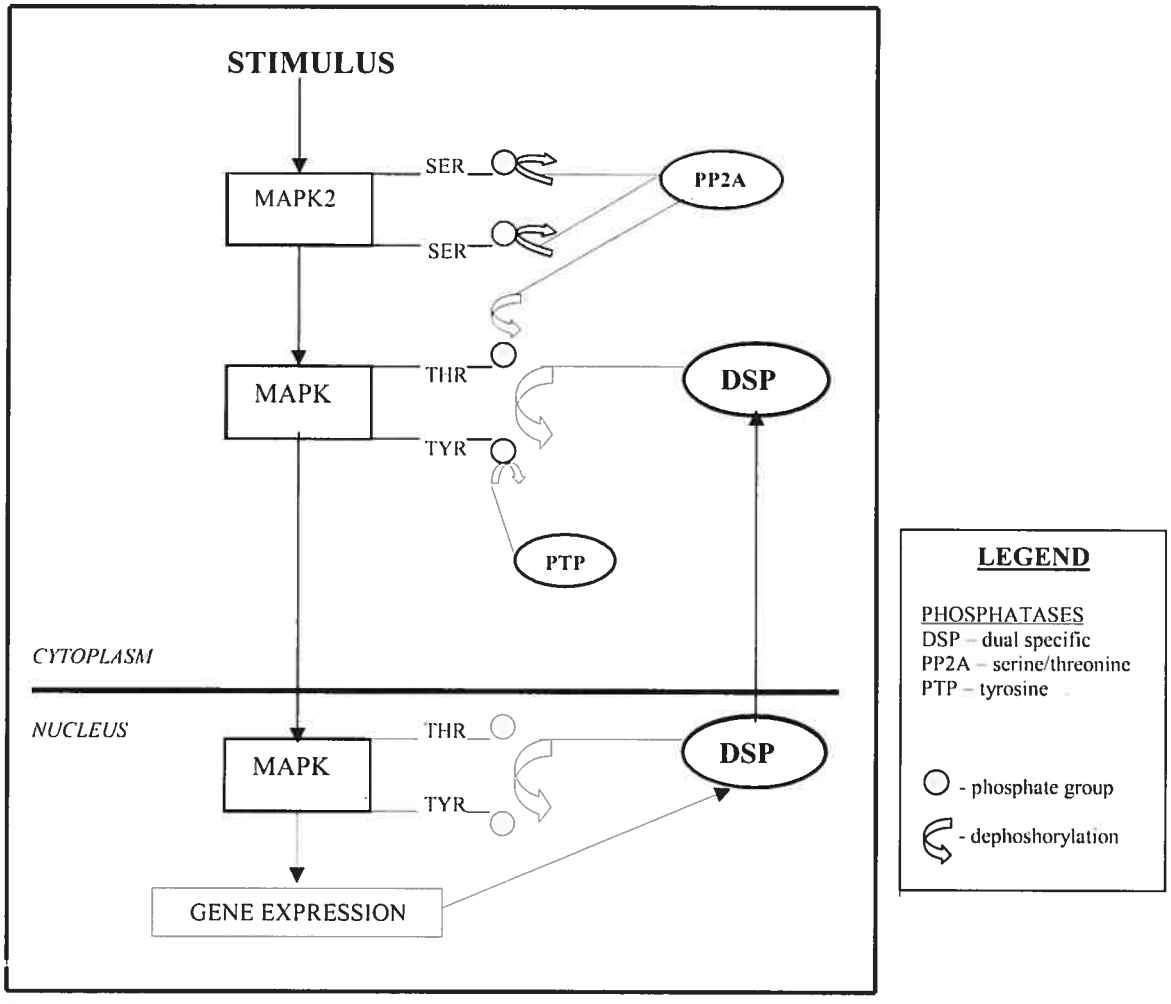

Figure 3 – Negative MAPK regulation.
(modified from Haneda, 1999)

REVIEW OF LITERATURE

(I) HYPERTROPHY – ADAPTATIONS

a) General

The study of skeletal muscle hypertrophy draws on experimental evidence from protocols utilizing resistance training, loaded contractions, functional overload, stretch, and pharmacological intervention agonists. Exposure to increased workload produces morphological, biochemical and physiological adaptations.

The most obvious and documented adaptation is an increase in whole muscle girth or muscle fiber cross sectional area regardless of fiber type. The augmentation of myofibrillar protein synthesis appears to occur in two stages. The first and more rapid stage is represented by increases in RNA activity involving translational and posttranslational mechanisms whereas the second stage, requiring more stimulation time, is exemplified by increased mRNA levels mainly attributable to transcriptional mechanisms (Carson, 1997). In addition to changes in the quantity of contractile proteins, there are also qualitative changes. In rodents and humans, resistance training produces isoform switching whereby the slower myosin heavy chain phenotype (IIa protein and mRNA) is expressed at a higher percentage (Baldwin and Haddad, 2001). Newly synthesized myosin can be incorporated nonuniformly throughout the thick filament, especially at the ends, allowing for the possible dominant expression of the slower phenotype in heterogeneous skeletal muscle fibers (Russell, 1992). These changes contribute to several physiological changes strongly correlated with hypertrophy including increases in peak tetanic force (Po), IEMG, rate of force development (dP/dT), and Vmax, the latter possibly due to the adaptive expression of fast myosin light chains

(Fitts and Widrick, 1996). An increase in muscle glycogen and decrease in mitochondrial density is predictable, however, other changes in muscle support systems such as metabolic enzymes, lipid stores, myoglobin content, and capillary density are inconsistent (Kraemer, 1996) and may depend on the specific resistance protocol used in the study with factors such as intensity, duration, subject fitness level, speed and type of contraction all affecting fiber recruitment and adaptation of energy systems.

Skeletal muscle hypertrophy and phenotype switching likely occur by different mechanisms since different Ras proteins are activated in each pathway (Murgia, 2000). Hypertrophy, including that which occurs in other cell types such as bone, cardiac and smooth muscle, involves the cytoskeleton, mechanotransduction mechanisms, and is highly dependant upon the amount of applied force (Goldspink, 1999). As previously mentioned, increases in protein synthesis can be accomplished by posttranscriptional mechanisms, for example, an increase in p70 activity leading to higher rates of translation initiation (Baar and Esser, 1999), and transcriptional mechanisms, by activating transcription factors and their capacity for gene transcription (Carson, 1997) (Pearson, 2001). Phenotype switching is also influenced by force or contractile activity (stretch-sensitive promoter sequence in the myosin gene) (Goldspink, 1999), but, unlike hypertrophy, phenotype switching necessarily involves posttranscriptional changes, specifically alternative gene splicing, and requires prolonged acute or chronic stimulus application (Carson, 1997). Before significant isoform switching becomes detectable whereby incorporation favors the higher available concentration of the transcribed slow isoforms versus the fast type isoforms, it appears that chronic stimulation is sufficient due to the relatively long half-life of the MHC protein (Russell, 2000). Acutely, a prolonged

stimulation bout results in fatigue and activity-induced increases in intracellular calcium levels. A pathway involving calcineurin, a calcium-calmodulin regulated phosphatase, is important for promoting the slow fiber type program (Delling, 2000). Nevertheless, the importance of a mechanical load signal is demonstrated by the inability of the over-expression of calcineurin alone without contractile activity to achieve phenotype switching and skeletal muscle fiber hypertrophy (Dunn, 2000). Moreover, the exercise protocols necessary for promoting expression of the slow isoforms is not limited to chronic stimulation but includes abbreviated mechanical loading (Dunn, 2001).

b) Hyperplasia

Although hypertrophy of existing muscle fibers is considered the main method of adaptation to resistance training, stretch, or compensatory regimens, there is evidence to suggest that hyperplasia, an increase in skeletal muscle fiber number, may contribute to the overall increase in muscle mass. Increases in cross-sectional area are occasionally associated with hyperplasia yet studies that demonstrate evidence of hyperplasia are almost certain to display significant increases in mean fiber area (Antonio and Gonyea, 1993). Hyperplasia and hypertrophy are likely distinct processes (Nishi, 2002) (Foulstone, 2004) (Ishido, 2006) but both require satellite cell activity.

Hyperplasia had been dismissed partly due to limits in methodology. The two most common techniques for quantitative measuring of muscle fiber number have their drawbacks, thus limiting precision and accuracy. Histological cross-section counts are based on the wrongful assumption that all fibers run from origin to insertion (Monti, 1997) (Patel and Lieber, 1997), therefore, the use of serial sectioning is required to

confirm the discontinuity of new myofiber formation and eliminate the possibility that split myofibers undergoing repair are responsible for the erroneous appearance (Antonio and Gonyea, '93). The second technique, direct counting after nitric acid digestion, sometimes results in total fiber number underestimation since small fibers may be missed (Antonio and Gonyea, '93). Indirect (qualitative) evidence of hyperplasia is imprecise since it requires the use of estimative formulas and correction factors (Antonio and Gonyea, '93).

Nevertheless, hypertrophy and hyperplasia require different signaling pathways although the involvement of MAPKs and IGF is common to both (Foulstone, 2004). Also, satellite cells are necessary for both processes by either fusing into an existing myofiber or fusing with other satellite cells to form a new myofiber. Evidence from skeletal muscle overload suggests that the hyperplasia process would likely involve satellite cell proliferation and differentiation (Ishido, 2006), each with distinct characteristics and regulation (Nishi, 2002) (Foulstone, 2004). Increased specialized expressions of adhesion molecules may help direct new myofiber formation via satellite cell fusion (Ishido, 2006).

c) Satellite Cells

Satellite cells, or adult myoblasts, are a population of mononuclear myogenic precursor cells located between the basal lamina and the muscle fiber sarcolemma. Their migration, proliferation, and differentiation are needed for the repair and growth processes because adult myonuclei are post-mitotic. They are normally dormant (or quiescent) but can be induced to divide mitotically as daughter cells can migrate across the plasma membrane into the cytosol, fuse with other satellite cells, and form a myotube.

After differentiation, they are considered true functional myonuclei because their genetic machinery can then contribute to protein production.

Resistance exercise results in myotrauma and an inflammatory-like response as satellite cells are regulated by the interaction of the immune system, cytokines, hormones and growth factors (Vierck, 2000). Locally, the combination of growth factors and ECM molecules needed for satellite cell activation are produced by many cell types including myoblasts or myofibers themselves, and macrophages, which also help with other aspects of the regeneration process such as angiogenesis and cellular debris removal (Grounds, 1998) (Vierck, 2000). However, satellite cells have a limited capacity to divide since, after a finite number of population doublings, they enter replicative senescence, a state of growth arrest characterized by refractoriness to mitogens. This may be altered with resistance training. Acute local over-expression of IGF-1 in skeletal muscles, a situation mimicking the existing environment after resistance training, prolonged the *in vitro* replicative life of satellite cells by facilitating G1/S cell cycle progression resulting in additional cycles (Chakravarthy, 2000). Also, MAPKs induce production and formation of cyclin-cdk complexes involved with overcoming the G1 checkpoint (Whitmarsh and Davis, 2000).

Their migration from distant sites towards those that require regeneration can be attributed to their chemotactic properties. Satellite cell motility increases in response to positive concentration gradients of HGF and FGF, growth factors that are released during muscle injury and exercise (Bischoff, 1997). Their responsiveness is increased by the expression of functional growth factor receptors and specific proteoglycans, which prevent growth factor proteolytic degradation (Grounds, 1998). Combining their

chemotactic properties with their expression of proteinases capable of degrading the muscle fiber lamina, the possibility exists of transverse migration towards adjacent fibers or muscles (Bischoff, 1997) (Allen, 1999). The importance of recruiting new satellite cells is shown by the observations that myonuclear number increased proportionally to fiber cross-sectional area and is in agreement of the theoretical idea that a single myonucleus can only support a finite volume of cytoplasm (Allen, 1999). Since MAPKs increase activity of the rate-limiting enzyme in nucleotide synthesis (Whitmarsh and Davis, 2000), one can speculate that they help produce the extra genetic machinery available with satellite cell divisions and aid in the hypertrophy response.

d) Extracellular Matrix

The ECM, composed of a variety of collagenous and noncollagenous proteins and polysaccharides, can provide mechanical support, regulate the activity of secreted growth factors, proteases and protease inhibitors, selectively influence macromolecular transport, and affect myoblast activity. Consequently, one can safely predict that exercise can affect the ECM environment. Studies are beginning to show that mechanical stress can cause qualitative and quantitative changes in the ECM although specific functional roles of individual macromolecular changes can only be speculated upon, especially in relation to the role of or the effect on the MAPKs during this process.

Microarray analysis of work overload-induced hypertrophied rat soleus muscle demonstrated an up-regulation of gene production of mRNA transcripts encoding for extracellular matrix proteins or matrix modifying enzymes (Carson, 2002). A family of matrix metalloproteinases (MMPs) is responsible for ECM degradation of specific

collagen types. Their activity is inhibited by a family of tissue inhibitors (TIMPs) and is also regulated by the status of integrins. Many ECM molecules, including native and degraded collagen, are ligands for integrins and thus affect cell growth and signaling via focal adhesion complexes (Kovanen, 2001). MMP and TIMP expression and activation can be altered by exercise resulting in ECM remodeling and helps explain the change in collagen subtype expression relative to a constant total collagen amount (Kovanen, 2001). In vascular smooth muscle cells, mechanical strain caused modifications of proteoglycan synthesis, proteoglycan interactions with other ECM molecules, and a concomitant increase in versican-hyaluronan aggregation (Lee, 2001).

At a minimum it is apparent that the ECM responds dynamically in response to exercise. Although specific roles for or effects on the MAPKs is unknown, reorganization of the ECM after mechanical loading may lead to alterations of the mechanotransduction properties via changes in integrin ligand binding.

(II) CELL SIGNALING - MECHANOTRANSDUCTION

The initial events responsible for the relay of mechanical and chemical signals during mechanical loading are unclear. It appears that they are not separate signaling processes but are instead coordinated, linked via integrins and other protein complexes.

a) Mechanical and chemical signaling (via integrins)

The total force generated by a muscle fiber is not entirely transmitted serially to the MTJ since a significant percentage of the total force generated can be accounted for

by lateral transmission (Street, 1983). Biophysical and qualitative evidence allows for the argument that via several possible sites, both radial and longitudinal forces can be transmitted laterally (Patel and Lieber, 1997) (Paul, 2002). Costameric protein systems, cytoskeletal complexes that link the functional sarcomere to the sarcolemma, are considered to be the site of lateral transmission although there may be other potential paths (Monti, 1999) (Patel and Lieber, 1997) (Paul, 2002). Two major protein systems have been identified and partially characterized, one of which includes integrin. They are the dystrophin/dystrophin-associated protein (DAP) system and the vinculin/talin/integrin system (Figure 4). Both systems provide a pathway between actin and the extracellular

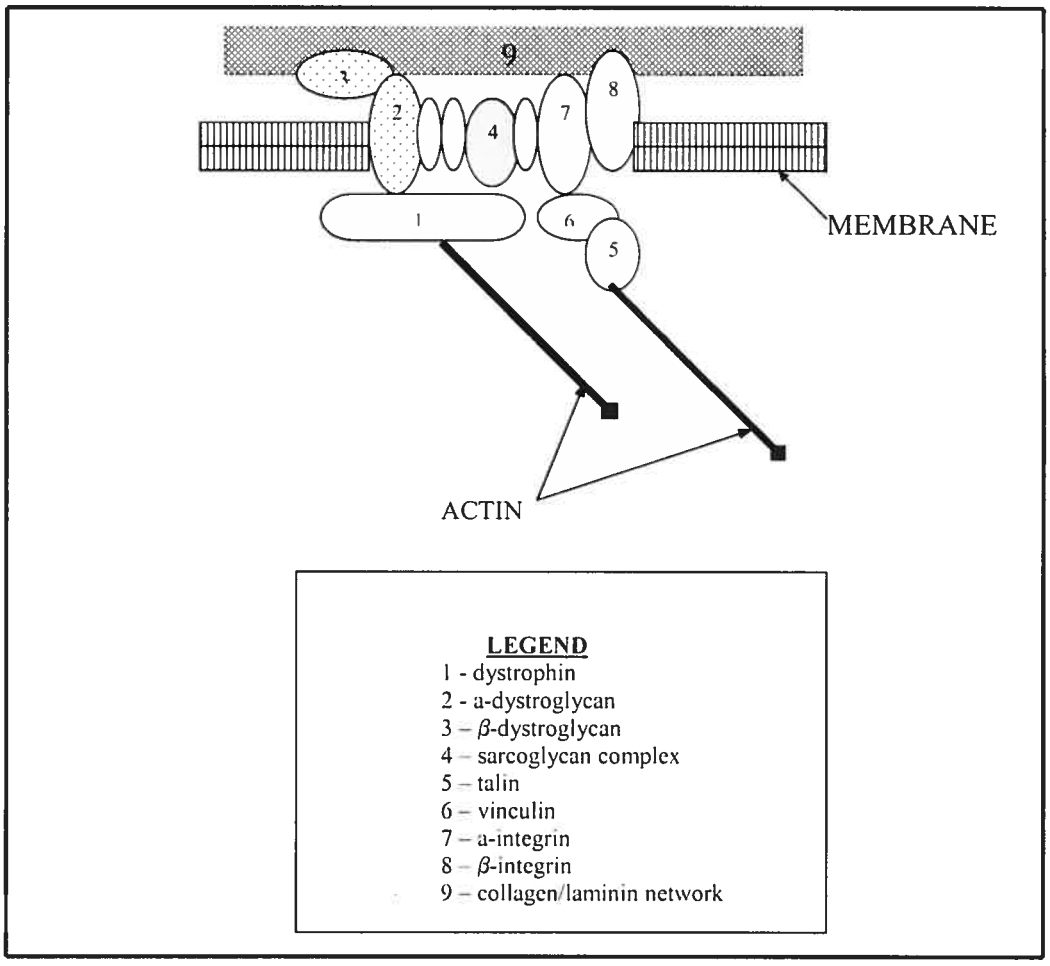

Figure 4 – Force transmission to the ECM
(from Monti, 1999).

matrix and, recent experimental evidence suggests that they may function in a coordinated manner rather than separately (Monti, 1999).

Integrins have been extensively studied due to their dual functional role as both adhesive and signaling molecules, their probable importance in mechanotransduction or force transmission, and thus their possible role in the hypertrophy response (Carson and Wei, 2000). Integrins may act as mechanotransducers during mechanical loading by activating MAPKs downstream (MacKenna, 1998) (Katsumi, 2005). The duality of these transmembrane receptors allows them to adhere to several protein types in the ECM and participate in cellular signal transduction. Ligand binding and signaling specificity depends on the α and β subunit isoforms and it has yet to be determined if mechanical loading affects integrin isoform expression (Carson and Wei, 2000). The cytoplasmic tails of integrins lack enzymatic activity, thus the required association with adapter proteins resulting in a macromolecular assembly called the (FAC) along with connections to the cytoskeleton, cytoplasmic kinases, transmembrane growth factor receptors, and ion channels helps to accomplish the task of transducing signals (Carson and Wei, 2000) (Giancotti and Ruoslahti, 1999).

Extracellular ligand binding activity and intracellular signaling activity can regulate each other and help meet the needs of the cell. In adherent cells, genetic programs such as differentiation, proliferation, and apoptosis are governed by cellular shape, which in turn are highly dependant upon the attachments of the integrin molecule and focal adhesions (Ruoslahti, 1997) (Chicurel, 1998a). Moreover, FAC formation changes during acute static stretching of cardiac myocytes and chronic stretching of

skeletal muscle (Carson and Wei, 2000). The reorganization of ECM proteins, integrins, and cytoskeletal proteins during mechanical perturbations is reinforced by a positive feedback system, which further promotes integrin clustering (Giancotti and Ruoslahti, 1999), and may help to increase or properly orient cellular tension. Also, integrin binding and mechanical tension correlated highly with rapid relocation of mRNA and ribosomes to FACs, possibly in order to enhance site-specific changes in protein synthesis (Chicurel, 1998b). Mechanical stress can be transmitted to the nucleus and cause movement or reorientation (Chicurel, 1998b).

There are other examples of how mechanical and chemical signals are linked. Mechanical forces acting through integrins help activate myosin ATPase activity and increase isometric tension (Chicurel, 1998a). Also, FAK and ERK2 phosphorylation correlated positively with initial levels of integrin-ligand binding and cell adhesion (Asthagiri, 1999). Finally, there are many downstream targets for integrin-mediated signaling that can affect protein synthesis via changes in translation and transcription (Carson and Wei, 2000).

In a review of mechanical force transduction in vascular smooth muscle cells, it has been proposed that in addition to integrins, receptor tyrosine kinases, GPCRs, and ion channels can act as mechanosensors (Li and Xu, 2000). Indeed integrins are needed for optimal activation of growth factor receptors (Giancotti and Ruoslahti, 1999). It should also be mentioned that in other cell types whereby force can be applied in different directions and perhaps utilize mechanosensors other than integrin, the nature of the force determines the specificity of MAPK activation (Lew, 1999).

b) Non-receptor signaling

An interesting study proposes a damage sensor model, without receptor signaling, as the initial event of mechanotransduction (Grembowicz, 1999). It was determined that temporary plasma membrane disruption correlated quantitatively with *c-fos* expression in mechanically injured monolayers. The model proposes that the initial event after plasma membrane disruption is a flux of normally impermeable molecules such as growth factors and ions. Stretch-sensitive ion channels can also act as mechanosensors (Li and Xu, 2000) and may contribute to this model.

c) IGF-1

Systemic and locally produced IGF-1 can affect protein synthesis in skeletal muscle. Moreover, after mechanical loading, a splice variant of IGF-1 (referred to as MGF) is produced locally and likely acts in an autocrine/paracrine fashion (Goldspink, 1999). Mechanical loading can cause local IGF-1 availability to increase without a change in serum IGF-1 due to either increased local IGF-1 production, significant decreases in inhibitory IGFBP-4 mRNA, or increases in synergistic androgen availability (Bamman, 2001). In skeletal muscle or skeletal myoblasts, MAPKs help relay the IGF-induced mitogenic or anabolic response (Coolican, 1997) (Adi, 2002) (Sarbassov, 1997) (Weyman, 1997) (Wu, 2000) (Haddad and Adams, 2004). Other growth factors may also play an important role in muscle hypertrophy.

d) MAPK Signalling Pathways

As shown in Figure 5, the MAPKs (ERK, JNK, and p38 subfamilies) are part of the intracellular signaling network. The paths of the subfamilies run parallel to each other, each with its own sensitivity to a given extracellular or intracellular stimulus. They are part of a phosphorylation cascade preceded by a series of kinases from three upstream levels (MAPK2, MAPK3, and MAPK4) with possible regulation at every level.

From the cell surface, receptor tyrosine kinases and G-protein linked receptors are able to detect extracellular stimuli, some of which are exercise related, and can initiate the process of MAPK activation through to level one, the MAPK4 group. G-proteins from the Ras/Rho family help mediate the signal to MAPK4. Also, enzymes and adaptor proteins aid receptor tyrosine kinases in the signal relay. The enzyme p120Ras-GAP can modify cytoskeletal structures (actin and focal contacts) and the adaptor protein GRB2 can bind with FAK, a component of the integrin signaling pathway (Denhardt, 1996). G-protein linked receptors can interact with the Ras/MAPK cascade via RhoA and PKC, aided by PKA, cAMP, Ca++, diacylglycerol and IP3. This can help maximize MAPK activation and attract protein complexes to the plasma membrane.

The MAPK4 group is not well defined and may include any protein between the receptor and the MAPK3 level, for example, GAP, GEF, PKC, P13-K, PAK, PKA, phospholipases.

The MAPK3 group has 14 members including Raf isoforms, MEKK1-3, and TAK. Amplification of signals is possible due to the diverse regulatory motifs found within group members. Thus, they can receive input from a variety of sources, both Ras-dependant and independent, and each can activate one or more MAPK2 proteins,

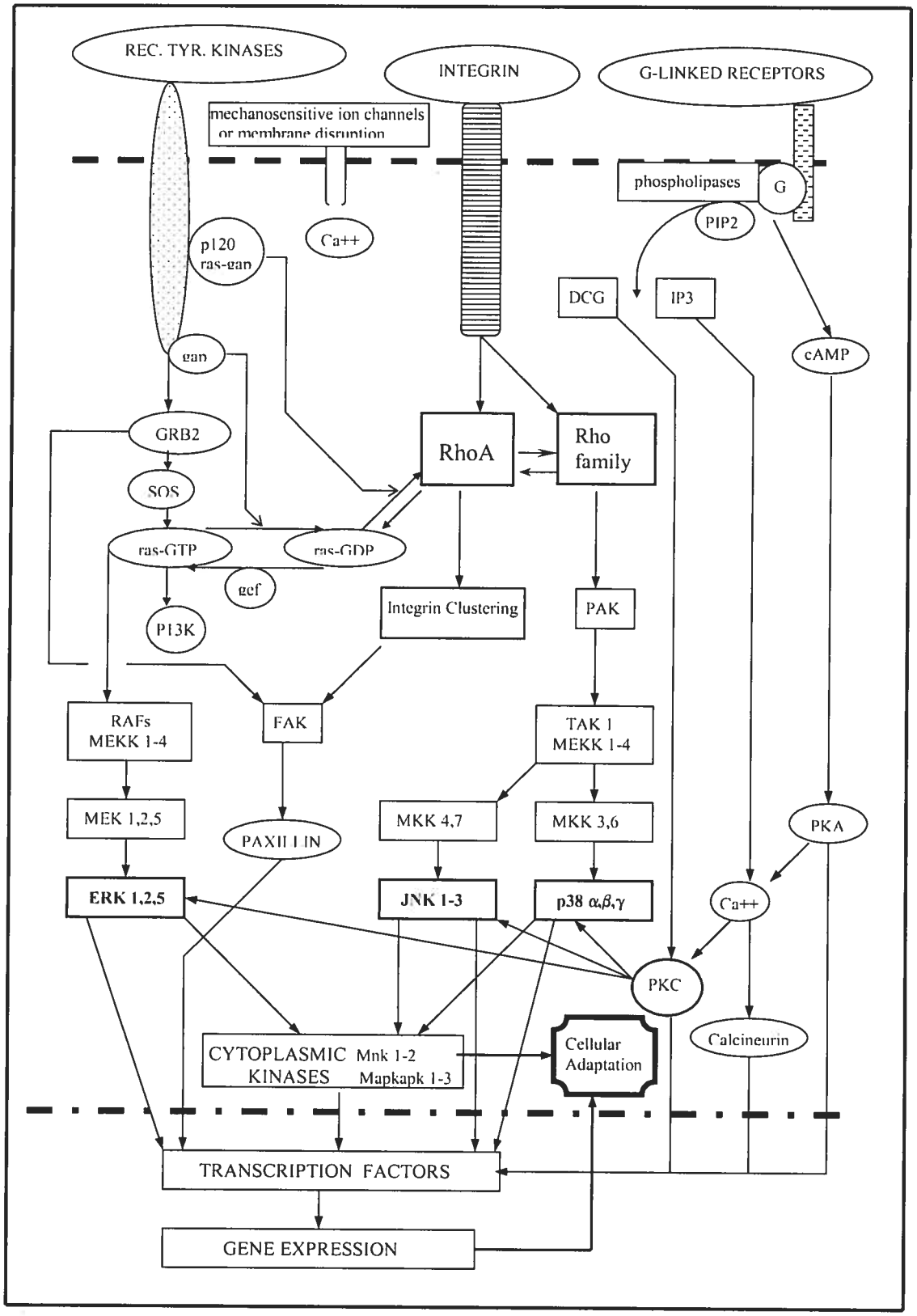

Figure 5 - Mechanotransduction and Mapk pathways

although different affinities for each substrate exists. Of interest, MEKK1 may play a significant role in mechanotransduction since it can activate both the ERK and JNK pathways beyond a threshold concentration and also colocalizes with cytoskeletal components (Pearson, 2001) (Denhardt, 1996).

At the MAPK2 level, which includes seven members (MEK1-2, MKK1-2, 4-6, SEK, and JNKK), amplification is not possible because of more specific kinase preferences for both serine/threonine and tyrosine residues on native MAPKs.

The MAPK subfamilies (ERK, JNK, and p38) are together a superfamily of protein kinases uniquely identified by a dual phosphorylation motif. Within subfamilies exist isoforms, which sometimes have opposing reactions to a given stimulus, especially the p38 family (Pearson, 2001). This demonstration of specificity must be taken into account when designing experiments and selecting detection methods, thus helping avoid conflicting results.

In mammalian cells, activation of the MAPK pathways has been shown to occur in response to various stimuli, some of which are related to physical activity. A lowered pH activates all three main pathways (Xue and Lucocq, 1997) whereas osmotic stress activates JNK and ERK (Kultz and Burg, 1998). Heat shock only activates JNK through a Ras-independent intracellular path involving the mitochondrion (Adler 1995). Interleukin-1, a cytokine associated with microtrauma, caused a mild activation of p38, a moderate activation of JNK activators MKK4/7, and a high activation of JNK isoforms in many cell types including skeletal muscle (Finch, 2001). Reactive oxygen species/redox potential activates all three MAPK subfamilies, possibly involving kinases and cytokines associated with cytoskeleton reorganization (Adler, 1999) and this signaling response

may play a role in cardiac myocyte hypertrophy (Xie, 1999). Finally, a variety of hormones via GPCRs in many cell types (Liebmann, 2001) and IGF in myoblasts (Coolican, 1997) induce MAPK activity and influence growth processes.

Whether the protein targets are found in the membrane, cytoplasm, or nucleus, MAPKs share common substrates and is likely accomplished by the complementary binding of MAPK domains to the different docking motifs found on the substrates. Thus, the result can be pre and posttranscriptional modifications. Transcriptional control can be achieved via cytoplasmic targets. For example, ERK can regulate transcription by activating cytoplasmic p90rsk (or MAPKAPK-1) and the subsequent phosphorylation of possible targets such as CREB, *c-fos*, SRF, etc. (Pearson, 2001). An example of posttranscriptional modifications is the interaction of ERK and p38 with protein kinases Mnk1-2 and MAPKAPK2-3. Each can then phosphorylate the eukaryotic translation initiation factor elF-4E and Hsp 25/27 respectively (Denhardt, 1996) (Pearson, 2001). Phosphorylation of Hsp 27 is implicated with actin polymerization and cytoskeletal repair processes (Denhardt, 1996) (Pearson, 2001). JNK interacts directly mainly with nuclear targets.

MAPKs can translocate to the nucleus and regulate the activities of transcription factors. ERK and JNK phosphorylate members of the AP-1 family (*c-fos, c-jun,* ATF-2) yet they have opposing effects on the DNA binding affinity of *c-jun* (Pearson, 2001). The TCF family (Elk-1, SAP1-2) is regulated by all three subfamilies and phosphorylation can result in an increased formation of ternary complexes also involving DNA and SRF (Pearson, 2001). Transcription factors can participate in homotypic and heterotypic protein interactions in order to achieve altered gene expression (Cox, 1999)

and are aided by the influence of MAPKs on histone activity and DNA accessibility (Whitmarsh and Davis, 2000). This flexibility allows for *cis* and *trans* control of transcription, which causes coordinated changes in the transcription rate and/or isoform switching of specific muscle genes, for example, the synergistic interaction of the MyoD and MEF2 family of transcription factors (Cox, 1999). In addition, MAPKs can interact with transcription factors from cytokine and metabolic signaling networks (Pearson, 2001) and interact with calcineurin during phenotype switching (De Windt, 2000) (Wu, 2000).

e) MAPKs and Physical Activity

Review tables (Widegren, 2001) summarize how different exercise models result in a rapid global increase of MAPK activity in human and rat skeletal muscle, and, when investigated, of upstream or downstream kinases. MAPK activation does not require systemic influence (Ryder, 2000) (Wretman, 2000) (Boppart, 2001) (Carlson, 2001), but appears dependent upon tension and intramuscular signaling. Besides variations in biochemical methodology procedures or time of activation measurement, differences in measured activity can be attributed to the exercise model used in the study.

In one study, only running and not HFES or LFES caused a biphasic activation of ERK (Nader and Esser, 2001). The type of contraction (concentric vs. eccentric vs. passive stretching) had different effects on ERK and p38 phosphorylation (Wretman, 2001). Also, the fiber type classification of the muscle can inherently influence MAPK activity. Phosphorylation of p38 was higher in fast twitch muscle although the total amount of ERK and p38 proteins was higher in slow type muscle (Wretman, 2000).

However, in another study, JNK and p38 activation was higher in slow fiber muscle (Boppart, 2001). Recently, there is evidence showing that force generation *per se* is not directly responsible for p38 activation (Dentel, 2005).

Most of the discrepancy can be explained by examining the force output of the working muscle. The fact that studies demonstrate higher MAPK activation during eccentric contractions vs. concentric or isometric contractions (Boppart, 1999) is likely attributable to the larger forces generated by eccentric contractions since it has been shown that there is a high correlation between JNK/ERK activation and the amount of tension generated by the muscle, regardless of the type of contraction and if it were developed actively or passively (Martineau and Gardiner, 2001). The same reasoning can be used to explain why HFES produces higher MAPK activation over LFES (Nader and Esser, 2001). Furthermore, the role that muscle stiffness and differing fiber lengths can play during tension generation and resistance to deformation (Patel and Lieber, 1997) may explain MAPK activation differences between slow and fast type muscles.

Mechanical tension is known to have hypertrophic effects in many cell types that can sense mechanical forces including skeletal muscle (Goldspink, 1999), smooth muscle (Gerthoffer and Gunst, 2001) and cardiomyocytes (Schaub, 1997). In endothelial cells, ERK activation responded in a force-dependant manner (Jo, 1997). JNK is dramatically activated in a dose dependant manner in response to tension in rat skeletal muscle (Martineau and Gardiner, 2001) (Boppart, 2001), and stretch in rat smooth muscle cells (Hamada, 1998). Therefore, there may likely be a high correlation between the amount of MAPK activation and hypertrophy.

III) MAPK REGULATION

a) Scaffolds

Scaffold proteins form oligomer complexes and are responsible for MAPK regulation by serially organizing MAPK cascades for efficient control and also restricting locality or substrate specificity whenever receiving or outputting a signal (see figure from Garrington and Johnson, 1999). JNK oligomerizes with members of the JIP scaffold family, each with tissue and subcellular location specificities (Garrington and Johnson, 1999) (Pearson, 2001). Scaffolds may be responsible for the co-localization of MEKK1 with various cytoskeletal components, including focal adhesions, and may aid in transferring signals to and from the cytoskeleton (Pearson, 2001). Positive and negative regulation is possible via posttranslational control (phosphorylation or proteolysis) of scaffold proteins or their binding partners (Garrington and Johnson, 1999).

b) Phosphatases

A major point of intracellular regulation occurs at the level of the MAPK. The duration and magnitude of MAPK activity reflects the balance between MAPK activators, usually upstream, and negative regulators. MAP kinase phosphatases (MKPs) are responsible for the negative regulation or dephosphorylation of activated MAPK and also of upstream MAPK2. Since phosphorylation of both residues is required for MAPK activation, dephosphorylation of one residue (by tyrosine-specific or serine/threonine-specific phosphatases) or both residues (by dual specificity tyrosine/threonine phosphatases) will accomplish MAPK deactivation. However, it is noteworthy to

mention that recent evidence indicates dual specificity phosphatases can also act as selective activators of JNK (Shen, 2001).

Little is known about the importance of tyrosine-specific or serine/threonine-specific phosphatases except that they appear to be constitutively expressed in the cytosol and are probably partly responsible for rapid MAPK inactivation (Camps, 2000). On the other hand, the family of (DSP) is transcriptionally induced by MAPKs relatively quickly (30-120 minutes) and is better characterized with parameters such as tissue specificity, substrate specificity, half-life, and subcellular compartmentalization (Camps, 2000) (Haneda, 1999). They act predominantly in the nucleus but some can also perform their function in the cytosol (Haneda, 1999).

The high level of interactivity between subfamilies and isoforms of MAPKs and MKPs represents an enormous unknown complexity of the MAPK regulation system. Inactivation of an individual MAPK isoform can be accomplished by different induction pathways and MKPs, and at different time periods and intracellular locations as demonstrated by differences in cytosolic and nuclear ERK kinetics (Reffas and Schlegel, 2000). Various phosphatases, cytosolic or nuclear, can be catalytically activated by the same MAPK (Hutter, 2000).

Although there is functional overlap in terms of MAPK substrate specificity, preferential binding exists for every phosphatase within both families. The physical complex formed by the binding of a MAPK to a MKP is highly specific and may be a result of the physical orientation of a MAPK with docking sites in scaffold proteins (Sharrocks, 2000). Also, MAPKs have a required docking site in order to bind and activate MKP-1 (Slack, 2001). Furthermore, all MKPs possess an amino terminus

substrate-binding site and a catalytic domain at the carboxyl terminal. In MKP-3, the ERK2 binding and catalytic regions partly overlap and are functionally coupled as structural determinations proved that the upregulation of phosphatase activity after ERK2 substrate binding to MKP-3 was caused by allosteric conformational changes at the active site (Farooq, 2001). However, MAPK binding does not necessarily trigger the catalytic activity of a MKP. The correlation between binding strength and catalytic activity appears to be questionable (Chen, 2001). Use of MKPs with mutated binding sites provide the explanation that JNK has different sites of interaction as compared to ERK and p38 (Chen, 2001) (Slack, 2001).

Also, the expression levels of MKPs or MAPKs can influence the kinetics of catalysis. An ERK-dependant pathway induced MKP-1 but, JNK and p38, and their associated transcriptional events were more sensitive to the inhibitive action of submaximal levels of MKP-1 (Franklin and Kraft, 1997). In another example, catalytic activation of MKP-1 by a MAPK increases in a dose-dependant manner, however, activation of MKP1 by p38α alone saturates at a lower concentration (Slack, 2001). This demonstrates the existence of a nonlinear system with thresholds, and how it can respond specifically to a given stimulation protocol.

Substrate binding is not influenced by protein kinase activity, therefore, it is possible that negative regulation occurs by preventing the phosphorylation of an inactive MAPK after binding to MKP in addition to the dephosphorylation of an active MAPK (Camps, 2000). Such regulation can contribute to the presence of a refractory period by not allowing a constant stimulatory signal or restimulation to activate the MAPK system.

Physiological outcomes vary depending on whether MAPK activation is acute/phasic or chronic/sustained. In rat hepatocytes, acute and not sustained MAPK activation promoted progression through the G1/S phase and an increase in DNA synthesis (Tombes, 1998) whereas in hamster embryo fibroblasts, sustained activation of ERK1 was required for G1 phase progression (Weber, 1997).

c) Desensitization/Refractory Period

Studies using various cell types demonstrate that MAPK activation (and/or immediate-early gene activation) cannot proceed indefinitely while exposed to continuous stimulation and is subject to periods of refractoriness. Desensitization can be classified as homologous if responsive to stimuli other than the original or heterologous if unresponsive to any form of stimuli including the original. The former appears to be prevalent and suggests that sufficient MAPK is usually available for stimulation.

Continuous stretching and continuous overload of skeletal muscle resulted in respective desensitization of *c-fos* and *c-jun* mRNA levels (Dawes, 1996) and MAPK phosphorylation (Carlson, 2001). Similarly, continuous stretching in smooth muscle (Franklin, 1997) and continuous shear stress in endothelial cells (Jo, 1997) resulted in MAPK homologous desensitization. However, when referring to a repetition of intermittent bouts of stretching, depending on the time of reapplication, there was either a cumulative effect surpassing original peak levels, a reattainment of peak levels, or an attenuated response (*c-fos* only) (Dawes, 1996). Therefore, there is questionable evidence concerning the presence of a refractory period.

Other forms of stimulation in various cell types show how desensitization is probably a general feature of all cells. However, the cell type and the agonist or stimulation protocol used strongly suggest specificity in the desensitization process. For example, in ovary cells, insulin-induced desensitization of ERK activation resulted in a 2-hour refractory period and was found to be pathway specific, upstream of ERK but downstream of Raf (Fucini, 1997). Also, in mouse fibroblasts, anisomycin caused homologous desensitization of JNK, p38, and IE genes (Hazzalin, 1998). Finally, there is evidence of a connection between phosphatases and desensitization. Mechanical wounding of plant cells resulted in a 30-minute MAPK refractory period and, the timing and amount of phosphatase transcript strongly correlates with the length of the refractory period and the levels of MAPK activation (Meskiene, 1998).

Although there are many possible mechanisms that are not yet well understood, the main mechanism of cell desensitization usually involves receptor endocytosis. GPCRs are strongly implicated because they are responsible for the transduction of a vast array of extracellular stimuli. The time frame of desensitization depends on the particular mechanism(s) that comes into effect. Uncoupling of the receptor complex results in rapid desensitization (seconds) as compared to internalization of cell surface receptors (minutes) whereas a decrease in the total cellular receptor pool via transcriptional changes takes longer (hours) (Ferguson, 2001). The process and time frame of resensitization appears to be closely linked to cellular desensitization. After endocytosis, replacement of surface receptors requires minutes if internalized receptors are recycled or hours if dependent upon mobilization of naïve receptors or de novo synthesis (Ferguson, 2001). Endocytosis is regulated and affected by the levels of β-arrestin and other proteins

(Ferguson, 2001). Moreover, β-arrestin has been found to satisfy the requirements necessary to be classified as a scaffold protein in JNK and ERK signaling cascades mediated via GPCRs (Pierce, 2001). Since β-arrestin plays a significant role in both the activation and desensitization process by contributing to the assembly of signaling protein complexes and subsequent intracellular trafficking, it is possible that these processes are not distinct but are indeed well coordinated.

The role, if any, of GPCR regulation during mechanical load induced MAPK signaling is unknown.

PURPOSE OF STUDY

Specific physiological responses depend on MAPK activation profiles. We investigated the time course of activation of MAPKs (JNK and ERK) in response to an acute bout of eccentric contractions. Kinetic data analysis has shown that MAPK activation profiles can be oscillatory (Kholodenko, 2000). ERK, in particular, has exhibited a biphasic induction profile in muscle cells, for example, in myogenic cell lines (Wu, 2000) and in skeletal muscle in response to continuous overload (Carlson, 2001) and to endurance running (Nader and Esser, 2001). Therefore, it is not unreasonable to expect to find a biphasic response although, to our knowledge, no study using a brief single bout of mechanical loading as a stimulus has yet to find such a response. In addition, as a follow-up to a study previously performed in our lab (Martineau and Gardiner, 2001), a quantitative analysis was performed in order to assess the relationship between activated MAPKs during the time course and tension-related muscle performance variables.

In order to test for the presence of a refractory period, the stimulation protocol was repeated 2 hours after the initial bout. We hypothesized that a refractory period would prevent the activation of JNK and would not be able to reattain initial levels of activation. The absence or presence of a refractory period will contribute to the understanding of MAPK-relayed mechanotransduction and the hypertrophy response.

METHODS AND MATERIALS

ANIMAL CARE

38 female Sprague-Dawley rats weighing a minimum of 180 grams were obtained from Charles River (St.-Constant, QC) and randomly assigned to a group. They were placed 2 per cage into standard rodent wire mesh cages and housed in a temperature-controlled animal care facility with a 12 hr. light/dark cycle and food and water availability *ad libitum*. Care and treatment of the animals was approved by the animal ethics committee of the Université de Montréal and were in accordance with the principles of the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care).

SURGERY (*In situ* nerve-muscle preparation)

The plantaris muscle and sciatic nerve of the left leg were surgically isolated. The plantaris muscle was isolated from the other ankle extensors while taking care to not disrupt its vasculature, innervation, tendon, and not have any tension applied to the muscle or sciatic nerve during the preparation. Before all surgical procedures were performed, animals were anaesthetized with an intra-peritoneal injection of ketamine and xylazine (62 mg/kg ketamine and 8 mg/kg xylazine) and included maintenance doses of the same mixture (25% of initial dose) every 45 minutes. All soft tissue exposed to the air was kept moist with frequent applications of saline solution.

Initially, with a scalpel, a 1" longitudinal incision of the dermis is made over the belly of the left leg extensors. Between the ankle/foot and the area approaching the pelvis, fascia, fat, and the dermis were separated from the muscle group. Weights were

used to pull the separated dermis apart and allow easier access to the frontal part of the leg. This was also used later as a reservoir for mineral bath oil. The hamstring (leg extensors) was pulled upwards with tweezers so as to separate it from the sciatic nerve meanwhile using scissors to make an incision in order to create a window and allow for visualization of the sciatic nerve. Micro-scissors were used to cut away surrounding fascia. A glass probe tested for successful isolation by ensuring that a minimum of 1 cm of the sciatic nerve would be accessible to electrodes.

After inserting and sliding blunt-end scissors centrally through the fascia notch, the hamstrings could be separated from the gastrocnemius by cutting it lengthwise from the original incision to the area approaching the common extensor tendon(s). The calcaneus was clipped leaving a bone chip attached to the common tendon and a 4" long 2-0 silk ligature was tied and hooked around at the bone tendon junction. The extensors were gently pulled away from the leg by using a hemostat to pull on the silk loop. Denervation of the foot was accomplished by cutting the tibial nerve running along the medial side of the leg. The soleus was cut near the proximal tendon, separated from the plantaris and then cut again near the common tendon. The medial gastrocnemius was cut near the tendon and separated from the plantaris after using a glass probe to locate anatomical notches within the center of the gastrocnemius. Denervation of the gastrocnemius followed. To minimize damage to the plantaris, the lateral gastrocnemius remained partially attached to it without affecting physiological measurements. Before closing, the surgical area was covered with saline saturated cotton balls. Finally, using a scalpel, bilaterally, parallel to the spine, 2 slits of the paraspinal muscles were made halfway between the pelvis and rib cage.

MUSCLE PHYSIOLOGY

The animal was secured in the prone position within a stereotaxic frame with the left foot immobilized by a clamp and the left knee pinned in a slightly flexed position to the frame. The silk ligature loop was attached to the lever arm of a muscle puller servomotor (Cambridge LR 350). The dermis of the hindlimb was pulled to form a bath and filled with heated mineral oil, monitored and maintained between 35-37°C. Core temperature was monitored by a rectal probe and maintained between 35-37°C using a heating pad.

Before performing the eccentric contraction protocol (Figure 6), the optimal length (*Lo*) for muscle twitch tension development was determined starting from a relaxed length. The muscle was indirectly stimulated via the sciatic nerve using a platinum bipolar electrode. All tension developments were visualized on an oscilloscope and recorded with a microcomputer (Figure 7). Supramaximal (5V) single square pulses of 0.05 ms in duration were delivered every second by a Grass S88 Stimulator. In order to remove slackness in the silk/tendon attachment, one isometric tetanic contraction (100Hz) (100ms) was performed. *Lo* was redetermined at a more accurate length and was held there for 30 seconds before the onset of the contraction protocol.

Time course of JNK activation was measured after a 5-minute bout of eccentric contractions. This protocol for maximal MAPK activation was performed earlier in our laboratory (Martineau and Gardiner, 2001). Indirect stimulation of the sciatic nerve with 5V, 150 ms of 0.05 ms single square pulses delivered at 100Hz once every second resulting in 300 tetanic contractions. During the eccentric contractions, utilizing a ramp function simultaneously with a computer-controlled servomotor, the muscle was

stretched to a new length of *Lo* + 3mm at a constant velocity of 20 mm/sec and held there for 350 ms. The muscle returned to the starting length also over a 150 ms time period and constant velocity, allowing 350 ms before the next stimulation. This results in a 50:50 duty cycle. Depending on the group, after the stimulation protocol, the muscle was kept at the predetermined *Lo* at physiological core and muscle temperature for 0, 30, 60, 120, or 180 minutes before muscle excision. One unstimulated control group was included in order to discount the effects of stretch, *Lo* determination, and MAPK baseline activation. For this group, after *Lo* was determined including one tetanic isometric contraction, the plantaris was held at *Lo* for 5 minutes without stimulation.

To determine if a refractory period existed, one group was stimulated a second time, 120 minutes after the first bout. The time course of activation demonstrated that the JNK activation half-life was 120 minutes. The abovementioned protocol to find *Lo* was used again to determine a new optimal starting length just prior to the 120-minute mark at which time, the second bout of eccentric contractions commenced followed immediately by muscle excision. To control for the 2-hour period as a possible source of influence on maximal MAPK activation during which the muscle is held at *Lo*, without an initial stimulation bout at time 0, one group had the plantaris held at *Lo* for 120 minutes before redetermining *Lo* and then performing the ensuing stimulation protocol. Should a refractory period exist, this control group was necessary in order to prove that maximal MAPK levels were attainable after the rat plantaris was held at *Lo* for 2 hours.

Muscle excision was performed within 10 seconds following the end of the stimulation protocol for the relevant groups. Immediately following excision, muscles were frozen in liquid nitrogen and stored at -80°C for subsequent biochemical analysis.

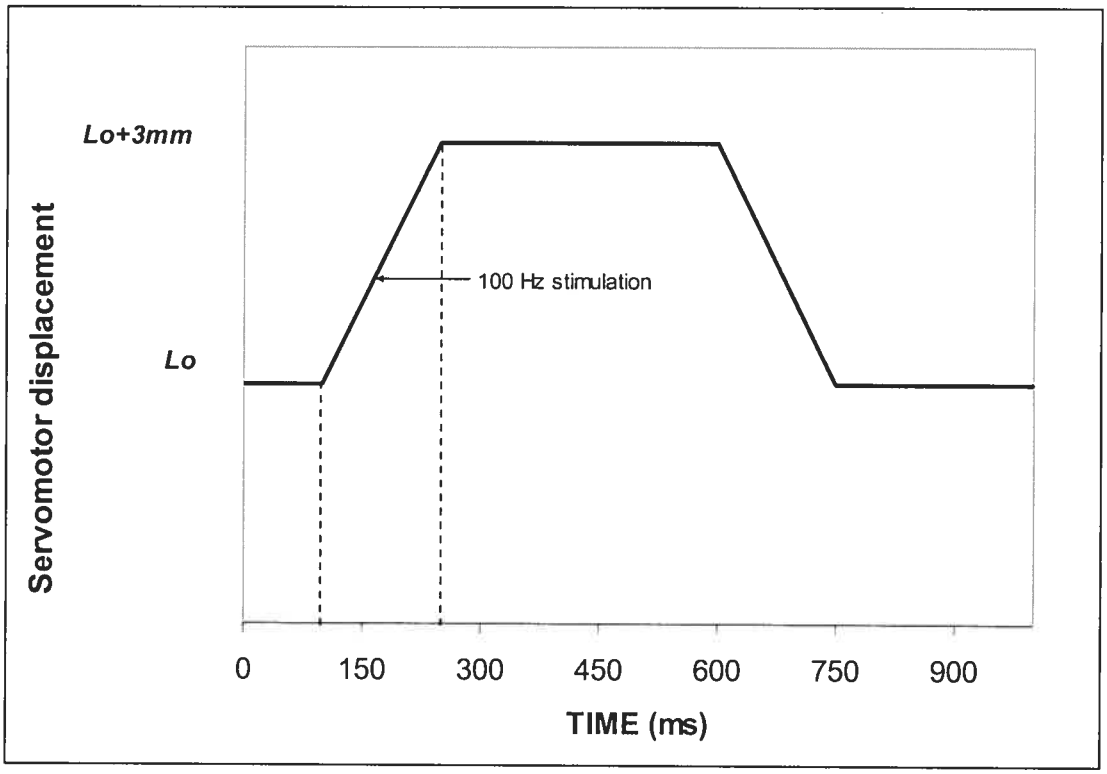

FIGURE 6. Graphical representation of the eccentric contraction protocol.

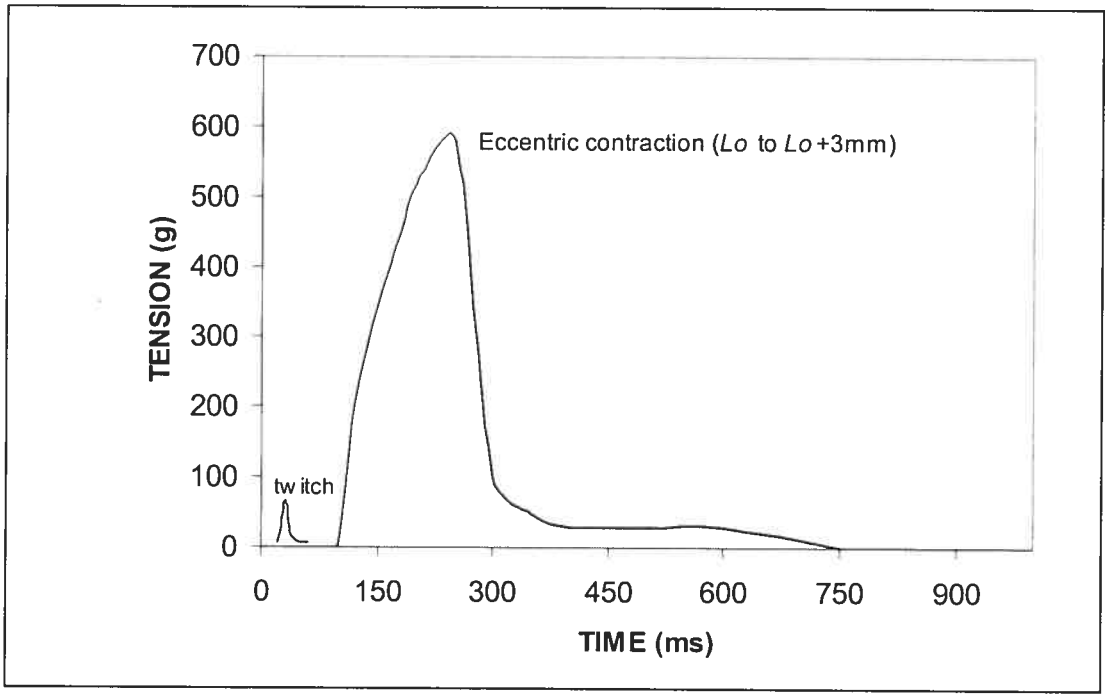

FIGURE 7. Typical peak eccentric contraction.

BIOCHEMICAL PROCEDURES

MAPK activation requires dual phosphorylation, which can be measured by phospho-specific antibodies using Western immunoblot techniques as described previously (Martineau and Gardiner, 2001).

Homogenization and sample preparation

Frozen whole plantaris muscles, after tendon removal, were quickly weighed followed by use of a nitrogen-chilled mortar and pestle to grind them into a fine powder and transferred to a set of chilled microcentrifuge tubes and kept in liquid nitrogen. Approximately 80 mg of the available powder from each sample was transferred to a separate set of chilled microcentrifuge tubes. This set was kept in liquid nitrogen until solubilized by the addition of 0.8 ml of ice-cold modified RIPA buffer (50mM HEPES, 150 mM NaCl, 5% glycerol, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 5mM EGTA, 2mM $MgCl_2$, pH 7.4) containing a cocktail of protease inhibitors (Mini-Protease, Boehringer; 2 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (100μM sodium orthovanadate, 10mM sodium fluoride, 1mM sodium pyrophosphate). Each homogeneous sample was immediately vortexed for 30 seconds and placed on ice for 1 hour during which samples were vortexed every 10 minutes. Then, to remove insoluble material, samples were centrifuged for 1 hour at 4500 *g*, and 4°C. The supernatants were decanted into a 3rd set of chilled microcentrifuge tubes and the pellets were discarded. Using triplicate measurements, the protein concentration of the supernatants was measured by Bradford protein assay (Bio-Rad) using known concentrations of bovine serum albumin as a standard. Samples of equal total protein concentration were prepared for SDS-PAGE by diluting an appropriate calculated amount

of the supernatant in reducing sample Laemmli buffer (60mM Tris, 10% glycerol, 5% ß-mercaptoethanol, 2% SDS, pH 6.8) followed by 1 minute of boiling to break disulfide bonds. Unused amounts of each aliquot were frozen and stored for repeats of the electrophoresis and Western blot procedure.

Electrophoresis and Western blot

The procedure was performed twice, once for the time course of activation, and another to test for the presence of a refractory period.

Each muscle sample, containing 180 μg of total protein in a 90 μl total volume, was resolved by loading onto a 16 cm long 9% polyacrylamide gel, separated at 10°C overnight, and simultaneously electrotransferred to a polyvinylidene difluoride membrane (Millipore). After a 15 second water rinse, all proteins on the membrane were visualized by staining with Ponceau S for 2 minutes, and followed by a second water rinse, in order to confirm equal sample loading and a successful transfer. The stain was removed by rinsing for 2 minutes with TBST. The membrane was blocked with Triton (TBST; 50mM Tris, 150 mM NaCl, pH 7.4, 0.5% Triton X-100) and 3% bovine serum albumin in Tris-buffered saline for 1.5 hours, rinsed with TBST, and then incubated overnight at 4°C in primary antibody. Using a concentration of 1:2000 in TBST plus 1% BSA and 0.5% NaN_3, monoclonal antibodies acting against p-JNK1, p-ERK1, p-ERK2, and p-38 (Santa Cruz Biotechnology) were used. Membranes were washed with TBST in a methanol tray, agitated lightly for 5 minutes, and the wash repeated. Next, for 1.5 hours, the membrane was incubated at room temperature in a secondary antibody solution (horseradish peroxidase-conjugated anti-mouse IgG) (Jackson Immunoresearch) using a

concentration of 1:10, 000 in TBST plus BSA. Visualization is accomplished by bathing membranes in an oxidizing, chemiluminescence substrate (ECL, Amersham) and exposed to blue-light sensitive film (ECL Film, Amersham) for 5-40 minutes. Using a flatbed scanner and NIH Image software, bands were quantified by densitometry.

Membranes were washed, stripped, reblocked, and reincubated to obtain phosphorylation values for the previously mentioned MAPK target substrates.

STATISTICS

ANOVA was used to test for between-group differences for the time course of activation and stimulation/restimulation data. Analyses were confirmed with Newman-Keuls post hoc tests and Dunnett post hoc tests for baseline comparisons (STATISTICA) (Appendix B). ANOVA was also used to test for between-group differences of all control variables (Appendix C).

Using Excel, curved and linear regression was performed to assess the relationship between various tension-related muscle performance variables and MAPK activation. Among the variables investigated included maximal peak tension and average peak tension (over first 60 contractions and entire 300 contractions), each unadjusted or normalized by maximal twitch or maximal tetanic tension, and/or muscle weight. STATISTICA was used to create 3-D surface plots for the variable judged to give the highest correlation values throughout the time course, especially taking into consideration the correlation values during maximal activation at 0 minutes. 3-D surface plots provided a visual representation of the relationship between variables including some noticeable peaks and valleys.

RESULTS

TIME COURSE OF MAPK ACTIVATION

p54-JNK (Fig. 8) – The activation profile did not demonstrate multiphasic induction which, to our knowledge, had never been previously shown for JNK in muscle. Peak activation (approximately 30-fold baseline levels) was reached immediately after the stimulation bout at 0 minutes and remained significantly higher than baseline throughout the time course, reaching a low at 120 minutes corresponding to values just below half-peak. The 0-minute group was significantly different from all groups. As previously demonstrated (Martineau and Gardiner, 2001), the JNK response amplitude due to mechanical loading is much higher than that of ERK1/2.

p44-ERK (Fig. 9) - The activation profile demonstrated a biphasic induction in agreement with previous studies for ERK in muscle (Carlson, 2001) (Nader and Esser, 2001) (Wu, 2000). Peak activation (approximately 3-fold baseline levels) was reached immediately after the stimulation bout at 0 minutes and quickly returned to and remained at near baseline levels until 180 minutes post-stimulation whereby it again became significantly higher than baseline. The 0-minute group was significantly different from all groups. The 180-minute group was significantly different from all groups except the 30-minute group, thus confirming a biphasic induction profile.

p42-ERK (Fig. 10) - The activation profile demonstrated a biphasic induction in agreement with previous studies for ERK in muscle (Carlson, 2001) (Nader and Esser, 2001) (Wu, 2000). Peak activation (approximately 2-fold baseline levels) was reached immediately after the stimulation bout at 0 minutes and quickly returned to and remained

at near baseline levels until 180 minutes post-stimulation whereby it again became significantly higher than baseline. The 0-minute group was significantly different from all groups except the180-minute group. The 180-minute group was significantly different from the 30-and 120-minute groups, thus confirming a biphasic induction profile.

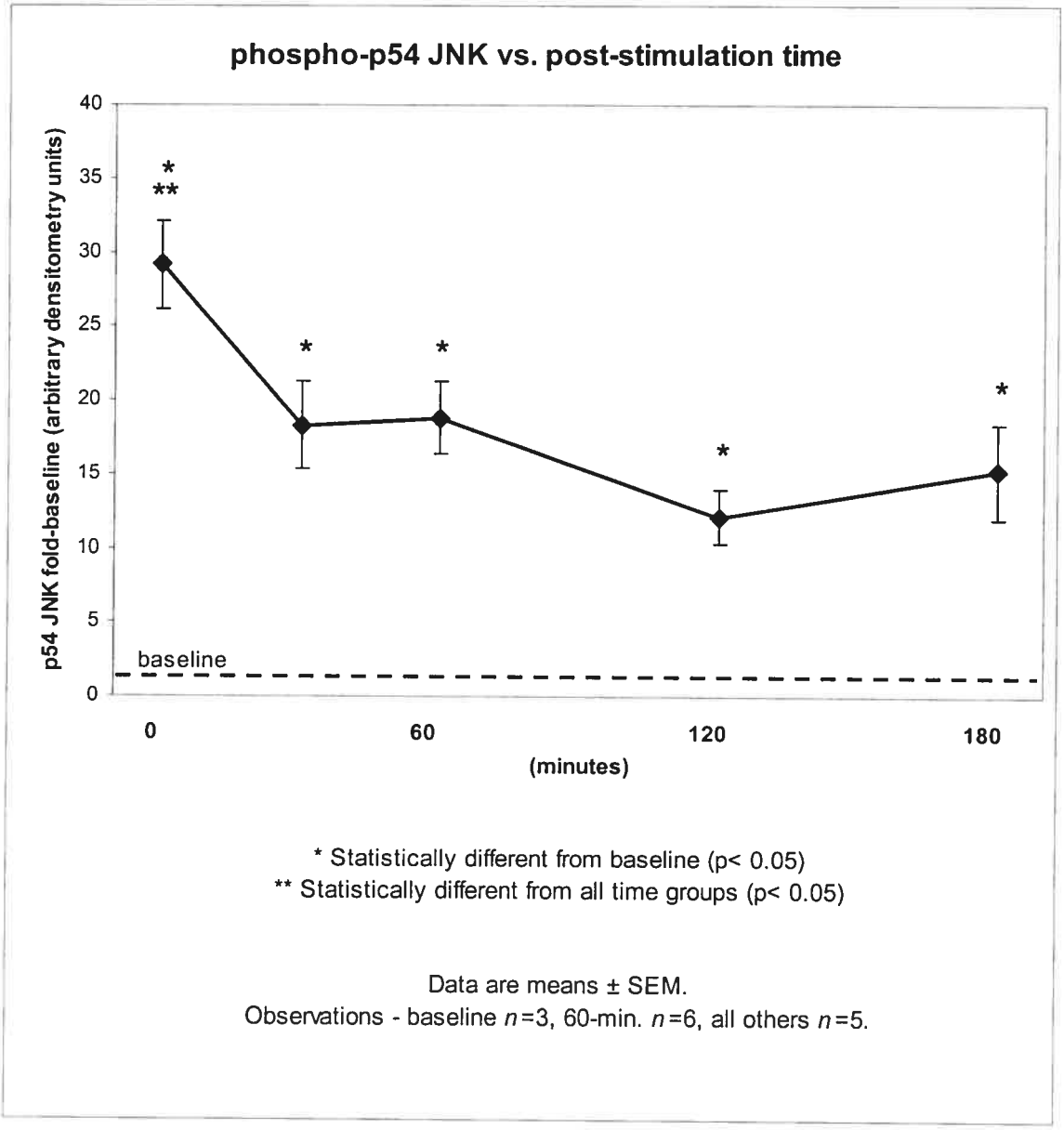

FIG. 8 – p-54 JNK vs. post-stimulation time.

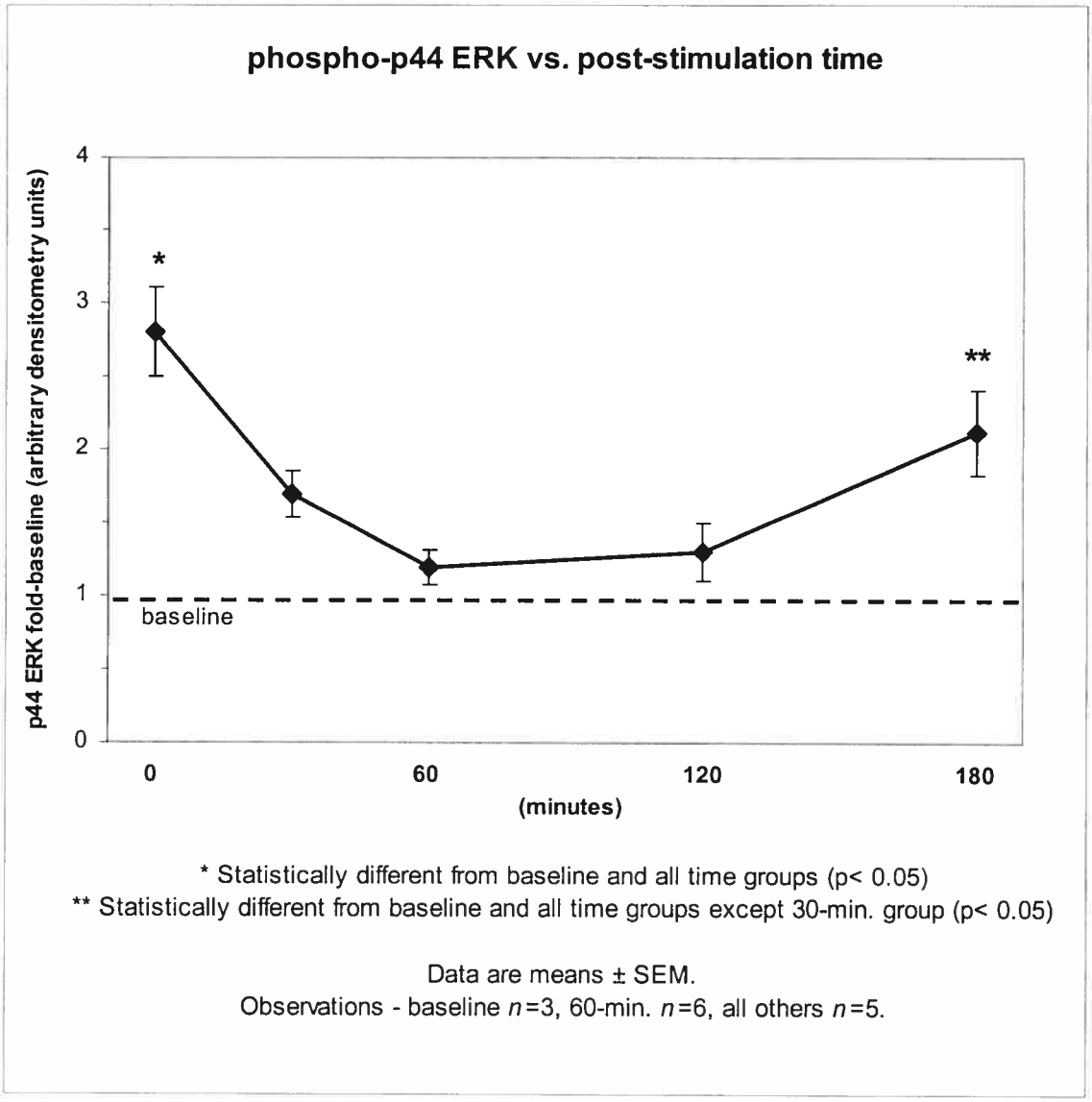

FIG. 9 – p-44 ERK vs. post-stimulation time.

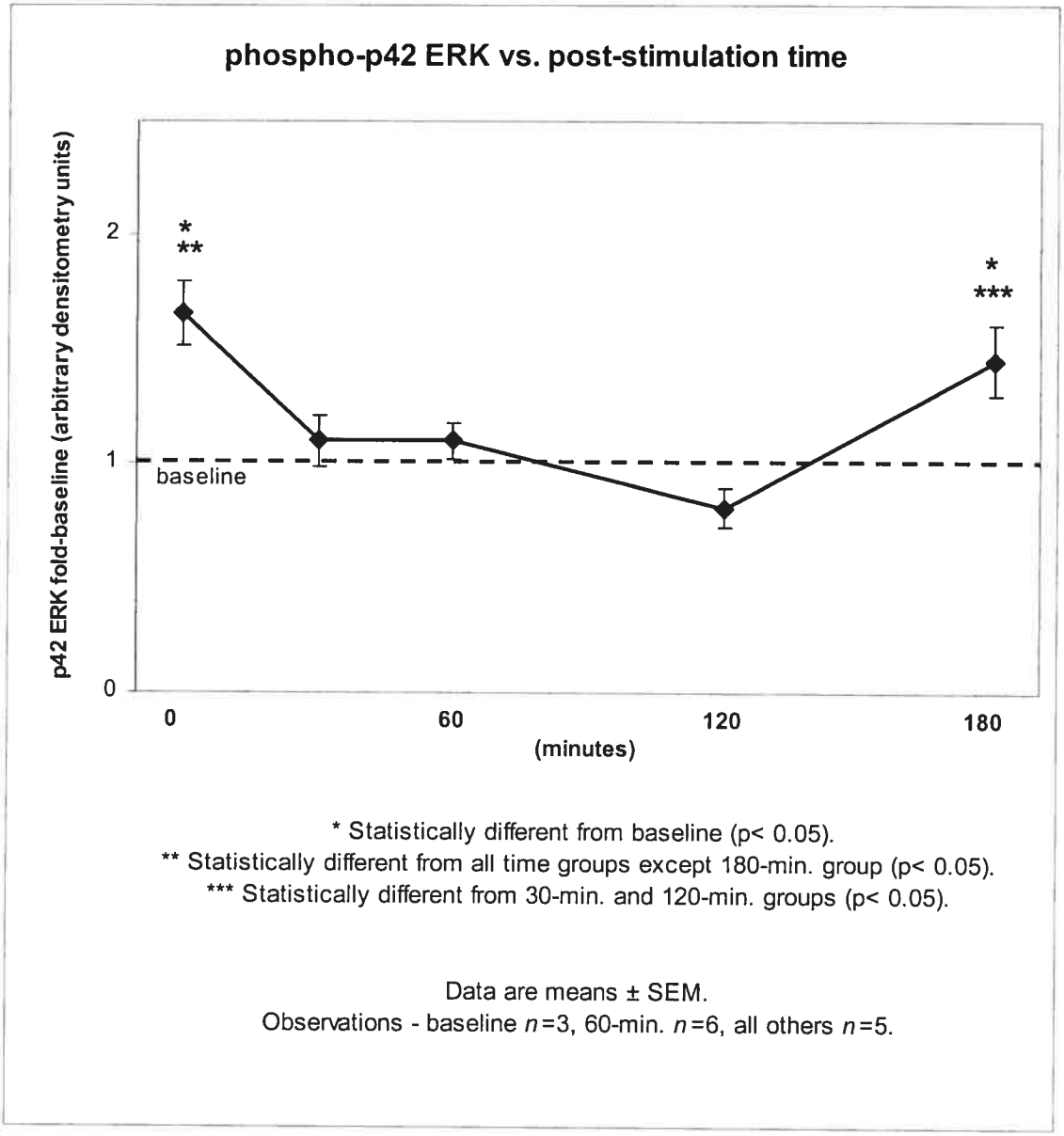

FIG. 10 – p-42 ERK vs. post-stimulation time.

ABSENCE OF A REFRACTORY PERIOD FOR p54-JNK (Fig. 11) – When considering the stimulation/restimulation data, only the 2-hour post stimulation group is statistically different from the others. All of the other groups produce similar maximal p54-JNK activation levels, including the restimulation group, thus contradicting our hypothesis of an existing refractory period. As expected, the control group (120-min. + stimulation) was one of the groups that produced similar maximal p54-JNK activation levels. This experimental design proved that JNK sensitivity to a maximal bout of eccentric contractions in rat plantaris muscle was unaltered regardless of the prior history, whether it was subject to a stimulation bout 2 hours earlier or held at *Lo* for 2 hours.

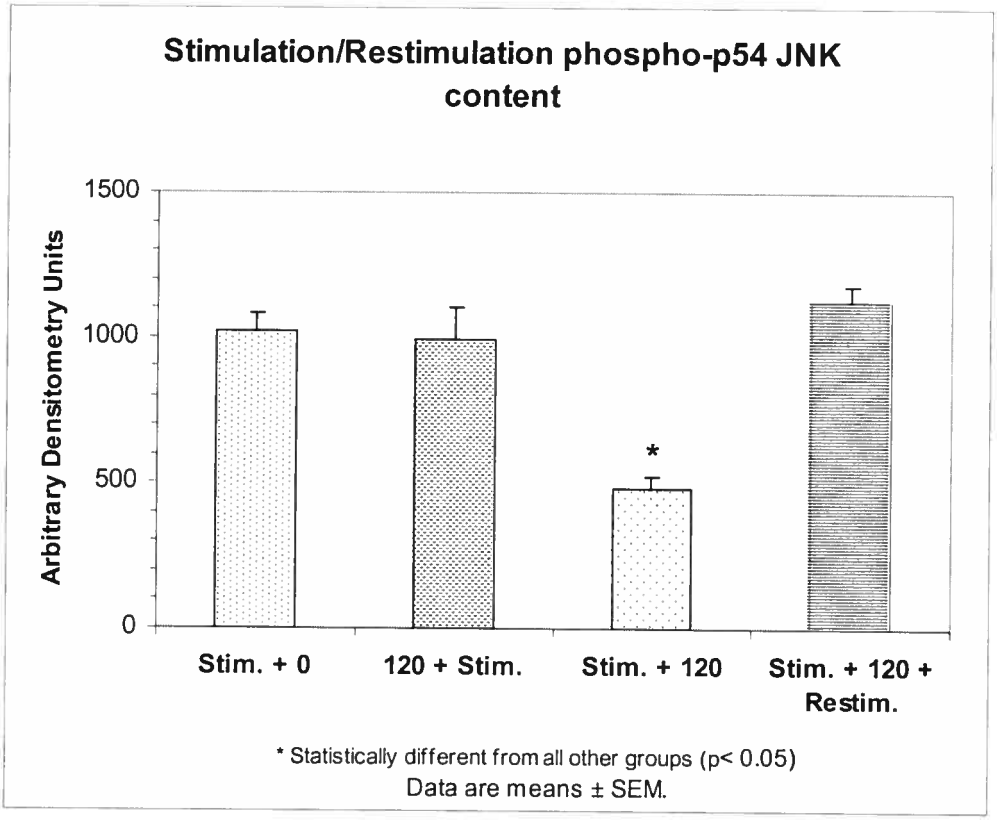

FIG. 11 – Test for refractory period.

CORRELATIONS

General Observations – Using linear regression correlation coefficients, there were certain trends that became apparent after studying the relationships between MAPK activation and possible contributing tension-related variables and/or intrinsic muscle parameters. They were as follows: 1) initial maximal MAPK activation was positively correlated with muscle work output, and 2) during the time course of activation study following the bout of stimulation, the relationship between muscle work output variables and MAPK activation demonstrated oscillatory behaviour as phosphorylated MAPK values presumably diminish gradually towards baseline values. To elaborate on the latter, many of the tension-related correlation values were much lower and/or negative at 30- and 120-minutes post-stimulation vs. 0- and 60-minutes post-stimulation. Another trend became apparent but can only be considered as a strong possibility due to the low sample size of the baseline group (n=3). When considering JNK and ERK2, baseline levels of MAPK activation were positively correlated with muscle weight.

In addition, it was not possible to conclude if the relationships are indeed linear or curvilinear since for the most part the differences in the correlation coefficients between the linear and curvilinear fits were minimal, thus rendering a more thorough statistical analysis to test for this determination unnecessary. However, it should be noted that for almost every linear regression comparison, at least one of the curvilinear values either matched or surpassed the linear value. Moreover, among the curvilinear functions (logarithmic, exponential, power), it was difficult to consider one of these functions as the distinguishable best curvilinear fit function by examining which one consistently provided the best correlation values throughout the data. Depending on the specific

tension-related variable examined, any one of the three curvilinear functions provided the best value. Whenever correlation values are quoted, the linear values will be used. Although there does not appear to be a significant difference, more points may distinguish which type of fit is indicative of the true relationship between the studied parameters.

p54-JNK (Table 1) (Figures 12,13) – JNK phosphorylation showed a near perfect positive relationship with muscle weight at baseline ($r^2 = 0.98$ for muscle weight) suggesting that muscle weight may have an effect on JNK activation values during the time course. Indeed, the relationship between JNK activation and muscle weight became moderately negative ($-0.38 <= r^2 <= -0.60$) through the first 60 minutes of the time course. This suggested an inverse relationship when pertaining to mechanotransduction sensitivity and this was proven by the overall higher correlation values when data was normalized to take muscle weight into account. In general, maximal p-54 JNK activation correlated much higher when surveying peak tensions over the entire 300-contraction protocol instead of limiting the investigation to the first 60 seconds. The highest correlation value for maximal p54-JNK activation was produced when plotting it against one of the 300-second variables (T-300/twitch/wt, $r^2 = 0.73$). A 3-D surface plot (p-54 JNK vs. T-300/twitch/wt. vs. time) visually demonstrated a weakening of the relationship over time and the presence of a valley at approximately 90-minutes post-stimulation.

p-44 ERK (Table 2) (Figures 14,15,16) – ERK1 demonstrated a negligible relationship with muscle weight at baseline and throughout most of the time course. Moreover, as expected because of this, peak tensions normalized by muscle weight generally showed lower correlation values with ERK1, especially maximal levels at 0-

minutes. Therefore, contrary to JNK and ERK2, maximal ERK1 activation correlated strongly with absolute tension and not relative tension.

Unlike JNK, ERK1 activation correlated higher when surveying peak tensions for the first 60 contractions instead of over the entire 300-second protocol, especially at 0 minutes. Peak tensions normalized by maximal twitch or tetanic tension did not, in general, produce higher correlation values. The highest value during maximal ERK1 activation was produced by plotting a regression utilizing unnormalized peak tensions over the first 60 contractions (Avg. Pk. T-60, $r^2 = 0.93$), and this parameter also was judged to provide the highest values throughout the time course. The global negative/negligible correlations at 30- and 120-minutes vs. the global positive correlations at 0- and 60-minutes post-stimulation suggested an alternating relationship when pertaining to mechanotransduction sensitivity. A 3-D surface plot (p-44 ERK vs. Avg. Pk. T-60 vs. time) visually demonstrates how the relationship quickly weakens at 30 minutes and essentially becomes the inverse at 120 minutes ($r^2 = -0.83$).

p-42 ERK (Table 3) (Figures 17,18) – ERK2 phosphorylation showed a moderate relationship with muscle weight at baseline ($r^2 = 0.64$ for muscle weight) suggesting that muscle weight may have an effect on ERK2 activation values during the time course. The fair to moderate relationship between ERK activation and muscle weight alternated between negative and positive correlations throughout the time course ($-0.31 <= r^2 <= 0.95$). This suggested an alternating relationship when pertaining to mechanotransduction sensitivity and this was proven by the global negative correlations at 30- and 120-minutes vs. the global positive correlations at 0- and 60-minutes post-stimulation. Similarly to ERK1, ERK2 activation generally correlated higher when surveying peak tensions for the

first 60 contractions instead of over the entire 300-second protocol, especially at 0 minutes. In addition, peak tensions normalized by maximal twitch or tetanic tension did not, in general, produce higher correlation values. For the parameter with the highest correlation value during maximal activation (T-60/wt., $r^2 = 0.86$) and judged to demonstrate the strongest relationship throughout the time course, the values followed form to the abovementioned alternating relationship in mechanotransduction sensitivity. A 3-D surface plot (p-42 ERK vs.T-60/wt. vs. time) visually demonstrated how the positive relationship remains strong at 60 minutes ($r^2 = 0.76$) until a fair reversal in mechanosensitivity occurs at 120 minutes ($r^2 = -0.42$).

Table 1. p54-JNK time course correlation values (R^2)

Assessment of linear regression between p-JNK and tension-related variables.

PARAMETER	BASELINE	0 min.-MAX	30 min.	60 min.	120 min.	180 min.
	linear/curved	linear/curved	linear/curved	linear/curved	linear/curved	linear/curved
Max.PeakTension	-	neg.	(-.16)/l (-.16)	neg.	neg.	neg.
T-60	-	neg.	neg.	neg.	(.84)/l (.87)	(.38)/e (.38)
T-60/twitch	-	(.27)/p (.38)	(-.23)/l (-.23)	(.11)/l (.11)	(.88)/p (.90)	(.15)/l (.15)
T-60/tetanic	-	neg.	(-.78)/l (-.77)	neg.	(.89)/l (.93)	neg.
T-300	-	(.36)/e (.41)	(-.12)/l (-.11)	neg.	(.46)/p (.61)	(.26)/p (.30)
T-300/twitch	-	(.55)/e (.60)	(-.17)/l (-.19)	neg.	(.60)/p (.76)	(.21)/l (.21)
T-300/tetanic	-	(.32)/e (.37)	(-.37)/l (-.39)	(-.21)/l (-.21)	(.55)/p (.69)	neg.
Muscle wt.	(.98)/e (.98)	(-.45)/p (-.51)	(-.38)/e (-.45)	(-.60)/l (-.60)	neg.	neg.
T-60/wt.	-	(.28)/p (.41)	(.34)/p (.41)	(.23)/e (.24)	(.38)/p (.56)	(.59)/l (.59)
T-60/twitch/wt.	-	(.53)/e (.62)	neg.	(.37)/l (.37)	(.34)/p (.48)	neg.
T-60/tetanic/wt.	-	(.27)/p (.37)	neg.	(.36)/l (.36)	(.44)/p (.62)	(.16)/e (.19)
T-300/wt.	-	(.60)/e (.66)	neg.	neg.	(.19)/p (.37)	(.52)/l (.52)
T-300/twitch/wt.	-	***(.73)/e (.79)***	***neg.***	***(.15)/e (.16)***	***(.23)/p (.41)***	***(.35)/l (.35)***
T-300/tetanic/wt.	-	(.58)/p (.65)	neg.	neg.	(.21)/p (.40)	(.19)/l (.19)

In bold italics (T-300/twitch/wt.) – group of correlation values judged to be highest.

neg. = negligible (R^2<0.10)
curvilinear regression (l = logarithmic, p = power, e = exponential)

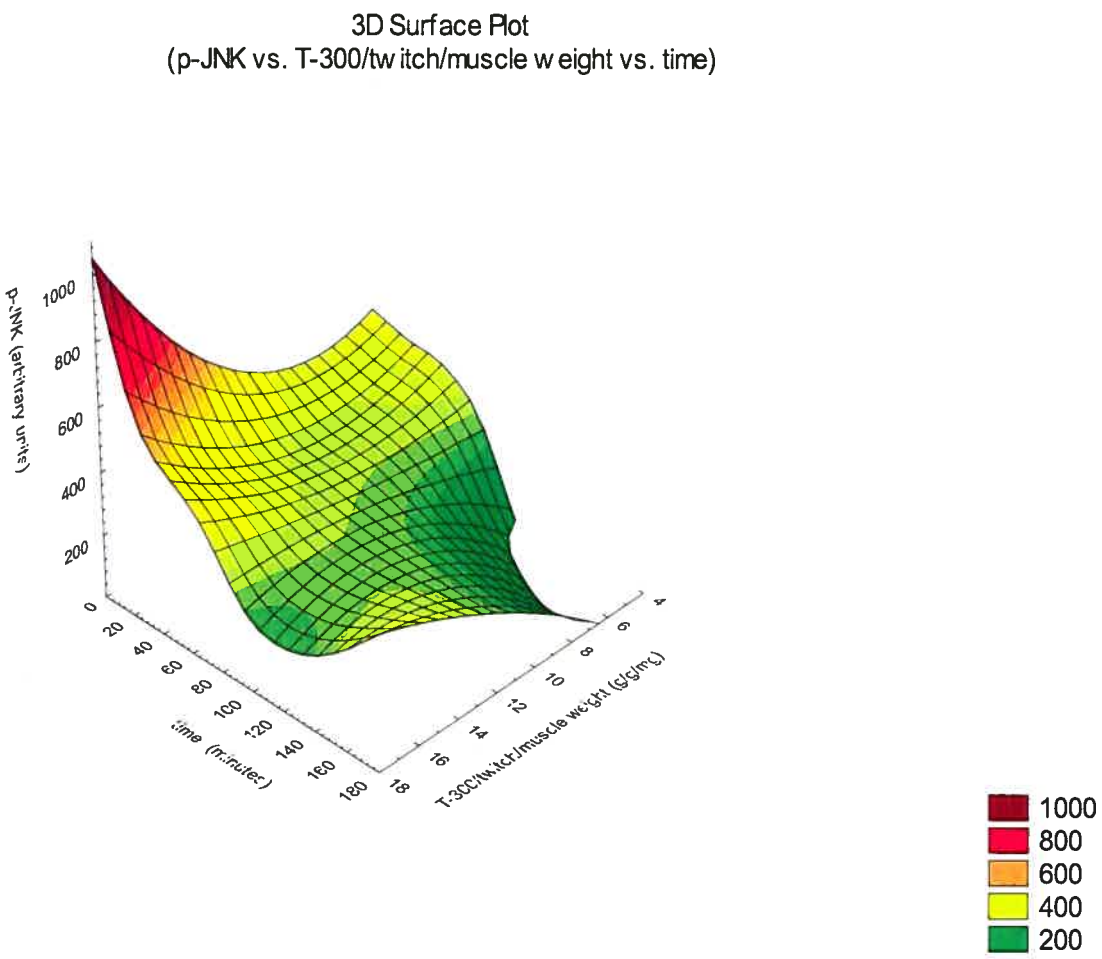

FIG. 12 – p54 JNK 3-D Surface Plot.

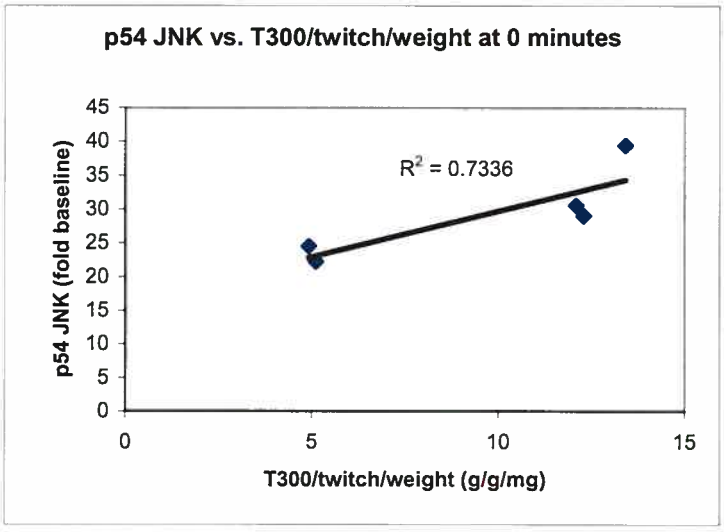

FIG. 13 – p54 JNK Regression Analysis at 0 minutes.

Table 2. p-ERK1 time course correlation values (R^2)

Assessment of linear regression between p-ERK1 and tension-related variables.

PARAMETER	BASELINE	0 min.-MAX	30 min.	60 min.	120 min.	180 min.
	linear/curved	linear/curved	linear/curved	linear/curved	linear/curved	linear/curved
Max.PeakTension	-	(.23)/l (.22)	(-.17)/l (-.17)	neg.	neg.	(-.33)/l (-.37)
T-60	-	*(.93)/l (.93)*	*neg.*	*(.38)/l (.39)*	*(-.83)/e (-.86)*	*(-.13)/l (-.14)*
T-300	-	(.67)/e (.74)	(-.21)/l (-.20)	(.13)/l (.10)	(-.87)/e (-.85)	neg.
T-60/twitch	-	(.83)/e (.87)	neg.	(.40)/p (.49)	(-.42)/e (-.44)	(-.12)/l (-.15)
T-60/tetanic	-	(.63)/e (.66)	(-.15)/l (-.15)	(.15)/e (.15)	(.75)/e (-.78)	(-.17)/l (-.19)
T-300/twitch	-	(.48)/e (.56)	neg.	(.25)/l (.25)	(-.55)/e (.60)	(-.12)/l (-.14)
T-300/tetanic	-	(.45)/e (.53)	(-.17)/l (-.18)	neg.	(-.80)/e (-.81)	neg.
Muscle wt.	neg.	neg.	neg.	(-.52)/e (-.55)	neg.	(-.29)/l (-.29)
T-60/wt.	-	(.77)/e (.77)	neg.	(.71)/p (.79)	(-.27)/e (-.33)	neg.
T-60/twitch/wt.	-	(.42)/l (.45)	neg.	(.57)/p (.71)	(-.11)/e (-.13)	neg.
T-60/tetanic/wt.	-	(.44)/l (.47)	(-.16)/l (-.17)	(.58)/p (.66)	(-.28)/e (-.35)	(-.12)/l (-.14)
T-300/wt.	-	(.61)/e (.68)	(-.25)/l (-.27)	(.52)/l (.55)	(-.30)/e (-.39)	neg.
T-300/twitch/wt.	-	(.41)/p (.52)	neg.	(.53)/l (.64)	(-.15)/e (-.21)	neg.
T-300/tetanic/wt.	-	(.42)/p (.53)	(-.16)/l (-.19)	(.52)/l (.54)	(-.27)/e (-.36)	neg.

In italics (Avg. Pk. T-60) – group of correlation values judged to be highest.

neg. = negligible (R^2<0.10)
curvilinear regression (l = logarithmic, p= power, e = exponential)

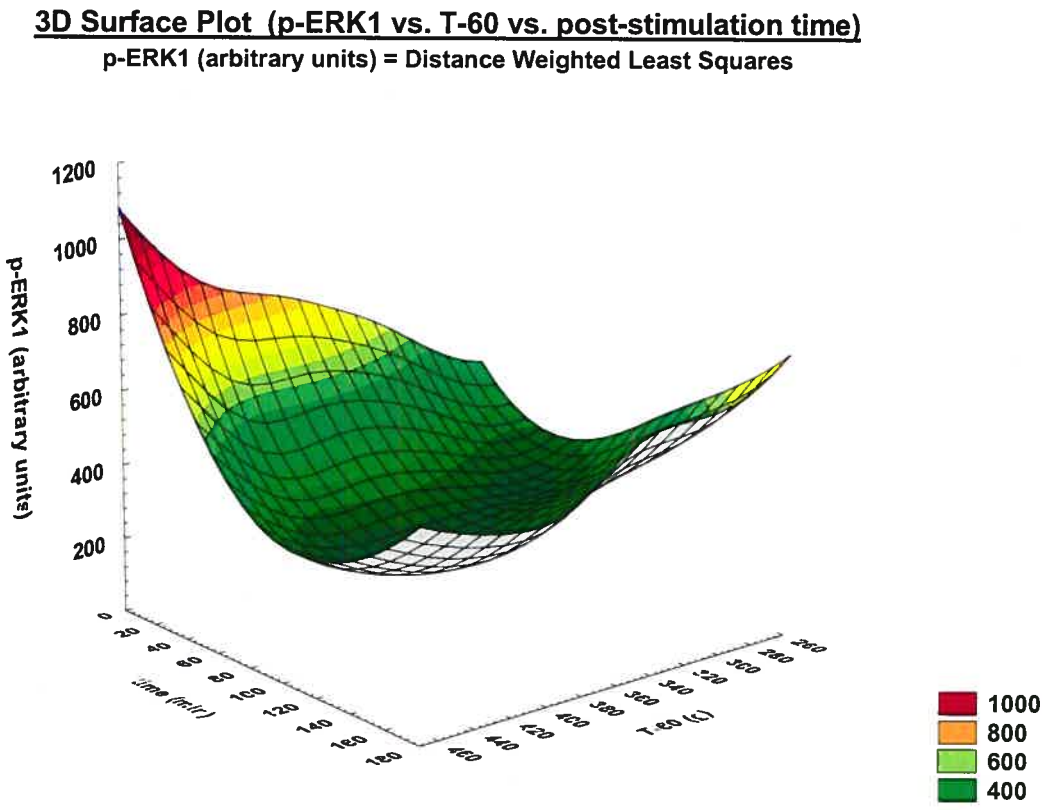

FIG. 14. – ERK1 3-D Surface Plot.

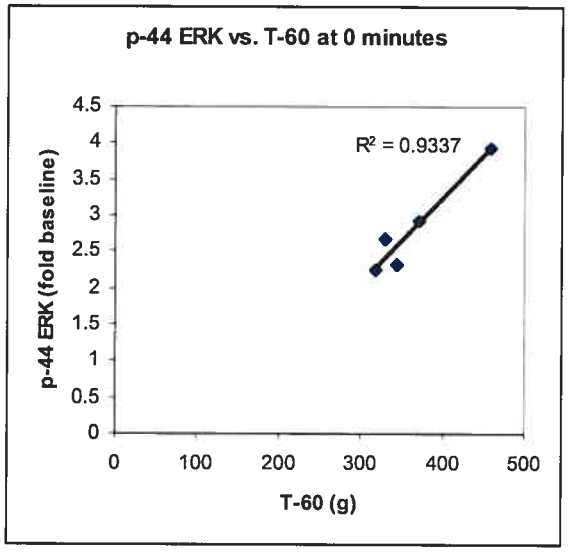

FIG. 15. – ERK1 Regression Analysis at 0 minutes.

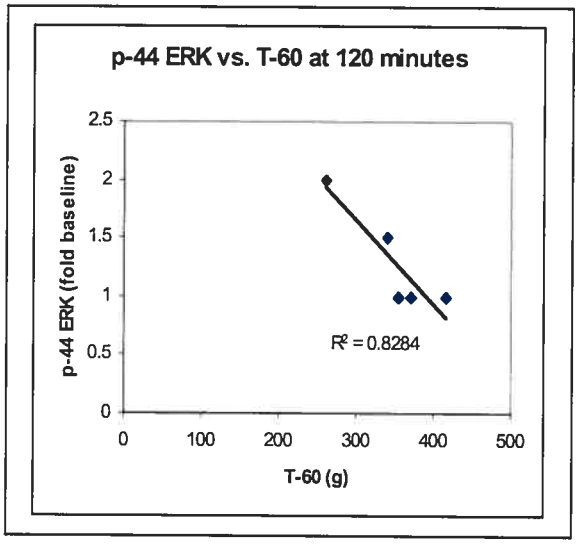

FIG. 16.- ERK1 Regression Analysis at 120 minutes.

Table 3. p-ERK2 time course correlation values (R^2)

Assessment of linear regression between p-ERK2 and tension-related variables.

PARAMETER	BASELINE	0 min.-MAX	30 min.	60 min.	120 min.	180 min.
	linear/curved	linear/curved	linear/curved	linear/curved	linear/curved	linear/curved
Max.PeakTension	-	(.25)/l (.24)	(.55)/l (.59)	neg.	(.57)/l (.57)	(-.19)/l (-.23)
T-60	-	(.51)/l (.51)	(.69)/l (.71)	(.60)/l (.60)	neg.	neg.
T-60/twitch	-	(.71)/e (.72)	(.67)/l (.68)	(.73)/p (.76)	neg.	neg.
T-60/tetanic	-	(.17)/e (.16)	neg.	(.26)/l (.26)	neg.	neg.
T-300	-	(.48)/e (.48)	(.55)/l (.55)	(.33)/l (.33)	neg.	neg.
T-300/twitch	-	(.44)/e (.45)	(.24)/l (.23)	(.60)/l (.60)	(-.18)/e (-.24)	neg.
T-300/tetanic	-	(.26)/e (.27)	neg.	neg.	neg.	neg.
Muscle wt.	(.64)/e (.60)	(-.33)/e (-.39)	(.35)/p (.37)	(-.31)/e (-.36)	(.94)/l (.93)	(-.26)/l (-.26)
T-60/wt.	-	***(.86)/e (.87)***	***(.13)/l (.13)***	***(.76)/p (.80)***	***(-.42)/e (-.48)***	***neg.***
T-60/twitch/wt.	-	(.68)/e (.74)	neg.	(.75)/p (.80)	(-.54)/e (-.61)	neg.
T-60/tetanic/wt.	-	(.47)/e (.52)	(-.19)/e (-.20)	(.47)/p (.53)	(-.41)/e (-.48)	neg.
T-300/wt.	-	(.62)/e (.64)	neg.	(.69)/l (.69)	(-.47)/e (-.52)	neg.
T-300/twitch/wt.	-	(.52)/e (.56)	neg.	(.80)/p (.86)	(-.63)/e (-.69)	neg.
T-300/tetanic/wt.	-	(.41)/p (.47)	(-.11)/p (-.13)	(.57)/p (.59)	(-.54)/e (-.60)	neg.

In bold italics (T-60/wt.) – group of correlation values judged to be highest.

neg. = negligible (R^2<0.10)
curvilinear regression (l = logarithmic, p = power, e = exponential)

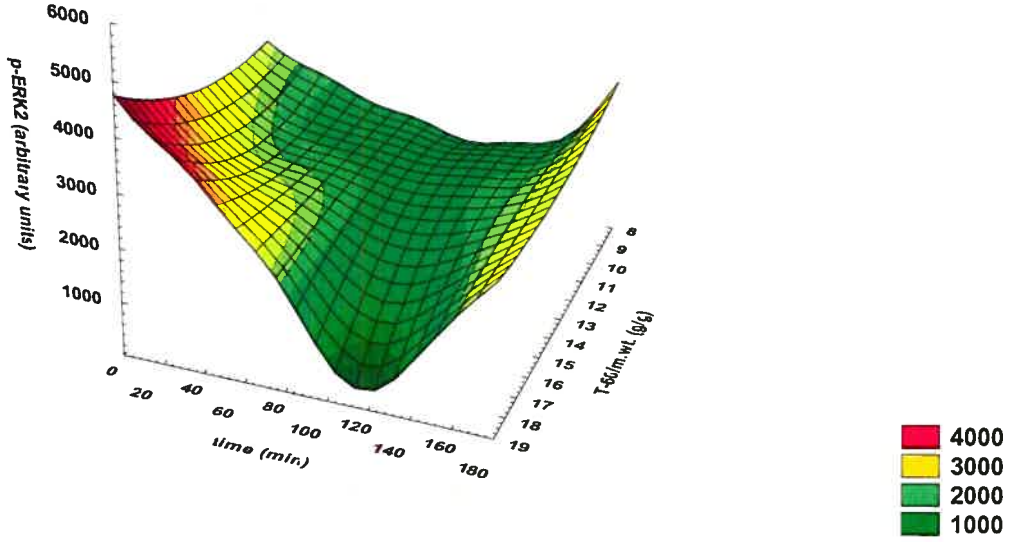

FIG.17. - ERK2 3-D Surface Plot.

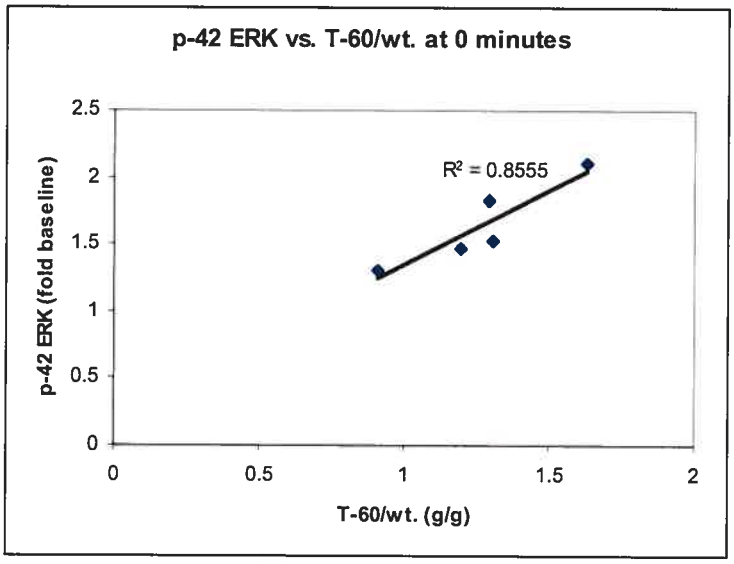

FIG. 18. – ERK2 Regression Analysis at 0 minutes.

DISCUSSION

A time course of MAPK activation in response to an acute bout of eccentric contractions was performed in order to help characterize the profile and the possible role of MAPK signaling during mechanically induced skeletal muscle hypertrophy. Cellular outcomes are usually heavily influenced by MAPK signaling and can generally be divided into either on/off cellular decisions or graded responses. Cellular growth involves on/off decisions of proliferation or differentiation, regulated by cell cycle control checkpoints that require either acute/phasic or chronic/sustained MAPK activation depending on the stimulus and cell type (Tombes, 1998) (Weber, 1997) (Wu, 2000). Thus, the time course of MAPK activation is likely an upstream regulator of protein synthesis and cell growth in mechanically loaded skeletal muscle. Cellular processes associated with early hypertrophy events such as protein nuclear translocation, qualitative or quantitative changes in transcription, ribosome recruitment, formation of cyclin-cdk complexes, and satellite cell processes (Carson, 1997) (Chakravarthy, 2000) (Chicurel, 1998) (Coolican, 1997) (Goldspink, 1999) (Haddad and Adams, 2002) (Whitmarsh and Davis, 2000) may be influenced by MAPK signaling.

A 3-hour time course was performed including measurements of individual phosphorylation values for p54-JNK, p44-ERK, and p42-ERK. As expected, there is a dose-dependant relationship between muscle tension and MAPK activation. Several studies have shown a magnitude dependant relationship between mechanical stress and MAPK or IE gene activation (Boppart, 2001) (Dawes, 1996) (Martineau and Gardiner, 2001) (Hamada, 1998) (Jo, 1997). However, the results of this study suggest that there are detailed differences for each MAPK with regards to the strength and duration of the

response, biphasic behaviour, and with muscle-related physiological parameters that can predict baseline and activation quantitative values. The major finding of this study is the lack of an existing refractory period for JNK activation 2 hours after an initial stimulation bout, thus allowing for speculation on the role of the JNK response and its downstream effects.

TIME COURSE OF MAPK ACTIVATION

This study has produced the observation that the activation profile for JNK is different from that of ERK1 and ERK 2. Each MAPK member investigated in this study differed in their temporal profile and/or their maximum response amplitude. It is very common to have different response amplitudes and/or different time course patterns between JNK and ERK1/2 in mechanically stressed skeletal muscle (Aronson, 1997) (Carlson, 2001) (Martineau and Gardiner, 2001) (Widegren, 2001), smooth muscle (Hamada, 1998), or endothelial cells (Jo, 1997). This strongly suggests individual roles and thus, different modes of regulation for the parallel MAPK signal pathways in the hypertrophy process.

The MAPK time courses demonstrate evidence of either variable, sustained activation or biphasic on/off states. MAPK signaling, whether it is acute/phasic or chronic/sustained, can influence on/off cellular processes that involve checkpoints (Tombes, 1998) (Weber, 1997) (Wu, 2000). Satellite cell recruitment involves several on/off processes and their activation in skeletal muscle is considered to be a vital aspect of the hypertrophy process in skeletal muscle (Adams, 1999) (Allen, 1999) (Rosenblatt, 1994) (Vierck, 2000). MAPK pathways and IGF-1 together can promote some of the

distinct phases of proliferation, differentiation, fusion and hypertrophy in skeletal muscle or skeletal myoblasts (Coolican, 1997) (Adi, 2002) (Sarbassov, 1997) (Weyman, 1997) (Wu, 2000) (Haddad and Adams, 2004). Although experiments using rat skeletal muscle loading have shown so far that the onset of IGF/MGF will likely occur after our 3-hour time course investigation (Haddad and Adams, 2002) (Adams, 1999) (Adams 2002), MAPKs and IGF/MGF may nevertheless interact sometime post-stimulation to increase satellite cell cycle progression and replicative capacity by overcoming cell cycle check points (Chakravarthy, 2000) (Adams, 2002).

Also, indirect temporal evidence from a survey of studies utilizing mechanical stress implicates MAPK activation with affecting downstream signals that promote protein synthesis. Our three-hour time course of MAPK activation appears to precede the onset of increases in translational protein synthesis (Baar, 1999) (Hernandez, 2000) (Nader, 2001) and the expression of growth-related genes (Chen, 2002). Nevertheless, these effects may be mediated via temporally related AP-1 activation (Jo, 1997) and/or temporally related induction of IE genes *c-fos* and *c-jun* (Aronson, 1997) (Hamada, 1998).

p-54 JNK – As previously reported, it is very likely that JNK signaling is associated with mechanically induced hypertrophy (Martineau and Gardiner, 2001) (Aronson, 1997) (Boppart, 1999) (Boppart, 2001) (Hamada, 1998) (Jo, 1997). One bout of eccentric contractions produced immediate and massive p54-JNK activation (approximately 30-fold baseline levels), peak levels which confirm the much higher response amplitude to mechanical stress of JNK versus ERK1/2 (Martineau and Gardiner,

2001) (Hamada, 1998) (Boppart, 2001). Other studies provided unclear results concerning the maximum response levels of JNK versus ERK1/2 (Aronson, 1997) (Carlson, 2001) (Jo, 1997). Nevertheless, as will be discussed later, this study reinforces the previously discovered dose-dependant relationship between mechanical stress and JNK activation (Martineau and Gardiner, 2001) (Boppart, 2001) (Hamada, 1998), and hence, the probable involvement of JNK with the hypertrophy response.

There was quick negative regulation, assumed to be due to MKPs, producing significantly lower levels of p54-JNK within 30 minutes post-stimulation. However, the initial rapid trend towards baseline levels did not continue and would require a time period longer than 3 hours since p54-JNK levels remained significantly above baseline for the entire time period investigated. Activation levels did not drop lower than those at its estimated half-life time of 2 hours (approximately 13-fold baseline) and the minor oscillations throughout the time course were not significant. Thus, based on all of these results, the JNK time course of activation can be considered as a prolonged 'on' response but with varying fold-baseline levels (intermediate levels) of activation. It would be of interest to investigate the relationship or correlation, if any, between the JNK time course of activation and MKPs activation patterns.

Several other studies that utilized different protocols also demonstrated a more prolonged JNK activation response in comparison to the ERK1/2 profile (Aronson, 1997) (Hamada, 1998) (Jo, 1997) while the results of others appear contradictory (Carlson, 2001). Sustained JNK activation may be important for augmenting transcriptional or translational protein synthesis since its time course precedes the reported onset time of these processes. For example, using p70 levels and polysome profiles as indicators, the

first evidence of an increase in translational protein synthesis in rat skeletal muscle occured 3-6 hours post-stimulation (Baar, 1999) (Hernandez, 2000) (Nader, 2001). Also in rat muscle, substantially more growth-related genes were upregulated 6 hours versus 1 hour after an acute bout of resistance training via transcription and translation (Chen, 2002). Prolonged JNK signaling may be a prerequisite for these adaptations by permitting enough time to potentially influence downstream regulators that have been temporally related in past studies. More specifically, JNK activation preceded one of the AP-1 activation phases (Jo, 1997) and *c-jun* activation was also sustained for long time periods (Angel and Karin, 1991) (Aronson, 1997) (Dawes, 1996) (Hamada, 1998). Moreover, JNK and *c-jun* activation were both dose-related to stretch (Dawes, 1996) (Hamada, 1998).

Up to now, the myogenic program is considered to be JNK-independent (Wu, 2000). Therefore, studies investigating the myogenic program in myoblasts or developed skeletal muscle have focused on ERK1/2 rather than JNK. Since sustained MAPK activation in certain protocols helped bypasss cell cycle checkpoints (Weber, 1997) (Wu, 2000) and satellite cell activity was required for hypertrophy (Rosenblatt, 1994), it is possible that the prolonged JNK response plays a role during the initial and extended post-stimulation phase of satellite cell proliferation. Also, JNK activation was sustained for a minimum of 6 hours before myoblasts commenced differentiation (Gredinger, 1998). Prolonged JNK activation may act cooperatively with transient ERK signaling to promote satellite cell differentiation after the proliferation phase.

JNK has been implicated as a strong candidate for interacting with calcineurin to help mediate its cardiac hypertrophic and/or phenotype switching effects (De Windt,

2000). Calcineurin had strong interactions with a group of transcription factors linked with genes responsible for skeletal muscle hypertrophy and phenotype switching (Delling, 2000) (Dunn, 2000) (Dunn, 2001). The prolonged activation profile of JNK in our study does not appear to bring forth evidence against the abovementioned proposed interaction of JNK with calcineurin. Traditionally, it has been thought that an extended duration of physical activity concomitant with sustained elevated calcium concentrations is required for calcineurin activation. This concept has been refined by the findings that any relative increase in physical activity, including abbreviated high frequency stimulation or overload, can respectively result in immediate or sustained calcineurin activation and promote slow-fiber type conversion, especially in the fast plantaris muscle (Dunn, 2001). From this, assuming a positive correlation between calcineurin activation and JNK activation (De Windt, 2000), and assuming that our protocol was similar enough to those which have resulted in a prolonged increase of calcineurin activation (Dunn, 2001), one cannot argue against the possible interconnection between JNK with calcineurin. Although this is only indirect temporal evidence, contradictory findings have not been brought forward in this study. Future studies may support or provide evidence against the possible interactions of JNK with the calcineurin pathway during skeletal muscle hypertrophy and phenotype switching.

ERK1/2 (p-44 ERK/p-42 ERK) – The time course of activation for both p42-ERK and p44-ERK exhibited a very similar temporal pattern and relatively small response amplitude when compared to JNK. The ERK1/2 time profile exhibited an on/off form of activation, which is significantly different from the prolonged 'on'

response of JNK. As previously mentioned and reported in several studies (Martineau and Gardiner, 2001) (Hamada, 1998) (Boppart, 2001), the maximal response amplitude of p42-ERK (approximately 2-fold baseline) and p44-ERK (approximately 3-fold baseline) was considerably lower than that of JNK (approximately 30-fold baseline). Regardless, as will be discussed later, ERK1/2 did respond in a dose-dependant manner with regards to tension. Dissimilarities in the type of activation and response amplitude suggest a difference of roles and regulation for these parallel pathways during the hypertrophy signaling process.

The higher ERK1/2 baseline values as compared to JNK support the idea that their kinetics or mechanism of activation differ (Martineau and Gardiner, 2001). This idea is further reinforced in this study since there is an inverse relationship between baseline and maximal activation values. For example, p42-ERK has both the highest baseline value and the lowest response amplitude. Although beyond the scope of this study, the narrow response range observed in our study may be partly explained by speculating on two interpretations. Firstly, as will be explored later, the ERK family may be part of a pathway responsible for sensing and relaying signals caused by force transmission in a different direction than that of JNK. The second explanation involves enzyme kinetics, more specifically, thresholds of activation and enzyme saturation. It has been reported that due to higher sensitivity, at lower tension levels ERK but not JNK is activated and, as tension levels increase, JNK becomes much more responsive (Martineau and Gardiner, 2001). Perhaps as ERK activation approaches saturation, JNK necessarily becomes more sensitive to increasing levels of tension. This was also the case for the activation of the p46-JNK isoform during concomitant higher tension and saturating p54-

JNK levels (Martineau and Gardiner, 2001). It is not uncommon to find dose-dependant changes in sensitivity and activation thresholds as this would reflect the dynamics of an existing physiological nonlinear system involving the interactions of MAPKs and MKPs (Franklin and Kraft, 1997) (Chen, 2001) (Slack, 2001). The two explanations may be linked by evidence of the effects of mechanical tension on kinase kinetics (Chicurel, 1998).

A major and unique finding in this study is the biphasic induction of both ERK1 and ERK2 in rat plantaris muscle 3 hours after exposure to an abbreviated mechanical stimulation bout. Other studies that discovered an ERK biphasic induction profile differed in the experimental protocol and findings including use of a myogenic cell line (Wu, 2000), or exposure to endurance training (Nader and Esser, 2001), or use of continuous mechanical overload to discover a biphasic induction of ERK2 in rat soleus muscle only (Carlson, 2001). One possible function of this biphasic on/off form of signaling is that it may be coupled to distinct satellite cell phases.

The myogenic and anabolic program in myoblasts or satellite cells consists of on/off stages including proliferation, differentiation, fusion and myotube hypertrophy. ERK activation was necessary for some of these processes and ultimately for skeletal muscle hypertrophy (Haddad and Adams, 2004). It appears that biphasic or transient ERK activation was a common and required feature in hypertrophy-related studies in order to promote and regulate these phases (Weyman, 1997) (Wu, 2000) (Adi, 2002). Moreover, the dual roles and biphasic activation of ERK1/2 was coupled to that of IGF-1 and coincided with or preceded many downstream processes and signals (Haddad and Adams, 2002) (Adi, 2002). The opposing myogenic and mitogenic phases had precursor

signaling markers with transient activation states that alternated inversely to each other (Rosenthal and Cheng, 1995), therefore, ERK is a likely upstream regulator of temporally separate myogenesis and mitogenesis (Wu, 200), and these effects may be mediated by *c-fos* (Coolican, 1997) (Gredinger, 1998). ERK and *c-fos* both exhibit a transient form of activation (Dawes, 1996) (Angel and Karin, 1991) (Aronson, 1997) (Izumo, 1988) and *c-fos* activation has been related to work overload in heart muscle (Izumo, 1988). Also, indirect temporal evidence suggests that other downstream targets may be affected by ERK activity. For example, ERK activation preceded one of the spikes in AP-1 activation (Jo, 1997) and several transcription factors responded in a biphasic manner after static stretch (Rauch, 2005).

ABSENCE of a REFRACTORY PERIOD

MAPK signaling in all-or-none physiological outcomes have been studied previously. The main characteristic for this type of response system is strong bistability (on/off) of MAPK activation and relevant cascade of downstream protein activities, whereby positive feedback loops help provide features of irreversibility that may include ultrasensitivty or hysterisis (Bagowski and Ferrell, 2001) (Xiong and Ferrell, 2003). This feature allows for only stimuli of a certain level of strength, transient or not, to achieve to completion an all or none cellular response (Bagowski and Ferrell, 2001) (Xiong and Ferrell, 2003). In such a response system, enough time would be provided for the necessary protein interactions that may involve MAPKs, mitogens, cyclins, cdks, and transcription factors to achieve a subsequent on/off response, for example, satellite cell cycle progressions.

On the other hand, as further confirmed in this study, mechanical stress causes MAPK signaling or downstream IE gene activation to respond in a graded manner (Boppart, 2001) (Dawes, 1996) (Martineau and Gardiner, 2001) (Hamada, 1998) (Jo, 1997). Such a response system must have characteristics different from the before mentioned digital response system (Hazzalin and Mahadevan, 2002) including the likely predominance of negative regulation from MKPs over positive feedback. Negative feedback regulation can affect MAPK signaling by desensitizing the cellular response to a stimulus (Brecht, 1999) (Ferguson, 2001) (Fucini, 1999) (Hazzalin, 1998) (Meskiene, 1998) (Pierce, 2001) (Polakiewicz, 1998) and MKPs are implicated with inducing a refractory period (Meskiene, 1998). There is evidence that continuous overload or stretch causes MAPK desensitization (Carlson, 2001) (Franklin, 1997) (Jo, 1997) or IE gene desensitization (Dawes, 1996). When considering intermittent stimuli and IE gene induction, the presence of a refractory period is questionable and may depend on the time of stretch reapplication (Dawes, 1996). In some instances, refractory periods may help ensure specificity of cellular responses. If a stimulus is homologous to the original, a refractory period ensures that the signaling system is reset in order to permit full sensitivity to the repeated stimulus. If it is heterologous, a refractory period would prevent from interference with the original stimulus until completion of the signaling response.

Thus, one of the goals of this study was to be the first to test for the presence of a JNK refractory period in skeletal muscle and begin the process of modeling the standard MAPK response due to repeat bouts of mechanical stimulation. A repeat of the protocol produced maximal p54-JNK activation in the restimulation group, which was not

significantly different from the 0-minute group. The lack of evidence for desensitization implies that the ability of skeletal muscle to sense and relay signals following a 2nd bout of mechanical stimulation remains intact. Therefore, the absence of a refractory period near its half-life time or, more specifically, the reproduction of statistically similar maximal p54-JNK activation, allows us to conclude that, amidst negative regulation, the magnitude-dependant relationship between tension and p54-JNK activation appears unaltered.

The net effect of a 2nd round of maximal JNK activation on downstream events can only be speculated on. The most trivial assumption is that of a greater adaptation. There is evidence that acute changes in signaling may result in long-term gains in muscle mass (Baar and Esser, 1999). Intracellular relocation of important components (Chicurel, 1998) and changes in transcriptional or posttranscriptional processes (Carson, 1997) (Goldspink, 1999) are general source areas of adaptation and may once again contribute to a further increase in mRNA and protein production (Carson, 1997) (Goldspink, 1999), and a larger influx of newly synthesized myosin protein into the filaments (Russell, 2000). It is possible that a larger time requirement would be necessary to achieve this greater adaptation since there appears to be a lag time between MAPK signaling and increases in protein synthesis (Baar, 1999) (Hernandez, 2000) (Nader, 2001) (Chen, 2002). If not, one can argue that there is no net effect, possibly because of a bottleneck effect. The existence of a point of limitation anywhere downstream such as limited availability of downstream substrates, the inability to further increase rates of transcription or translation, or unnecessary increases in myofibrillar protein synthesis, could classify the 2nd wave of signaling as being redundant.

Nevertheless, there is support for the trivial assumption of a net increase in protein synthesis. It has been proposed that an amplitude-dependant MAPK signaling response is translated to frequency modulation by directly affecting transcription rates (Hazzalin and Mahadevan, 2002). In addition, a correlation between acute changes in translational control signaling and long-term gains in muscle mass has already been demonstrated (Baar and Esser, 1999). Using the abovementioned information as a model and extrapolating it to graded MAPK signaling in mechanically loaded skeletal muscle, a higher burst in JNK activation from a fully sensitive 2^{nd} bout would result in higher amounts of protein synthesis. Since higher levels of generated tension cause higher levels of mechanical deformation and structural microtrauma (Lieber, 1994) (Boppart, 1999), and hence a higher requirement for myofibrillar hypertrophy, the model would satisfy the theoretical need to have matching levels of MAPK signaling and protein synthesis in order to produce proper muscular adaptation. Thus, the 2^{nd} wave of maximal JNK signaling produced by the experimental restimulation group has the potential to cause further increases in myofibrillar protein synthesis.

Another aspect of interest is the possible connection between tension, force transmission paths, and spatially organized MAPK signaling. Evidence of scaffolds and organized localization patterns of MAPK signaling has been reviewed (Garrington and Johnson, 1999) (Pearson, 2001) (Samaj, 2004). More specifically, MAPKs and scaffolds may interact with contractile or cytoskeletal proteins that likely participate in the tension generating and sensing process (Chicurel, 1998) (Samaj, 2004). Due to the similar maximal JNK activation values in both the 0-minute group and the restimulation group, using electron microscopy and immunodetection techniques, it is possible to confirm or

refute that both groups exhibit a similar activation-mapping pattern. All of the above can imply that MAPK activation occurs in a spatially structured arrangement.

CORRELATIONS

The importance of the MAPK pathways, including JNK, was proven when constitutive expression of MKP-1 attenuated the hypertrophy response in cardiomyocytes (Bueno, 2000). Without contribution from systemic factors (Ryder, 2000) (Wretman, 2000) (Boppart, 2001) (Carlson, 2001), tension was required and sufficient to promote hypertrophy (Goldspink, 1999) and helped mediate the effects of calcineurin (Dunn, 2000). As expected, average maximal tension, either over the first 60 seconds or the entire 300-second protocol, is strongly correlated with MAPK activation.

Similar to the analysis previously conducted in our lab (Martineau and Gardiner, 2001), linear regression correlation coefficients were used to measure the relationship between initial maximum MAPK activation and various muscle tension-related variables. Although some of the variables utilized were similar to the abovementioned study, new variables were used to explore other intrinsic parameters that may possibly affect the sensitivity of MAPK activation. Namely, variables correcting for intrinsic parameters such as muscle mass and maximal tetanic tension were added to the list of variables correcting for peak twitch tension only. Therefore, to summarize, we utilized several absolute tension variables and relative tension-related variables.

The use of numerous relative tension-related variables provided a thorough attempt to define relative mechanical stress. We believe that it is best defined as muscle workload per unit of muscle mass. Due to the nature of skeletal muscle, maximal

stimulation can cause high levels of tension to be simultaneously generated and sensed by muscle itself (Chicurel, 1998) (MacKenna, 1998). The ability of muscle to transmit force laterally in multiple directions and at various sites throughout its volume (Street, 1983) (Monti, 1999) (Patel and Lieber, 1997) (Paul, 2002) requires us to consider mechanical stress in terms of three dimensions. Since mammilian muscle density has been used as a constant when performing calculations (Patel and Lieber, 1997), according to the formula d=m/v, mass and volume are linearly proportionally to each other. Therefore, muscle mass can be used as a quantitative equivalent of volume, which represents an estimate of the three-dimensional capacity of muscle to dissipate mechanical stress. Hence, as a measure of relative mechanical stress, tension was divided by muscle mass for many of the variables.

Correcting for intrinsic physiological properties such as peak twitch tension or maximal tetanic tension in addition to correcting for muscle mass may help provide a more accurate definition of relative mechanical stress. Regardless, all of the variables can be classified as muscle work output variables since the amount of tension generated by the plantaris muscle is represented in some form within every variable (except for the variable used to correlate MAPK activation with muscle mass alone). In general, initial maximal MAPK activation was positively correlated with average maximal tension or rather, average muscle work output. Other studies have also shown a correlation between tension and MAPK activation (Boppart, 2001) (Martineau and Gardiner, 2001) (Hamada, 1998) (Jo, 1997). Our study provides further support for the important signaling role of MAPKs during mechanotransduction and the muscle hypertrophy adaptive process.

It was our goal that one of the variables, absolute or relative, would be distinguishable by consistently exhibiting the highest correlation value throughout the study. This was not the case and supports the notion that MAPK activation is specific and may be dependant on specific force transmission pathways. Our results suggest that depending on the MAPK, absolute or relative tension can represent the more accurate predictor of mechanical stress. In other words, longitudinal and lateral force transmission each contributes to purposeful tension converging at the tendon yet they may have different mechanisms (Street, 1983) (Monti, 1999) (Patel and Lieber, 1997) (Paul, 2002), requiring different inhibitors (MacKenna, 1998), which may be proven in the future to affect MAPK activation. Different sites of force transmission vary qualitatively and quantitatively in their protein content including the type of integrins (Paul, 2002). In fact, during mechanical loading, MAPK activation was influenced by the type of upstream matrix substrate and integrin binding (MacKenna, 1998). Co-localization of MAPKs with certain structural proteins (Samaj, 2004) further supports the idea that MAPK activation may be related to the spatial organization of muscle and may be dependant upon specific force transmission pathways.

CORRELATIONS and BASELINE MAPK VALUES – Although the low sample number used for baseline data (n=3) makes it difficult to accept findings as being significant, some interesting trends appear to have been discovered. The ERK family had much higher baseline activation values as compared to the very low value for JNK. This demonstrates that ERK1/2, especially ERK2, is activated during resting tension. As previously reported, ERK1/2 are more sensitive at lower tension levels (Martineau and

Gardiner, 2001). This may highlight the capacity or physiological range of certain force transmission pathways associated with ERK activation. One can speculate that since certain force transmission pathways are utilized during resting tension (Street, 1983), ERK1/2 activation may be associated with these predominately non-contractile elements. On the other hand, JNK activation may be more dependant upon force transmission pathways that are affected by contractility.

Also, ERK2 and JNK showed moderate and high correlations respectively with muscle mass during resting tension. Thus, this was taken into consideration when determining relative mechanical stress during stimulation. Beyond the scope of this study, another possible significance for the apparent trend between baseline JNK/ERK2 activation and muscle mass is the possible involvement of MAPKs with baseline protein synthesis.

CORRELATIONS and MAPK ACTIVATION VALUES – Lateral and longitudinal force transmission pathways contribute to useful net force production at the tendon. There is evidence to suggest that the lateral pathways can contribute a majority of the percentage of muscular force production (Street, 1983). Force dissipated in three dimensions throughout the volume of muscle would support the notion of a larger capacity to transmit tension versus simple linear transmission (Street, 1983) (Monti, 1999) (Patel and Lieber, 1997) (Paul, 2002). Instead, force that is produced by myofibrillar contractility can be dissipated in multiple directions per unit volume of a muscle lattice network.

As expected, since their baseline values correlated highly with muscle mass, JNK and ERK2 both correlated strongly and positively with a relative mechanical stress variable. In other words, the activation values correlated highly with variables that divide some representation of mechanical tension by the measured mass of the muscle. The fact that they correlate well to the linear inverse of muscle mass suggests that JNK and ERK2 may be associated with measuring stress in lateral transmission pathways. However, their mechanism may be different and their involvement may not be limited only to lateral transmission. Firstly, the same MAPK can be implicated in both axial and transverse forms of force transmission in skeletal muscle yet each form has a different signaling mechanism (Kumar, 2002). Indeed, different structural elements and proteins are involved with various possible pathways (Street, 1983) (Monti, 1999) (Patel and Lieber, 1997) (Paul, 2002) and MAPKs can localize with specific cytoskeletal proteins (Samaj, 2004). In another example, during mechanical stretch in cardiac fibroblasts, ERK2 and JNK were activated by a different variety of upstream matrix substrates and integrins (MacKenna, 1998). Furthermore, the low JNK activation baseline values at resting tension and the greater sensitivity of JNK at higher tensions suggest that it is strongly implicated with contractile elements. Finally, since mechanical tension can affect the chemical kinetics of various molecules and kinases within a cell (Chicurel, 1998), JNK or its upstream activators may be more responsive to this effect.

Contrarily, ERK1 is different from both JNK and ERK2 in that it correlates the highest with absolute tension and not relative mechanical stress. Since the volume effect of force dissipation does not appear to be a necessary correction factor, this suggests that ERK1 may be predominately associated with linear force transmission, possibly with the

MTJ site and its relevant molecules. The MTJ is a site involved only with linear force transmission, receiving tension produced in series from the sarcomeres, and expresses a specialized group of integrins and proteins (Paul, 2002). A hypothetical model is proposed in Diagram 1.

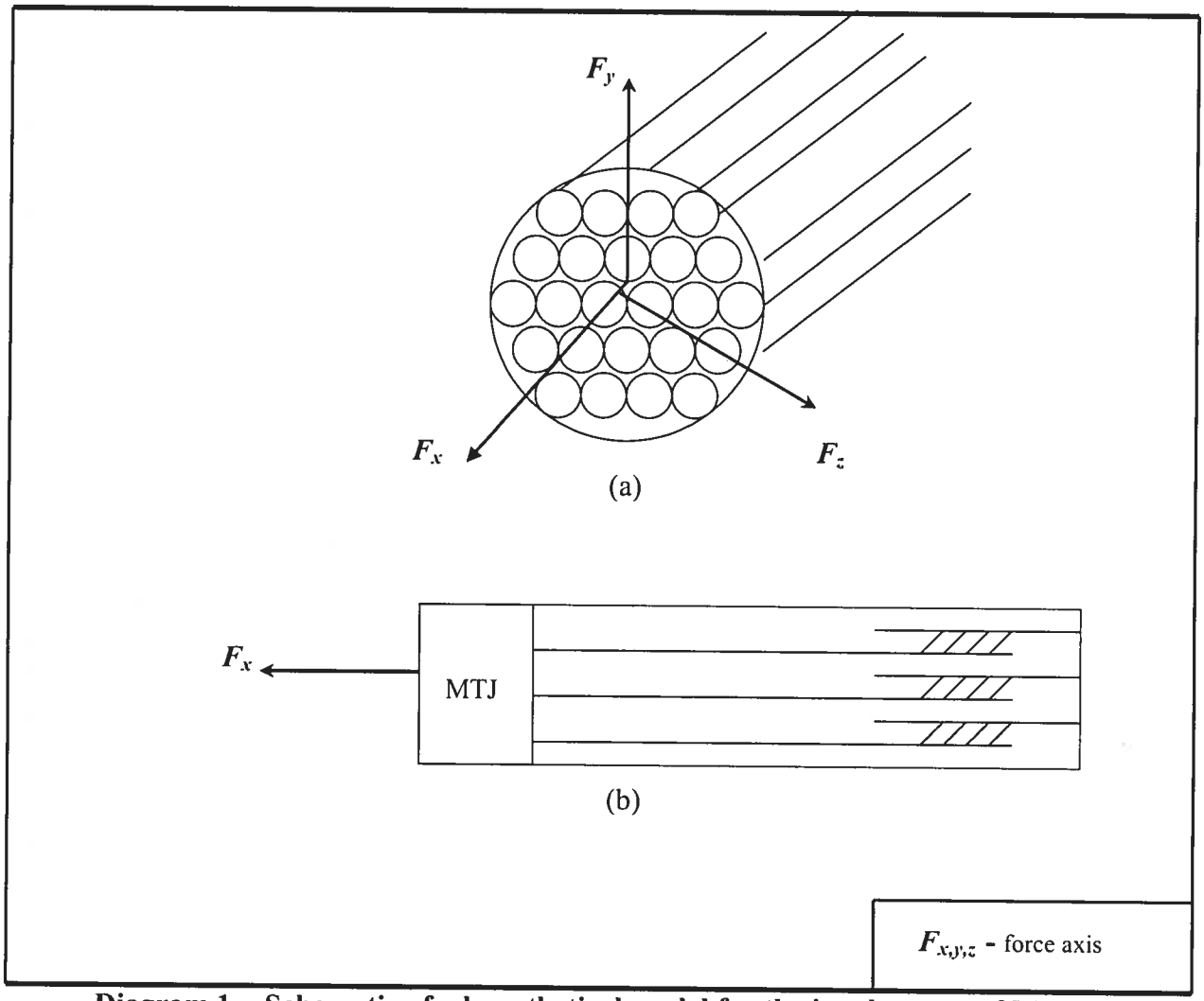

Diagram 1. - Schematic of a hypothetical model for the involvement of MAPK signaling with axis of force dissipation.

As discussed, this hypothetical model helps demonstrate a connection between what is suggested by the correlation values and information found in the literature.

(a) JNK and ERK2 may be implicated with indicating levels of mechanical stress in lateral transmission pathways. This would involve multi-directional force transmission per unit volume.

(b) ERK1 may be involved only with linear force transmission between sarcomeres and the MTJ.

PATTERN of CORRELATION VALUES and MAPK TIME COURSE - The experimental design allowed for this type of analysis to be extended throughout the time course of activation in order to permit comparisons of all the post-stimulation groups with the maximal activation group. Thus, we determined if variables with an initially high correlation value during maximal MAPK activation immediately post-stimulation would maintain this relationship at all the time points. Our results have indicated the presence of oscillatory or flip-flop behaviour between tension-related variables and MAPK activation during the time course of activation. In other words, in general, positive correlations at 0- and 60-minutes post-stimulation alternated with negligible or negative correlations at 30- and 120-minutes post-stimulation.

The only speculative explanation is that positive correlations coincide with upswings in MAPK activation, negative correlations coincide with negative regulation, and negligible relationships reflect transitional periods of regulation. MKPs are responsible for rapid negative regulation of MAPKs, usually within minutes (Camps, 2000) and together with MAPKs form an oscillatory signaling system (Kholodenko, 2000). Thus, it may be discovered that MKPs oscillate in a pattern that mirrors that of the MAPKs, albeit with a slight delay or lag period. Although MKP measurements were not made, one can speculate that MKP activation values may correlate positively with MAPK activation and muscle workload.

For example, the following is a scenario that would satisfactorily explain much of the results. A high workload stimulation bout results in high, immediate maximal MAPK activation and thus a strong positive correlation at 0-minutes. This causes a strong

counteraction of MKPs quickly producing high negative regulation (MAPK deactivation) thus canceling off the positive correlation and moreover, especially in the case of ERK1/2, producing negligible or negative correlations. In other words, assuming an oscillatory wave response, a higher workload would produce larger positive and negative amplitudes (i.e. higher peaks during/after activation phases and lower valleys during/after deactivation phases). The cycle can then repeat itself with varying amplitudes as it progresses through the time course of activation until eventually smaller oscillations predominate and values return to baseline levels.

FUTURE EXPERIMENTS/LIMITATIONS OF STUDY

All three major parts of this study possess specific areas that can be explored in future experiments. Some of these have already been mentioned. The findings in this study have a major limitation in that all the results are derived from the plantaris muscle in young female Sprague-Dawley rats.

CORRELATIONS – There are several follow-up experiments, mainly by simply increasing the sample size and thus its statistical power, which can clarify the possible existence of certain quantitative relationships discovered in this study. Firstly, from our small sample size (n=3), the limited data suggests that baseline activation values for JNK and ERK2 is positively correlated with muscle weight. This has important implications for understanding and identifying the regulatory mechanism responsible for baseline myofibrillar protein synthesis or the mechanical signaling state at resting 'taut' tension. Increasing the sample size to one that has strong statistical power would help confirm this relationship, especially since this directly affects the need to utilize a weight-adjusted approach for certain MAPKs with regards to activation sensitivity due to mechanical loading. It would be interesting to determine if resistance training would have an impact on some aspect related to the baseline signaling state, for example, baseline MAPK activation and/or resting tension values.

Also, as mentioned previously, we were unable to determine if the relationship between MAPK activation and different tension-related variables in response to maximal mechanical loading exhibits a linear or curvilinear relationship. Regardless of the variable, the linear and curvilinear correlation values were very similar and would require

a substantial increase in sample size in order to distinguish with confidence, if there exists a difference at all between the two classes. Differences greater than those found in this study would require the use of an advanced statistical analysis in order to confirm or refute its significance.

However, in our lab, a previous study utilizing a much wider range of tension values already determined convincingly that the JNK activation response is a curvilinear power function (Martineau and Gardiner, 2001). It is very possible that the narrow range of tension values used in this study did not allow us to differentiate and that data spread out over a wider range is necessary to distinguish between linear and curvilinear response functions. The previous study investigated JNK activation over a wide range of tension values from passive to maximal eccentric. Our narrow range focused only on maximal eccentric contractions, which corresponds to the steeper, higher end of the p54-JNK/tension curve produced in that study.

One can explore the effects of resistance training on mechanotransduction. MAPK activation values, muscle mass, rates of fatigue, and all tension values (resting, twitch, tetanic, average peak activation) may be affected by physiological training adaptations. This may alter correlation values and mechanosensitivity in general.

TIME COURSE/IMPLICATED PATHWAYS - There is a set of experiments that can be conducted in the future which focus on data related to the time course following a mechanical stimulation bout.

Firstly, the MAPK activation profile can be complemented with a MKP time course. MKPs are known to act as negative regulators of MAPKs and often show coincidental onset time of activation/deactivation depending on the experimental protocol

(Meskiene, 1998) (Reffas and Schlegel, 2000). Assuming the temporal profiles of a given MAPK and a potential negative regulator, for example JNK and MKP-1, showed coincidental activation/deactivation, such indirect temporal evidence would help confirm the importance of MKPs during negative MAPK regulation in skeletal muscle mechanotransduction. This would also help clarify the role of MKPs during the biphasic ERK1/2 response. Further confirmation would require, for example, the use of a MKP inhibitor to help prove the requirement of MKPs for MAPK regulation.

Secondly, a time course that features downstream substrates or upstream signals may yield insightful information should there be coincidental or preceding time of onset of activation. Downstream substrates or signaling molecules may be responsible for cellular processes involved in increasing protein synthesis such as translation, transcription, and cell-cycle control. Likely candidates would include p-70, elF-4E, AP-1, cyclins, cdk, *c-fos* and *c-jun* mRNA/protein. All of these have been strongly implicated with the response of skeletal muscle to mechanical loading. Potential upstream signals such as *Ras* and *Rho* proteins can also be investigated. Furthermore, a temporal connection can be made between the biphasic ERK1/2 response and upstream signals that may produce this characteristic or downstream markers that may help mediate its effects. The time course of activation of calcineurin and IGF/MGF may help to reveal the relationship, if any, of these important molecules with the MAPK pathways. It is currently unclear if they act upstream, downstream, or parallel to MAPK pathways during mechanotransduction.

Therefore, any kinetic information involving any of the abovementioned signals or markers would help piece together a model for the sequence of early events during adaptive hypertrophy. The implication that mechanically induced MAPK activation acts

as an upstream regulator of a given substrate or molecule can be partially confirmed by inhibiting the specific MAPK and observing differences in these potential downstream activities. Similarly one can inhibit a potential upstream signal and determine its effect, if any, on MAPK activation. Additionally, any relationship, if any, between tension and downstream, upstream or parallel signal activation would also be determined. One can also investigate the effects of using a different protocol that produces lower tension, say isometric contractions, on downstream events such as *c-fos* and *c-jun*.

It may be necessary to extend the time course beyond three hours in order to obtain some useful information relating to adaptive cellular processes. The timeline of many early events has yet to be fully determined and, moreover, past experiments involving some of the abovementioned substrates such as IGF and p70 have required time courses exceeding three hours (Haddad and Adams, 2002) (Adams, 1999) (Adams 2002) (Baar, 1999) (Hernandez, 2000) (Nader, 2001). Time course studies that exceed three hours may also provide the added benefit of revealing prolonged 'on' signaling or other significant MAPK oscillations that were not present within the three-hour time frame. This hypothetical discovery would then necessitate an expanded viewpoint of the role of MAPKs during intermediate or long-term events.

Another important aspect that can be taken into consideration for future experiments is the use of different time points since there is evidence in the literature that each MAPK possesses different time periods during oscillatory signaling (Kholodenko, 2000). This would help eliminate the risk of missing a peak or valley during oscillatory signaling may represent the difference between having comparisons of certain time groups reach statistical significance or not. It is unknown if MAPK time periods are

influenced by the protocol, more specifically, the amount of workload performed or tension produced by skeletal muscle.

Also, an interesting and thoroughly informative future experiment would be one that is able to precisely control muscle tension (or workload) and thus, allow for the production of isotension curves throughout the time course. These isocurves would connect several group samples that have performed identical mechanical workloads. From this we could compare the effect of different workloads on the time course of activation profile. This would help confirm some of the trends or potential relationships discovered in our study, namely the alternating positive and negative correlations between workload and MAPK activation during the time course. In addition, a general predictive kinetic equation that takes into account as many factors as possible such as MAPK/MKP kinetics, post-stimulation time, tension, and relative workload could conceivably be formulated.

Finally, in order to study potential force transmission pathways associated with a certain MAPK, one can use specific inhibitors that can parcel out linear and lateral directions of transmission. Every site of force transmission has a unique expression of proteins, especially integrins. For example, inhibition of an integrin located only at the MTJ, the site associated with linear transmission, may alter MAPK signaling during mechanical stimulation. The findings may help confirm the suggestion of our results that ERK1 may be implicated with linear force transmission. Furthermore, using electron microscopy and immunodetection techniques, visualization of MAPK co-localization can bring forth important evidence concerning the role of cytoskeletal proteins in

mechanotransduction and perhaps further confirm the dependance of MAPK signaling on force transmission pathways.

RESTIMULATION/REFRACTORY PERIOD - Other future experiments can focus on the re-stimulation part of the time course. One can choose an earlier time to invoke a 2nd stimulation bout in order to test for the possible presence of an additive effect that would result in JNK activation values (or ERK1/2 or other signals) that are significantly higher than the initial maximal values immediately following the 1st stimulation bout. Past time course experiments involving mechanical stretch have shown that an additive effect may be present for the induction of IE genes (Dawes, 1996), therefore, *c-fos* and *c-jun* induction can be tested during repeat bouts of mechanical loading. On the other hand, although without any indication from our study, an earlier time of re-stimulation may actually reveal the presence of a MAPK refractory period. Evidence thus far suggests that re-stimulation at an earlier time point will likely either reproduce maximal MAPK activation or perhaps produce an additive effect.

CONCLUSION

In summary, MAPKs are likely involved with mechanically induced intracellular signaling and early hypertrophy events in skeletal muscle. Tension is known to be necessary for hypertrophy. A quantitative analysis demonstrated that phosphorylation of JNK and ERK2 correlated with relative tension and ERK1 correlated with absolute tension.

However, each MAPK possibly has distinct functions and modes of regulation. Firstly, there exist specific differences in the abovementioned tension-related variables that can predict MAPK activation. Secondly, during the time course of activation, JNK exhibited a prolonged 'on' response whereas ERK1/2 exhibited a biphasic response. Also, ERK1/2 displayed much lower response amplitudes as compared to JNK.

Finally, a 2nd mechanical stimulation bout 2 hours after the first demonstrated an absence of a refractory period for mechanically induced JNK activation. Maximal JNK activation was re-attained with the same protocol. Thus, there is no evidence for mechanical desensitization in rat plantaris muscle.

Our findings can contribute to understanding diseases such as muscular dystrophy and in the design of training protocols and disuse atrophy countermeasures.

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APPENDIX A

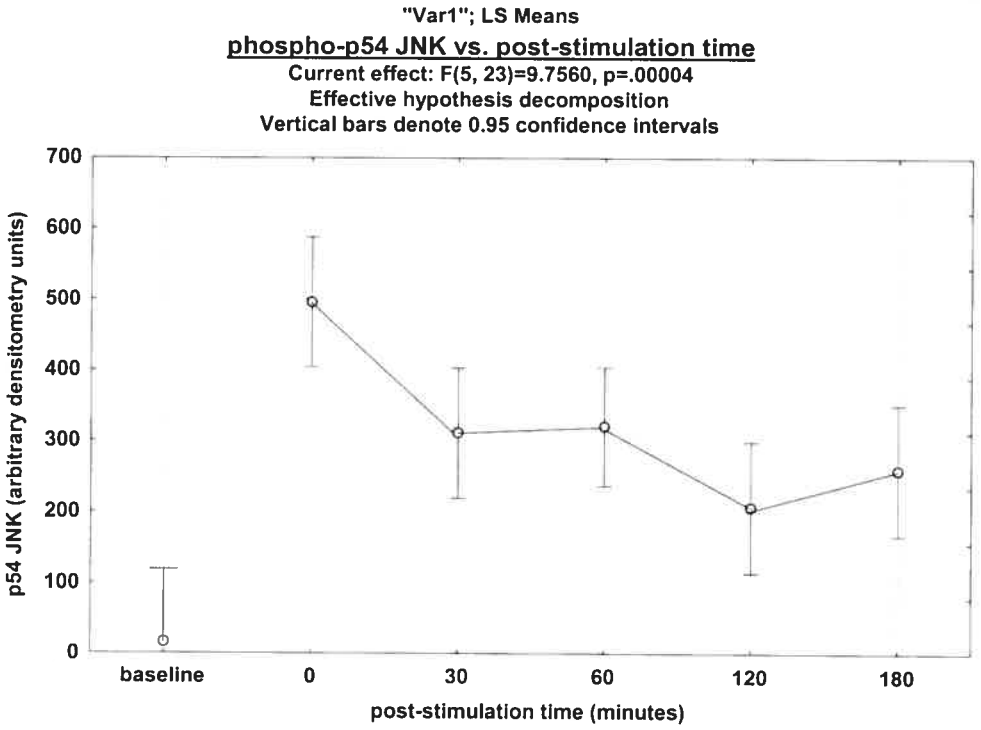
TABLE A1 - SUMMARY of RAW DATA

	Preparatory parameters				Developed tension (g)			Fold-baseline activation		
Group/Sample#	Body weight (g)	Muscle weight (mg)	L_o twitch tension (g)	L_o tetanic tension (g)	Maximal peak tension	Avg. peak tension after 60 seconds	Avg. peak tension after 300 seconds	P54-JNK	P42-ERK	P44-ERK
Baseline/1	190	221	48	200	-	-	-	-	-	-
Baseline/2	215	398	72	354	-	-	-	-	-	-
Baseline/3	201	259	60	298	-	-	-	-	-	-
0 min./1	205	265	66	285	572	317	86	24.5	1.48	2.2
0 min./2	228	376	78	297	400	343	148	22.2	1.30	2.3
0 min./3	205	284	65	275	423	370	228	29.1	1.53	2.9
0 min./4	182	254	62	304	406	329	211	39.4	1.82	2.7
0 min./5	205	282	74	339	601	459	252	30.6	2.10	3.9
30 min./1	198	230	70	275	414	315	189	22.6	0.75	1.3
30 min./2	190	244	62	273	444	335	217	18.9	1.21	1.9
30 min./3	194	271	77	359	466	370	193	26.4	1.24	2.0
30 min./4	198	285	68	265	432	326	184	11.1	0.95	2.0
30 min./5	217	302	73	322	599	416	241	12.5	1.34	1.3
60 min./1	205	239	68	287	405	299	143	26.6	1.04	1.2
60 min./2	207	265	81	310	392	365	219	15.6	1.07	1.4
60 min./3	199	280	72	333	399	374	230	19.6	1.34	1.5
60 min./4	203	328	83	284	422	307	199	17.3	0.85	0.7
60 min./5	210	263	66	332	556	429	257	23.8	1.33	1.4
60 min./6	205	324	75	302	518	353	210	10.0	0.94	1.0
120 min./1	216	266	64	305	531	262	134	6.9	0.85	2.0
120 min./2	190	209	63	281	380	340	214	12.8	0.62	1.5
120 min./3	185	282	79	303	415	356	229	9.8	0.93	1.0
120 min./4	176	230	59	266	411	372	236	13.6	0.59	1.0
120 min./5	200	310	65	281	580	416	221	17.5	1.06	1.0
180 min./1	208	254	74	312	412	363	215	19.7	1.81	2.9
180 min./2	202	260	63	317	622	466	258	23.2	1.53	2.1
180 min./3	200	215	53	261	478	286	122	7.3	1.62	2.5
180 min./4	215	272	67	305	530	382	208	17.7	0.94	1.2
180 min./5	217	300	68	281	536	408	252	8.0	1.33	1.9

APPENDIX B

MAPK TIME COURSE ACTIVATION DATA

RAW DATA & ANOVA ANALYSIS



Sample #	baseline	0 min.	30 min.	60 min.	120 min.	180 min.
1	14	418	384	452	118	335
2	23	377	322	266	217	395
3	15	495	448	333	167	124
4	-	669	188	294	231	301
5	-	521	213	404	297	137
6	-	-	-	171	-	-
Average	17	496	311	320	206	258
St. Dev.	4.9	113	111	100	68	122
S.E.M.	2.9	50	50	41	30	54

Table B1. Raw Data - Arbitrary p54-JNK values throughout post-stimulation time course.

Sample #	baseline	0 min.	30 min.	60 min.	120 min.	180 min.
1	-	24.5	22.6	26.6	6.9	19.7
2	-	22.2	18.9	15.6	12.8	23.2
3	-	29.1	26.4	19.6	9.8	7.3
4	-	39.4	11.1	17.3	13.6	17.7
5	-	30.6	12.5	23.8	17.5	8.0
6	-	-	-	10.0	-	-
Avg. fold baseline	-	29.2	18.3	18.8	12.1	15.2
St. Dev.	-	6.7	6.5	5.9	4.0	7.2
S.E.M.	-	3.0	2.9	2.4	1.8	3.2

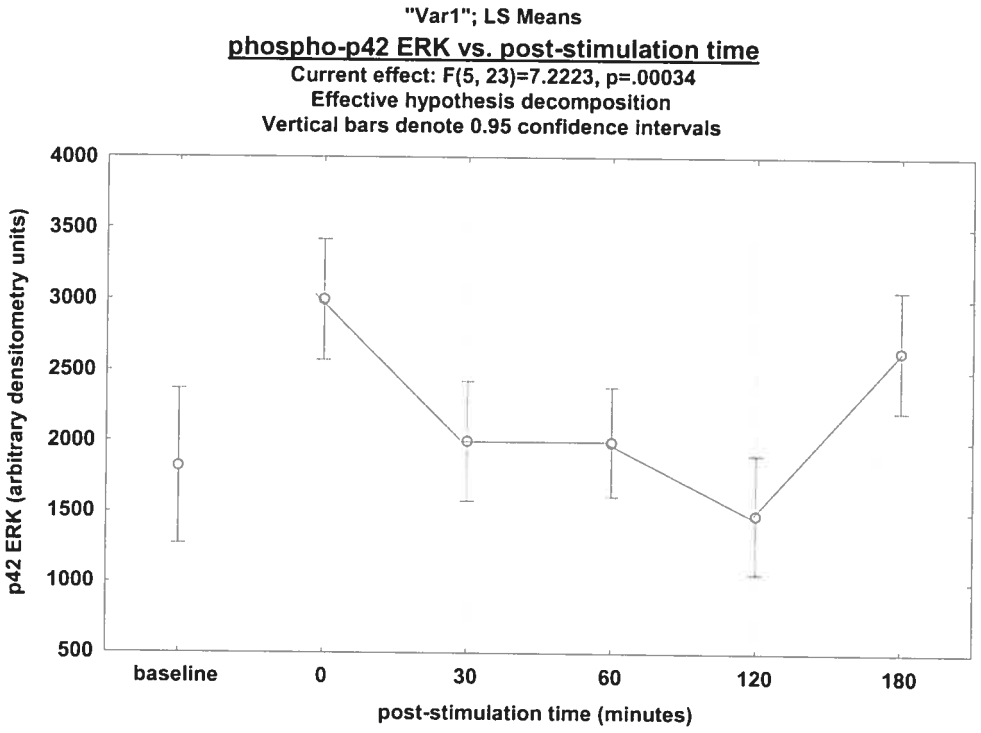
Table B2. Raw Data – Fold baseline p54-JNK values throughout post-stimulation time course.

Newman-Keuls test; variable (p54 JNK) (Spreadsheet9 in Workbook7)
Probabilities for Post Hoc Tests
Error: Between MS = 10839., df = 21.000

Cell No.	(time)	{1} 496.00	{2} 311.00	{3} 320.00	{4} 206.00	{5} 258.40
1	0		0.024621	0.013012	0.001843	0.007305
2	30	0.024621		0.890886	0.258856	0.425798
3	60	0.013012	0.890886		0.319247	0.614722
4	120	0.001843	0.258856	0.319247		0.427532
5	180	0.007305	0.425798	0.614722	0.427532	

Dunnett test; variable p54JNK (Spreadsheet1 in full time course)
Probabilities for Post Hoc Tests (M>Control)
Error: Between MS = 9899.0, df = 23.000

Cell No.	Var1	{4} 17.333
1	0	0.000026
2	30	0.001153
3	60	0.000619
4	baseline	
5	120	0.031742
6	180	0.006406



Sample #	baseline	0 min.	30 min.	60 min.	120 min.	180 min.
1	1798	2706	1375	1897	1555	3299
2	2091	2377	2200	1956	1133	2795
3	1579	2790	2256	2438	1704	2954
4	-	3310	1737	1540	1077	1718
5	-	3799	2441	2425	1935	2422
6	-	-	-	1720	-	-
Average	1823	2996	2002	1996	1481	2638
St. Dev.	257	560	436	367	370	603
S.E.M.	148	250	195	150	165	270

Table B3. Raw Data - Arbitrary p42-ERK values throughout post-stimulation time course.

Sample #	baseline	0 min.	30 min.	60 min.	120 min.	180 min.
1	-	1.48	0.75	1.04	0.85	1.81
2	-	1.30	1.21	1.07	0.62	1.53
3	-	1.53	1.24	1.34	0.93	1.62
4	-	1.82	0.95	0.85	0.59	0.94
5	-	2.10	1.34	1.33	1.06	1.33
6	-	-	-	0.94	-	-
Average fold baseline	-	1.65	1.1	1.1	0.8	1.45
St. Dev.	-	0.32	0.24	0.20	0.20	0.33
S.E.M.	-	0.14	0.11	0.08	0.09	0.15

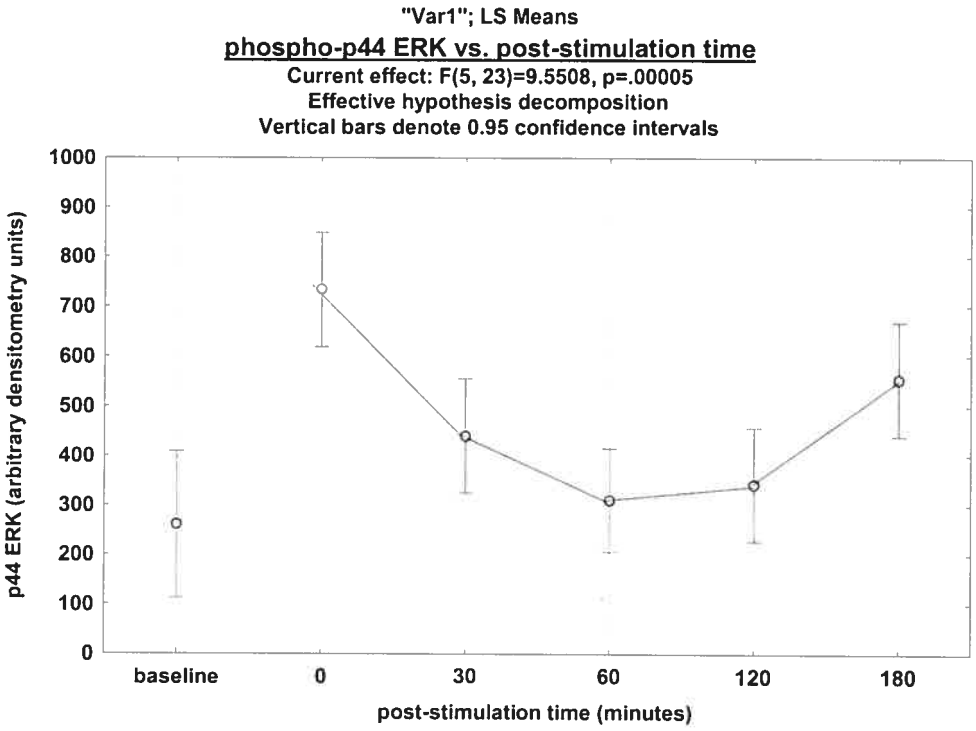
Table B4. Raw Data – Fold-baseline p42-ERK values throughout post-stimulation time course.

Newman-Keuls test; variable (p42 ERK) (Spreadsheet9 in Workbook7)
Probabilities for Post Hoc Tests
Error: Between MS = 2232E2, df = 21.000

Cell No.	(time)	{1} 2996.4	{2} 2001.8	{3} 1996.0	{4} 1480.8	{5} 2637.6
1	0		0.007635	0.013213	0.000476	0.235645
2	30	0.007635		0.984539	0.202868	0.042255
3	60	0.013213	0.984539		0.094227	0.097487
4	120	0.000476	0.202868	0.094227		0.003999
5	180	0.235645	0.042255	0.097487	0.003999	

Dunnett test; variable p42ERK (Spreadsheet1 in full time course)
Probabilities for Post Hoc Tests (M>Control)
Error: Between MS = 2096E2, df = 23.000

Cell No.	Var1	{4} 1822.7
1	0	0.004074
2	30	0.638600
3	60	0.638753
4	baseline	
5	120	0.985663
6	180	0.044013



Sample #	baseline	0 min.	30 min.	60 min.	120 min.	180 min.
1	277	584	329	312	522	744
2	263	607	483	367	402	549
3	241	759	530	392	268	654
4	-	692	523	176	262	324
5	-	1024	333	355	254	501
6	-	-	-	258	-	-
Average	260	733	440	310	342	554
St. Dev.	18	177	101	81	118	160
S.E.M.	10	79	45	33	53	71

Table B5. Raw Data - Arbitrary p44-ERK values throughout post-stimulation time course.

Sample #	baseline	0 min.	30 min.	60 min.	120 min.	180 min.
1	-	2.2	1.3	1.2	2.0	2.9
2	-	2.3	1.9	1.4	1.5	2.1
3	-	2.9	2.0	1.5	1.0	2.5
4	-	2.7	2.0	0.7	1.0	1.2
5	-	3.9	1.3	1.4	1.0	1.9
6	-	-	-	1.0	-	-
Avg. fold baseline	-	2.8	1.7	1.2	1.3	2.1
St. Dev.	-	0.7	0.4	0.3	0.45	0.65
S.E.M.	-	0.3	0.16	0.12	0.2	0.29

Table B6. Raw Data – Fold-baseline p44-ERK values throughout post-stimulation time course.

Newman-Keuls test; variable (p44 ERK) (Spreadsheet9 in Workbook7)
Probabilities for Post Hoc Tests
Error: Between MS = 16945., df = 21.000

Cell No.	(time)	{1} 733.20	{2} 439.60	{3} 310.00	{4} 341.60	{5} 554.40
1	0		0.004420	0.000419	0.000613	0.038545
2	30	0.004420		0.267314	0.239572	0.170920
3	60	0.000419	0.267314		0.700308	0.030732
4	120	0.000613	0.239572	0.700308		0.040045
5	180	0.038545	0.170920	0.030732	0.040045	

Dunnett test; variable p44ERK (Spreadsheet1 in full time course)
Probabilities for Post Hoc Tests (M>Control)
Error: Between MS = 15500., df = 23.000

Cell No.	Var1	{4} 260.33
1	0	0.000089
2	30	0.106598
3	60	0.625978
4	baseline	
5	120	0.474367
6	180	0.007768

APPENDIX C

ANOVA ANALYSIS of TENSION-RELATED VARIABLES

Max. Peak Tension (grams)

0-MAX.	30 min.	60 min.	120 min.	180 min.
572	414	405	531	412
400	444	392	380	622
423	466	399	415	478
406	432	422	411	530
601	599	556	580	536
-	-	518	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	2402	480.4	9557.3
Column 2	5	2355	471	5477
Column 3	6	2692	448.6667	4924.667
Column 4	5	2317	463.4	7552.3
Column 5	5	2578	515.6	6022.8

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	13299.68	4	3324.921	0.502106	0.734533	2.840096
Within Groups	139060.9	21	6621.949			
Total	152360.6	25				

Avg. Pk. T-60 (grams)

0-MAX	30 min.	60 min.	120 min.	180 min.
317	315	299	262	363
343	335	365	340	466
370	370	374	356	286
329	326	307	372	382
459	416	429	416	408
-	-	353	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	1818	363.6	3233.8
Column 2	5	1762	352.4	1688.3
Column 3	6	2127	354.5	2275.9
Column 4	5	1746	349.2	3179.2
Column 5	5	1905	381	4326

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3323.146	4	830.7865	0.285593	0.884003	2.840096
Within Groups	61088.7	21	2908.986			
Total	64411.85	25				

Avg. Pk. T-300 (grams)

0-MAX.	30 min.	60 min.	120 min.	180 min.
86	189	143	134	215
148	217	219	214	258
228	193	230	229	122
211	184	199	236	208
252	241	257	221	252
-	-	210	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	925	185	4546
Column 2	5	1024	204.8	570.2
Column 3	6	1258	209.6667	1459.867
Column 4	5	1034	206.8	1724.7
Column 5	5	1055	211	2959

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2282.605	4	570.6513	0.257719	0.901653	2.840096
Within Groups	46498.93	21	2214.235			
Total	48781.54	25				

Avg. Pk. T-60/twitch (grams/grams)

0-MAX.	30 min.	60 min.	120 min.	180 min.
4.8	4.5	4.4	4.1	4.9
4.4	5.4	4.5	5.4	7.4
5.7	4.8	5.2	4.5	5.4
5.3	4.8	3.7	6.3	5.7
6.2	5.7	6.5	6.4	6.0
-	-	4.7	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	26.4	5.28	0.507
Column 2	5	25.2	5.04	0.243
Column 3	6	29	4.833333	0.902667
Column 4	5	26.7	5.34	1.073
Column 5	5	29.4	5.88	0.887

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.290128	4	0.822532	1.125044	0.371451	2.840096
Within Groups	15.35333	21	0.731111			
Total	18.64346	25				

Avg. Pk. T-60/tetanic (grams/grams)

0-MAX.	30 min.	60 min.	120 min.	180 min.
1.1	1.15	1.04	.86	1.16
1.15	1.23	1.18	1.2	1.47
1.35	1.03	1.12	1.17	1.09
1.1	1.23	1.08	1.4	1.25
1.35	1.29	1.29	1.48	1.45
-	-	1.17	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	6.05	1.21	0.01675
Column 2	5	5.93	1.186	0.01008
Column 3	6	6.88	1.146667	0.007747
Column 4	5	6.11	1.222	0.05812
Column 5	5	6.42	1.284	0.02908

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.054858	4	0.013715	0.582002	0.679022	2.840096
Within Groups	0.494853	21	0.023564			
Total	0.549712	25				

Avg. Pk. T-300/twitch (grams/grams)

0-MAX.	30 min.	60 min.	120 min.	180 min.
1.3	2.7	2.1	2.1	2.9
1.9	3.5	2.7	3.4	4.1
3.5	2.5	3.2	2.9	2.3
3.4	2.7	2.4	4.0	3.1
3.4	3.3	3.9	3.4	3.7
-	-	2.8	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	13.5	2.7	1.055
Column 2	5	14.7	2.94	0.188
Column 3	6	17.1	2.85	0.403
Column 4	5	15.8	3.16	0.503
Column 5	5	16.1	3.22	0.492

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.948385	4	0.237096	0.454	0.768416	2.840096
Within Groups	10.967	21	0.522238			
Total	11.91538	25				

Avg. Pk. T-300/tetanic (grams/grams)

0-MAX.	30 min.	60 min.	120 min.	180 min.
0.3	0.69	0.5	0.44	0.69
0.5	0.79	0.71	0.76	0.81
0.83	0.54	0.69	0.76	0.47
0.69	0.69	0.7	0.89	0.68
0.74	0.75	0.77	0.79	0.89
-	-	0.7	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	3.06	0.612	0.04497
Column 2	5	3.46	0.692	0.00902
Column 3	6	4.07	0.678333	0.008457
Column 4	5	3.64	0.728	0.02877
Column 5	5	3.54	0.708	0.02532

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.038985	4	0.009746	0.431248	0.784454	2.840096
Within Groups	0.474603	21	0.0226			

Muscle Weight (grams)

0-Max.	30 min.	60 min.	120 min.	180 min.
265	230	239	266	254
376	244	265	209	260
284	271	280	282	215
254	285	328	230	272
282	302	263	310	300
-	-	324	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	1461	292.2	2348.2
Column 2	5	1332	266.4	865.3
Column 3	6	1699	283.16667	1274.9667
Column 4	5	1297	259.4	1629.8
Column 5	5	1301	260.2	951.2

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4422.7051	4	1105.6763	0.7856845	0.5472795	2.8400998
Within Groups	29552.833	21	1407.2778			
Total	33975.538	25				

Avg. Pk. T.-60/muscle weight (grams/grams)

0-max.	30 min.	60 min.	120 min.	180 min.
1.20	1.37	1.25	0.99	1.43
0.91	1.37	1.38	1.63	1.79
1.30	1.37	1.34	1.26	1.3
1.30	1.14	0.94	1.62	1.40
1.63	1.38	1.63	1.34	1.36
-	-	1.09	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	6.34	1.268	0.06647
Column 2	5	6.63	1.326	0.01083
Column 3	6	7.63	1.271667	0.057657
Column 4	5	6.835	1.367	0.07282
Column 5	5	7.31	1.462	0.03507

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.132635	4	0.033159	0.676678	0.615668	2.8401
Within Groups	1.029043	21	0.049002			
Total	1.161678	25				

Avg. Pk. T.-60/twitch/muscle weight (grams/grams/grams)

0-max.	30 min.	60 min.	120 min.	180 min.
18.1	19.6	18.4	15.4	19.3
11.7	22.1	17.0	25.8	28.5
20.1	17.7	18.6	16.0	25.1
20.9	16.8	11.3	27.4	21.0
22.0	18.9	24.7	20.6	20.0
-	-	14.5	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	92.8	18.56	16.738
Column 2	5	95.1	19.02	4.127
Column 3	6	104.5	17.41667	20.30167
Column 4	5	105.2	21.04	30.128
Column 5	5	113.9	22.78	15.277

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	96.48282	4	24.12071	1.381754	0.274192	2.8401
Within Groups	366.5883	21	17.45659			
Total	463.0712	25				

Avg. Pk. T.-60/tetanic/muscle weight (grams/grams/grams)

0-max.	30 min.	60 min.	120 min.	180 min.
4.2	5.0	4.4	3.2	3.7
3.1	5.1	4.5	5.7	4.6
4.8	3.8	4.0	4.1	4.2
4.3	4.3	3.3	6.1	4.1
4.8	4.3	4.9	4.8	5.2
-	-	3.6	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	21.2	4.24	0.483
Column 2	5	22.5	4.5	0.295
Column 3	6	24.7	4.116667	0.357667
Column 4	5	23.9	4.78	1.387
Column 5	5	21.8	4.36	0.323

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.386205	4	0.346551	0.619878	0.653295	2.8401
Within Groups	11.74033	21	0.559063			
Total	13.12654	25				

Avg. Pk. T. 300/muscle weight (grams/grams)

0 max.	30 min.	60 min.	120 min.	180 min.
0.32	0.82	0.60	0.50	0.85
0.39	0.89	0.83	1.0	0.99
0.80	0.71	0.82	0.81	0.57
0.83	0.65	0.61	1.02	0.76
0.89	0.80	0.98	0.71	0.84
-	-	0.65	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	3.23	0.646	0.07223
Column 2	5	3.87	0.774	0.00893
Column 3	6	4.49	0.748333	0.023257
Column 4	5	4.04	0.808	0.04657
Column 5	5	4.01	0.802	0.02367

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.086563	4	0.021641	0.629541	0.646809	2.8401
Within Groups	0.721883	21	0.034375			
Total	0.808446	25				

Avg. Pk. T. 300/twitch/muscle weight (grams/grams/grams)

0-max.	30 min.	60 min.	120 min.	180 min.
4.9	11.7	8.8	7.9	11.3
5.1	14.3	10.4	16.3	15.8
12.3	9.2	11.4	10.3	10.6
13.4	9.5	7.2	17.4	11.4
12.1	10.8	14.9	11.1	12.3
-	-	8.6	-	-

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	47.8	9.56	17.578
Column 2	5	55.5	11.1	4.215
Column 3	6	61.3	10.21667	7.417667
Column 4	5	63	12.6	16.59
Column 5	5	61.4	12.28	4.237

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	34.74551	4	8.686378	0.878814	0.493324	2.8401
Within Groups	207.5683	21	9.884206			
Total	242.3138	25				

Avg. Pk. T. 300/tetanic/muscle weight (grams/grams/grams)

0-max.	30 min.	60 min.	120 min.	180 min.
1.2	3.0	2.1	1.7	2.7
1.3	3.2	2.7	3.6	3.1
2.9	2.0	2.5	2.7	2.2
2.7	2.4	2.1	3.9	2.5
2.6	2.5	2.9	2.5	3.0
		2.2		

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	10.7	2.14	0.673
Column 2	5	13.1	2.62	0.232
Column 3	6	14.5	2.416667	0.113667
Column 4	5	14.4	2.88	0.782
Column 5	5	13.5	2.7	0.135

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.628282	4	0.407071	1.088101	0.387849	2.8401
Within Groups	7.856333	21	0.374111			
Total	9.484615	25				