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A study of early-response mechano-chemical signaling pathways in rat skeletal muscle of different fiber-type composition

par

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A study of early-response mechano-chemical signaling pathways in rat skeletal muscle of different fiber-type composition

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Mémoire accepté le .....

Résumé

Afin de mieux comprendre les effets physiologiques de l'exercice sur les muscles squelettiques, il faut caractériser les mécanismes intracellulaires de signalisation qui permettent aux fibres musculaires d'interpréter et de répondre aux divers stimuli qui leur sont imposés. De nouvelles techniques de laboratoire nous permettent maintenant d'étudier les effets de l'exercice à un niveau moléculaire, plus spécifiquement, au niveau de la transcription des gènes et de la synthèse protéique.

L'augmentation de tension provoquée par l'étirement d'un muscle induit l'hypertrophie des fibres qui le composent. Une déformation physique de la matrice extracellulaire déclenche l'activation d'une voie de signalisation intracellulaire qui influence l'expression des gènes. Ce mécanisme est appelé mécanotransduction.

La famille de 'mitogen-activated protein kinases' (MAPKs) représente une voie de signalisation moléculaire importante pour la régulation et la coordination de diverses activités cellulaires. Bien que les mécanismes de mécanotransduction dans le muscle squelettique demeurent inconnus, des études antérieures ont démontré que les MAPKs sont activées dans les cellules musculaires suite à un stress mécanique. L'étude des MAPKs pourrait nous fournir d'amples informations sur la régulation du phénotype des muscles et l'hypertrophie musculaire.

Cette étude a pour but de déterminer si la mécanosensibilité d'un muscle varie selon les types de fibres qui le composent. Pour répondre à cette question, nous avons mesuré l'activation des MAPKs dans divers muscles du rat suite à l'application aigüe d'un stress mécanique.

Les muscles soléaire (SOL), plantaire (PLN), extensor digitorum longus (EDL) et gastrocnémien médial (MG) ont été chirurgicalement isolés, in situ, et exposés à un protocole d'étirement passif pour une durée de 5 minutes. L'analyse biochimique de l'activité du p54-JNK et ERK1/2 MAPK suite à ce protocole d'étirement démontre que même si le SOL, PLN et EDL varient de façon significative quant aux différents types de fibres qui les composent, leur sensibilité à un stress mécanique est semblable. Par contre, lorsque le même étirement passif est appliqué au MG, un muscle composé de deux compartiments contenant chacun des types de fibres différents, ces compartiments répondent de façon hétérogène quant à l'activation des MAPKs. Cependant, lorsque des contractions musculaires isométriques servent de stimuli mécaniques pour déclencher l'activation des MAPKs, la réponse hétérogène disparaît.

Ces observations suggèrent que bien qu'ils soient différents, les muscles traduisent les signaux mécaniques en signaux chimiques de la même façon. Nous proposons que l'étirement passif du MG a pour effet

d'imposer plus de tension sur un compartiment en comparaison avec l'autre. Ceci est probablement la conséquence d'une différence dans l'architecture des fibres composants les compartiments et non celle d'une différence dans le type de fibre comme tel.

MOTS CLÉS: mécanotransduction, mitogen-activated protein kinases, fibres musculaires, étirement passif, signalisation intracellulaire

Summary

A central concern in understanding the biological effects of exercise on the adaptations of skeletal muscle is to elucidate the signaling mechanisms that enable muscle cells to interpret and respond to an imposed exercise stimulus with altered gene transcription and protein synthesis. Techniques in molecular biology have been applied to exercise physiology research and continue to provide new information as to the nature of the intracellular pathways that transduce signals inherent to the exercise stimulus to the gene regulatory centre of the cell.

Mechanical loading is a prime determinant of muscle volume and phenotype. Physical perturbations of the muscle extracellular matrix can influence biochemical activities at the nuclear level through a mechanism described as mechanotransduction.

The mitogen-activated protein kinase (MAPK) signal transduction pathways are among the most widespread mechanisms of eukaryotic cell regulation. While mechanotransduction and mechano-chemical signaling pathways in skeletal muscle are not well characterized, the mechanical response of MAPKs is a reflection of the upstream processes. The investigation of MAPK activation in response to mechanical stress in skeletal muscle may yield important information about the regulation of muscle phenotype and hypertrophy.

This study investigates whether muscles exhibiting a wide range of metabolic profiles and fiber-type compositions display differences in their ability to detect and transduce mechanical stimuli. To address this question, activation of p54-JNK and ERK1/2 MAPKs was assessed in different muscles of the rat hindlimb subjected to a standardized mechanical stress.

Using an in situ isolated muscle preparation, the soleus (SOL), plantaris (PLN), extensor digitorum longus (EDL) and medial gastrocnemius (MG) muscles were subjected to 5 minutes of standardized passive stretch corresponding to 300 g of tensional force per gram wet muscle weight. Biochemical analysis of MAPK phosphorylation following this 5 minute stretch protocol indicates no difference in activation between SOL, PLN and EDL despite large metabolic and fiber-type differences. Stated otherwise, muscles are equally capable of transducing a mechanical signal acting across the cell surface into a chemical signal within the intracellular compartment. However, in response to stretch of the whole MG, the deep and superficial compartments displayed heterogeneous MAPK activation. This heterogeneity was abolished by mechanical stimulation in the form of isometric contractile activity.

Based on these observations, we conclude that there are no differences in the detection of mechanical forces and in the transmission of mechano-

signaling down to the MAPK cascades as a result of metabolic profile or fiber-type composition across muscles. However, at the tissue level, architectural factors contribute to the "physiological mechanosensitivity" of a muscle and are likely responsible for the heterogeneous compartmental response observed in MG.

KEY WORDS: mechanotransduction, mitogen-activated protein kinases, skeletal muscle, passive stretch, intracellular signaling

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Figure 1 **Conceptual Overview of Stretch-Activated Signaling.**

The biological response of passive stretch in a muscle fiber entails several steps. First, the stretch has to be sensed at the sarcolemma. Integrins are membrane-associated proteins thought to be involved in the "sensing" of the mechanical stretch stimulus. The subsequent "transduction" of the stretch signal is believed to occur through focal adhesion complexes. FACs are associated with numerous intracellular signaling molecules. The nuclear response elicited is specific to the inducing stimulus and the nature of the activated signaling cascades.....11

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The activation of receptor tyrosine kinases by external stimuli such as mechanical stress, leads to the activation of kinase cascades which then phosphorylate and modify the activity of specific target proteins, such as transcription factors (SRF, c-jun, MEF2). The MAPK cascades consist of at least three related signaling pathways. Classified into the c-Jun NH_2-terminal kinase (JNK) family, the extracellular-regulated kinase (ERK)

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Abbreviations

Ca^{++}= calcium ion

CREB= cyclic AMP-responsive element binding protein

DAG= diacylglycerol

ECM= extracellular matrix

ERK= extracellular-regulated kinase

FAC= focal adhesion complex

FAK= focal adhesion kinase

G-protein= guanine nucleotide-binding protein

IEG= immediate early gene

IGF-1= insulin-like growth factor-1

IP_3= inositol triphosphate

JNK= c-jun NH_2-terminal kinase

MAPK= mitogen-activated protein kinase

MEF2= myocyte enhancer-binding factor 2

MHC= myosin heavy chain

MRF= muscle regulatory factor

PGF_2= prostaglandin F_2

PIP_2= phosphatidylinositol bisphosphate

PKC= protein kinase C

SR= sarcoplasmic reticulum

TF= transcription factor

Chapter 1-Literature Review

Skeletal muscle is a dynamic tissue that possesses a remarkable capability to adapt both structurally and functionally to the type of activity it is required to perform. It is well established that the properties of skeletal muscle, which together determine its phenotype, are able to adapt to different functional demands through morphological, biochemical and molecular modifications (Booth 1988, Roy et al. 1985, Vrbova 1979). These modifications are expressed in such a way as to optimize muscle function in response to altered levels of activity.

Generally, chronic exposure to reductions and increases in activity levels results in muscle adaptations of atrophy and hypertrophy, respectively (Roy et al. 1991). However, it is difficult to make broad generalizations regarding muscle adaptation since the exercise stimulus is highly complex with many potential sites of regulation. While the exercise stimulus coordinately affects the function of several physiological systems, for the purpose of this thesis, only the events occurring within and affecting skeletal muscle will be discussed.

The adaptations in muscle properties which occur in response to increased activity are highly specific to the type, intensity, frequency, and duration of the exercise performed. A recent study by Nader and Esser (2001) provides experimental evidence demonstrating that different forms of exercise result in the activation of specific signaling pathways.

Generally, exercise can be classified as either endurance-type or resistance-type. Typically, the effects of endurance-type exercise on muscular adaptations involve modifications of the biochemical properties of the muscle fibers so as to increase the muscle's resistance to fatigue (Gollnick and Saltin 1982, Henriksson et al. 1986). For example, levels of the oxidative enzymes succinate dehydrogenase and citrate synthase increase while glycolytic enzyme activities decrease (Henriksson et al. 1986). Increases are also observed in the number of capillaries and mitochondria, reflecting an increased metabolic capacity of the muscle (Holloszy and Booth 1976). Another important adaptation involves changes in the proteins that regulate calcium handling within the muscle fibers. These adaptations, in addition to the many more that have not been mentioned, complement the eventual transformation in the fiber's myosin heavy chain (MHC) isoform profile from fast to slow (Dunn and Michel 1997).

The myosin molecule, which exists in several different isoforms, is of major importance in determining the contractile properties of the muscle cell. A change in MHC profile is achieved by the increased synthesis of IIa and IIx MHC isoforms to replace existing IIb MHC isoforms as the fiber slowly acquires the less energy costly type I MHC (Jaschinski et al. 1998). By altering the MHC profile of their fibers in a coordinated fashion with other 'accessory' myofibrillar proteins (such as myosin light chains,

energetic enzymes, troponins and other calcium-handling proteins), muscles essentially change fiber type in an effort to become more efficient at completing new functional tasks (Termin and Pette 1992).

By contrast, the effects of resistance-type exercise on the adaptations of muscle tend to promote the development of increased muscle strength and mass (Alway et al. 1990, Sale et al. 1987). There appears to be a general consensus among exercise physiologists that muscle hyperplasia does not occur to a significant degree in humans (MacDougall et al. 1984, McCall et al. 1996). Therefore, it is assumed that the increase in muscle mass observed in response to resistance-type training is a result of the hypertrophy of existing muscle fibers, and not the production of new ones (Abernethy et al. 1994).

Intrinsic properties of muscles, other than strength and mass also change with resistance exercise. For instance, heavy resistance training has been shown to result in a decrease in capillary density (Tesch et al. 1984). Increases in myokinase and creatine phosphokinase, enzymes related to the phosphagen system, have also been reported with resistance training (Costill et al. 1979). In studies conducted by MacDougall (1979) it was shown that resistance training in humans reduced the mitochondrial volume density and mitochondrial-to-myofibrillar volume ratio. This is

thought to be due to disproportionate increases between contractile protein and the number of mitochondria.

Changes to the neural component, which include the disinhibition of protective mechanisms associated with Golgi tendon organs (Fleck and Falkel 1986), precision and efficiency of motor unit recruitment (McDonagh et al. 1983), and synchronization of firing (Milner-Brown et al. 1973) represent important adaptations to resistance training. A discussion of such neural adaptations falls outside the scope of this thesis.

In summary, muscle displays a quality of plasticity whereby adaptations are specific to the imposed stimulus. Whereas the physiological and biochemical adaptations occurring in activated muscle in response to increased activity have been well described, a characterization of the molecular events that promote these specific adaptations remains elusive.

1.1 MECHANICAL STRETCH AS A SIGNAL FOR HYPERTROPHY

Much of what is known regarding the mechanisms that lead to stretch-induced hypertrophic growth is derived from studies in cardiac cells. Elegant studies in cultured cardiomyocytes have elucidated a cascade of molecular events induced by mechanical stretch, which include:

activation of phospholipases to release diacylglycerol and phosphatidylinositol (Komuro et al. 1991, Sadoshima and Izumo 1997), activation of protein kinases (Sadoshima and Izumo 1997), induction of immediate early gene expression (Komuro et al. 1990, Sadoshima et al. 1992), and increased rates of protein synthesis (Komuro et al. 1990, Sadoshima et al. 1992). Although the conversion of a mechanical signal to a hypertrophic response in skeletal myofibers is not as well characterized, the mechanisms by which changes in mechanical force regulate cell function may share important similarities across different cell types, even though the adaptational responses on a phenotypic level may differ.

Early evidence for the hypertrophic effect of stretch on skeletal muscle comes from the studies of Vandenburgh and Kaufman (1979) who developed a technique for mechanically stretching cultured muscle cells in vitro. They demonstrated that static stretch induces a hypertrophic response in cultured chick skeletal muscle cells, providing the first evidence that skeletal muscle cells respond to externally applied mechanical tension in the absence of neuronal or hormonal influence. Later, studies by Goldspink and colleagues (1991) demonstrated that stretching the rabbit tibialis anterior, by cast immobilization of the ankle joint, could increase the wet weight of the affected muscle by 20%.

Aside from its hypertrophy-promoting effects, passive muscle stretch has also been shown to promote atrophy-countering effects. As alluded to earlier, chronic disuse of muscle causes atrophy and a slow-to-fast transformation of the contractile and phenotypic parameters of the affected muscles (Loughna et al. 1986). Studies using non-weight-bearing models of disuse muscle atrophy have shown that immobilizing the non-weight-bearing muscle in a slightly lengthened position prevented reductions in fiber mass and size (Goldspink 1977). Furthermore, passively stretching an unweighted muscle has been shown to counteract the slow-to-fast fiber adaptations that typically occur with models of suspension and immobilization (Leterme et al. 1994). The model of spinal cord transection is a model of complete inactivity that results in severe muscle atrophy and a shift toward a faster myosin phenotype (Lieber et al. 1986). Hindlimb exercise achieved passively by securing the paralyzed hindlimbs on foot-holds of a motor-driven bicycle has been shown to minimize the muscle atrophy associated with the spinal cord transection (Dupont-Versteegden et al. 1998).

The element of stretch is an important exercise-associated stimulus. Any resistance training that involves dynamic, as opposed to isometric contractions includes contractions of the eccentric (lengthening) type. During dynamic resistance training in humans, the eccentric component appears to be more powerful than the concentric component at eliciting

muscle hypertrophy (Hather et al. 1991, Hortobagyi et al. 1996, Jones and Rutherford 1987). The high tensional forces generated by eccentric contractions have been known to cause structural damage to muscle tissue resulting in pain and discomfort (Friden and Lieber 1998). A brief description of the damage includes disruptions of the cytoskeleton, membrane damage, Z-line streaming, sarcoplasmic reticulum (SR) damage, and localized areas of increased calcium, causing inflammation and the infiltration of macrophages and neutrophils (Friden and Lieber 1992, Tidball 1995). It is possible that some of these muscle damage-associated events may serve a secondary role as activators of signaling pathways for altered gene expression and increased protein synthesis.

While mechanical events at the extracellular level clearly have an important influence on biochemical activities at the nuclear level, a fundamental unanswered question remains. How do muscle cells sense and convert a physical stimulus into a chemical signal capable of initiating an intracellular signaling response that affects gene expression?

1.2 SENSING OF THE STRETCH SIGNAL

In classic stimulus-response coupling, a soluble, signal-bearing molecule binds a designated receptor and initiates an intracellular response. However, with stretch-activated signaling, the mechanism of action is less clear. There are many different kinds of receptors on the cell surface. The most well characterized are G-protein coupled receptors and tyrosine kinase receptors, both of which bind soluble ligands (Liebmann 2001). However, there is another class of cell surface receptor that binds to the ECM and is important in keeping the cell anchored. Such proteins are known as integrins. Currently, hypotheses as to the identity of the 'molecular mediators' capable of sensing tensional forces acting across the cell surface and translating them into biochemical signals capable of modulating gene expression implicate integrin proteins (Laser et al. 2000, MacKenna et al. 1998, Ross et al. 1998, Schmidt et al. 1998).

Integrins and Focal Adhesions

Integrins were initially thought of solely as molecules necessary for adhesive interactions between cells and the ECM. Recent work has indicated that integrins are bidirectional signaling molecules (Lewis and Schwartz 1995). The tensegrity-based theory proposed by Ingber and colleagues (1993) suggests that integrins act as a mechanoreceptor and that mechanical signals can be transduced through the cytoskeleton.

Integrin proteins can transduce both chemical and mechanical signals into intracellular signaling pathways in what is referred to as "outside-in signaling" (Disatnik and Rando 1999). One of the earliest changes initiated by integrin engagement is clustering of integrins at focal adhesions and tyrosine phosphorylation of proteins such as paxillin, talin, and the cytosolic enzyme focal adhesion kinase (FAK) (Vuori 1998). FAK phosphorylation is considered to be a critical step in the downstream signaling pathways known to modulate many cellular functions including gene and protein expression, cell proliferation, differentiation, and death (Clark and Brugge 1995, Schwartz et al. 1995). A conceptual overview of stretch-activated signaling through integrins is presented in figure 1.

The function of integrin-mediated signaling has been extensively examined in cardiac muscle and has been linked to certain types of cardiac hypertrophy (Ross et al. 1998, Shyy and Chien 1997). Studies in cardiomyocytes have shown that activation of FAK appears to be sensitive to mechanical stretch and is capable of activating mitogen-activated protein kinases (MAPKs) (Seko et al. 1999). Activation of mechanically-sensitive signaling cascades begins with a tug on an integrin molecule which causes stress-dependent changes in the cytoskeletal structure, followed by integrin clustering, and the transmission of the

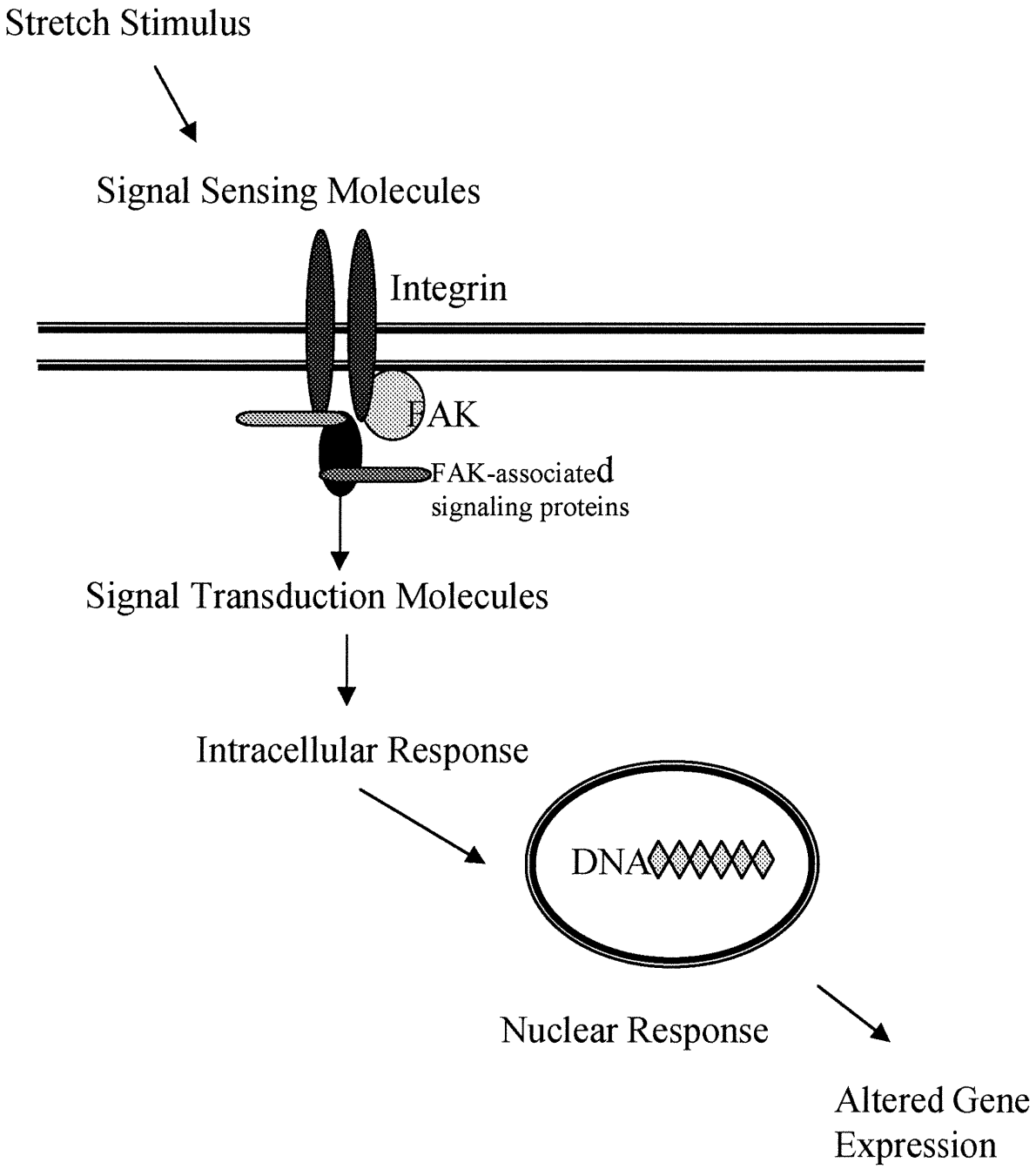

Figure 1- Conceptual Overview of Stretch-Activated Signaling

stretch signal over a specific molecular pathway (Clark and Brugge 1995). Specific signaling molecules and cascades that have been connected to integrin clustering include the activation of protein kinase C (PKC), MAPKs, inositol triphosphate kinase (IP_3 kinase), and the plasma membrane-localized Ras family of GTPases (Clark and Brugge 1995, Kapron-Bras et al. 1993, Plopper et al. 1995, Schmidt et al. 1998).

Whereas several key players have been implicated in the pathways that connect stretch to hypertrophic growth, the complexity of interaction between signaling pathways make deciphering them a difficult task.

1.3 STRETCH-ACTIVATED INTRACELLULAR SIGNALING MESSENGERS

The nature of the molecular messengers that form the signaling pathways responsible for relaying mechanical signals received at the cell surface to the nucleus are cell-type specific. The same mechanical stimulus might activate different molecular pathways in skeletal versus cardiac versus smooth muscle. However, irrespective of the biological tissue, candidate signaling proteins must fulfill two important criteria: 1) the putative pathway must be altered or induced by mechanical stress, and 2) the pathway must influence the reprogramming of gene expression leading to the activation of critical muscle proteins. Figure 2 illustrates possible

intracellular signaling molecules that may be involved in the mechanism of stretch-induced muscle hypertrophy.

Much of what is known regarding stretch-induced singnaling in muscle comes from studies in cardiac myocytes. Stretch-induced signaling in skeletal muscle is a relatively new area. Nevertheless, there appears to be a growing interest among exercise physiologists in delineating exercise-induced signal transduction pathways that regulate gene expression in skeletal muscle. In the quest to characterize such pathways, it is sometimes easier to retrace a pathway backwards from the induction of a nuclear event rather than starting with events at the cell surface and following a downstream approach.

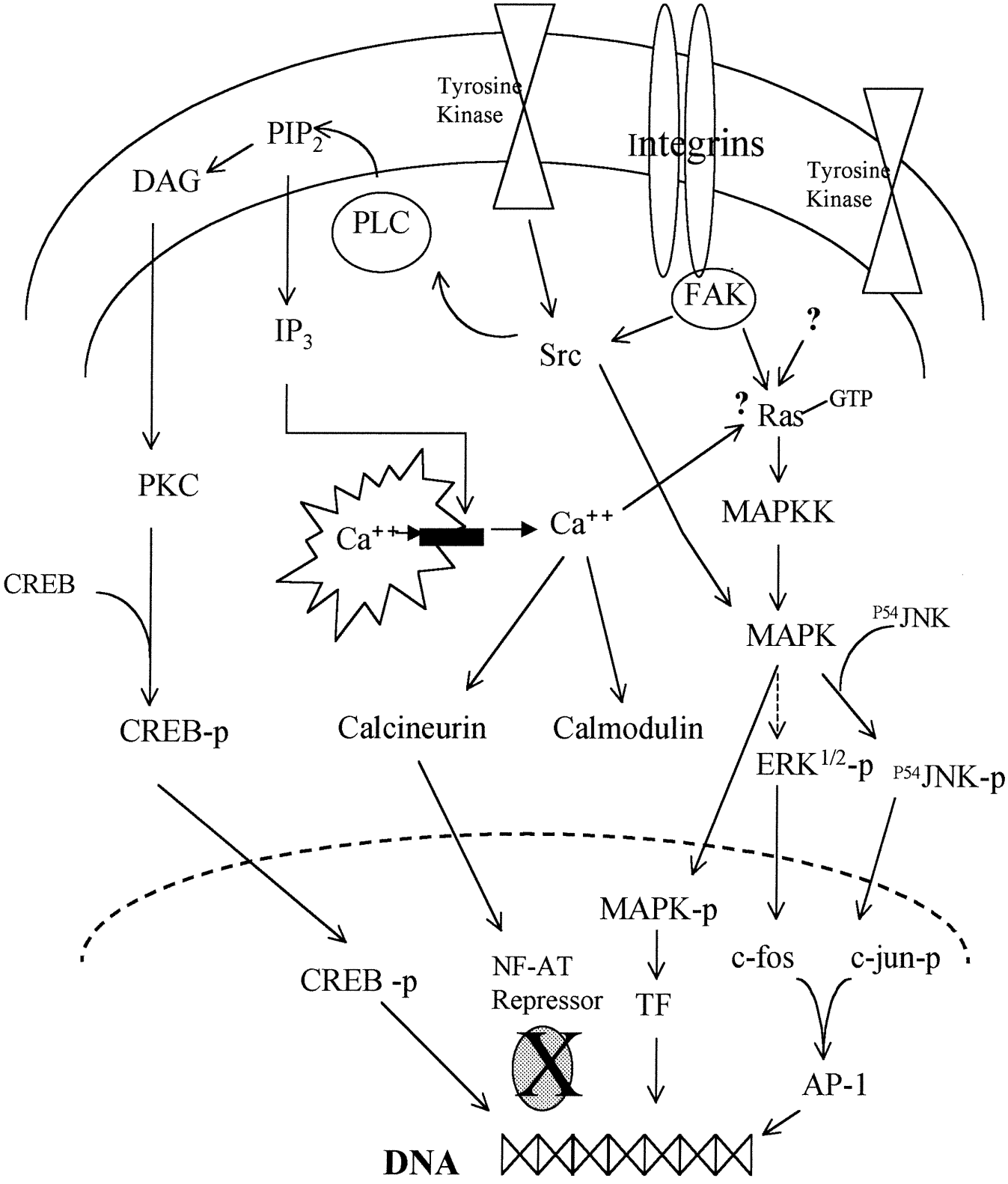

Figure 2 - Intracellular Signaling Pathways

Immediate Early Genes

IEGs function as the targets of intracellular signaling cascades. IEGs are induced rapidly in a variety of cell-types following an assortment of stimuli. There is typically a downregulation of the expression of numerous IEGs in mature, post-mitotic cells. Osbaldeston et al. (1995) demonstrated that following mechanical stretch of adult rabbit latissimus dorsi muscle, *c-jun* and *c-fos* mRNA is rapidly and transiently induced and furthermore, this induction gives rise to increased levels of the corresponding proteins. Similarly, Goldspink et al. (1995) also observed increases in *c-fos* and *c-jun* mRNA levels and rates of protein synthesis in the extensor muscles of the rabbit following static stretch, in vivo. The *c-fos* and *c-jun* proteins are components of the activator protein-1 (AP-1) transcription factor complex (Chiu et al. 1988). Aronson and colleagues (1998) have shown that following exercise in humans, *c-jun* mRNA is increased. Furthermore, this increase is associated with increased c-jun NH_2-terminal kinase (JNK) MAPK activity suggesting the JNK pathway as a link between mechanical stimulus at the cell surface and transcriptional responses at the cell nucleus.

Muscle Regulatory Factors

The muscle regulatory factors (MRFs) and the myocyte enhancer-binding factor 2 (MEF2) are families of transcriptional regulatory proteins that are important in regulating the expression of muscle-specific genes. These

factors are usually active during development to establish general myofiber phenotypes. It has been proposed that these MRFs might not be permanently repressed in adult myofibers and may become functionally activated to influence muscle plasticity (Eppley et al. 1993).

Jacobs-El, Zhou and Russell (1995) demonstrated that the expression of several members of the MRF family, namely Myf-5 and MRF4, is increased in the skeletal muscle of mature rats within hours following static stretch. The function of MEF2 factors as 'receivers' of signals from calcium-regulated signaling proteins such as calmodulin kinase and calcineurin (Wu et al. 2000), and even MAPK pathways (Zhao et al. 1999) has been suggested.

Calcium

One of the most diverse intracellular signaling molecules is calcium. The calcium ion functions as a second messenger in many cellular responses to stimuli (Berchtold et al. 2000). By means of active transport systems, calcium is maintained at a very low concentration in the cytosol. When a signal transiently causes calcium channels of either the SR or the plasma membrane to open, there is a large chemical gradient favoring the diffusion of calcium into the cytosol.

In cardiomyocytes, calcium appears to be important in the pathway leading to hypertrophic protein synthesis (Calaghan and White 1999, Ruwhof and van der Laarse 2000). Similarly, in smooth muscle, calcium influx through mechanosenstive ion channels results in JNK activation under regulation by calmodulin and calcineurin (Kushida et al. 2001). Calcineurin is a cytoplasmic calcium-regulated phosphatase. Its proposed mode of gene modulation is via the dephosphorylation of various gene transcription/translation repressors (see figure 2). A recent study has demonstrated the implication of calcineurin in the induction of skeletal muscle hypertrophy and muscle fiber type conversions associated with overload (Dunn et al. 1999). However, it has also been shown that activation of the calcineurin pathway alone is not sufficient for muscle hypertrophy (Naya et al. 2000). Therefore, muscle hypertrophy may require the activation of additional signaling pathways such as the MAPK pathways.

Prostaglandins

The eicosanoids are a family of substances produced from arachidonic acid and include the prostaglandins. The synthesis of prostaglandins begins when a stimulus activates the enzyme phospholipase A_2. This enzyme splits arachidonic acid off from the cell plasma membrane phospholipids and the arachidonic acid is metabolized by the enzyme cyclooxygenase thus initiating the synthesis of prostaglandins (Thompson

and Palmer 1998). Prostaglandins are known to be synthesized and released from exercising skeletal muscle and their involvement in the mechanisms of stretch-induced muscle hypertrophy has been proposed (Vandenburgh et al. 1993). Prostaglandins regulate protein turnover in skeletal muscle by acting as "mechanical second messengers", with PGE_2 and PGF_2 stimulating protein degradation and protein synthesis rates, respectively (Vandenburgh et al. 1990).

Mitogen-Activated Protein Kinases

The MAPK cascade is a point of convergence for many intracellular signaling molecules involved in the transduction of signals to the nucleus. Their implication in the regulation of cellular protein synthesis has been described in response to mechanical stimuli for various cell type, including bone (Mikuni-Takagaki 1999), cartilage (Hung et al. 2000), smooth muscle (Smith et al. 1997), vascular endothelial cells (Malek and Izumo 1995), and cardiac myocytes (Yamazaki et al. 1996). The MAPK family consists of at least three related signaling cascades, as depicted in figure 3. Classified into the JNK family, the extracellular-regulated kinase (ERK) family, and the p38 family, these MAPKs are independently responsive to distinct stimuli such as hormones, growth factors, mitogens, and cellular stressors. Although the role of each MAPK member is highly cell-type and context dependent, in skeletal muscle,

The MAPK signaling cascades

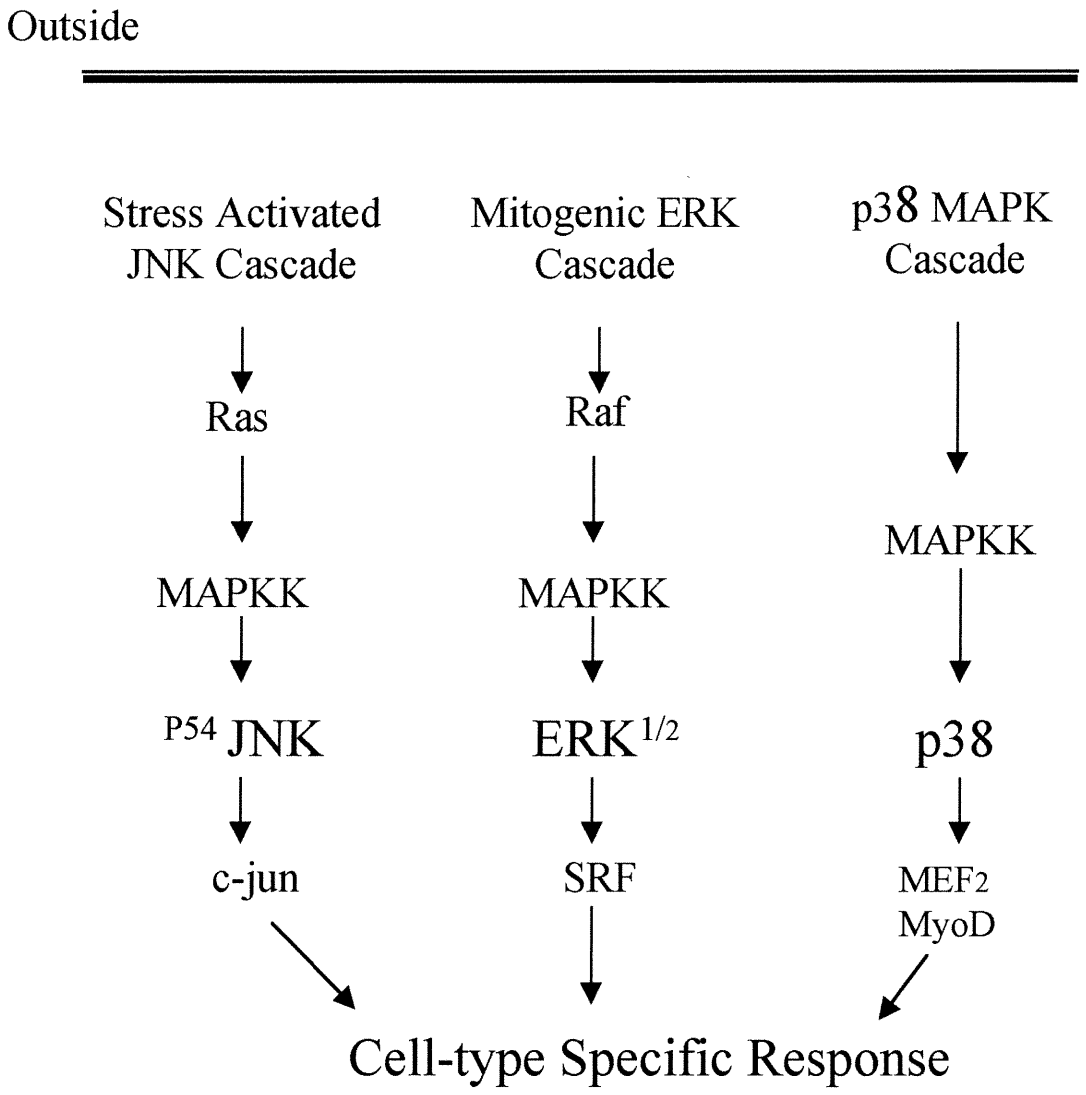

Figure 3- The Family of Mitogen-Activated Protein Kinases

studies suggest JNK confers the greatest responsiveness to mechanical stress (Goodyear et al. 1996, Martineau and Gardiner 2001).

Several reports have shown that the mechanical stretching of skeletal muscle, applied either passively or resulting from contractile activity, is capable of inducing the activation of the MAPK intracellular signaling pathways, (Boppart et al. 2001, Goodyear et al. 1996, Martineau and Gardiner 2002 (in press), Widegren et al. 2001, Wretman et al. 2000). Moreover, JNK activation in human skeletal muscle is much greater following eccentric contractions (15-fold baseline activation) than following concentric contractions (3.5-fold baseline activation) (Boppart et al. 1999). Interestingly, results of studies using one-legged exercise protocols demonstrate that MAPK phosphorylation is restricted to muscles of the activated leg, thereby eliminating the possibility of a systemic influence and reinforcing the case for a localized mechanical event as the signal for stretch-induced MAPK phosphorylation (Aronson et al. 1998). Further evidence in support of this notion comes from observations that increased specific gene expression (Michel et al. 1994) and protein synthesis (Chesley et al. 1992) are restricted to the overloaded or contracting muscle.

Recently, Boppart and colleagues (2001) reported a differential activation of MAPKs in isolated slow- and fast-twitch rat muscle in response to

passive stretch suggesting that type I fibers are more sensitive than type II fibers to an absolute magnitude of passive tension. However, with consideration to documented tensional-dependent phosphorylation response of the MAPKs (Martineau and Gardiner 2001) and due to the lack of standardization of the stretch stimulus used in this study, it is possible that the slow-twitch muscles were subjected to higher tensional forces which might explain their elevated response. Furthermore, the only other study to investigate MAPK induction in fast- and slow-twitch muscle following electrical stimulation, in vitro, reported a lack of fiber-type difference for the activation of ERK (Wretman et al. 2000). Left as such, the issue of whether the ability to detect mechanical forces is fiber-type specific, remains equivocal. Thus, further investigation into the sensitivity of different muscles to a normalized mechanical stress is warranted.

For the past few years, our laboratory has been investigating the mechanism by which mechanical forces acting through skeletal muscle cells activate intracellular signaling pathways that regulated gene expression and muscle phenotype. The investigation of MAPK activation in response to mechanical stress in skeletal muscle may yield important information about the mechanotransduction process.

The study presented here does not attempt to explain the mechanisms involved in muscle mechanotransduction. Rather, the purpose of this study is to gain a better understanding of a distinct segment of the mechanotransduction pathway thought to be involved in the molecular relay of a mechanical stimulus to the cell nucleus. Specifically, the aim of this study is to test whether muscles exhibiting a wide range of metabolic profiles and fiber-type composition exhibit differences in the detection and transmission of mechanical stimuli. This was assessed by measuring activation of the p54 JNK and ERK 1/2 MAPKs in response to in-situ standardized mechanical stimulation of physiological magnitude imposed on the rat soleus (SOL), plantaris (PLN), extensor digitorum longus (EDL), and the two distinct compartments of the medial gastrocnemius (MG).

Chapter 2- Inter- and intra-muscle comparisons of MAPK mechanosensitivity: evidence for an absence of fiber-type dependency

ABSTRACT

Mechanical loading is a prime determinant of muscle volume and phenotype. However, it is not well understood how mechanical stimulation impacts gene expression. The purpose of this study was to test whether muscles exhibiting a wide range of metabolic profiles and fiber-type composition exhibit differences in the detection and transmission of mechanical stimuli. This was assessed by measuring activation of the mitogen-activated-protein kinases (MAPKs), specifically p54 JNK and ERK 1/2, in response to in-situ standardized mechanical stimulation of physiological magnitude imposed on the rat soleus (SOL), plantaris (PLN), extensor digitorum longus (EDL), and the two distinct compartments of the medial gastrocnemius (MG). While mechanotransduction and mechano-signaling pathways are not well characterized, the mechanical response of MAPKs, in particular p54 JNK and ERK 1/2, is a reflection of these upstream processes. In response to 5 min of static passive stretch, p54 JNK and ERK 1/2 activation were not different between SOL, PLN and EDL despite large metabolic and fiber-type differences. However, in response to stretch of the whole MG, the deep and superficial compartments exhibited heterogenous MAPK activation, whereby the response of the deep compartment was more than double that of the superficial. This heterogeneity was abolished by mechanical stimulation in the form of isometric contractile activity,

suggesting that architectural factors may be responsible rather than metabolic or fiber-type differences. It is concluded that there are no differences as a result of metabolic profile or fiber-type composition across muscles in the detection of mechanical stimulation and in the transmission of mechano-signaling down to the MAPK cascades.

KEYWORDS

skeletal muscle

mitogen-activated protein kinase

mechanotransduction

passive stretch

fiber-type

INTRODUCTION

Pattern of activity regulates muscle phenotype, and mechanical loading is a factor of this regulation (7, 11). However, the mechanisms by which physical forces are detected and converted into biochemical signaling, and how these forces impact gene regulation are poorly understood. A comparison of the response to mechanical stimulation of muscles having different metabolic profiles and fiber-type composition may be revelative of the functioning of these processes.

While mechano-signaling pathways as a whole are not well characterized, the mechanical-responsiveness of the mitogen-activated protein kinases (MAPKs) has received considerable attention. MAPKs are sensitive to mechanical stimulation in numerous cell types, including skeletal muscle where recently, p54 JNK MAPK activation has been shown to be quantitatively related to tension (10). Furthermore, activation of this kinase is quantitatively related to peak developed tension and, in the presence of constant peak tension, to the tension-time-integral (10). ERK 1/2 also exhibit mechanical responsiveness. However, the mechanical responsiveness of p38 in muscle is equivocal.

The goal of the present study was to test whether differences exist in the mechanotransduction or early signaling processes of different muscles

displaying a wide range of metabolic properties and fiber-type compositions. This was addressed by comparing the activation of p54 JNK and ERK1/2 MAPKs in soleus (SOL), plantaris (PLN), and extensor digitorum longus (EDL) muscles, as well as within the two metabolically and functionally distinct compartments of the medial gastrocnemius (MG) muscle, in response to a standardized mechanical stimulation. Passive stretch was employed as a form of mechanical stimulation in order to better parcel out the effects of mechanical stress from the confounding metabolic processes associated with contraction.

An influence of muscle fiber-type composition on mechanical sensitivity of MAPKs has previously been suggested from the comparison of JNK activation to an in-vitro supra-physiological stretch in rat soleus and extensor digitorum longus muscles (3). By contrast, the same two muscles were shown to exhibit a similar ERK response to in-vitro contractile activity (14). The results of the present study suggest that metabolic profile or fiber-type composition do not affect muscle detection of mechanical stimuli or transmission of mechano-signaling down to the level of JNK and ERK MAPKs, but that architectural factors may be very influential to mechanosensitivity. These findings may contribute to the understanding of the regulation of muscle phenotype.

METHODS

Thirty four female Sprague-Dawley rats (Charles River Laboratories), weighing 195±5 g, were anaesthetized by intraperitoneal injection of ketamine and xylazine (61.5, 7.7 mg/kg, respectively). Animals underwent acute surgical isolation and mechanical stimulation of one of four hindlimb muscles, following which they were killed by anesthetic overdose. All procedures were approved by the animal ethics committee of the Université de Montréal and were in accordance with the guidelines of the Canadian Council of Animal Care.

Mechanical stimulation experiments were conducted on the soleus (SOL), plantaris (PLN), extensor digitorum longus (EDL) or medial gastrocnemius (MG) muscles. The in-situ muscle preparation previously described elsewhere (10) was employed for the plantaris and adapted to the three other hindlimb muscles. Briefly, the left PLN, SOL, or MG was isolated from the other extensors. For PLN, the proximal end was left attached to the MG. Partial MG isolation from the lateral gastrocnemius (LG) was performed by a cut through the natural division for the tibial and sural nerves, distally to the common tendon. Following isolation, the calcaneus was clipped, leaving a bone chip attached to the tendon and a silk ligature was securely tied around the bone-tendon interface. The animal was placed in the prone position within a stereotaxic frame with

the left foot clamped and the left knee pinned in a slightly flexed position. Isolation of the right EDL consisted of excising the tibialis anterior muscle and cutting the flexor retinaculum in order to free the EDL tendon. A silk ligature was sutured through the tendon. The animal was placed within the stereotaxic frame in the supine position with right knee pinned and foot clamped. For all muscles, the silk thread was attached to the lever arm of a muscle puller servomotor (305B-LR; Aurora Scientific). Care was taken to ensure no tension was placed upon the isolated muscle. The skin of the hindlimb was pulled into a bath filled with heated mineral oil. Animal core temperature and muscle bath temperature were carefully monitored and maintained at 36-37°C.

Standardized mechanical stimulation was imposed upon the four muscles by applying a tension-normalized mechanical stretch to the isolated muscle. Normalization was based on muscle weight since the four experimental muscles do not differ significantly in length (29±1.5 mm). Since actual weights of experimental muscles could not be measured prior to the experiment and since muscles are excised rapidly at the expense of precision immediately following the experiment, the following estimated muscles weights based on measurements of carefully dissected muscles from age and weight-matched control rats were employed: 130, 250, 110 and 675 mg, respectively for SOL, PLN, EDL and MG.

In order to minimize mechanical stimulation before the onset of the stretch protocol, length for optimal twitch tension development (L_o) was not empirically determined by twitch stimulation. Instead, immediately prior to the experiment, muscles were stretched to a standardized starting position producing a passive tension of 30 g/g of estimated wet muscle weight, and considered to approximate L_o, as based on pilot studies. The experimental procedure then consisted of stretching muscles to a tension of 300 g/g beyond starting tension, (i.e. 330 g/g or 43, 82, 36 and 223 g for SOL, PLN, EDL and MG, respectively) and approximating 10 x L_o tension. This tension was maintained precisely constant through instantaneous length adjustments made by the computer-controlled muscle puller, thereby compensating for any force decay of the muscle-tendon unit attributable to muscle creep or stress-relaxation. This tension was held for exactly 5 min, immediately following which the experimental muscle was excised and frozen in liquid nitrogen. For each experiment, the corresponding contralateral muscle served as a non-stimulated control, since pilot experiments demonstrated no effect of the in-situ preparation on the signaling pathways measured. Frozen muscles were stored at -80°C prior to biochemical analysis. The frozen MGs were visually separated into red (MGr) and white (MGw) portions, corresponding to deep and superficial compartments, respectively, in a cryostat using a cold razor blade, and individual MG compartments were processed separately.

In addition to the normalized passive stretch experiments described above, further control experiments were performed on the MG. First, passive stretch was applied to the intact gastrocnemius complex (MG + LG). L_o was estimated for the entire gastrocnemius muscle and passive stretch normalized to L_o + 300 g/g was applied for 5 min. Following this, the complex was rapidly separated and the MG was frozen. MG compartments were separated as described above.

Second, a cyclical passive stretch protocol featuring a length excursion of 6 mm beyond L_o, corresponding to approximately 20% of muscle length, was applied for 5 min using a sinusoidal function with a period of 800 ms delivered at a frequency of 1 Hz. This length-controlled stretch required precise determination of L_o by twitch stimulation. Through a bipolar electrode placed under the nerve, 5V single square pulses of 0.05 ms in duration were delivered once every 3 s while the muscle was slowly lengthened and twitch tension monitored. Electrical stimulation was ceased following determination of L_o and muscles were allowed to rest 5 min before onset of the stretch protocol.

Third, mechanical stimulation in the form of isometric contractile activity was applied to isolated MG. The muscle was set to L_o by twitch stimulation, as described above. Following a 5 min rest period, the muscle

was indirectly stimulated to contract tetanically at 100 Hz for 150 ms repeated once every second for 1 min. Muscles were excised 4 min following cessation of stimulation in order to keep sampling time identical to other experiments.

Tissue processing, electrophoretic separation of proteins, and measurements of phosphorylated p54 JNK, phosphorylated ERK 1/2, and total p54 JNK contents by immunoblotting using phospho-specific antibodies against JNK (Santa Cruz Biotechnology) and ERK (New England BioLabs) and an anti-JNK2 antibody (Santa Cruz Biotechnology), were performed as previously described in detail (10). Briefly, frozen muscles were weighed and powdered in liquid nitrogen. Approximately 100 mg of powder was solubilized in 10 volumes of ice-cold modified RIPA buffer containing a cocktail of protease and phosphatase inhibitors. Homogenates were vortexed frequently for 1 h at 4°C then centrifuged at 4500 g for 1h at 4°C. The protein concentration of the supernatant was measured by Bradford protein assay (Bio-Rad). Muscle lysates containing 180 µg of protein were prepared for SDS-PAGE by dilutions with Laemli reducing sample buffer followed by a 10-min immersion in near-boiling water. Samples were resolved on 9% gels and simultaneously transferred to a single PVDF membrane (Millipore). Equal sample loading was confirmed by Ponceau S stain. The membrane was blocked and incubated overnight at 4°C with primary antibody solution followed by a 90-minute

incubation at room temperature in secondary antibody solution. Revelation was performed by enhanced chemiluminescence (Amersham) with film exposure times ranging from 5 to 45 minutes. Films were scanned and bands quantified by NIH Image software. The same blot was probed with anti-phospho-JNK and anti-phospho-ERK, and then stripped and reprobed with anti-JNK2. In this way, three content measurements were obtained from each sample.

Measurements of fiber bundle length and sarcomere length and the calculation of sarcomere number were performed on a number of control muscles. Muscles were fixed at resting length in 10% formaldehyde with gentle agitation over three days. Following fixation, the MG was bluntly separated into its red and white compartments. Muscles were bathed in a 20% nitric acid solution until visible dissociation of fiber bundles and then stored in a 50% glycerol solution. The length of bundles, containing approximately 20 fibers each, was measured under a dissection microscope. For the MG, only bundles containing fibers tapered at both ends (i.e. not cut) were measured. From these bundles, isolated fibers were observed under 1000 x magnification and the number of sarcomeres was counted in 50 μm segments at various points along the length of the fiber to obtain an estimate of mean sarcomere length. The total number of sarcomeres per muscle fiber for each muscle or compartment was obtained by dividing the mean fiber length by the mean sarcomere length.

For all experiments, data were analyzed by one-way ANOVA and Scheffe post-hoc test. Statistical significance was established at 0.05.

RESULTS

Standardized mechanical stimulation in the form of normalized static stretch of 300 g/g wet muscle weight applied for 5 min to SOL, PLN, EDL, MGw or MGr resulted in a significant ($p<0.05$) increase in content of phosphorylated p54 JNK (Figure 1). Baseline p54 phosphorylation levels measured in corresponding unstimulated, control muscles were not significantly different between these muscles (Figure 1A-inset). Similarly, total content of JNK, independent of phosphorylation state, was not different between these muscles (Figure 2A). The p54 JNK response of SOL, PLN, and EDL ranged between 4.4- to 6-fold baseline levels and was not significantly different between these muscles despite their wide range of metabolic profiles and fiber-type compositions. In contrast to this, the two compartments of the MG exhibited a clearly heterogenous p54 JNK response to 300 g/g of static tension applied to the whole MG (Figure 1A). The response of MGw was 5-fold baseline levels and not significantly different from SOL, PLN, or EDL, while the response of MGr was 13-fold baseline levels and significantly different ($p<0.05$) from all other muscles.

The ERK response to the normalized stretch was qualitatively similar to the JNK response but of smaller amplitude. In SOL, PLN, and EDL, stretch-stimulation resulted in significant ($P<0.05$) phophorylation ranging

from 1.9 - to 2.2 -fold baseline levels, with no statistical difference between muscles. For the MG muscle, a distinct compartmental response was again observed. MGr showed a response of 4.5-fold baseline levels that was significantly different from the response observed in MGw and all other muscles (Figure 3A).

To address the heterogeneity in compartmental response observed in MG, three additional sets of experiments were performed on this muscle. To verify whether isolation of the MG from the LG could account for the heterogeneous response, stretch was applied to the entire gastrocnemius muscle complex (MG + LG). This variation of the surgical procedure did not alter the heterogeneous response (data not shown). In order to determine whether the heterogeneous response could be induced by a different type of passive stretch, a more physiological protocol, consisting of sinusoidal cyclical stretch, was administered. This protocol also resulted in the conservation of the heterogeneous compartmental JNK response (Figure 4).

Finally, to address whether mechanical stimulation in the absence of length change would also induce a heterogeneous compartmental response, isolated MGs were stimulated to contract isometrically for 1 min. Results show a 7- and 6-fold increase in JNK activation above baseline levels for MGr and MGw, respectively, and thus abolishment of compartmental

heterogeneity (Figure 5).

Fiber length was found to be similar between SOL, PLN and EDL with mean sarcomere numbers per fiber of 5079 ± 417, 4854 ± 87 and 4712 ± 351, respectively. However, when comparing the two compartments of the MG, fiber lengths of the MGw and MGr were significantly different with MGw containing 50% more sarcomeres than MGr (6059 ± 280 and 4049 ± 380, respectively). A difference in mean sarcomere length between the two compartments was also observed (2.42 and 2.03 μm for MGr and MGw, respectively), despite fixation of the whole muscle at near resting length prior to separation of the compartments.

DISCUSSION

The activation of mechanically sensitive JNK and ERK MAPK was compared in response to standardized mechanical stimulation in the form of normalized passive stretch between three rat hindlimb muscles spanning a wide range of fiber types and metabolic profiles but featuring simple architecture. In addition, mechanically-induced activation of JNK and ERK was compared in two metabolically distinct compartments of a single complex muscle. The results of this study demonstrate that mechanically-induced activation of muscle is similar in SOL, PLN and EDL. However, in response to the same standardized stimulation applied to the whole MG, the two compartments of this muscle exhibit striking heterogeneity in MAPK activation such that the deep, red region displays significantly higher sensitivity than the superficial, white region.

p38 activation in response to mechanical stimulation was not investigated as the mechanical responsiveness of this family of kinases remains in question in skeletal muscle. We have previously reported no activation in response to 5 min. of contractile activity (10) and others have reported only very subtle activation in response to contractile activity (8) or passive stretch (13). Contrasting reports of contraction-induced p38 phosphorylation in muscle (3, 12, 15) investigated the response at time periods exceeding 10

min. following the initial stimulus. This suggests that p38 follows a delayed time course of activation relative to JNK and ERK.

The finding that SOL, PLN, and EDL did not differ in MAPK activation to a standardized stress refutes the hypothesis that mechanosensitivity differs between muscles composed of different fiber types. This is consistent with the report that ERK activation in response to in-vitro contractile activity is similar in rat EDL and SOL (14) and concurs with a study demonstrating no difference in JNK activation between EDL and SOL in response to a static stretch of ~250 g/g or of ~500 g/g (3). This latter study reported a difference in response to extreme passive stretch tension on the order of 1000 g/g. This finding may have been due to differences in rate of force decay, or alternatively may indicate an uncoupling between the sub-maximal and maximal response of mechanically-induced signaling at a non-physiological stretch tension.

An absence of difference in mechanosensitivity among these three muscles indicates that the process of mechanotransduction and the ensuing signaling events, at least as far as the MAPK cascades, are not dependent on fiber-type composition. As MAPK cascades impact transcriptional and translational regulation (2, 4), these data would suggest that a mechanical hypertrophic stimulus will be relayed to the myonuclei and ribosomes

equally well in all muscle fibers. It is unknown whether differences in events distal to the MAPKs result in divergent responses to an identical stimulus.

In contrast to the results of the inter-muscle comparison, the comparison of MAPK activation in distinct compartments of a single complex muscle resulting from passive stretch of the entire muscle demonstrates intra-muscle heterogeneity. This heterogeneity was observed in response to the same passive stretch protocol applied to the SOL, PLN, and EDL muscles, as well as in response to a more physiological protocol consisting of cyclical sinusoidal stretches. Additionally, the heterogeneous response between MGr and MGw was conserved when stretch was applied to the entire gastrocnemius complex (MG+LG). However, the heterogeneity was abolished when mechanical stimulation was induced by isometric contractile activity of the whole muscle, a protocol expected to induce similar levels of maximal peak tension per cross-sectional area in all fibers. One minute of contractile activity was chosen to minimize the potential impact of different rates of fatigue between the two compartments on peak tension development. Contractile activity resulted in MAPK activation in both compartments of the same magnitude as that induced by passive stretch in the MGw and in the other three muscles, which indicates a significantly smaller response in the MGr than that induced by the passive

stretch protocols.

The divergent response to active and passive mechanical stimulation suggests that there are no differences in MAPK mechanosensitivity between the fibers of the two compartments of the MG in response to similar mechanical stimulation. The heterogeneity observed in response to passive stretch stimulation is likely the result of different magnitudes of mechanical stimulation applied to the fibers of the two compartments during stretch of the whole muscle. The finding of significantly different sarcomere lengths in the two compartments of an MG fixed prior to separation, which is in line with reports of different L_o for the two compartments (5, 6), suggests that at any given length, MGr is under more tension than MGw. Furthermore, differences in the length of the fibers of these two compartments, whereby MGw fibers are 50% longer than MGr fibers, may also contribute to a disparity in stress subjected onto the two populations of fibers.

In addition to fiber length, fiber cross-sectional area may perhaps also influence mechanosensitivity, whereby a smaller fiber may be subjected to more stress than a larger fiber. The range of fiber cross-sectional area between SOL, PLN, and EDL (3050 μm^2 to 4050 μm^2; (1)) had no effect on the MAPK response in these muscles. However, in the MG, a similar range in

fiber cross-sectional area (3000 μm^2 in MGr vs. 4000 μm^2 in MGw (1)) in conjunction with the discrepancy in fiber length, results in an almost two-fold range in fiber volume between these two compartments. Differences in fiber length or sarcomere number for the two compartments of MG have previously been reported (9). If two fibers differing in length but not in cross section are simultaneously pulled through the same length excursion, the shorter fiber with fewer sarcomeres will be subjected to a greater strain. Therefore, while it is valid to normalize actively developed forces according to cross-sectional area, the normalization of passively applied forces requires that, in addition to cross-section, deformation of fibers in relation to their length or sarcomere number be considered.

The results of the inter- and intra-muscle comparisons support that there are no intrinsic differences in MAPK mechanosensitivity between muscle fibers of similar sizes but of various fiber types in response to normalized mechanical stimulation. However, the divergent responses of the compartments of the MG to stimulation by passive stretch and by isometric contractions reveal that at the tissue level in-vivo, architectural factors such as fiber length, diameter, angle relative to the muscle force vector, and motor unit distribution, all contribute to the "physiological mechanosensitivity" of a fiber in response to mechanical stimulation in the form of normal movement composed of passive stresses and anisometric

active stresses. Thus fibers of different muscles as well as fibers of a same muscle will exhibit heterogeneity in their signaling response to overload in a rehabilitation or training setting due to architectural factors, irrespective of fiber type.

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FIGURE LEGENDS

Figure 1A. Effects of 5 min. of normalized (L_o + 300) passive stretch on phospho-JNK content. Data expressed as fold increase over baseline levels. For all muscles, stretch-induced activation is significantly different from baseline activation ($p<0.05$). * denotes significantly different stretch activation of the MGr as compared to other muscles ($p<0.05$). Inset graph shows baseline phospho-JNK content measured in unstimulated muscles and expressed in arbitrary densitometry units (a.d.u.). Data expressed as means ± SEM for a sample size of 6 for each muscle in the stretch condition and a sample size of 3 for muscles in the baseline condition.

Figure 1B: representative blot of phospho-JNK in SOL, PLN, EDL, MGr and MGw following passive stretch for 5 min. (top) and baseline phospho-JNK content measured in unstimulated control muscles (bottom). For presentation purposes, two representative samples for each muscle were taken from a single overexposed blot. All samples were simultaneously analyzed on a single blot. The same blot was probed with anti-phospho-JNK and anti-phospho-ERK, and then stripped and reprobed with anti-JNK2. In this way, three content measurements were obtained from each sample.

Figure 2A. Total muscle JNK content, irrespective of phosphorylation state, expressed in arbitrary densitometry units. Measurements were performed on the same muscle samples used for determination of phospho-JNK content. Data expressed as means ± SEM with a sample size of 6 for each muscle.

Figure 2B: representative blot of total JNK protein content for each muscle. For presentation purposes, two representative samples for each muscle were taken from a single blot.

Figure 3A. Effects of 5 min. of normalized (Lo + 300 g/g) passive stretch on phospho-ERK content. Data expressed as fold increase above baseline levels. Stretch-induced activation is significantly different from baseline activation ($p<0.05$), for all muscles except MGw. Different capital letters (**A-C**) denote significantly different stretch activation as compared to other muscles ($p<0.05$). Inset graph represents baseline phospho-ERK content, expressed in arbitrary densitometry units (a.d.u.). Different letters (**a-c**) denote significant differences ($p<0.05$) in baseline activation. Data expressed as means ± SEM with a sample size of 6 for each muscle in the stretch condition and a sample size of 3 for muscles in the baseline condition.

Figure 3B: representative blot of phospho-ERK content in SOL, PLN, EDL, MGr and MGw following 5 min. of passive stretch (top) and baseline phospho-ERK content measured in unstimulated control muscles (bottom).

For presentation purposes, two representative samples for each muscle were taken from a single blot.

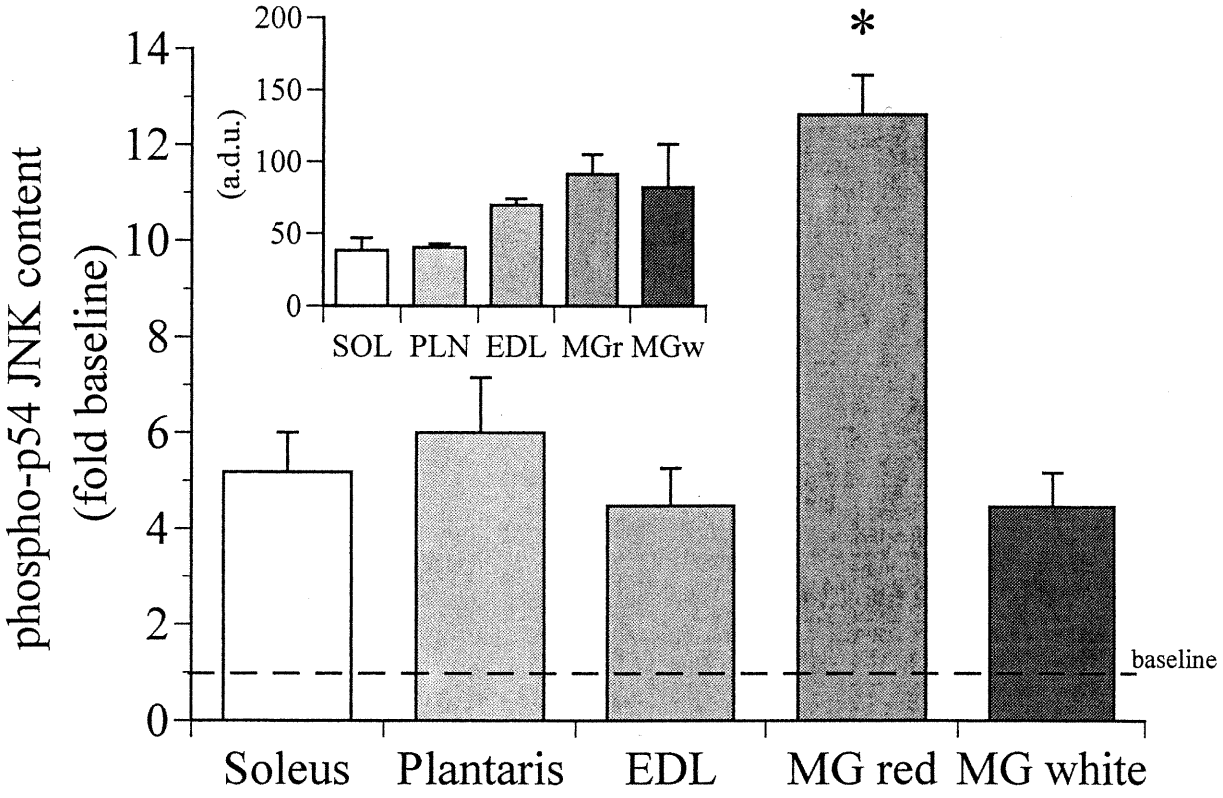
Figure 4. Phospho-JNK content in the deep, red compartment (MGr) and the superficial, white compartment (MGw) of medial gastrocnemius muscle in response to cyclical stretch of 6 mm beyond L_o , repeated once every second for 5 min. A heterogeneous response is also observed to result from this type of stimulation. Data expressed as means ± SEM for a sample size of 2. * denotes a significant difference between the two compartments of the same muscle ($p<0.05$).

Figure 5. Phospho-JNK content in response to 1 min. of tetanic isometric contractions of MG (100 Hz, 150 ms/s, repeated once per second) measured at 5 min. following the onset of contractions. A heterogeneous response is not observed to result from active isometric stimulation. Data are means ± SEM for sample sizes of 4 (baseline groups; light bars) and 3 (stimulated groups; dark bars).

Figures

Figure 1

A


phospho-p54 JNK content
(fold baseline)
14
12
10
8
6
4
2
0
200
150
100
50
0
(a.d.u.)
SOL PLN EDL MGr MGw
*
baseline
Soleus
Plantaris
EDL
MG red
MG white

B

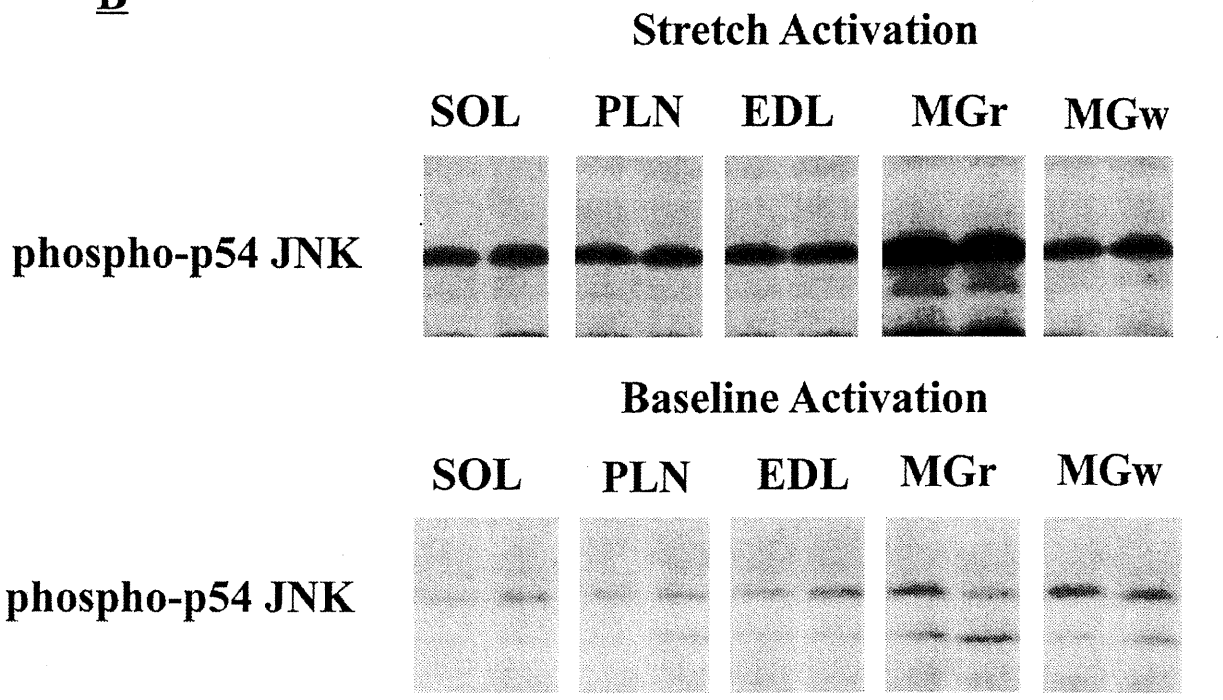
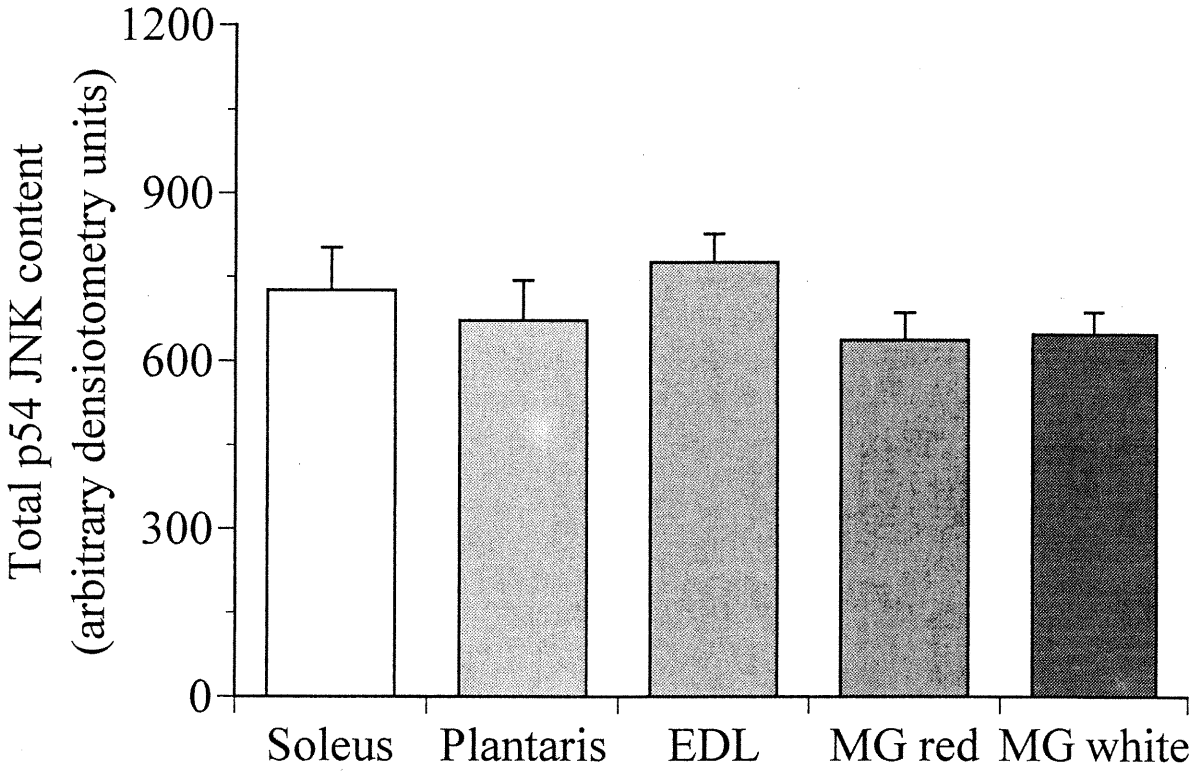

Stretch Activation
SOL
PLN
EDL
MGr
MGw
phospho-p54 JNK
Baseline Activation
SOL
PLN
EDL
MGr
MGw
phospho-p54 JNK

Figure 2

A


Total p54 JNK content
(arbitrary densiotometry units)
1200
900
600
300
0
Soleus
Plantaris
EDL
MG red
MG white

B

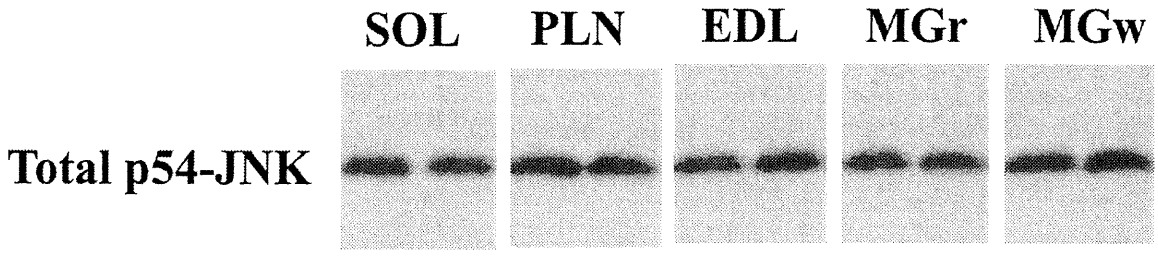
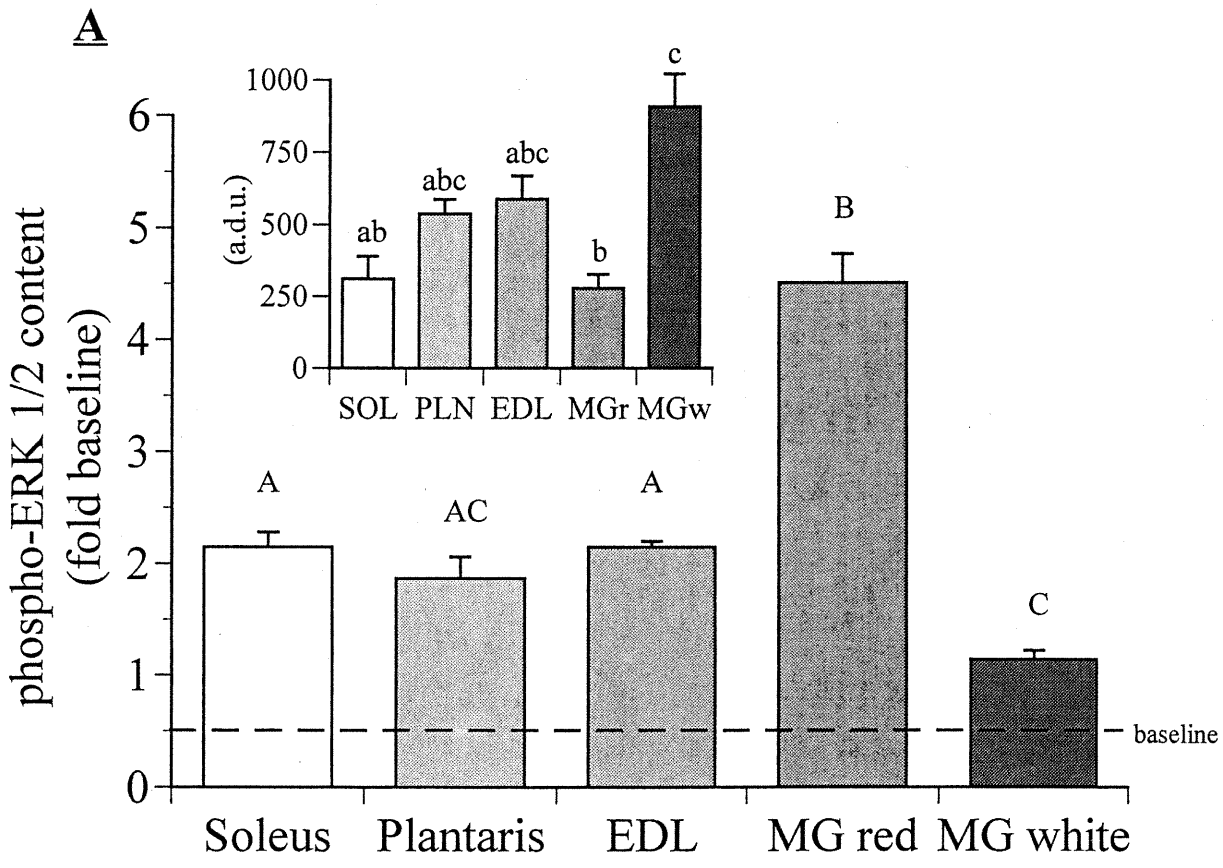

SOL
PLN
EDL
MGr
MGw
Total p54-JNK

Figure 3


A
phospho-ERK 1/2 content
(fold baseline)
0
1
2
3
4
5
6
A
AC
A
B
C
baseline
Soleus
Plantaris
EDL
MG red
MG white
1000
750
500
250
0
(a.d.u.)
ab
abc
abc
b
c
SOL
PLN
EDL
MGr
MGw

B

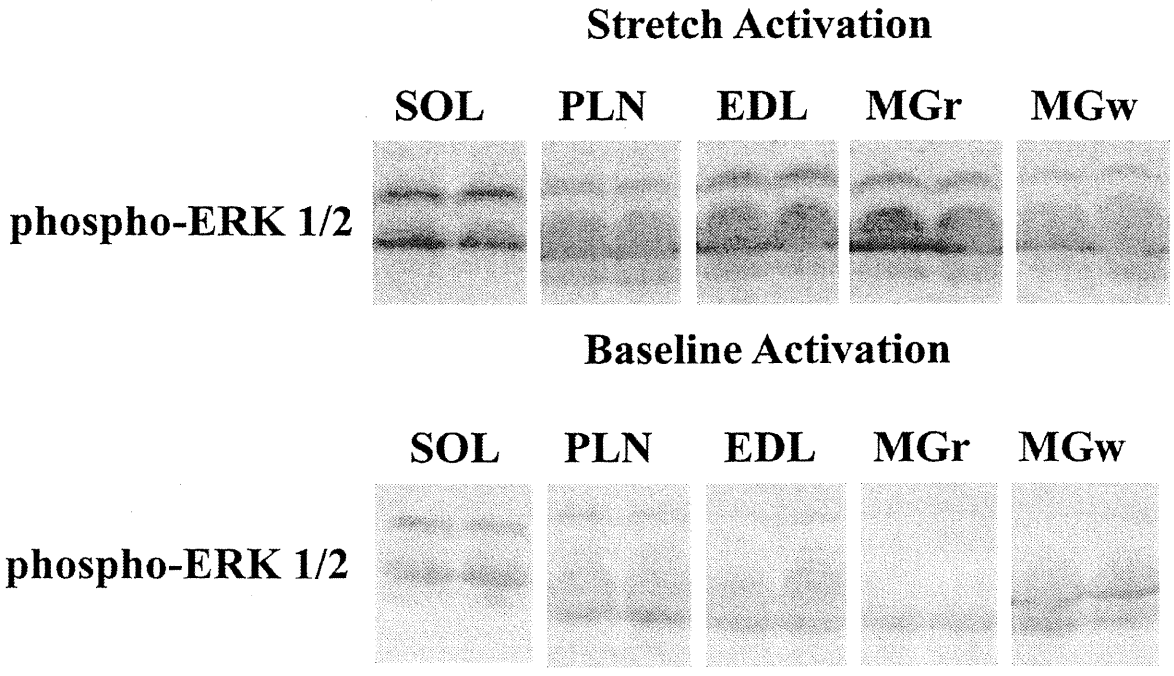
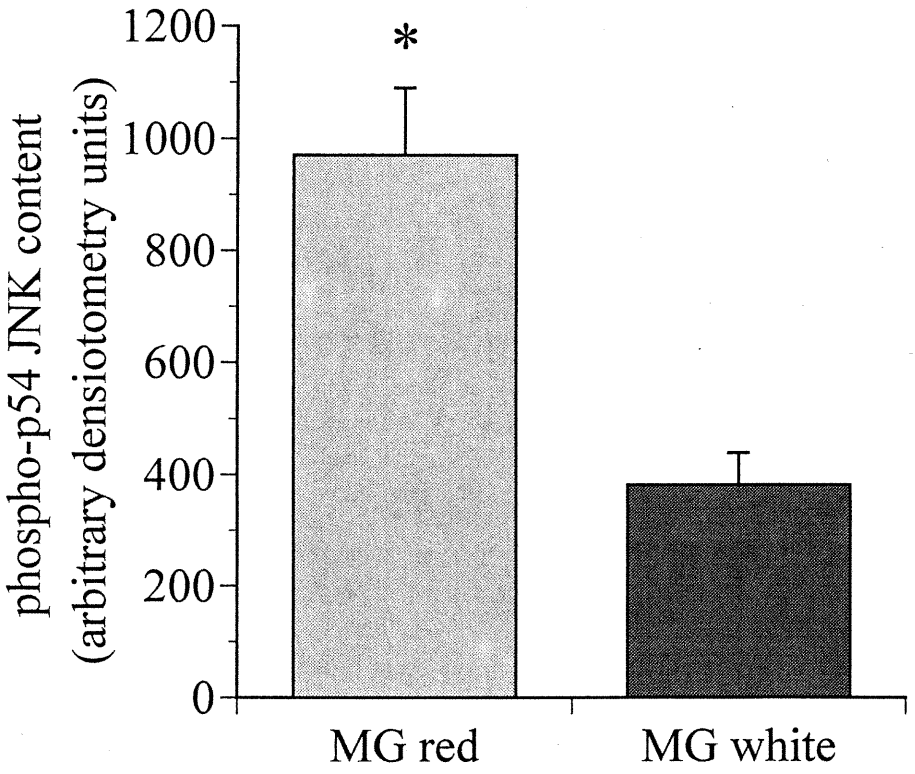

Stretch Activation
SOL
PLN
EDL
MGr
MGw
phospho-ERK 1/2
Baseline Activation
SOL
PLN
EDL
MGr
MGw
phospho-ERK 1/2

Figure 4


1200
1000
800
600
400
200
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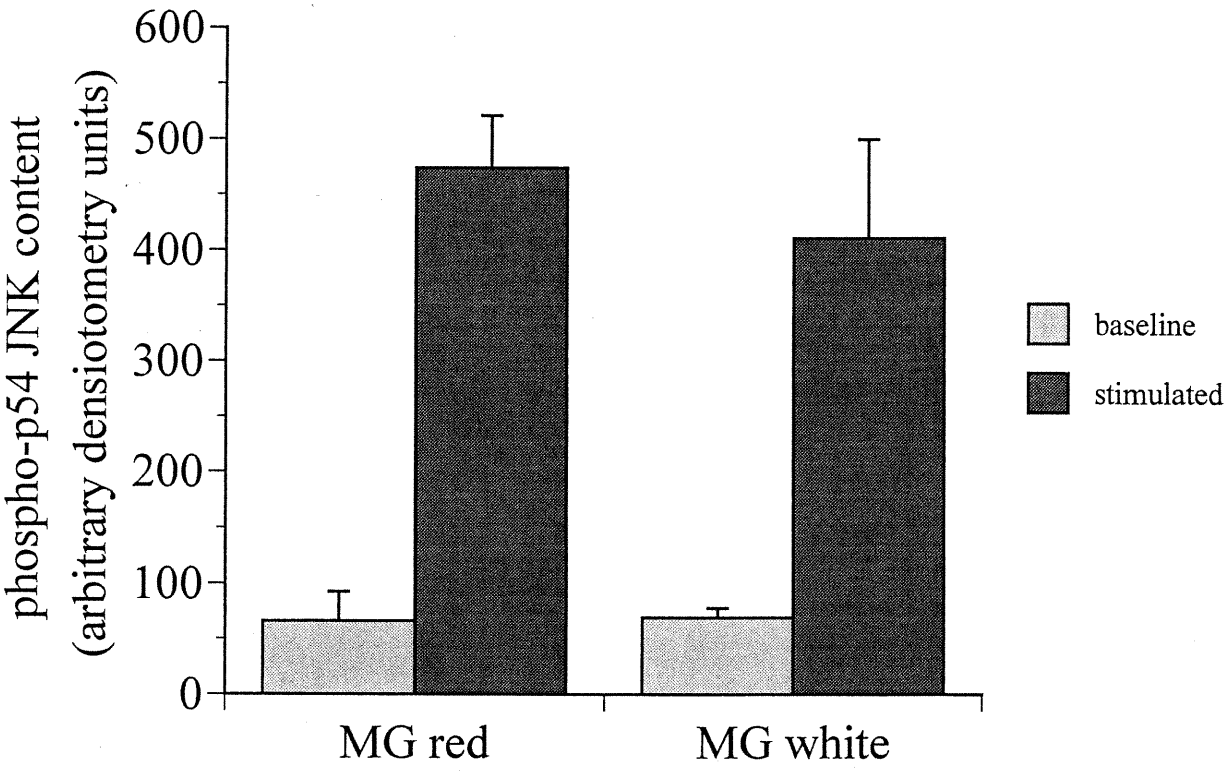
*
phospho-p54 JNK content
(arbitrary densiotometry units)
MG red
MG white

Figure 5


phospho-p54 JNK content
(arbitrary densiotometry units)
600
500
400
300
200
100
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MG red
MG white
baseline
stimulated

Chapter 3- Conclusions

This study employed a model of passive stretch, conceivably the simplest and most easily studied form of mechanical stress, to stimulate MAPK activation in rat hindlimb muscles of differing fiber type, size, architecture, and stiffness.

A feature unique to this study was the use of a computer-controlled muscle lever system to monitor the tension applied to the muscle-tendon unit. The characteristic tendency for muscle tension to decay with time, a phenomenon described in viscoelastic tissue as force-relaxation, has been attributed to factors related to compliance of the aponeurosis (Huijing et al. 1989) and muscle creep, defined as the tendency for tissue to elongate over time when subjected to a constant tensile force. An obvious advantage of this stretch protocol is that any decrease in tension generated by slack or the "stretching-out" of the muscle contractile proteins is compensated through incremental adjustments in the amplitude of muscle stretch by the servomotor such that constant tension is maintained throughout the experimental period.

The data from this study suggest two important points: 1) in terms of their JNK and ERK MAPK activation, muscles of different fiber composition respond to a standardized mechanical stress in a similar fashion, and 2) stretch of the MG, whether passive or dynamic, elicits a nonhomogeneous

response from its deep and superficial compartments such that the deep portion appears more sensitive.

Regional differences in fiber characteristics and distributions in deep and superficial compartments of MG are documented (De Ruiter et al. 1995a, Gardiner et al. 1991, Roy et al. 1985). The MG is a compartmentalized muscle consisting of a white superficial (lateroposterior) portion in which no type I fibers are present and a red deep (medioanterior) portion consisting of a mixture of type I and type II fibers (De Ruiter et al. 1995a). Reports on the nonhomogeneous architecture of the MG have described two compartments with two pennation patterns and two different optimal lengths, (De Ruiter et al. 1995a, Gareis et al. 1992, Huijing et al. 1989). Hence, it is conceivable that a muscle weight-normalized stretch applied to the MG might misrepresent the true magnitude of tension imposed upon the different MG compartments such that the deep compartment 'sees' greater tension as a result of its more complex structural arrangement.

Another potential explanation relates to fiber area. DeRuiter and colleagues (1995) observed type II fibers in deep MG that were more numerous and smaller in cross-sectional area than fibers in the superficial MG. Similarly, in three other studies whereby different muscle-fiber populations and corresponding fiber areas were quantified, the fibers of the deep MG are

reported as being smaller in cross-sectional area compared to fibers in superficial MG (Armstrong and Phelps 1984, Roy et al. 1985), compared to fibers of soleus and EDL (Armstrong and Phelps 1984, Kraemer et al. 2000), and compared to plantaris fibers (Armstrong and Phelps 1984). However, it is doubtful that muscle-fiber size alone is the principle factor responsible for the notably heightened MAPK activation response in deep MG. Furthermore, if indeed fiber area represented a primordial element in the determination of muscle sensitivity to mechanical stress, we might expect a diminished response in soleus due to the characteristically large proportion of type I fibers (typically fibers with the largest cross-sectional area) populating this muscle. This was not observed.

A third possible explanation for the significantly greater MAPK activation in the MGr relates to its placement and functional role within the MG. It has been proposed that deep fibers of the MG may be involved primarily in producing submaximal sustained postural contractions and are likely to be activated in vivo during activities requiring relatively low power outputs for long periods of time. In contrast, the superficial compartment is likely recruited to generate the more dynamic, powerful contractions during locomotion (De Ruiter et al. 1995b). It is conceivable that several of these possible explanations are concomitantly applicable and together explain the observed deep MG response.

The observations of this study may have far-reaching implications in a rehabilitation context. Anatomically, the MG compartments are innervated by separate branches of the primary muscle nerve (Gardiner 1993). Also, the number and soma size characteristics of the motorneurons supplying the two nerve branches are different (Vanden Noven et al. 1994). Thus each neuromuscular compartment contains a distinct population of motor units. There is likely to be a differential recruitment of motor units from these two regions of the MG for different functional tasks. This non-uniform organizational design of compartmentalized skeletal muscles could have implications for force-development and thus may predispose certain localized areas to neuromuscular damage and dysfunction.

While the results of this in-situ study suggest that there is no difference in the potential of different muscles to sense and transduce mechanical signals, at least to the point of MAPK activation, it would be unwise to make inferences pertaining to whole muscle adaptational responses to an exercise stimulus. Exercise is a physiologically complex stimulus that integrates the action of the cardiovascular, endocrine, musculoskeletal, and central nervous systems. Due to the highly standardized, in-situ nature of this study, muscles were removed from the context and connectedness of integrated physiological function where input from other systems would

otherwise occupy an influential role. While the conclusions of this study have little direct application to the in vivo reality of muscle function and adaptation, this mechanistic study might serve as a reference for future studies related to muscle signaling in response to mechanical stress.

To conclude, activation of MAPK signaling molecules in passively stretched muscles in situ appears to be unrelated to muscle fiber-type composition. In reference to the heterogeneous response of the compartments of the MG to mechanical stimulation by passive stretch, we suggest that at the tissue level, in vivo, architectural factors such as fiber length, diameter, and motor unit distribution collectively influence the physiological mechanosensitivity of a muscle. This hypothesis gains further support from the observation that following isometric contractions of the MG by electrical stimulation of the sciatic nerve, the heterogeneous compartmental response was abolished. Stimulation by isometric contractions is independent of the fiber length, fiber diameter and motor unit distribution within the different MG compartments. Therefore, the present findings suggest that muscle sensitivity to a standardized mechanical stress is similar among rat hindlimb muscles of differing fiber type and size.

A deeper understanding of the molecular mechanisms by which mechanical stimulation of muscle regulates muscle mass could have potentially important clinical implications with the development of countermeasures to prevent or diminish muscle wasting associated with injury, disease and ageing.

Appendices

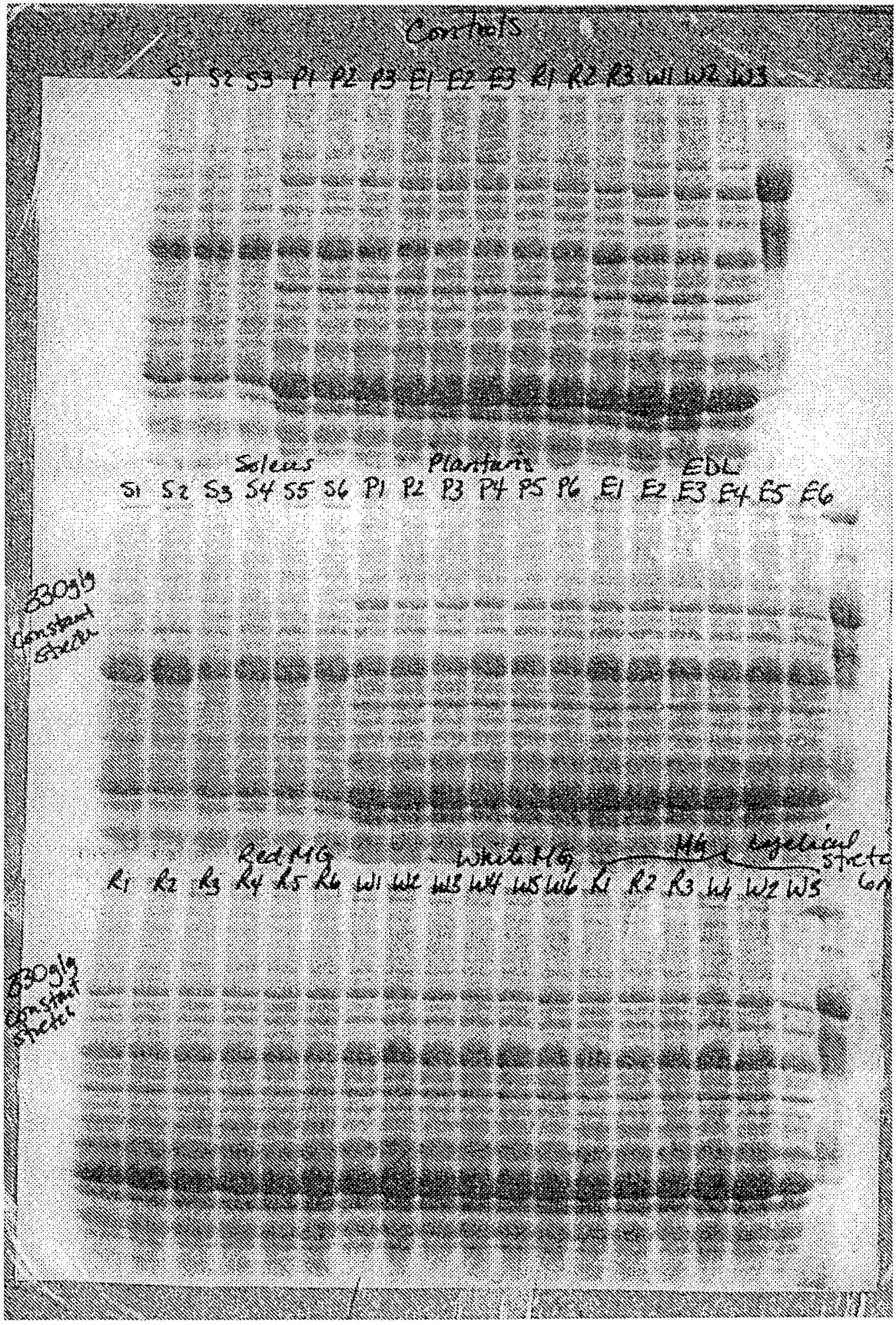
Appendix 1: Biochemical Procedures

Frozen muscles were weighed then powdered in liquid nitrogen then solubilized in ice-cold modified RIPA buffer (50 mM HEPES, pH 7.4,150 mM NaCl, 5 mM EGTA, 2 mM $MgCl_2$, 5% glycerol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Mini-Protease, Boehringer; 2 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (100 µM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride). Homogenates were vortexed frequently for 1 h at 4°C then centrifuged at 4,500 g for 1h at 4°C to remove insoluble material. The protein concentration of the supernatants was measured by Bradford protein assay (Bio-Rad) using known concentrations of bovine serum albumin (BSA) as standards. Muscle lysate samples of equal total protein concentration were prepared by dilutions with Laemmli buffer (60 mM Tris, 10% glycerol, 5% ß-mercaptoethanol, 2% SDS, pH 6.8) followed by a 10-minute immersion in near boiling (90-95°C) water.

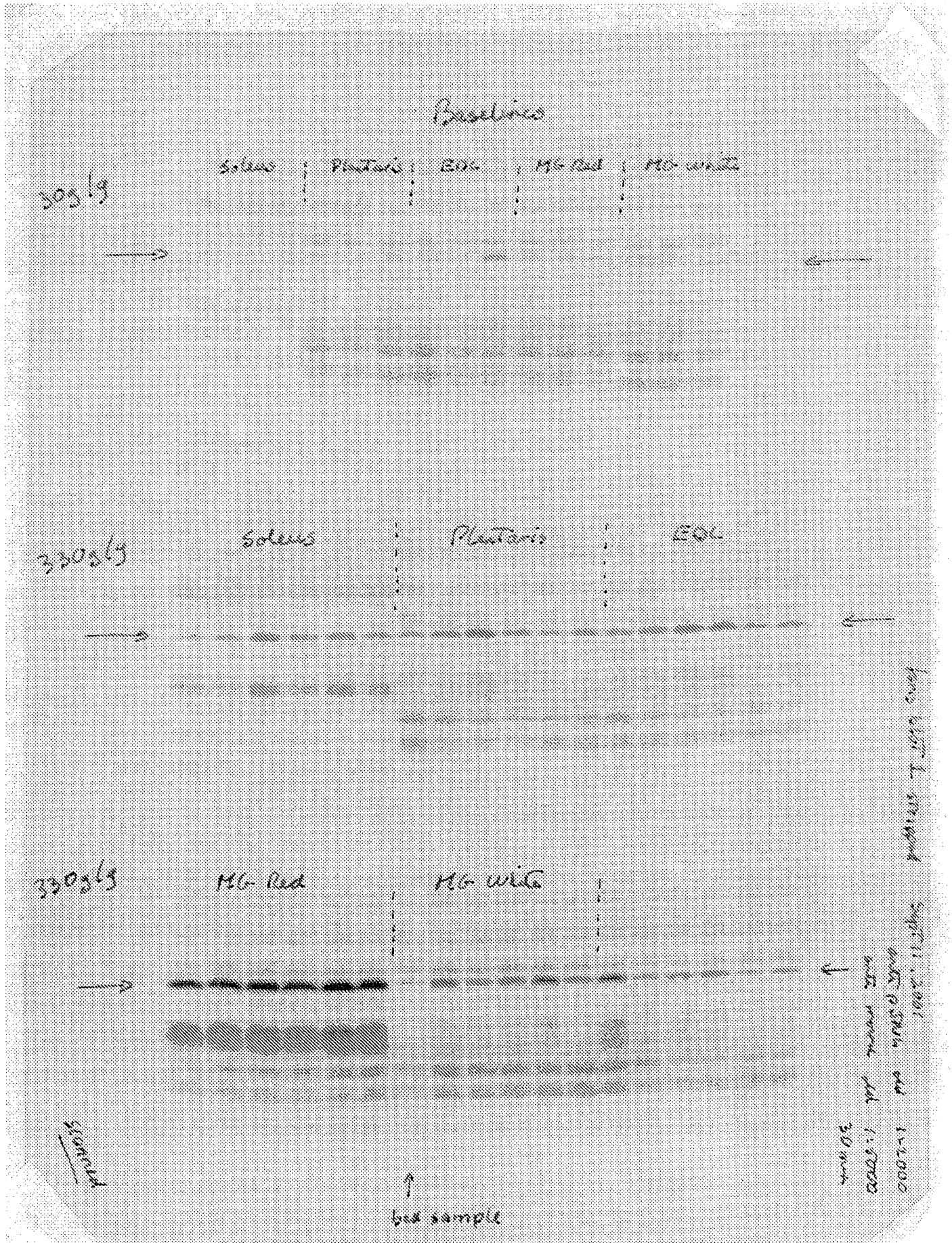
Muscle lysate samples containing 180µg of protein were resolved by 9% SDS-polyacrylamide gel electrophoresis overnight and transferred to a polyvinylidene difluoride membrane (Millipore). Staining with Ponceau S was used to verify equal sample loading. The membrane was blocked in Tris-buffered saline and Triton (TBST; 50mM Tris, 150mM NaCl, pH 7.4,

0.5% Triton X-100) and 3% BSA then incubated overnight at 4°C with primary antibody (either monoclonal anti-p54 JNK, anti-p42 ERK, total JNK, total ERK, Santa Cruz Biotechnology) of concentration 1:2000 in TBST plus 1% BSA plus 0.5% NaN3. After washes with TBST, the membrane was incubated for 90 min at room temperature with secondary antibody (Jackson Immunoresearch) of concentration 1:5000 in TBST and BSA. The signal was visualized by enhanced chemiluminescence (ECL, Amersham). Films were scanned and band intensity was quantified by densitometry using NIH Image software.

Appendix 2: Membrane Stained with Ponceau S



Appendix 3: Film showing p54-JNK Phosphorylation



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