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Study of the sensitivity of skeletal muscle to mechanical stimulation, as assessed by mechanically-responsive biochemical events

(Etude de la sensibilité du muscle squelletique à la stimulation mécanique)

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Summary

The purpose of this work was to broaden our limited understanding of how skeletal myocytes sense mechanical forces and how these forces are transduced into intracellular signals which regulate gene expression. A biochemical marker of the magnitude of mechanical stimulation induced by contractions or passive stretch in skeletal muscle was identified for quantitative use in conjunction with the rat in-situ hindlimb nerve-muscle preparation in order to study mechanotransduction. This marker, activation of the p54 JNK mitogen-activated-protein-kinase, was employed to parcel out the characteristics of mechanical stimulation to which myocytes are most sensitive. By stimulating muscles to contract concentrically, isometrically or eccentrically for 5 min, peak tension was recognized as a more important determinant of p54 JNK activation, measured immediately following stimulation, than the tension-time integral (TTI) or the rate of tension development (dT/dt). By systematically varying TTI and dT/dt with different passive stretch protocols, it was observed that, under conditions of constant peak tension, myocytes are insensitive to dT/dt while TTI is linearly related to p54 JNK activation. The study of the force-transmitting structures known as focal adhesions revealed that mechanical stimulation, in the form of stretch or contractile activity, induces a rapid tension-dependent decrease of the solubility of β1 integrins. This was interpreted as an increase in the concentration of strongly cytoskeleton-associated integrins, serving a cytoprotective function by increasing the number of force-transmitting proteins at focal adhesions. By using models of chronically increased and decreased muscle loading, it was observed that mechanosensitivity, the magnitude of the p54 JNK response to standardized mechanical stimulation, is inversely related to muscle size. Furthermore, it was also observed that the unstimulated concentration of strongly cytoskeleton-associated β1 integrins is inversely related to mechanosensitivity. These findings support the

hypothesis that mechanically-induced signaling is dependent on the number of integrins through which tension is transmitted, which is consistent with the apparently central role of integrins in mechanotransduction. The study of the functionality of mechanically-stimulated signaling in dystrophic muscle revealed that contraction-induced p54 JNK activation is depressed while β1 integrin tension-responsiveness is normal. These findings suggest that mechanotransduction is unaffected by a defect of the dystrophin-glycoprotein complex, but that such a defect may interfere with signaling.

As a whole, the findings of these studies significantly contribute to the understanding of the sensitivity of myocytes to mechanical stimulation and of mechanotransduction. These findings may help clarify the role of mechanical forces in the regulation of cellular volume, metabolism and cell survival in normal and diseased muscle and perhaps in other adherent cell-types. The link between mechanosensitivity and concentration of strongly cytoskeleton-associated β1 integrins introduces the prospect of artificial modulation of mechanosensitivity as a countermeasure for disuse atrophy or as a treatment for cardiac hypertrophy.

Sommaire

Cette thèse comporte cinq études qui visent à contribuer à la compréhension du mécanisme cellulaire par lequel les forces mécaniques sont détectées et déclenchent des signaux contrôlant l'expression génique dans les myocytes. Afin d'étudier la mécano-transduction, un marqueur biochimique de la stimulation mécanique induite par la contraction ou par l'étirement pouvant être utilisé de façon quantitative en conjonction avec la préparation in-situ du nerf sciatique et de la musculature du membre inférieur du rat, a été identifié. Ce marqueur, l'activation de la kinase p54 JNK, a été utilisé pour décrire les caractéristiques de la stimulation mécanique auxquelles les myocytes sont le plus sensibles. En stimulant des muscles à se contracter de façon concentrique, isométrique ou excentrique pendant 5 min, la tension maximale s'est révélée comme étant plus déterminante de l'activation de la p54 JNK, mesurée immédiatement après la période de contraction, que l'intégrale tension-temps (TTI) ou que la vitesse maximale de développement de tension (dT/dt max). En variant systématiquement la TTI et la dT/dt max par différents protocoles d'étirements passifs, il a été possible de déterminer que, dans des conditions de tension maximale constante, les myocytes sont insensibles à la dT/dt max tandis qu'il existe une relation linéaire entre la TTI et l'activation de la p54 JNK. L'étude des adhésions focales, qui sont les structures servant à la transmission des forces mécaniques, a montré que la contraction ou l'étirement induisent une diminution rapide de la solubilité d'intégrines β1, qui est proportionnelle à la tension maximale de stimulation. Cette réponse a été interprétée comme une augmentation cytoprotectrice de la concentration d'intégrines fortement liées au cytosquelette, servant à augmenter le nombre de protéines qui participent à la transmission des forces à l'adhésion focale. L'utilisation de modèles d'augmentation et de diminution chroniques du niveau de charge musculaire a permis d'observer une relation négative entre la mécano-sensibilité, soit l'importance de

l'activation de la p54 JNK en réponse à un niveau de stimulation mécanique standardisé, et la taille du muscle. Par ailleurs, ces études ont mis en évidence une relation négative entre la mécano-sensibilité et la concentration basale d'intégrines β1 fortement liées au cytosquelette. Ces résultats sont en faveur de l'hypothèse selon laquelle la signalisation déclenchée par la stimulation mécanique dépend du nombre d'intégrines participant à la transmission de la tension. Ceci concorde avec le rôle important que joueraient les intégrines dans la mécano-transduction. L'étude du fonctionnement de la signalisation déclenchée par la stimulation mécanique dans le muscle dystrophique a montré que l'activation de la p54 JNK stimulée par la contraction est diminuée tandis que la réactivité mécanique de l'intégrine β1 demeure normale. Ces résultats suggèrent que la mécano-transduction n'est pas affectée par une déficience au niveau du complexe dystrophine-protéines associées à la dystrophine, mais qu'une telle déficience peut avoir un effet néfaste sur la signalisation.

Les résultats de ces études contribuent à la compréhension de la sensibilité des myocytes à la stimulation mécanique ainsi que du mécanisme de la mécano-transduction, et devraient aider à mieux comprendre le rôle de la stimulation mécanique dans la régulation du volume cellulaire, du métabolisme et de la survie cellulaire dans les muscles normaux et dystrophiques, et probablement dans d'autres types de cellules adhérentes. La relation entre la mécano-sensibilité et la concentration d'intégrines β1 fortement liées au cytosquelette ouvre la possibilité de moduler artificiellement la mécano-sensibilité dans le but de lutter, par exemple, contre l'atrophie musculaire et l'hypertrophie cardiaque.

Keywords

mechanical stimulation
tension
mechanotransduction
mechanosensitivity
intracellular signaling
mitogen-activated-protein-kinase (MAPK)
cytoskeleton
integrin
dystrophin-glycoprotein-complex
in-vivo

Mots Clés

stimulation mécanique
tension
mécano-transduction
mécano-sensibilité
signalisation intracellulaire
"mitogen-activated-protein-kinase (MAPK)"
cytosquelette
intégrine
complexe dystrophine-protéines associées à la dystrophine
in-vivo

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List of Abbreviations

ANOVA:	analysis of variance
AP-1:	activator protein-1
BIO 14.6:	cardiomyopathic strain of syrian hamster
CSEP:	Canadian Society for Exercise Physiology
DGC:	dystrophin-glycoprotein complex
dT/dt:	rate of force development (first derivative of tension)
ECM:	extracellular matrix
ERK:	extracellular-regulated kinase (pERK: phospho-ERK)
FAK:	focal adhesion kinase
Grb2:	growth-factor-receptor-bound 2
IEG:	immediate-early genes
IGF-1:	insulin-like growth factor 1
JNK:	c-Jun-N-terminal kinase (pJNK: phospho-JNK)
L_o:	muscle length for optimal twitch tension development
LVG:	strain of golden syrian hamster
MAPK:	mitogen-activated protein kinase
MG:	medial gastrocnemius
MG:	mechanically-gated
PIP_2:	phosphatidyinositol biphosphate
RIPA:	radioimmunoprecipitation assay (buffer)
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2:	src homology-2
SOS:	son of sevenless
TTI:	tension-time integral
TTX:	tetrodotoxin

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Chapter 1

Introduction

Introductory statement

Mechanical forces exert a profound impact on cellular function and gene expression. The regulation of numerous cellular processes is influenced by these forces, including the control of cell morphology and volume, metabolism, cell-cycle progression, and survival. While recognition of the extent of the impact of mechanical forces on cellular function is growing rapidly, remarkably little is known about how these forces exert their effects. Namely, the mechanism of mechanotransduction, through which intracellular signaling pathways which ultimately regulate gene expression are triggered in response to mechanical stimulation remains to be completely understood. The goal of this thesis is to contribute to the elucidation of this fundamental mechanism.

The study of mechanotransduction can reveal the mechanical cues to which cells are responsive and lead to an understanding of how cells sense their environment. This knowledge can be used to identify mechanically-sensitive processes and to determine the importance of mechanical stimulation to their regulation, to increase the efficacy of interventions designed to stimulate these processes, and to design new therapeutic approaches to target these processes which mimic an alteration in the amplitude, frequency, or volume of mechanical stimulation imposed onto cells.

The mechanical environment of cells

All cells experience mechanical forces resulting from the physical interaction with their environment (Watson, 1991; Ingber, 1997). The fundamental process of cell adhesion can be considered a form of mechanical stimulation since, in order to spread and exhibit any form of specialized morphology, adherent cells must exert forces against the underlying extracellular matrix or

against neighboring cells though adhesion junctions, and are necessarily subjected to equal and opposite reaction forces (Ingber, 1997). The forces resulting from cell adhesion and cell spreading are relatively small and tonic but nevertheless have a profound influence on cellular function. For example, the regulation of cell cycle progression and cell survival are well known to be dependent on the mechanical interaction between cells and their environment, and inhibition of these interactions such as results from incompatibility between cell adhesion molecules and ECM composition, blocking of cell adhesion molecules with antibodies or peptides, or disruption of structural components of the cytoskeleton, can impair cell proliferation and differentiation, and even result in apoptotic cell death (Shyy and Chien, 1997; Ruoslahti, 1997; Giancotti and Ruoslahti, 1999).

A number of cell types routinely experience, and are responsive to much larger mechanical forces than simply result from adhesion and the maintenance of morphology. These cells types have been collectively termed mechanocytes (Goldspink and Booth, 1992) and include, among others, vascular endothelial (Chien et al., 1998) and smooth muscle cells (Williams, 1998), kidney podocytes (Endlich et al., 2001) and mesangial cells (Riser et al., 2000), airway epithelial and smooth muscle cells (Liu et al., 1999), osteocytes and osteoblasts (Duncan, 1995), chondrocytes (Grodzinsky et al., 2000), tendon fibroblasts (Eastwood, 1998), and cardiac (Yamazaki et al., 1995) and skeletal muscle fibers (Roy et al., 1991). These various cell types differ in the types of mechanical forces that they experience. For example, vascular and airway smooth muscle cells are predominantly subjected to strain forces resulting from the cyclical changes in blood or gas volume which they must accommodate (Williams, 1998; Liu et al., 1999). By contrast, vascular endothelial and airway epithelial cells are subjected to shear forces resulting from the flow of blood and gasses, respectively, over their apical surface, in addition to strain forces (Ballermann et al., 1998; Liu et al., 1999). Cells of the

skeletal system predominantly experience compressive and shear forces (Duncan, 1995; Turner and Pavalko, 1998; Grodzinsky et al., 2000). Finally, striated muscle cells experience strain (Yamazaki et al., 1995), and perhaps also shear forces. The exact nature of the forces to which the different mechanocytes are subjected remains to be fully characterized. Furthermore, little is known about whether all mechanical forces are equally well-perceived and induce similar cellular effects.

Cellular responses to mechanical stimulation

As stated in the introductory statement, numerous cellular processes are regulated by mechanical forces. However, mechanocytes appear to exhibit cell-type specificity in their cellular responses to mechanical stimulation. While the extent of the regulatory role of mechanical forces in various mechanocytes remains to be fully characterized, it stands to reason that every cellular process contributing to the structural and functional specialization of a mechanocyte is regulated by these forces, and that, in the absence of mechanical stimulation, mechanocytes lose or do not develop their specialization. Following this reasoning then, all processes that confer enhanced mechanical resistance to mechanocytes and all processes related to the primary function of the mechanocyte must be regulated by mechanical forces. Since common strategies for resisting to mechanical forces are likely to be employed across mechanocytes, or at the very least across mechanocytes subjected to similar types of forces, it can be expected that responses designed to regulate mechanical resistance are also shared. These might include acute and chronic remodelling of the cytoskeleton such as the rapid and local stiffening exhibited by focal adhesions and the cytoskeleton of cultured fibroblasts in response to mechanical stimulation (Wang et al., 1993; Wang and Ingber, 1995; Choquet et al., 1997; Glogauer et al., 1997; Wang, 1998).

Conversely, since the primary function of mechanocytes is cell-type specific, then it can be expected that a number of responses will also be cell-type specific. For example, the primary function of vascular endothelial cells is to impermeabilize blood vessels, and, as such, their morphology, cellular orientation, and rate of progression through the cell-cycle are all heavily dependent on flow patterns (D'Amore, 1992; Davies, 1995; Ballerman et al., 1998; Chien et al., 1998; Berk et al., 2001). In bone and connective tissue cells, processes involved in the secretion of collagen or other matrix molecules are tightly regulated by mechanical stimulation (Duncan, 1995). In striated muscle where force generation is the primary function and the most important determinant of force generation capacity is cross-sectional area (Roy et al., 1991), chronically increased loading elicits hypertrophy while decreased loading results in atrophy (Roy et al., 1991; Goldspink, 1999). This intimate relationship between fiber volume and magnitude of mechanical stimulation, which applies equally well to skeletal muscle fibers as to cardiac myocytes, can be viewed as the result of a mechanism for maintaining a balance between capacity and demand for force generation capacity. Mechanical stimulation also affects muscle fiber length since chronic immobilization of skeletal muscle in a fully extended position elicits an increase in the number of sarcomeres in series, while immobilization in a shortened position results in a loss of sarcomeres in series (Goldspink, 1999). Chronic alterations in blood volume produce similar effects in cardiac muscle (Rossi and Carillo, 1991). These effects likely serve to maximize myofilament overlap and force production at the favored operational muscle length. While the regulation of fiber volume is the most obvious process influenced by mechanical stimulation in striated muscle, there is evidence that numerous other processes are also regulated by mechanical forces. It is known, for example, that maintenance of the integrity of the contractile apparatus of cultured striated muscle cells prevented from contracting is dependent on externally applied forces (Simpson et al., 1995; 1996).

Furthermore, striated muscle cells in culture also possess the ability to alter their orientation and morphology in function of the direction of applied force (Vandenburgh et al., 1996), a property which has implications for tissue architecture. Finally, muscle metabolism is also sensitive to mechanical forces as non-insulin-dependent uptake of glucose by muscle, a phenomenon well known to be stimulated by contraction (Ivy, 1987), occurs in response to passive stretch in culture (Hatfaludy et al, 1989) and in vivo (Ihlemann et al., 1999).

Mechanotransduction

While mechanical forces are undeniably important regulators of cellular function, likely more important even than growth factors (Ingber, 1998), very little is known about how they achieve their cellular effects. The complex array of intracellular signaling pathways which are activated by mechanical stimulation are poorly characterized, and the fundamental mechanism through which mechanical forces trigger this intracellular signaling, known as mechano-chemical coupling or mechanotransduction, is not well understood.

Over recent years, significant progress has been made towards identifying cellular structures which are involved in mechanotransduction. There exists important evidence to suggest that mechanotransduction takes place at cytoskeletal structures called focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Pommerenke et al., 1996; Ingber, 1997; Shyy and Chien, 1997; Li et al., 1997; MacKenna et al., 1998; Schmidt et al. 1998; Chen et al., 1999; Jalali et al., 2001; Geiger and Bershadsky, 2001). These structures mediate cellular adhesion to the ECM and bidirectional force transmission between the cytoskeleton and the ECM (Burridge and Chrzanowska-Wodnicka, 1996).

Focal adhesions are constructed around integral membrane ECM-binding proteins, known as integrins (Hynes, 1987; 1992), which are attached via a complex protein scaffold to the sub-membranous cortical actin cytoskeleton (Burridge and Chrzanowska-Wodnicka, 1996). Cell adhesion by focal adhesions is mediated by large numbers of relatively low-affinity integrin interactions with ECM proteins. Contractile bundles of actin, which support the shape of a cell, insert into, and exert tension against the cytoplasmic face of focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Ingber, 1997). In striated muscle, the intra-sarcomeric cytoskeleton transmits the forces generated by the contractile apparatus to the ECM by way of lateral connections between Z-discs and focal adhesions, known as costameres, and by longitudinal connections between the terminal Z-discs and focal adhesions, known as myotendinous junctions (Small et al., 1992; Patel and Lieber, 1997; Berthier and Blaineau, 1997; Monti et al., 1999).

A large number of signaling proteins cluster at focal adhesions, and the number of signaling pathways with which focal adhesions are known to interact has grown steadily over recent years (Burridge and Chrzanowska-Wodnicka, 1996; Dedhar and Hannigan, 1996; Shyy and Chien, 1997; Longhurst and Jennings, 1998; Geiger and Bershadsky, 2001). Integrins are central to focal adhesion signaling (Hynes, 1992). The formation of new integrin-ECM interactions during focal adhesion formation triggers multiple signaling events involved in clustering and assembly of focal adhesion components, cytoskeleton remodelling, and in regulation of gene expression (Burridge and Chrzanowska-Wodnicka, 1996; Dedhar and Hannigan, 1996; Shyy and Chien, 1997; Longhurst and Jennings, 1998; Geiger and Bershadsky, 2001). It appears that during mechanical stimulation of focal adhesions, integrin-ECM interactions also trigger the resulting signaling, thereby reconciling the signaling commonalities observed between stimulation and focal adhesion formation (Shyy and Chien, 1997); the recent demonstration

that mechanically-induced signaling is abolished if integrins are inhibited from forming new interactions with the ECM strongly supports that the turnover of integrin-ECM low affinity interactions is the cue for focal adhesion signaling (Jalali et al., 2001) and suggests that mechanical forces are perceived as an increase in the rate of this turnover.

The initial signaling event triggered by integrins has proven difficult to elucidate. It is believed that binding of an integrin to an ECM ligand induces a conformational change within the molecule, and that this change is propagated across the membrane to the cytoplasmic domain of the integrin (Humphries, 1996; Tozer et al., 1996). The biophysics of such a propagation are not fully understood. As the short cytoplasmic domain of integrins does not exhibit any enzymatic activity, it is believed that a propagated conformational change exposes a binding site for signaling molecules. The recent observation that the src homology-2 (SH2) domain-containing adaptor protein Shc binds to integrin in response to mechanical stimulation (Chen et al., 1999) appears to confirm this hypothesis and enables a link to be made between integrins and mechanical activation of signaling pathways such as the mitogen-activated protein kinases.

In addition to focal adhesions, mechanical forces are transmitted through other types of adhesion structures which mediate cell-ECM and cell-cell adhesion (Gumbiner, 1996). In some cell types, cell-ECM adhesion is mediated by hemidesmosomes as well as by focal adhesions. Cell-cell adhesions are predominantly mediated by cadherin-containing adherens junctions, and in some cell types, by desmosomes. While little is known about the signaling function of these structures, a mechanotransduction role should not be discounted (Aplin et al., 1998; Vleminckx and Kemler, 1999; Green and Gaudry, 2000).

A number of diverse cell types, including striated muscle fibers (Ohlendiek, 1996), neurons (Powell and Kleinman, 1997; Mehler, 2000), and kidney podocytes (Raats et al., 2000), are characterized by specialized focal adhesions composed of a complex of proteins known as the dystrophin-glycoprotein complex (DGC), in addition to integrins and their scaffolding proteins. Much like the traditional components of the focal adhesions, the DGC forms a bridge between the cortical cytoskeleton and the ECM which is believed to mediate cellular adhesion (Engvall, 1994; Gumbiner, 1996; Ohlendiek, 1996) and, by extension, transmit mechanical forces. Interaction with the ECM occurs via the laminin-binding protein α-dystroglycan, and the large actin-binding protein dystrophin tethers the complex to the cytoskeleton. The function of the numerous proteins which make up the complex is largely unknown, and the relationship of the DGC to the traditional components of the focal adhesion is also unclear. This complex is thought to have an important structural role and confer mechanical resistance to cells. Evidence for this comes from the link between genetic deficiencies of various components of the DGC and predisposition to mechanical damage in striated muscle (Matsumura and Campbell, 1993; Petrof et al., 1993). Additionally, there is mounting evidence that, much like the focal adhesion, the DGC has important signaling functions and, furthermore, that signaling deficiencies may contribute to the etiology of the dystrophinopathies (Tidball et al., 1995; Hack et al., 1999; Wehling et al., 2001; Rando, 2001). A link between the DGC and the regulation of cell survival suggests that the DGC may have a mechanotransduction role. However, direct evidence to support this hypothesis is lacking.

There is an important body of literature supporting that mechanotransduction may take place at cellular locations other than the focal adhesion through alternate mechanisms. Such mechanisms are not mutually exclusive with mechanotransduction mediated by focal adhesions, but rather

may be complementary to this mechanism. For example, there exists evidence that forces applied locally at focal adhesions are instantaneously propagated by the complex and still poorly understood architecture of the cell to organelles such as the nucleus and ribosomes (Maniotis et al., 1997; Chicurel et al., 1998). These propagated forces have been observed to alter the structure of the nucleus (Maniotis et al., 1997), and this has been proposed to be a novel mechanism by which forces directly regulate transcription (Maniotis et al., 1997; Ingber, 1997; Ingber, 1998).

There also exists evidence that mechanically-gated (MG) ion channels, well known to regulate the excitability of specialized mechano-sensory cells (Hamill and McBride, 1996; Hamill and Martinac, 2001), may have a role in mechanotransduction in non-sensory cells. While these channels are well characterized electrophysiologically and pharmacologically (Hamill and McBride, 1996; Hamill and Martinac, 2001), very little is known about their structure or expression. Moreover, little is known about the biophysics of the gating mechanism. As the cell plasma membrane is not a load bearing structure (Ingber, 1997) and there is often a large excess of membrane (Hamill and Martinac, 2001), gating of these channels is more likely to be regulated by mechanical deformation of the cytoskeleton than by membrane stretch per se (Hamill and Martinac, 2001). This requires that the channels be tethered to the cytoskeleton, and such an interaction is supported by the finding that the sensitivity of MG calcium channels is modulated by changes in the stiffness of the cytoskeleton (Glogauer et al., 1997; Wu et al. 1999). While an influx of extracellular calcium through gadolinium-sensitive stretch-activated channels has been observed in response to physiological levels of mechanical stimulation in cultured cells (Rosales et al., 1997; Glogauer et al., 1997; Ikeda et al., 1998; Kushida et al., 2001), there is controversy regarding the importance of such an influx to the mechanical activation of signaling pathways (Ikeda et al., 1998; Kushida et al., 2001).

The fact that different types of mechanical forces are experienced by the various mechanocytes raises questions concerning the sensitivity of the mechanotransduction mechanism(s) to these different types of forces and concerning the possible existence of cell-type-specific specializations of the mechanotransduction apparatus conferring enhanced sensitivity to one type of stimulation at the expense of other types. While there are currently no satisfying answers to these questions, there is evidence that in cultured endothelial cells, shear and strain forces can elicit different responses (Ballerman et al., 1998; Chien et al., 1998). Additional questions which must be addressed deal with the temporal component of mechanical stimulation; while most of the research into mechanotransduction has been concerned with amplitude of stimulation, little is known about the sensitivity of the mechanism to frequency and volume of stimulation.

Mechanically-induced signaling

Only the briefest overview of the large number of mechanically-regulated signaling pathways will be presented here. In order to simplify this presentation, it is useful to group pathways based on their function: 1) pathways which produce an acute and proximal response, such as those involved in regulation of the cytoskeleton; 2) pathways which produce a chronic and distal response involving the regulation of gene expression.

In response to mechanical stimulation, acute and local cytoskeletal remodelling takes place which affects the mechanical behavior of the cell and its resistance to mechanical forces. This remodelling includes focal adhesion assembly (Balaban et al., 2001; Riveline et al., 2001), a local stiffening of focal adhesions and of the underlying cytoskeleton (Wang et al., 1993; Wang and Ingber, 1995; Choquet et al., 1997; Glogauer et al., 1997; Wang, 1998), and a local

accumulation of actin (Glogauer et al., 1997). These responses have only been studied in a limited number of cell types but, as discussed above, are likely to be universal responses in mechanocytes. Cytoskeletal remodelling and contractility are controlled by the Rho family of small GTPases, which includes Rho, Rac, and cdc42 (Tapon and Hall, 1997; Longhurst and Jennings, 1998; Aspenstrom, 1999). For example, Rho promotes cytoskeletal contractility through p160 Rho-associated kinase mediated control of myosin-light-chain phosphorylation, and promotes actin polymerization through p140 mDIA mediated control of profilin recruitment as well as through phosphatidylinositol 5-kinase mediated control of phosphatidyinositol biphosphate (PIP_2) production (Longhurst and Jennings, 1998; Geiger and Bershadsky, 2001). Upon mechanical stimulation, Rho translocates from a cytosolic to a membrane fraction, a process indicative of activation (Li et al., 1999; Numaguchi et al., 1999). However, the events upstream of a presumptive Rho activation in response to mechanical stimulation are unclear. It should be noted that the distinction between pathways producing an acute and local response and pathways controlling gene expression is blurred by evidence suggesting that Rho can affect gene expression (Wei et al., 2000; 2001).

The effects of mechanical stimulation on gene expression are mediated in large part by activation of mitogen-activated protein kinase (MAPK) pathways. These pathways are important points of convergence for growth factor and stress signaling, and have been reviewed in depth (Karin, 1998; Tibbles and Woodgett, 1999; Garrington and Johnson, 1999). Upon activation, MAPKs translocate to the nucleus where they phosphorylate a number of transcription factors such as activator protein 1 (AP-1) and Elk-1. MAPKs have been grouped into three families, each containing several isoforms. These families are the c-Jun N-terminal kinases (JNK), the extracellular signal-regulated kinases (ERK), and the p38 MAPKs. Response specificity is thought

to be achieved through spatial and temporal combinatorial signaling. MAPK activation has been observed to be mechanically-regulated in a large number of cell types including vascular smooth muscle (Zou et al., 1998), vascular endothelial cells (Berk et al., 1995), chondrocytes (Hung et al. 2000), cardiomyocytes (Sadoshima and Izumo, 1993), and skeletal myocytes (Aronson et al., 1997). While a great deal of progress has been made in elucidating the events upstream of mechanical activation of JNK and ERK, the details of these signaling cascades remain confused, due in part to an important cross-talk component. It must also be noted that most of the work in this area is based on a model of shear stimulation of vascular endothelial cells, and that pathways may differ in other cell types or in response to other forms of mechanical stimulation. One key finding is the participation of the p21 Ras GTPase in mechanical activation of both JNK and ERK pathways (Li et al., 1996; Chen et al., 1999). Ras also appears to be a point of convergence since it can be activated in response to mechanical stimulation by a complex composed of Shc, growth factor receptor-binding protein 2 (Grb2), and Son of sevenless (Sos)(Chen et al., 1999), or by Grb2 and Sos interacting with the non-receptor tyrosine kinases p60 Src or focal adhesion kinase (FAK)(Li et al., 1997; Jalali et al., 1998). The primary signaling events in response to mechanical stimulation remain for the most part elusive, but include the interaction of Shc and integrins (Chen et al., 1999)

Purpose of this thesis

The objective of the work described in this thesis is to provide insight into the mechanism of mechanotransduction for the purpose of furthering the understanding of this fundamental mechanism and of the mechanical regulation of muscle fiber volume. Physiological approaches are employed in order to gain insight into the biophysics of mechanotransduction in muscle. Specifically, by applying precisely measured forces in-situ and measuring the

outcome of this stimulation as the activation of a signaling event downstream of mechanotransduction, characteristics of mechanical stimulation to which the transduction mechanism is most sensitive are identified, biophysical constraints regulating the mechanism are established, the contribution of specific components of the cytoskeleton to the mechanism is addressed, and the regulation of the sensitivity of the mechanism through mechanically-induced remodelling of focal adhesions is explored.

Introduction to the manuscripts

This thesis is comprised of five related manuscripts contributing to the understanding of mechanotransduction and of the sensitivity of skeletal muscle to mechanical forces. This section provides a brief and candid introduction to each manuscript, addressing the background and aims of the studies, as well as their continuity. Because of the inherent difficulty in assessing a doctoral candidate's contribution to multi-authored manuscripts such as are included within this thesis, I have been instructed to also include a brief statement concerning my contribution and that of my co-authors for each of the manuscripts. All the studies presented here were funded by Phillip Gardiner with grants obtained from the Natural Sciences and Engineering Research Council of Canada.

"Insight into skeletal muscle mechanotransduction: Quantitative relationship between tension and MAPK activation"

This was the first study to build on a year of pilot work designed to identify an appropriate marker of mechanical stimulation in skeletal muscle which could be used as a downstream reporter or "assay" of the mechanotransduction process. Based on work by the Goldspink group which reported stretch-induced expression of immediate-early genes (IEG)

(Osbaldeston et al., 1995; Dawes et al., 1996) peaking 1 hour following onset of stretch and positively-related to the amplitude of stretch, we focussed on the MAPKs, the kinases responsible for activating IEGs and promoting IEG expression. MAPKs had also recently been reported by the Goodyear group to be activated in response to muscle contraction (Goodyear et al., 1996; Aronson et al., 1997). Finally, around the time that we undertook this work, the measurement of MAPK activity was simplified by the introduction of antibodies directed against the phosphorylated form of ERK, JNK, and p38 MAPKs. We focussed on ERK and JNK MAPKs, as these kinases were known to be downstream of signaling pathways regulated by focal adhesions. Using the in-situ nerve-muscle preparation of the rat hindlimb, well established in our laboratory, we subjected the rat medial gastrocnemius muscle to constant passive stretch of differing amplitudes and duration, as well as to intermittent tetanic isometric contractions. We observed rapidly-developed activation, on the order of a few minutes, of ERK 1/2 and p54 JNK. The activation of p54 JNK was much more pronounced than ERK 1/2 and was found to be positively related to stretch amplitude. The activation of p54 JNK became our marker of choice for all subsequent studies of mechanotransduction for the following reasons: 1) it was sensitive to both passively applied forces and to actively generated forces; 2) it exhibited a clear dose-response relationship to mechanical stimulation; 3) it was rapid, featuring minimal delay between stimulus and response; 4) it was well amplified and therefore easily detectable; 5) it was technically simple to measure. Parts of this pilot work were presented in 1999 at Experimental Biology and at the CSEP annual meeting (see Appendix 3 for abstracts).

Using p54 JNK activation as a marker of mechanical stimulation, we set out to test the hypothesis that tension was a determinant of stimulation. In order to subject muscles to very high tensions while maintaining a physiological length, it was necessary to develop tension actively. We designed an

experiment in which peak tension was varied over a wide physiological range by subjecting plantaris muscles to isometric, concentric and eccentric contractions using a newly-acquired muscle lever system. We observed peak tension-dependent activations of both p54 JNK and ERK 1/2, but not of p38. The relationship between peak tension and p54 JNK activation was highly significant, supporting the use of p54 JNK as a marker of tension-stimulation in subsequent studies. This work was presented at Experimental Biology 2000 and published in the Journal of Applied Physiology in 2001.

I am responsible for developing the hypothesis of this study, for its design, for the data collection, analysis and interpretation, as well as for the writing of the manuscript. Phil Gardiner instructed me in the physiological and electrophysiological techniques employed and contributed to the development of the hypothesis, the experimental design, the interpretation of the results, and to the correction of the manuscript.

"Skeletal muscle is sensitive to the tension-time integral but not to the rate of change of tension, as assessed by mechanically-induced signaling"

In the previous study it was determined that peak tension is an important determinant of mechanical stimulation in muscle, as assessed by activation of p54 JNK. It was also suggested that the tension-time integral (TTI) and the rate of tension development (dT/dt), temporal components of tension, could contribute to mechanical stimulation. This follow-up study was designed to formally test this hypothesis by systematically varying TTI and dT/dt under conditions of fixed peak tension. A simple method for accomplishing this was to cyclically stretch muscles to a fixed excursion amplitude. Stretching to a fixed tension would have been more precise, but the resulting variable length would have made it virtually impossible to exert control over dT/dt and TTI. Under conditions of controlled peak tension and dT/dt, TTI was found to be

linearly related to p54 JNK activation. Surprisingly, dT/dt was found to be unrelated to p54 JNK activation over a ten-fold range of dT/dt, under conditions of controlled peak tension and TTI. These findings were published in the Journal of Biomechanics in 2002.

I am responsible for developing the hypothesis of this study, for the data collection, analysis and interpretation, and for the writing of the manuscript. I also contributed to the design of the study. Phil Gardiner is largely responsible for the straight-forward design, and contributed to the interpretation of the results and to the correction of the manuscript.

"Contraction-stimulated p54 JNK activation is attenuated in δ-sarcoglycan-deficient skeletal muscle"

A goal of this thesis was to address whether the dystrophin-glycoprotein complex (DGC) is involved in mechanotransduction or mechanically-induced signaling, and whether a defect of this complex, as occurs in muscular dystrophy, impairs these processes. The DGC is believed to mediate cell adhesion to the basement membrane in parallel with integrin-based focal adhesions, and to contribute to the mechanical resistance of skeletal muscle cells. There is emerging evidence suggesting that, like the focal adhesion, the DGC may have signaling functions, and that the dystrophinopathies may be the result of deficient signaling rather than impaired mechanical resistance. δ-sarcoglycan deficient hamsters, available from a colony maintained at the Université de Montréal, were employed for this study and compared to age-matched golden syrian hamsters from Charles River. p54 JNK activation in response to intermittent tetanic contractions of the gastrocnemius complex in situ was found to be attenuated in dystrophic muscle, suggesting a role of the DGC in mechanotransduction or in mechanically-induced signaling. Tissues from this study were later analyzed for integrin solubility changes, described

in the next manuscript, and it was observed that integrin remodelling in response to contractions was intact in dystrophic muscles. This finding suggests that while some mechano-signaling is attenuated, mechanotransduction per se is not impaired in dystrophic muscle. Therefore, the DGC may play a role in the transduction of mechanically-stimulated signaling rather than in mechano-chemical coupling. This manuscript was accepted for publication in Muscle and Nerve in April 2002.

I am responsible for developing the hypothesis of this study, for its design, for the data collection, analysis and interpretation, as well as for the writing of the manuscript. Phil Gardiner contributed to the interpretation of the results, and to the correction of the manuscript.

"Association of β1 integrin to the cytoskeleton is increased by mechanical stimulation in a tension-dependent manner in rat skeletal muscle in-vivo"

This manuscript represents a formal description of novel observations made in the course of the studies presented in the fifth and final manuscript. After observing that the magnitude of the p54 JNK response to a standardized protocol of intermittent tetanic contractions was increased in atrophied muscles and decreased in hypertrophied muscles (see final manuscript), efforts were made to relate this change in mechanosensitivity to a change in the concentration of cytoskeletal proteins possibly involved in mechanotransduction or mechanically-induced signaling. In the course of evaluating the concentration of β1 integrin, this protein was observed to exist within two sub-populations which differ in their strength of association to the cytoskeleton, as assessed by detergent-solubility. Furthermore, it was observed that contractile activity induced an increase in the concentration of strongly cytoskeleton-associated β1 integrin, mirrored by a reciprocal decrease in the concentration of weakly associated β1 integrin. This manuscript

describes this remodelling phenomenon in greater detail, including the finding of a linear relationship between peak tension and the magnitude of the β1 integrin solubility shift. This work was presented at Experimental Biology 2002 and the manuscript was submitted for publication to the Journal of Biological Chemistry in April 2002 .

I am responsible for developing the hypothesis of this study, for its design, for the data collection, analysis and interpretation, as well as for the writing of the manuscript. Victor Gisiger, of the department of Cell Biology, contributed to the design of control experiments, to the interpretation of the results, and to the correction of the manuscript. Phil Gardiner also contributed to the correction of the manuscript.

"Chronic modulation of mechanosensitivity and concentration of detergent-insoluble β1 integrin in skeletal muscle in-vivo: Is mechanosensitivity dictated by the concentration of force-transmitting integrins?"

The first part of this manuscript reports on the study of contraction-induced p54 JNK activation in atrophied and hypertrophied muscles, conducted between 2000 and 2001. The second part of the manuscript describes a cytoskeletal adaptation occurring in parallel to muscle trophic remodelling, and which may account for the observed change in signaling response. A clear presentation of these findings required that the novel observation of cytoskeletal remodelling be formally reported elsewhere in the form of the preceding manuscript.

This study was designed to address the hypothesis that the trophic adaptations to chronically altered levels of activity are accompanied by alterations in mechanosensitivity, acting to minimize deviations in fiber size from an optimal size range. Aggressive models of disuse and overload were employed

to induce atrophy and hypertrophy, respectively. The magnitude of the p54 JNK response to intermittent tetanic contractions, a protocol chosen to induce the same relative level of stimulation in muscles of varying cross-sectional area but identical length, was found to be inversely related to muscle size. This finding provided an opportunity to identify cytoskeletal structures which may be involved in mechanotransduction or in mechanically-induced signaling by identifying cytoskeletal proteins which undergo a change of concentration directly or indirectly related to the change in mechanosensitivity. A number of focal adhesion proteins and pathways involved in cytoskeletal remodelling were studied and no relationship to mechanosensitivity was observed. One of these cytoskeletal proteins was β1 integrin, a protein believed to be central to mechanotransduction. Concentration of this protein had been measured in homogenates extracted with RIPA-buffer, a technique commonly used to extract cytoskeletal proteins, and analysis had been conducted on pooled muscles from the unstimulated control condition and from the intermittent contraction condition. Serendipitously, it was observed that in every group, the concentration of β1 integrin was systematically lower in stimulated muscles than in controls. This clearly indicated that a population of β1 integrin failed to be solubilized by the RIPA buffer, and sample pellets were therefore extracted by boiling in SDS-PAGE reducing sample buffer. Only by taking into account both RIPA-soluble and RIPA-insoluble populations of β1 integrin was it possible to observe a remarkable relationship between β1 integrin and mechanosensitivity and to propose a mechanism by which mechanosensitivity is regulated. The contraction-induced p54 JNK activation data from atrophied and hypertrophied muscles was presented at the CSEP annual meeting in 2001. At the time of writing this thesis, this manuscript was awaiting acceptance of the previous manuscript before submission.

I am responsible for developing the hypothesis of this study, for its design, for

the data collection, analysis and interpretation, and for the writing of the manuscript. Phil Gardiner instructed me in the muscle loading and unloading protocols employed, and contributed to the interpretation of the results and to the correction of the manuscript. Victor Gisiger's contribution to the interpretation of the results from the previous manuscript had an important impact on my interpretation of this study. Kristina Csukly, an M.Sc. candidate from Phil Gardiner's laboratory, contributed to surgeries and post-operative care of animals.

Chapter 2

Manuscript 1

Title

Insight into skeletal muscle mechanotransduction: Quantitative relationship between tension and MAPK activation

Authors

Louis C. Martineau and Phillip F. Gardiner

Journal

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Summary

The mechanism by which mechanical forces acting through skeletal muscle cells generate intracellular signalling, known as mechanotransduction, and the details of how gene expression and cell size are regulated by this signalling, are poorly understood. Mitogen-activated-protein-kinases (MAPKs) are known to be involved in mechanically-induced signalling in various cell types including skeletal muscle where MAPK activation has been reported in response to contraction and to passive stretch. Therefore, the investigation of MAPK activation in response to mechanical stress in skeletal muscle may yield important information about the mechanotransduction process. Using a rat plantaris in-situ preparation, a wide range of developed peak tensions were generated through passive stretch and concentric, isometric and eccentric contractile protocols, and the resulting phosphorylation of JNK, ERK and p38 MAPKs was assessed. Isoforms of JNK and ERK MAPKs were found to be phosphorylated in a tension-dependent manner, such that eccentric > isometric > concentric > passive stretch. Peak tension was found to be a better predictor of MAPK phosphorylation than time-tension integral or dT/dt. Differences in maximal response amplitude and sensitivity between JNK and ERK MAPKs suggest different roles for these two kinase families in mechanically-induced signalling. A strong linear relationship between p54 JNK phosphorylation and peak tension over a 15-fold range in tension (R^2=0.89, n=32) was observed, supporting that contraction-type differences can be explained in terms of tension, and demonstrating that MAPK activation is a quantitative reflection of the magnitude of mechanical stress applied to muscle. Thus, the measurement of MAPK activation, as an assay of skeletal muscle mechanotransduction, may help elucidate mechanically-induced hypertrophy.

Sommaire (Translated summary)

Le mécanisme de la mécano-transduction, par lequel les forces mécaniques passant à travers les myocytes squelettiques déclenchent une signalisation intracellulaire, est peu connu. De plus, il n'est pas clair comment cette signalisation exerce un contrôle au niveau de l'expression génique et de la régulation du volume cellulaire. Les "mitogen-activated-protein-kinases" (MAPKs) sont impliquées dans la signalisation déclenchée par la stimulation mécanique dans plusieurs types de cellules incluant les myocytes squelettiques où elles sont activées par les contractions et par l'étirement passif. L'étude de l'activation des MAPKs en réponse à la stimulation mécanique dans le muscle squelettique peut donc révéler d'importants renseignements au sujet du mécanisme de la méchano-transduction. Dans la présente étude, une large gamme de tensions maximales fut générée in-situ dans le muscle plantaire du rat par des protocoles d'étirement passif et de contractions concentriques, isométriques et excentriques, et la phosphorylation des MAPKs JNK, ERK, et p38 fut ensuite mesurée. Les résultats démontrent que certains isoformes de JNK et ERK sont phosphorylés suite à la stimulation mécanique et que le degré de phosphorylation dépend de la tension maximale suivant l'ordre: excentrique > isométrique > concentrique > étirement passif. De plus, la tension maximale est un meilleur prédicteur du degré de phosphorylation des MAPKs que l'intégrale tension-temps ou que la vitesse maximale du développement de tension. Des différences en termes d'amplitude de réponse et de sensibilité furent observées entre les JNK et ERK, suggérant des rôles distincts pour ces deux familles de MAPKs dans la signalisation déclenchée par la stimulation mécanique. Une relation linéaire hautement significative entre la tension maximale et le degré de phosphorylation de p54-JNK (R^2=0.89, n=32) à été observée permettant de conclure que les différences entre les types de contraction s'expliquent en termes de tension maximale, et que l'activation

de MAPK est un marqueur quantitatif de la stimulation mécanique percue par le muscle. L'activation de MAPK peut donc servir d'index de la mécano-transduction dans le muscle squelettique et pourrait aider à comprendre comment la stimulation mécanique contrôle le volume cellulaire.

Introduction

Mechanotransduction is the fundamental mechanism by which mechanical stress acting through a cell initiates intracellular signalling. Through this mechanism, forces promote cellular growth and survival (27, 10), influence metabolic responses (17), and govern tissue architecture in various cell types (32, 34, 5, 39). While all adhesion-dependent cells appear to be sensitive to mechanical forces (35, 27), this is especially evident in mechanocytes, or cells routinely subjected to mechanical forces such as vascular endothelial (21) and smooth muscle cells (25), airway smooth muscle cells (33), chondrocytes (37, 16), osteocytes (24), cardiomyocytes (32, 29, 38) and skeletal muscle cells (34, 13). Striated muscle cells are particularly responsive to mechanical stress as evidenced by the fact that cell volume is in large part dictated by physical forces; loading elicits hypertrophy while unloading elicits atrophy (26).

Despite the great deal of research in this area, mechanotransduction remains poorly understood. A number of mechanisms have been proposed to explain the mechano-chemical coupling on a molecular level, but none is generally accepted. Only slightly better understood are the details of the downstream signalling pathways through which mechanical stress affects gene expression.

While numerous pathways have been implicated, the involvement of the mitogen-activated protein kinase (MAPK) cascades in mechanically-induced signalling from the cytosol to the nucleus is consistent across various cell types (40, 15, 3, 18, 20, 16, 30, 31, 19, 29, 38). This function is compatible with the well-known role of the MAPKs as points of convergence for various signalling cascades regulating gene expression (11), including signalling cascades triggered by many soluble growth factors and their cell-surface receptors and cascades triggered by physical stresses through unknown transduction mechanisms. The MAPKs, classified into the

c-Jun-NH2-terminal kinase (JNK) family (also known as the SAPKs or stress-activated protein kinases), the extracellular-regulated kinase (ERK) family, and the p38 family, translocate to the nucleus upon activation through tyrosine and threonine phosphorylation by upstream MAPK kinases. There they phosphorylate a number of transcription factors such as ELK-1, activator-protein 1 (AP-1), and serum-response factor (SRF), thereby regulating gene expression (11).

Only a limited number of studies have examined mechanisms of mechanically-induced hypertrophy in skeletal muscle, despite the well-documented adaptability of this tissue in response to the forces acting through it. These studies have employed models of passive stretch, perhaps the simplest and most easily studied form of mechanical stress, to stimulate skeletal muscle hypertrophy and have tended to focus on the role of autocrine secretion of growth factors (12, 34). The events involved in the initiation of muscle hypertrophy are not known and knowledge of early events related to mechanical stimulation is limited to findings that passive stretch induces, in a stretch-amplitude-dependent manner, immediate-early genes (6) and MAPK phosphorylation (22), the later recently demonstrated by our laboratory.

As MAPK activation can be induced by tetanic muscle contraction (1, 28) as well as by passive stretch alone (22), it was hypothesized that activation is sensitive to tension either imposed passively on, or developed actively by the muscle, and that MAPK activation is a reflection of the mechanotransduction process. To demonstrate tension-dependent activation of MAPK, we compared contractile protocols which differed in their developed tension but not in their activation or fiber recruitment profiles.

The purpose of this paper was therefore to characterize the relationship

between tension and MAPK activation and to demonstrate that the measurement of MAPK activation can be used as a tool for gaining insight into the mechanotransduction process and perhaps the initiation of hypertrophic signaling. We report a quantitative relationship between peak tension and MAPK phosphorylation, specifically the p54 JNK isoform. The relationships between phosphorylation and other tension-related parameters such as rate of force development and aggregate tension, as measured by tension-time integral, were analyzed. While rate appears to have little influence on MAPK activation, duration of tension application in addition to peak tension should be factored into future models of mechanotransduction in skeletal muscle. Finally, a dose-response activation of MAPKs to mechanical stimulation supports a role of MAPKs in mechanically-induced gene regulation in skeletal muscle.

An abstract of this work has been presented elsewhere (23).

Materials and Methods

Animals

Forty female Sprague-Dawley rats (Charles River), weighing 195 ± 5 g, underwent an in-situ nerve-muscle preparation of the sciatic nerve and plantaris muscle. Animals were anaesthetized with an IP injection of ketamine and xylazine (61.5 mg/kg ketamine and 7.7 mg/kg xylazine) and maintained under anaesthesia by hourly injections of 25% of the initial dose. Following the experiment, animals were killed by anaesthetic overdose. Treatment of animals was certified by the animal ethics committee of the Université de Montréal and conformed to the regulations of the Canadian Council of Animal Care.

In-situ nerve-muscle preparation

The plantaris muscle of the left leg was surgically isolated from the other ankle extensors. Care was taken not to disrupt its vasculature, innervation or tendon, and not to apply any tension on the muscle during isolation. The other ankle extensors were denervated and tenotomized at the proximal end of their distal tendon in order to avoid separating the common tendon of the extensors and possibly damaging the plantaris tendon. The calcaneus was clipped, leaving a bone chip attached to the common tendon, and a silk ligature was firmly placed around the bone-tendon interface.

Following surgery, the animal was secured in the prone position within a stereotaxic frame. The left foot was immobilized with a clamp and the left knee was pinned to the stereotaxic frame in a slightly flexed position. The silk ligature around the common tendon was attached to the lever arm of a muscle puller servomotor (305B-LR; Aurora Scientific), without putting the isolated muscle under tension. The skin of the hindlimb was pulled into a bath which was filled with heated mineral oil maintained at 36-37° C. Core temperature was monitored by rectal probe and maintained at 35-36° C using a heating pad.

In order to determine optimal length for muscle twitch tension development (Lo), the isolated muscle was indirectly stimulated through a platinum bipolar electrode placed on the sciatic nerve, and developed tension was simultaneously visualized on an oscilloscope and recorded with a microcomputer. Always beginning from a completely relaxed length, the muscle was slowly lengthened while supramaximal (5V) single square pulses of 0.05 ms in duration were delivered once every 3 seconds by a Grass S88 stimulator. Lo determination to less than ± 1 mm required on average 60 twitches. Following determination of Lo, electrical stimulation was ceased and the muscle held at this length for 5 minutes before the onset of the

contractile protocol.

Contractile Protocols

Effect of different types of contractions on MAPK phosphorylation: Isometric, eccentric and concentric contractions generated using identical stimulation parameters were compared in terms of their efficacy for activating MAPKs. Electrical stimulation in the form of 150 ms of supramaximal single square pulses, 0.05 ms in duration, delivered at a frequency of 100 Hz, was applied to the sciatic nerve once every second for exactly 5-min (i.e. 300 tetanic contractions). Muscle length was either kept constant at Lo (isometric contraction), or varied from Lo to Lo + 3 mm (eccentric contraction with passive return to Lo) or Lo + 3 mm to Lo (concentric contraction with passive stretch return to Lo + 3 mm) over 150 ms synchronously with the electrical stimulation using the computer-controlled servomotor. A 3 mm length excursion corresponds to approximately 10% of muscle length. A ramp function was employed in order to keep velocity constant at 20 mm/s or approximately 2/3 Lo/s. Time spent at Lo was identical to time spent at Lo + 3 mm for both Eccentric and Concentric groups. This was accomplished by using a 50:50 duty cycle. Thus following the 150 ms length excursion, muscles were held at the new length for 350 ms before being returned to starting length over 150 ms. Refer to Figure 1 for a schematic representation of the various contractile protocols.

In order to parcel out the effects of activation, stretch, movement, and contraction type on MAPK phosphorylation, contractile groups were compared to three control groups: an unstimulated group maintained at Lo for the same duration as the contractile protocols (Static Lo); an unstimulated group maintained at Lo + 3 mm (Static Lo + 3 mm); and an unstimulated group which passively underwent the same length excursion as the eccentric and concentric groups (Passive Stretch Lo to Lo + 3 mm). Analysis was

performed by ANOVA with a Fisher post-hoc test.

Any phosphorylation resulting from the determination of Lo (not addressed here) may result in phosphorylation levels of the Static Lo control group to be greater than actual baseline levels. However, since all groups undergo this determination, between-group differences cannot be due to the effect of repeated muscle twitches.

Relationship between tension and MAPK phosphorylation: In order to investigate the relationship between tension-related parameters and MAPK phosphorylation, a wide range of tension (15-fold range) was generated. However, in order to keep stimulation parameters constant, and in the case of anisometric contractions, keep velocity and excursion constant, this range was generated by using isometric, eccentric and concentric contractile protocols which differed in the length at which contraction was initiated.

Thus, in addition to the isometric contractions performed at Lo described above, isometric contractions were performed at Lo - 3 mm, and at Lo + 3 mm. In addition to the eccentric contractions performed between Lo and + 3 mm, eccentric contractions were performed between Lo - 3 mm and Lo. In addition to the concentric contractions performed between Lo + 3 mm and Lo, concentric contractions were performed between Lo and Lo - 3 mm. All groups conformed to the 50:50 duty cycle design described above in section A. This symmetry allowed time spent reaching Lo and at Lo to be kept constant across all groups regardless of starting length. Refer to figure 1 for a schematic representation of the various contractile protocols. The Passive Stretch group described above was also included in the analysis for a total of 8 groups or 32 points for regression analysis. Refer to Figure 2 for representative tension/time tracings of the 8 groups.

Regressions were performed between MAPK phosphorylation (measurements obtained as described below) and a number of tension-related parameters obtained from the 32 samples. Parameters investigated included maximal peak tension and average peak tension (over first 10, 30 or 60 contractions, or entire 300 contractions) normalized by maximal twitch tension, maximal time-tension integral (TTI) and aggregate TTI (over first 10, 30 or 60 contractions, or entire 300 contractions) normalized by maximal twitch tension, and maximal peak rate of tension development (dT/dt) and average peak dT/dt (over first 10, 30 or 60 contractions, or entire 300 contractions). Regressions were performed using Mac Curve-Fit software.

Muscle excision

Muscle excision was performed within 10 s of the end of the experimental protocol and muscles were immediately frozen in liquid nitrogen and stored at -80° C for subsequent biochemical analysis.

Western immunoblots using phospho-specific MAPK antibodies

MAPK activation was assessed by measurement of dual phosphorylation using phospho-specific antibodies. As MAPK activation is controlled through this phosphorylation, measurements of phosphorylation should adequately reflect activity. A high degree of agreement between phosphorylation measurement and kinase assays has been demonstrated (4).

Homogenization and sample preparation: Frozen muscles were powdered in liquid nitrogen. Approximately 90 mg of muscle powder was transferred to a cooled microcentrifuge tube and kept in liquid nitrogen until the addition of 1 ml of ice-cold modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 5% glycerol, 5 mM EGTA, 2 mM $MgCl_2$, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (Mini-Protease, Boehringer; 2 mM phenylmethylsulfonyl fluoride) and

phosphatase inhibitors (100 μM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride). The sample was vortexed 30 seconds and placed on ice for 1 hour. Throughout this 1 hour period, samples were frequently vortexed. Samples were then centrifuged at 4500 g , 4° C, for 1 hour in order to remove insoluble material. The supernatants were decanted and pellets were discarded. Protein concentration of the supernatants was determined by Bradford protein assay (Bio-Rad). Samples of equal total protein concentration were then prepared for SDS-PAGE by diluting an appropriate amount of the supernatant in reducing sample buffer (60 mM Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, pH 6.8) and boiling for 1 minute.

Electrophoresis and Western Blot: Forty samples, each containing 200 μg of total protein, were loaded onto 16 cm long 9% acrylamide gels and separated overnight at 10° C. The 40 separated samples were simultaneously electro-transferred to a single piece of polyvinylidene difluoride (PVDF) membrane (Millipore), and visualized by Ponceau S stain to confirm successful transfer and equal sample loading of all lanes. Membranes were blocked with 3% BSA in Tris-buffered saline + Triton (TBST: 50 mM Tris, 150 mM NaCl, pH 7.4, 0.5% Triton X-100), bathed overnight at 4° C in primary antibody solution and bathed 1.5 hours at room temperature in secondary antibody solution. All washes were done in TBST. Monoclonal antibodies directed against phosphorylated JNK (reactive with p-JNK1, p-JNK2, and p-JNK3), ERK (reactive with p-ERK1 and p-ERK2) and p38 (reactive with p-p38 and p-p38β) (Santa Cruz Biotechnology) were prepared at a concentration of 1:2000 in TBST plus 1% BSA and 0.5% NaN_3 while HRP-conjugated anti-mouse IgG secondary antibodies (Jackson Immunoresearch) were prepared at 1:10000 in TBST plus BSA. 200 μg of whole cell lysate of UV-treated HeLa cells or heat-shocked NIH/3T3 cells (Santa Cruz Biotechnology) was used as a positive control for p38

phosphorylation. Membranes were then bathed in chemiluminescence substrate (ECL, Amersham) and exposed to blue-light-sensitive film (ECL Film, Amersham) for 5 to 20 minutes. Membranes were stripped, reblocked and reprobed in order to obtain ERK, JNK, and p38 phosphorylation values from every sample. Films were quantified by densitometry using a flatbed scanner and NIH Image software. In cases where the range in signal amplitude exceeded the dynamic range of film (approximately 16-fold resolution), short and long exposures of the same blots were quantified. A ratio was calculated from the results of samples which were within the linear range on both films and used as a conversion factor for the pooling of data from two films of the same blot.

Results

Effects of contraction type on MAPK phosphorylation

The phosphorylation of both JNK and ERK MAPKs was observed to be increased above unstimulated and unstretched control levels immediately following the three 5-minute contractile protocols compared: Eccentric (Lo to Lo + 3 mm), Isometric (Lo) and Concentric (Lo + 3 mm to Lo) (Figure 3). Refer to Table 1 for group descriptive statistics. Representative tension tracings are illustrated in Figure 2. For both JNK and ERK, eccentric contractions resulted in significantly more phosphorylation than isometric contractions, which in turn resulted in significantly more phosphorylation than concentric contractions.

Western blots reveal that both p54 and p46 isoforms of the JNK family are phosphorylated in response to tension. However, while p54 phosphorylation was observed over a wide range of tension, p46 phosphorylation was only observed in conjunction with a saturating phospho-p54 signal. Only p54

phosphorylation was quantified and reported here. As a result of the eccentric contractile protocol, p54 JNK phosphorylation surpassed 80-fold control levels, 10-fold isometric levels, or 40-fold concentric levels. The p44 ERK (ERK-1) isoform of the ERK family was observed to be phosphorylated in response to tension and this phosphorylation was quantified. The eccentric protocol resulted in ERK-1 phosphorylation of 5-fold control levels, the isometric protocol in 4-fold control levels, and the concentric protocol in 3-fold control levels. The amplitude of phosphorylation reported here is in line with typically reported levels of MAPK activation in response to mechanical stress (2, 15).

The Static Lo + 3 mm group or the Passive Stretch Lo to Lo + 3 mm control groups did not result in more JNK phosphorylation than the Static Lo baseline control group, indicating that the JNK phosphorylation resulting from the anisometric contractile groups cannot be attributed to the 3 mm length excursion imposed on the muscle. These same control groups resulted in ERK phosphorylation of 2-fold control levels. Thus, part of the ERK phosphorylation induced by the anisometric protocols may be attributed to the length excursion. These findings can also be interpreted as indicating that the ERK system is more sensitive to low tensions than is the JNK system.

Passive stretch or contractile activity in any form were not observed to induce phosphorylation of p38 MAPK above control values, as measured immediately following the 5-min protocol. However, increased p38 activity was observed at longer times following the 5-min protocol (data not shown).

See figure 4 for representative phospho-JNK, phospho-ERK and phospho-p38 western blots.

Relationship between tension and MAPK activation

Seven contractile protocols were used to generate maximal peak tensions ranging from 226 to 655 g or 3.3- to 11.2-fold twitch tension (Table 1). An unstimulated cyclical stretch group, the Passive Stretch Lo to Lo + 3mm group, generated on average peak tensions of 44 g or 0.8-fold twitch tension. When taken together, the 8 groups produced a 15-fold range in peak tension. Group descriptive data is summarized in Table 1. Representative tension tracings are illustrated in figure 2.

Phosphorylation values for p54 JNK (pJNK densitometry values linearized by log 10 transformation) and p44 ERK (pERK raw densitometry values) were subjected to regression and correlational analysis against the parameters of peak tension, tension-time integral (TTI), and rate of tension development (dT/dt). Positive tension-phosphorylation relationships were observed for both JNK and ERK. Results of linear regression analyses are summarized in Table 2. For both JNK and ERK, the relationship between normalized peak tension and phosphorylation was stronger than between TTI or dT/dt and phosphorylation. For all parameters measured, the correlation with JNK phosphorylation was consistently stronger than the correlation with ERK phosphorylation.

The strongest relationships observed, as assessed by correlation coefficient, were between peak tension measurements and JNK phosphorylation ($R^2 > 0.85$). Of these, normalized average peak tension over the first 60 seconds had the highest correlation coefficient ($R^2 = 0.89$). When group average raw densitometry data were plotted against average peak tension over the first 60 seconds, a simple power function ($y = 0.3 \times ^{4.67}$) produced a near-perfect fit ($R^2 = 0.998$). These relationships are illustrated in Figure 5.

Analysis performed according to contraction type revealed that the positive tension-phosphorylation relationship holds within the eccentric groups and within the isometric groups, as illustrated in Figure 5. This supports that MAPK phosphorylation is independent of contraction type.

Discussion

The present study suggests that mitogen-activated-protein-kinases (MAPKs) may be involved in mechanically-induced signalling in skeletal muscle. A strong relationship between peak tension, developed actively or passively, and p54 JNK MAPK phosphorylation demonstrates that MAPK phosphorylation can be used as quantitative marker of the magnitude of mechanical stress applied to a muscle. This study also supports that differences in MAPK phosphorylation resulting from controlled in-situ eccentric, isometric and concentric contractile protocols are due to differences in magnitude of developed tension between the different contractile protocols, independently of contraction type. Finally, the results of this study indicate that peak tension is a better predictor of MAPK phosphorylation than other tension-related parameters such as time-tension integral and peak rate of tension development.

The relationship between peak tension and phosphorylation is demonstrated to hold over a 15-fold range in tension produced with passive stretch and contractile protocols employing different types of contraction (i.e concentric, isometric, eccentric). The strength of this relationship, and the fact that the positive relationship holds even within the range of peak tensions generated by protocols employing the same type of contraction, both support that contraction-type differences are due to differences in tension and not contraction type per se.

The study of the mechanical sensitivity of MAPKs in skeletal muscle, as presented here, does not address directly the mechanism of mechanotransduction or the proximity of the MAPKs to the initial mechano-chemical coupling event. Rather, the purpose of the study is to demonstrate that MAPK activation can be used as a quantitative measurement of the magnitude of mechanical stress applied to a muscle. This in turn may be used to elucidate early mechano-sensitive events upstream of the MAPKs, including the mechanotransduction process itself. Mechanotransduction and the events leading to mechanically-induced MAPK activation are unclear and may occur through any of a number of mechanisms such as the release of growth factors stored in the extracellular matrix, influx of ions through stretch-sensitive channels, or direct activation of kinases through mechanical deformation. The measurement of mechanically-induced MAPK activation therefore represents a simple and reliable assay to investigate the effects of disrupting various cellular components and signalling pathways suspected of participating in mechanotransduction and mechanical regulation of gene expression.

Furthermore, by assessing the efficacy of different types of stimulation on MAPK phosphorylation, the measurement of mechanically-induced MAPK activation can be used to elucidate the nature of the mechanical stress to which muscle is most sensitive. Determination of the sensitivity of MAPKs to amplitude, frequency, velocity, and duration of mechanical stimulation can provide new insight into the design of protocols for the maintenance of muscle mass in situations of decreased loading, and also represents another approach for elucidating the mechanism of mechanotransduction. In the present study, the assessment of the relationship between various tension-related parameters (peak tension, TTI, peak dT/dt) and MAPK activation represents an application of this strategy. For example, the finding of a low correlation between peak dT/dt and phosphorylation suggests that

mechanically-induced signalling is largely independent of contraction velocity. This combined with a fair correlation between TTI and phosphorylation would suggest that two protocols developing identical peak tension at different frequencies but with identical TTI would induce similar levels of signalling. Furthermore, a high correlation between peak tension and MAPK phosphorylation and a low correlation between peak dT/dt and phosphorylation are both consistent with a mechanotransduction mechanism based on elastic deformation. The relative importance of these parameters in the modelling of mechanical sensitivity needs to be addressed directly with studies designed to vary TTI and dT/dt.

Delay kinetics, time-course, and time-dependent changes in sensitivity of mechanically-induced MAPK activation are other parameters which need to be addressed in order to fully model the sensitivity of muscle to mechanical stimulation. While these issues are beyond the scope of this study, the complexity of the delay kinetics can nevertheless be appreciated from the data presented here. The finding that average peak tension over the first minute is a slightly better predictor of MAPK phosphorylation than maximal peak tension or average peak tension over the first 10 or 30 seconds, combined with the interesting observation that a dramatically different rate of fatigue between contraction types is apparent between the 30th and 60th second of the protocol (Figure 6), suggest that phosphorylation measured immediately following the 5 minute protocols is determined by more than 30 seconds of stimulation.

While this study does not address directly the role of MAPKs in mechanically-induced gene regulation and muscle hypertrophy, the dose-response behavior of p54 JNK MAPK to mechanical stimulation, as presented here and as previously described in response to passive stretch (22), certainly supports a role for MAPKs in relaying a hypertrophic stimulus to

the nucleus and possibly triggering a program of events necessary for hypertrophy. This is further supported by the report of a rapid stretch-amplitude-dependent induction of c-jun and c-fos in response to passive stretch (6). A similar magnitude-dependent activation of JNK and induction of immediate early genes by mechanical stress has been reported in smooth muscle cells (15).

The MAPKs, in particular the JNK MAPKs, and calcineurin, an alternate pathway to the nucleus, are believed to be essential components of the hypertrophic response in cardiac muscle (7, 9). Recent evidence suggests that calcineurin may act through JNK (7). Inhibition of calcineurin in skeletal muscle has recently been shown to abolish the hypertrophy typically induced by synergist-ablation muscle overload (8). It should not be surprising to find that chronic inhibition of the JNK pathway will also abolish the potential for hypertrophy. This is a reasonable hypothesis not only in light of the rapid and transient activation of MAPKs in response to mechanical stress, but also in light of the longer contribution of autocrine growth factor action in the hypertrophic response (12).

MAPK-family-specific differences in response amplitude and sensitivity to tension suggest that while the JNK and ERK MAPKs may transduce a mechanically-induced signal in parallel, they may serve different functions in the regulation of muscle plasticity. JNK phosphorylation is not observed below a peak tension threshold on the order of 2 to 4-fold twitch tension. However, above this threshold, phosphorylation increases exponentially with tension to as much as 80-fold baseline values at around 10-fold twitch tension. By contrast, the observed signal amplitude of ERK phosphorylation is only 5-fold. However, an ERK response at the very low tensions produced by the passive stretch protocols employed here, suggests a higher sensitivity to tension. The lower response amplitude of ERK may be partly explained by a

higher baseline level of activity, due to its higher sensitivity. The finding of a tighter relationship, as assessed by correlation coefficient, between JNK phosphorylation and peak tension than between ERK and tension, suggests that p54 JNK may have a more important role in mechanically-induced gene regulation than p44 ERK.

In contrast to the JNK and ERK families of MAPKs, the p38 family was not found to be activated above control levels by 5 minutes of contractile activity. This suggests that p38 is either not involved in skeletal muscle mechanotransduction, or follows a different time course of activation. The latter appears to be the case as activation of p38 resulting from 5-min of contractile activity can be observed at later time points (data not shown). This is in accord with other reports of contractile-activity-induced MAPK activation: Goodyear et al. showed very low p38 activation (less than 2 fold control values) occurring significantly later than JNK activation in exercised rat muscle (14), and Widegren et al. showed p38 activation to be at least an order of magnitude lower than ERK activation in exercised human muscle (36).

In summary, the present study demonstrates a possible involvement of MAPKs in mechanically-induced signalling in skeletal muscle. A quantitative relationship between p54 JNK phosphorylation and peak tension supports that JNK activation is a reflection of mechanical stimulation applied to a muscle and may be used as an assay of the mechanotransduction process. Such an assay represents a useful tool for the elucidation of early signalling events involved in mechanically-induced muscle hypertrophy, and for the understanding of the nature of the mechanical stimuli to which muscle is sensitive.

Acknowledgments

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Figure Legends

Figure 1

Graphical representation of 1000 ms contractile protocols repeated 300 times. Heavy lines represent the three protocols employed in Study A, while the light lines represent the additional four protocols employed in Study B. Electrical stimulation is delivered between 0 and 150 ms, coinciding with the 3mm ramps of the Eccentric and Concentric protocols. Static control groups are identical to the Isometric Lo and Isometric Lo + 3mm protocols without electrical stimulation, while the Passive Stretch protocol is identical to the Eccentric or Concentric protocols without electrical stimulation. Note that the Concentric and Eccentric protocols used in Study A, differ only in the timing of the stimulation. Note also that all anisometric protocols feature identical time at Lo regardless of initial length.

Figure 2

Representative tension tracings from the 7 contractile protocols and 1 cyclical passive stretch protocol employed in Studies A and B. For each of the experiments depicted, a tracing of a twitch at Lo is followed by a tracing of the iteration at which maximal peak tension is achieved (iteration number indicated beside tracing). The 60th iteration is superimposed onto the maximal peak tension tracings. Note that the rate of fatigue resulting from the two concentric protocols is greater than that which results from the isometric or eccentric protocols. A highly repeatable contraction-type- and muscle-length-dependent potentiation effect is also observed.

Figure 3

Effect of contraction type on p54 JNK (**Figure 3A**) and p44 ERK MAPK (**Figure 3B**) phosphorylation, as measured by western blot. Data expressed as fold Static Lo unstimulated control levels. Additional unstimulated control groups are used to parcel out the effects of a length excursion and of the resulting tension at Lo + 3mm from contraction type. All groups have a sample size of 4. Different letters identify significant ($p<0.05$) between-group differences, as determined by ANOVA. Normalized maximal peak tensions generated during the anisometric protocols are overlaid to illustrate the positive tension-phosphorylation relationship. The effect of contraction type on MAPK activation is qualitatively similar for both p54 JNK and p44 ERK.

Figure 4

Representative films of phospho-JNK (**Figure 4A**), phospho-ERK (**Figure 4B**), and phospho-p38 (**C**) western blots. In **A**, lanes 1 and 2: unstretched, unstimulated controls at Lo; Lanes 3 to 6: concentric contractile protocol; Lanes 7 to 10: isometric contractile protocol; Lanes 11 to 14: eccentric contractile protocol. This film is overexposed in order to visualize control levels. In **B**, lanes 1 and 2: controls; lanes 3 and 4: concentric; lanes 5 and 6: isometric; lanes 7 and 8: eccentric. In **C**, lane 1 contains whole cell lysate of UV-treated HeLa cells while lane 2 contains whole cell lysate of heat-shocked NIH/3T3 cells. Both these lanes are positive for phospho-p38. Lanes 3 and 4: control; lanes 5 and 6: concentric; lanes 7 and 8: isometric; lanes 9 and 10: eccentric. None of the contractile protocols elicit phosphorylation of p38 above control levels.

Figure 5

p54 JNK phosphorylation can be reliably predicted from peak tension, as demonstrated by regression analysis. The average peak tension generated over the first minute normalized by the twitch tension is plotted against arbitrary densitometry data. **Inset graph**: densitometry data are log-transformed and all 32 points are fitted with a linear function. **Main graph**: 8 group averages are fitted using a simple power function. Between group differences, as determined by ANOVA, are illustrated by different letters for phosphorylation and by different numbers for tension. Note that the positive tension-phosphorylation relationship holds within the 2 eccentric protocols and within the three isometric protocols.

Figure 6

The rate of fatigue, expressed as % decrease from peak tension over a given time interval, is greater in experiments employing a concentric contractile protocol (n=8) than in experiments employing an isometric (n=12) or an eccentric protocol (n=8). This is especially evident between the 30th and 60th second. The fact that these differences affect the relationship between peak tension and MAPK phosphorylation suggests that the MAPK response, as measured at 5 minutes, is determined by more than 30 seconds of mechanical stimulation.

TABLE 1: Descriptive Statistics

Group	Sample Size	Body Weight (g)	Preparatory Parameters: Lo Twitch Tension (g)	Preparatory Parameters: Lo Passive Tension (g)	Experimental Parameters: Maximal Baseline Passive T (g)	Experimental Parameters: Maximal Developed Tension (g)	Experimental Parameters: Developed T Normalized by Twitch T
Static Lo	4	190 ± 2 (a)	62 ± 3 (a,b)	7 ± 1 (a)	7 ± 1 (a)	n/a	n/a
Static Lo + 3mm	4	191 ± 2 (a)	63 ± 5 (a,b)	6 ± 1 (a)	32 ± 5 (b)	n/a	n/a
Passive Lo to Lo + 3mm	4	196 ± 3 (a)	58 ± 5 (a)	7 ± 1 (a)	7 ± 1 (a)	44 ± 5 (a)	0.8 ± 0.1 (a)
Isometric Lo - 3mm	4	199 ± 3 (a)	60 ± 6 (a)	7 ± 0 (a)	2 ± 1 (a)	283 ± 3 (b,c)	4.8 ± 0.2 (c)
Isometric Lo	4	193 ± 3 (a)	71 ± 4 (b,c,d)	6 ± 1 (a)	7 ± 1 (a)	398 ± 18 (e)	5.6 ± 0.2 (d)
Isometric Lo + 3mm	4	195 ± 3 (a)	65 ± 2 (a,b,c)	6 ± 1 (a)	31 ± 3 (b)	337 ± 16 (d)	5.2 ± 0.1 (c,d)
Eccentric Lo to Lo + 3mm	4	193 ± 4 (a)	60 ± 3 (a,b)	6 ± 1 (a)	7 ± 1 (a)	630 ± 13 (g)	10.5 ± 0.3 (f)
Eccentric Lo - 3mm to Lo	4	195 ± 2 (a)	59 ± 4 (a)	7 ± 0 (a)	1 ± 0 (a)	508 ± 22 (f)	8.6 ± 0.3 (e)
Concentric Lo + 3mm to Lo	4	194 ± 2 (a)	64 ± 2 (a,b,c)	5 ± 1 (a)	25 ± 3 (b)	241 ± 6 (b)	3.8 ± 0.2 (b)
Concentric Lo to Lo - 3mm	4	195 ± 2 (a)	74 ± 4 (c,d)	6 ± 0 (a)	7 ± 0 (a)	299 ± 7 (c,d)	4.1 ± 0.3 (b)

All data expressed as mean ± SEM
Different letters denote significant differences ($p<0.05$) between groups.

TABLE 2: Assessment of linear regressions between tension-related variables and pJNK and pERK densitometry values

	log-transformed pJNK values (R^2)	pERK values (R^2)
Maximal peak tension / max. twitch tension	0.86	0.64
Average peak tension / max. twitch tension		
first 10 contractions	0.86	0.65
first 30 contractions	0.87	0.65
first 60 contractions	**0.89**	**0.65**
all 300 contractions	0.85	0.61
Maximal 1000ms TTI / max. twitch tension	0.75	0.53
Aggregate TTI / max. twitch tension		
first 10 contractions	0.75	0.54
first 30 contractions	0.70	0.51
first 60 contractions	0.71	0.49
all 300 contractions	0.65	0.43
Maximal peak dT/dt	0.50	0.42
Average peak dT/dt		
first 10 contractions	0.50	0.43
first 30 contractions	0.41	0.36
first 60 contractions	0.41	0.34
all 300 contractions	0.12	0.11

Figure 1

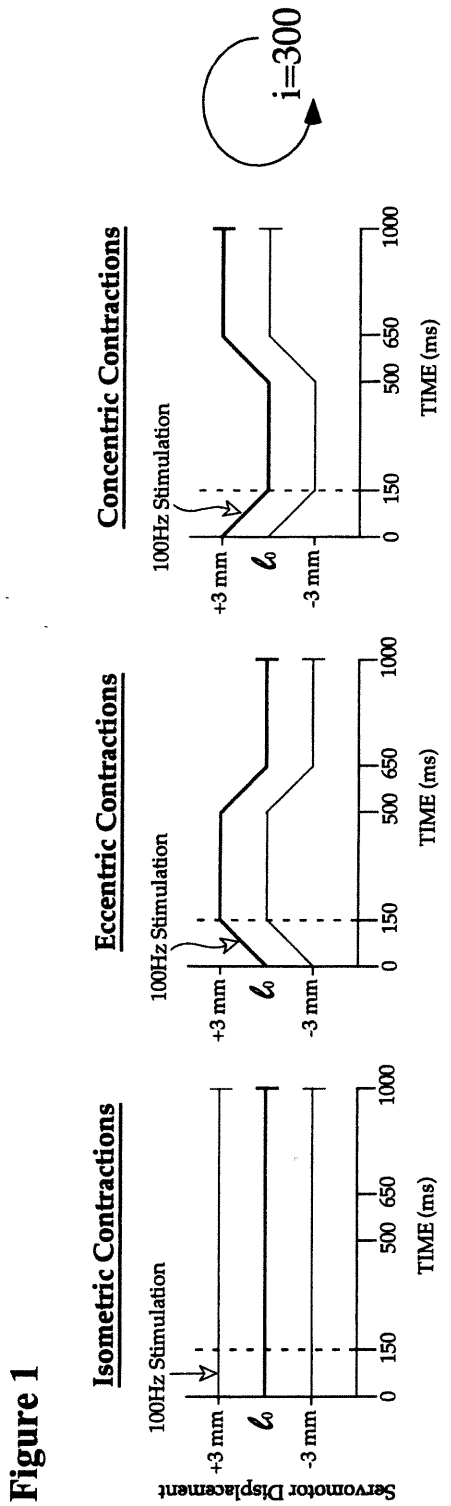
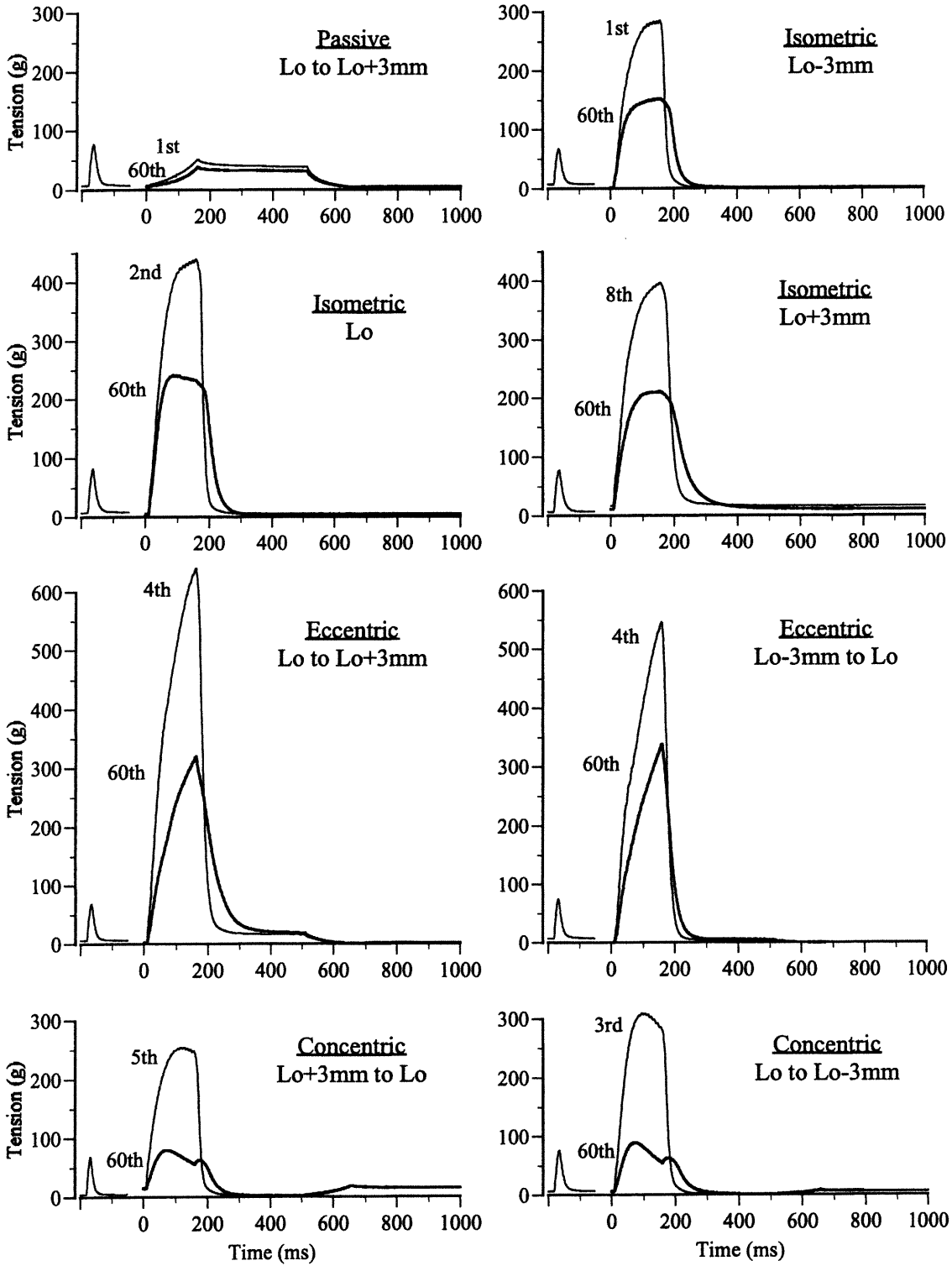

Isometric Contractions
Eccentric Contractions
Concentric Contractions
100Hz Stimulation
+3 mm
-3 mm
l_0
0
150
500
650
1000
TIME (ms)
Servomotor Displacement
i=300

Figure 2


Passive
Lo to Lo+3mm
1st
60th
Isometric
Lo-3mm
1st
60th
Isometric
Lo
2nd
60th
Isometric
Lo+3mm
8th
60th
Eccentric
Lo to Lo+3mm
4th
60th
Eccentric
Lo-3mm to Lo
4th
60th
Concentric
Lo+3mm to Lo
5th
60th
Concentric
Lo to Lo-3mm
3rd
60th
Tension (g)
Time (ms)
0
100
200
300
400
500
600
800
1000

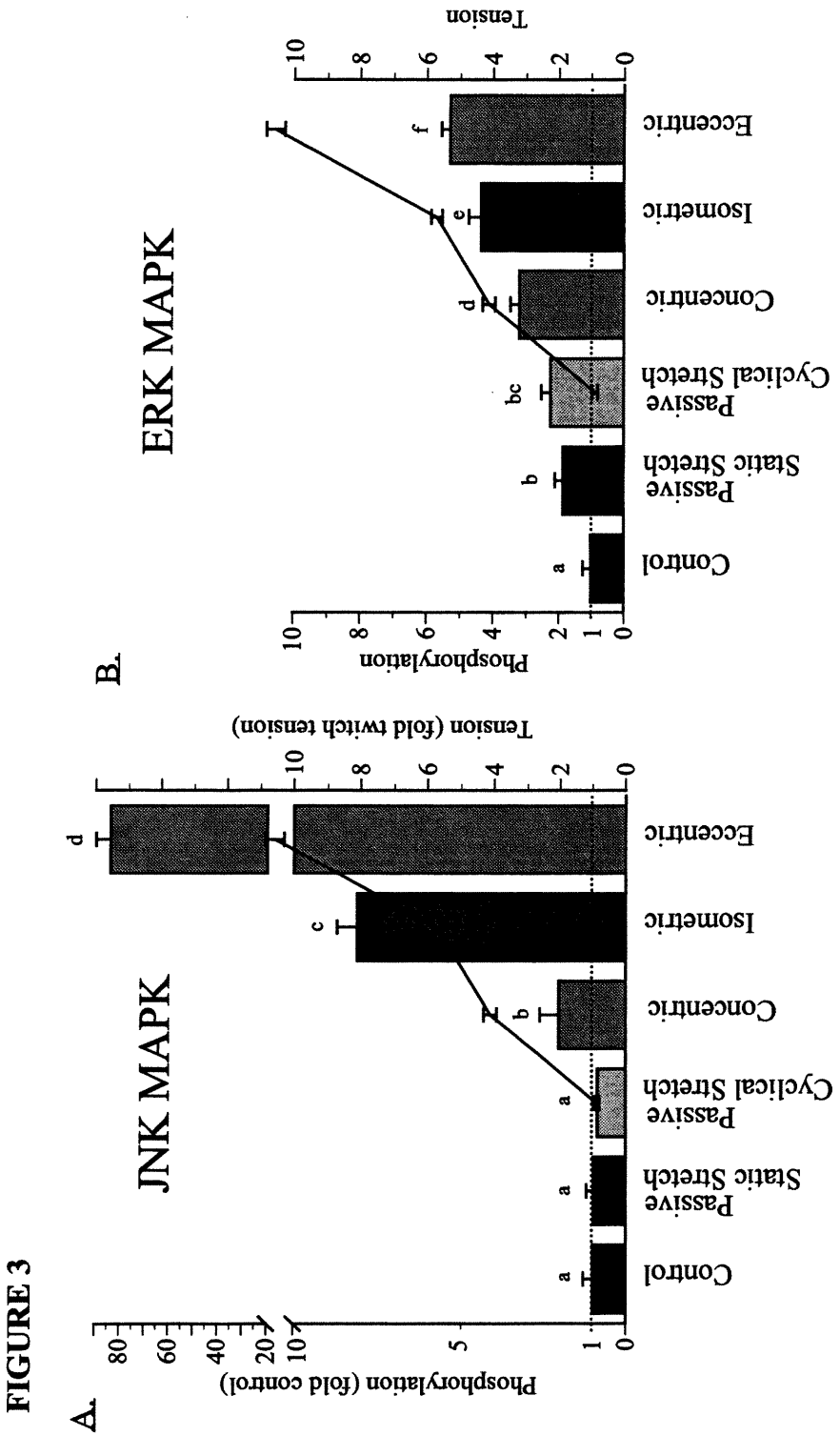

A.
JNK MAPK
Phosphorylation (fold control)
Tension (fold twitch tension)
Control
Passive Static Stretch
Passive Cyclical Stretch
Concentric
Isometric
Eccentric
B.
ERK MAPK
Phosphorylation
Tension

FIGURE 3

Figure 4

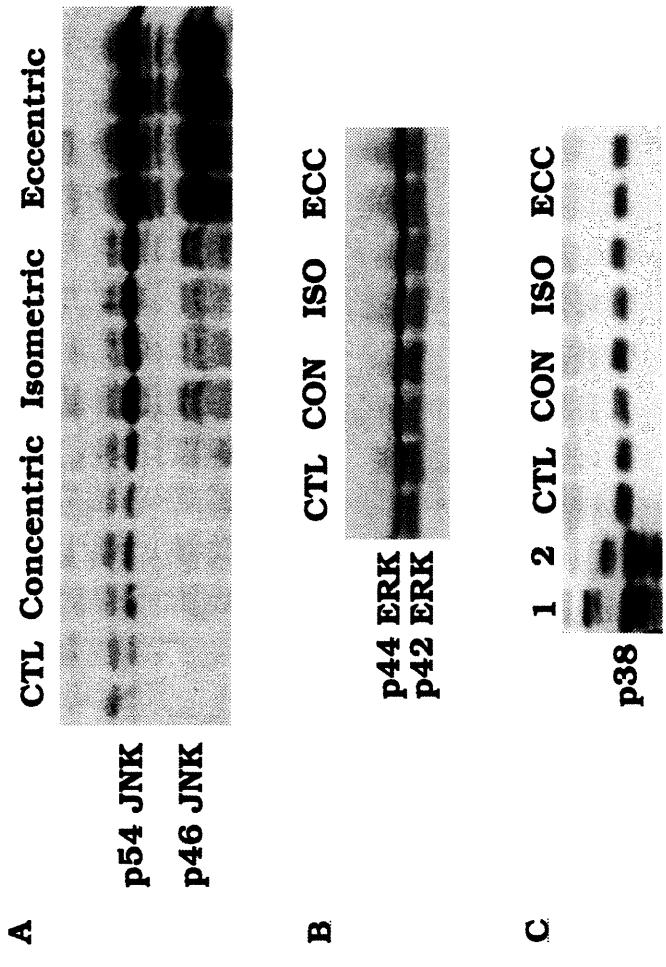
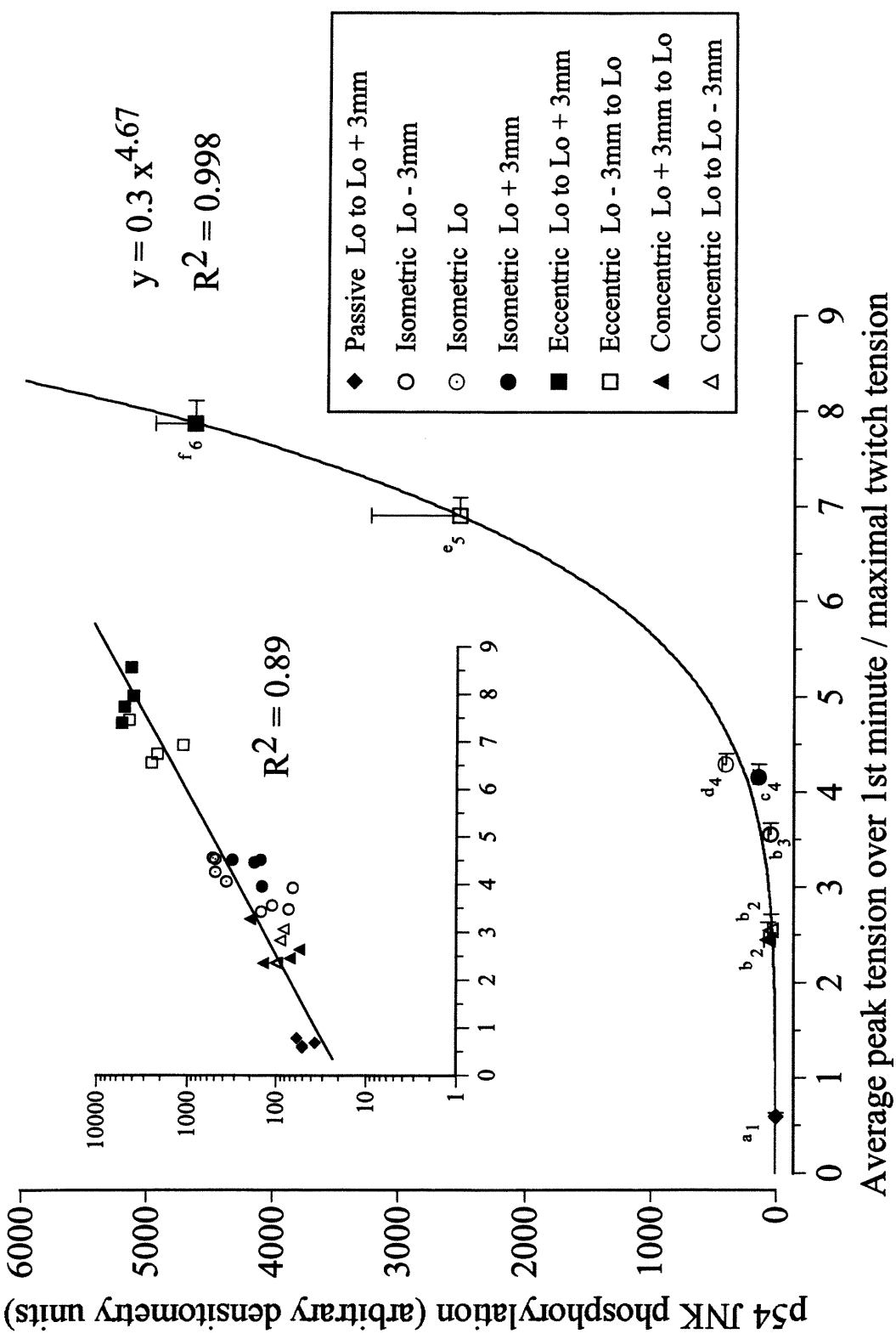

A
CTL Concentric Isometric Eccentric
p54 JNK
p46 JNK
B
CTL CON ISO ECC
p44 ERK
p42 ERK
C
1 2 CTL CON ISO ECC
p38

FIGURE 5


p54 JNK phosphorylation (arbitrary densitometry units)
Average peak tension over 1st minute / maximal twitch tension
y = 0.3 x^4.67
R2 = 0.998
R2 = 0.89
Passive Lo to Lo + 3mm
Isometric Lo - 3mm
Isometric Lo
Isometric Lo + 3mm
Eccentric Lo to Lo + 3mm
Eccentric Lo - 3mm to Lo
Concentric Lo + 3mm to Lo
Concentric Lo to Lo - 3mm

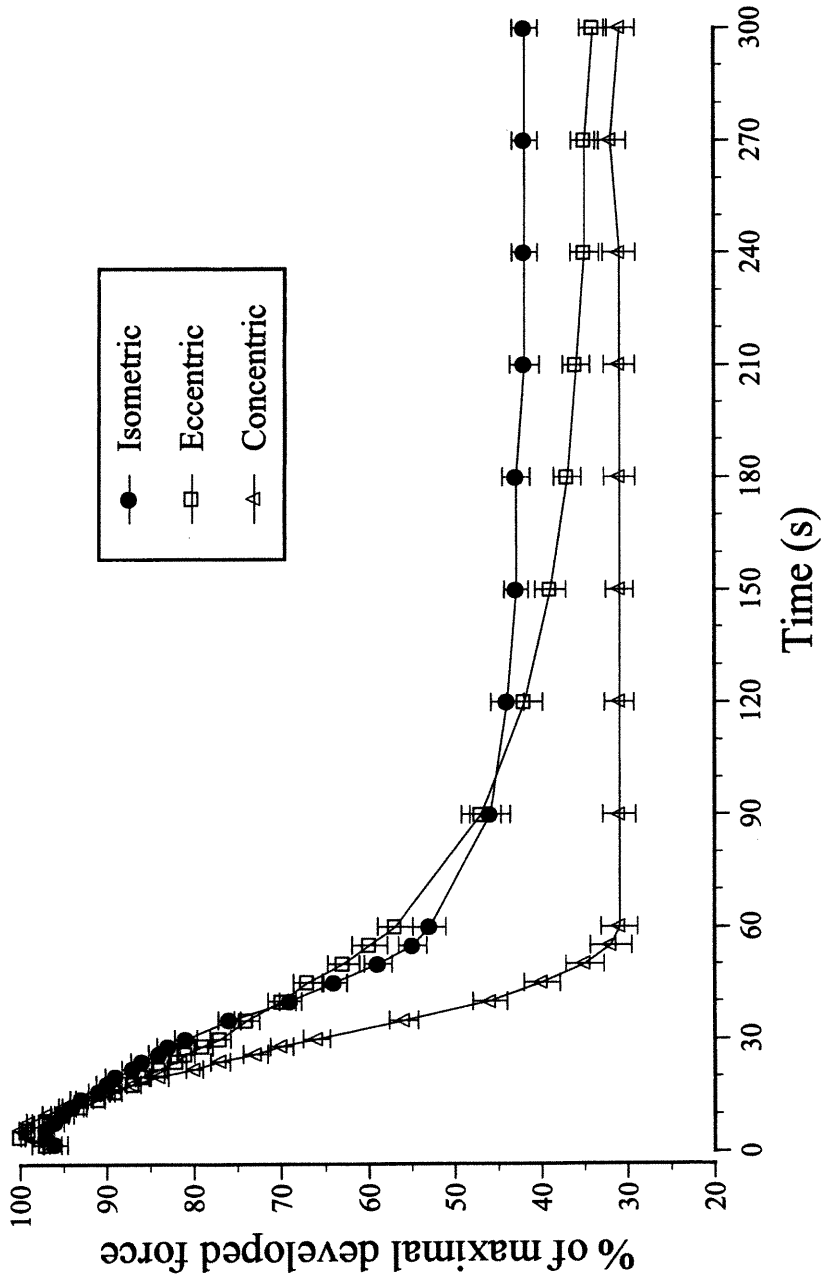

Isometric
Eccentric
Concentric
% of maximal developed force
Time (s)
100
90
80
70
60
50
40
30
20
0
30
60
90
120
150
180
210
240
270
300

FIGURE 6

Chapter 3

Manuscript 2

Title

Skeletal muscle is sensitive to the tension-time integral but not to the rate of change of tension, as assessed by mechanically-induced signaling

Authors

Louis C. Martineau et Phillip F. Gardiner

Journal

Journal of Biomechanics, 35(5): 657-663, 2002

Summary

Mechanical forces regulate many cellular processes. Mechanotransduction, however, is poorly understood. In skeletal muscle, mechanical forces have a major impact on the regulation of cellular volume, yet the nature of the mechanical stimulation to which muscle is most sensitive is unknown. It was recently demonstrated that activation of the mechanically-sensitive kinase p54-JNK is a quantitative marker of mechanical stimulation in skeletal muscle. This marker was shown to be more sensitive to peak tension than to other tension-related parameters such as the tension-time integral (TTI) and the rate of change of tension (dT/dt). The purpose of the present study was to parcel out the contribution of TTI and dT/dt to mechanical stimulation of muscle under conditions of constant peak tension. The rat medial gastrocnemius in-situ was subjected to one of four 5-min passive stretch protocols consisting of equal length excursions, but differing in displacement-time integral (4, 40 or 100%) and/or rate of stretch (0, 3, or 30 mm/s), and the resulting p54-JNK phosphorylation was assessed. A linear relationship between TTI and p54-JNK signaling was observed. However, no effect of dT/dt was observed. It is concluded that peak tension and TTI are necessary parameters for modeling the mechanical stimulus-response of muscle. Additionally, the mechanism of mechanotransduction is sensitive to peak tension and TTI, but not to dT/dt, and thus exhibits spring-like behavior. These findings may contribute to the refinement of disuse atrophy countermeasures.

Sommaire (Translated summary)

Les forces mécaniques exercent un contrôle sur plusieurs fonctions cellulaires. Par contre, le mécanisme de la mécano-transduction n'est pas bien compris. Dans le muscle squelettique, les forces mécaniques exercent un contrôle important au niveau de la régulation du volume cellulaire. Cependant, il n'est pas clair à quelles caractéristiques de la stimulation mécanique les myocytes squelettiques sont le plus sensibles. Il a récemment été démontré que l'activation de la kinase p54-JNK, sensible au stress mécanique, peut servir de marqueur quantitatif de la stimulation mécanique du muscle squelettique. Ce marqueur répond davantage à la tension maximale qu'à d'autres paramètres de tension, tels l'intégrale tension-temps (TTI) et la vitesse maximale de développement de tension (dT/dt). Le but de la présente étude est d'établir l'apport du TTI et du dT/dt à la stimulation mécanique du muscle en présence d'une tension maximale fixe. Le muscle gastrocnémien médial du rat fut étiré in-situ selon un de quatre protocoles d'étirement passif résultant en une même longueur d'étirement mais caractérisés par différentes valeurs de l'intégrale déplacement-temps (4, 40, et 100%) et par différentes vitesses d'étirement (0, 3, 30 mm/s). Suite à l'étirement, l'activation de la p54-JNK fut mesurée. Les résultats démontrent une relation linéaire entre le TTI et l'activation de la p54-JNK. Par contre, le dT/dt n'a eu aucun effet sur l'activation de cette signalisation. Il en est conclu que la tension maximale ainsi que le TTI sont des paramètres essentiels pour modeler la réponse du muscle squelettique à la stimulation mécanique. De plus, il est possible de conclure que le mécanisme de mécano-transduction est sensible à la tension maximale ainsi qu'au TTI, mais pas au dT/dt, et donc démontre des caractéristiques d'un ressort. Les résultats de cette étude pourront contribuer au raffinement des techniques de prévention de l'atrophie musculaire.

Introduction

Mechanical forces passively applied to, or actively generated by cells initiate intracellular signaling which influences many cellular functions such as the control of cell cycle progression and cell survival (Ruoslahti, 1997), metabolism (Ihlemann et al., 1999), and morphogenesis (Vandenburgh et al., 1991; Simpson et al., 1995; Goldspink, 1999). The trophic influence of mechanical forces is particularly evident in mechanocytes, or cells routinely subjected to mechanical stress, such as vascular endothelial and smooth muscle cells (Papadaki & Eskin, 1997), airway smooth muscle (Smith et al., 1998), chondrocytes (Wright et al., 1997), bone cells (Mikuni-Takagaki, 1999), and cardiac (Sadoshima & Izumo, 1993) and skeletal myocytes (Vandenburgh et al., 1991).

The process by which mechanical forces initiate intracellular signaling is termed mechanotransduction. The biophysical and molecular basis of this mechano-chemical coupling is not well understood. Events downstream of mechanotransduction are slightly better understood and mechanically-sensitive signaling pathways have been elucidated in numerous types of mechanocytes. While a large number of signaling molecules has been implicated, members of the mitogen-activated-protein-kinase (MAPK) families in particular appear to have a consistent involvement across cell types (Yamazaki et al., 1993; Berk et al., 1995; Aronson et al., 1997; Matsuda et al., 1998; Zou et al., 1998; Hung et al., 2000). This is in line with the universal role of MAPKs as a point of convergence and integration for a multitude of receptor-mediated and cellular-stress-activated pathways (Widmann et al., 1999).

In skeletal muscle, cell volume is regulated to a major extent by mechanical forces: loading of muscle cells elicits hypertrophy, while chronic unloading

results in atrophy (Roy et al., 1991; Goldspink, 1999). Little is known, however, about the mechanism through which loading state regulates remodelling, or even about the exact nature of the mechanical forces to which muscle cells are responsive. The answers to these questions are important to the refinement of existing disuse atrophy countermeasures and to the development of new molecular strategies for the control of cellular volume. Insight into skeletal muscle mechanotransduction may also contribute to a better understanding of this process in all mechanocytes.

Recently our laboratory has demonstrated that phosphorylation of a 54 kDa isoform of jun-N-terminal-kinase (p54-JNK) MAPK, a mechanically-sensitive kinase in skeletal muscle (Aronson et al., 1997; Martineau & Gardiner, 1999), is a quantitative marker of mechanical stimulation in skeletal muscle and can be employed as an assay of the mechanotransduction process (Martineau & Gardiner, 2001). In that study, phosphorylation of p54-JNK was found to be highly correlated (R^2=0.89, n=32) to average peak tension in skeletal muscle in-situ over a 15-fold range of tension produced both passively, in the form of stretch, and actively in the form of concentric, isometric and eccentric contractions. While the protocols employed were chosen to vary peak tension, resulting tension-time integral (TTI) and rate of change of tension (dT/dt) were also assessed and were found to be not as well correlated (R^2=0.75 and R^2=0.50, respectively) to p54-JNK activation. These parameters, which obviously covary, remain to be systematically manipulated in order to assess their contribution to mechanical stimulation of muscle and to gain insight into the biophysics of mechano-chemical coupling.

The purpose of the present study was to parcel out the sensitivity of muscle to rate of change of tension and to total tension, independently of peak tension, the apparent governing factor in mechano-stimulation of muscle. Specifically, this study tested the hypothesis that dT/dt and TTI modulate

mechanically-induced signaling, as assessed by p54-JNK activation. Using passive stretch as a form of mechanical stimulation, TTI and dT/dt were systematically varied over a large physiological range while keeping peak force constant. The findings of a linear relationship between TTI and p54-JNK signaling, but no effect of dT/dt on this signaling, indicate that muscle is not sensitive to the rate at which tension is developed, but only to peak and total tension.

Materials and methods

Thirty female Sprague-Dawley rats (Charles River), weighing 195 ± 5 g and anaesthetized by intraperitoneal injections of ketamine and xylazine (61.5 mg/kg and 7.7 mg/kg, respectively), underwent an in-situ nerve-muscle preparation of the sciatic nerve and medial gastrocnemius muscle (MG). Following the experiment, animals were killed by anaesthetic overdose. Treatment of animals was certified by the animal ethics committee of the Université de Montréal and conformed to the regulations of the Canadian Council of Animal Care.

The in situ model used here is similar to the one previously described in detail (Martineau & Gardiner, 2001) with the exception that the MG muscle was isolated instead of the plantaris muscle. Isolation from the other ankle extensors was achieved by excising the soleus, tenotomizing the plantaris and leaving its proximal half attached to the MG, and separating the lateral gastrocnemius by a cut from the natural division through which the tibial and sural nerves pass, distally to the common tendon. Briefly, the remainder of the protocol consisted of clipping the calcaneus and leaving a bone chip attached to the common tendon. A silk ligature was then firmly placed around the bone-tendon interface. The animal was secured in the prone position within a stereotaxic frame with the left foot clamped and the left

knee pinned in a slightly flexed position. The silk ligature was attached to the lever arm of a muscle puller servomotor (305B-LR; Aurora Scientific), without putting the isolated muscle under tension. The skin of the hindlimb was pulled into a bath filled with heated mineral oil maintained at 36-37° C. Core temperature was monitored by rectal probe and maintained at 35-36° C.

Optimal length for muscle twitch tension development (Lo) to less than ±1 mm was determined over a period of approximately 5-min by stimulating the sciatic nerve with supramaximal single square pulses of 0.05 ms in duration delivered every 3 seconds while the muscle was slowly lengthened from a completely relaxed position. Following this determination, the muscle was subjected to a single tetanic contraction (100Hz, 100ms) in order to firmly seat the silk ligature, after which the determination of Lo was repeated. Following this procedure, electrical stimulation was ceased and the muscle held at this length for 5-min before the onset of the experimental protocol.

The thirty animals were divided equally into five groups: four experimental groups which underwent a 5-min passive stretch protocol of the MG, and one control group which had the MG muscle maintained at Lo for the same duration in order to establish a baseline level of p54-JNK phosphorylation (Baseline group). All passive stretch protocols featured a 6 mm length excursion from Lo, corresponding to a strain of approximately 20%. In the MG of a 200 g Sprague-Dawley rat, this length excursion results in the generation of peak tensions capable of eliciting a large but submaximal activation of p54-JNK (Martineau & Gardiner, 1999). Stretch duration was set to 5-min as this is sufficient time for the development of peak phosphorylation (Martineau & Gardiner, 1999). Muscles were excised and frozen in liquid nitrogen within 20 seconds of the end of the 5-min period. Frozen muscles were stored at -80° C for subsequent biochemical analysis.

The four passive stretch protocols employed to parcel out the effects of the tension-time integral (TTI) and of the rate of change of tension (dT/dt) on tension-induced activation of p54-JNK MAPK are schematically summarized in Figure 1. Protocol 1 consisted of a static stretch held for the entire 5-min duration, thereby maximizing the time-under-tension (High TTI group). Protocols 2 and 3 consisted of cyclical stretches following sinusoidal functions featuring an identical displacement-time integral (DTI) when calculated over a 5-sec period. The stretch cycle of Protocol 2 required 400 ms and was repeated continuously at a frequency of 2 Hz while the stretch cycle of Protocol 3 required 4 seconds and was repeated at a frequency of 0.2 Hz. Thus, over a 5-sec period, both protocols produced a DTI equivalent to 40% of the DTI of Protocol 1. The average stretch velocity was 30 mm/s (6 mm in 0.2 s) for Protocol 2 (Mid TTI High Rate group) and 3 mm/s (6 mm in 2 s) for Protocol 3 (Mid TTI Low Rate group), or approximately 1 Lo/s and 0.1 Lo/s, respectively. Protocol 4 employed the same high rate sinusoidal function as Protocol 2 repeated at the same low frequency as Protocol 3, thereby producing a DTI equivalent to 4% of the DTI of Protocol 1, when calculated over a 5-sec period (Low TTI group). The length excursions of the muscle-tendon complex were precisely regulated by the computer-controlled muscle puller.

Western immunoblots to assess p54-JNK MAPK dual phosphorylation, a reflection of activation, were performed using a monoclonal phospho-specific antibody reactive with phospho-JNK-1, 2 and 3 (Santa Cruz Biotechnology). Blots were also probed with anti-JNK-2 (p54-JNK) antibody (Santa Cruz Biotechnology) in order to confirm that all samples contained similar quantities of total (phosphorylated and unphosphorylated) MAPK. The western blot procedure has been previously described in greater detail (Martineau & Gardiner, 2001). Briefly, frozen muscles were powdered under liquid nitrogen and solubilized by frequent vortexing over a period of 1 h in modified RIPA buffer containing a cocktail of protease inhibitors and

phosphatase inhibitors. Homogenates were centrifuged 1 h at 4500 x g and the protein concentration of the supernatants was determined by Bradford protein assay. Samples containing 200 µg total protein were prepared for SDS-PAGE by dilution and boiling in reducing sample buffer. Samples were separated on 9% acrylamide gels and electro-transferred to a single PVDF membrane. Successful transfer and equal loading were confirmed by Ponceau S stain. Membranes were blocked and then bathed overnight at 4° C in primary antibody solution and bathed 1.5 h at room temp. in secondary antibody solution. Revelation was performed by chemiluminescence with film exposure times ranging from 15 to 45 min. Films were scanned with a flatbed scanner and densitometry of results within the linear range of the technique was performed with NIH Image software. The entire western blot procedure was performed in duplicate and densitometry data were averaged from both blots. One-way ANOVA analysis with Fisher post-hoc was used to assess between-group differences in densitometry data and in the physiological variables summarized in Table 1.

Results

The ranges of TTI values and peak dT/dt values measured at the onset of the passive stretch protocols (24-fold and 10.7-fold, respectively) correspond very closely to the calculated ranges of displacement-time integral and rate of stretch (25-fold and 10-fold, respectively), as described in Table 1. Despite featuring identical length excursions, the stretch protocols varied slightly (~20% between the cyclical stretch protocols) in the peak tensions generated, due to an uneven contribution of creep and/or stress-relaxation (Fung, 1972). Furthermore, differences in time-under-tension and frequency of stretch between protocols resulted in different rates of tension loss due to creep and/or stress-relaxation, over the course of the 5-min protocols. However, differences in calculated % loss of peak passive tension, resting tension, TTI,

and peak dT/dt were negligible and do not affect the interpretation of the results.

Marked differences ($p<0.05$) in phosphorylation of p54-JNK were observed between groups, despite employing protocols which elicited similar peak tensions. All groups with the exception of the Low TTI group were different from the baseline control group (Figure 2A). The Mid TTI Low Rate and Mid TTI High Rate groups were different from both the High TTI group and the Low TTI group.

A significant effect of TTI on phosphorylation of p54-JNK was attested to by the difference between the 2 protocols employing the same rate of stretch but featuring a ten-fold difference in displacement-time integral (Low TTI vs Mid TTI High Frequency) and by the absence of difference between the 2 groups with identical displacement-time integrals (Mid TTI High Rate and Mid TTI Low Rate) (Figure 2A). A near-perfect ($R^2=0.99$) positive linear relationship was observed between group-average TTI (initial 5-sec TTI normalized by muscle force) and group-average p54-JNK phosphorylation (Figure 2B). This relationship held for the final 5-sec TTI ($R^2=0.97$) and therefore for TTI averaged over any duration.

No effect of dT/dt on phosphorylation of p54-JNK was observed. This was best illustrated by the absence of difference in phosphorylation between the two protocols employing the same displacement-time integral but featuring a ten-fold difference in rate of stretch (Mid TTI Low Rate vs Mid TTI High Rate) (Figure 2A).

Discussion

Peak tension has previously been demonstrated to be a governing factor in

mechanically-induced signaling in skeletal muscle, more important than other tension-related parameters such as the tension-time integral (TTI) and rate of change of tension (dT/dt) (Martineau & Gardiner, 2001). The current study was designed to parcel out the sensitivity of skeletal muscle to TTI and to dT/dt, under conditions of fixed peak tension. This information is necessary to the elucidation of the nature of the mechanical stress to which muscle is most sensitive and can provide insight into the biophysics of mechanotransduction in mechanocytes.

A strong relationship between mechanical stimulation of muscle, in the form of passive stretch or contractile activity, and activation of p54-JNK MAPK has previously been characterized (Martineau & Gardiner, 1999; 2001) and it was demonstrated that the magnitude of this signaling is quantitatively related to peak tension generated passively or actively. The quantitative nature of this relationship supports that mechanical activation of p54-JNK can be used as an assay of mechanical stimulation of muscle and of mechanotransduction. While other mechanically-sensitive kinases, including members of other MAPK families, have been identified in skeletal muscle and other tissues, their use as quantitative markers of mechanical stimulation must either be validated or is limited by early saturation and therefore lack of resolution, as in the case of extracellular-regulated kinase (ERK) MAPKs (Martineau & Gardiner, 2001).

The JNK MAPK cascade has traditionally been associated with various cellular stresses (Tibbles & Woodgett, 1999). However, numerous lines of evidence support that the mechanical sensitivity of this pathway is not related to cellular damage: 1) passive strain on the order of 20%, as employed in this study, does not produce any ultrastructural damage (Lieber et al., 1991); 2) p54-JNK is differentially activated by the stretch protocols employed here despite an identical length excursion and no effect of rate of stretch; 3) more

activation is observed to result from static stretch than from cyclical stretch; 4) the level of phosphorylation reported in this study is on the same order as phosphorylation induced by isometric contractile activity (Aronson et al., 1997; Martineau and Gardiner, 2001), a challenge known not to be damaging to muscle (Lieber et al., 1991). While it remains to be tested whether mechanical activation p54-JNK in skeletal muscle is correlated to nuclear events such as activation of stretch-sensitive immediate-early genes (Dawes et al., 1996) or to an increase in protein synthesis, a link between mechanical activation of JNK and gene expression has been made in other tissues (Li et al., 1996).

Mechanical stimulation in the form of passive stretch was employed in this study in order to precisely control the parameters of peak tension, TTI, and dT/dt while avoiding complications due to fatigue. The MG muscle, which was used in the original characterization of stretch-induced p54-JNK activation (Martineau & Gardiner, 1999), was selected for the current study because the peak forces necessary for p54-JNK activation can be passively generated in this muscle at near-physiological strain magnitudes.

Despite the well-established sensitivity of skeletal muscle to mechanical forces, the exact nature of the mechanical stimulus which regulates its cellular volume is unclear. While peak tension appears to be the prime determinant of mechano-stimulation (Martineau & Gardiner, 2001), the present study demonstrates that TTI is also a determining factor capable of modulating the resulting intracellular message. It can be speculated that the linear relationship between TTI and p54-JNK signaling observed in the present study adds a third dimension to the power relationship previously reported between peak tension and p54-JNK phosphorylation.

Although it is not surprising to find an effect of TTI, the lack of effect of

dT/dt, and therefore the lack of interaction between these two parameters, is a novel observation. This implies that, under conditions of equal creep and stress-relaxation, static stretch must be a more effective form of mechano-stimulation than any pattern of cyclical stretch due to its greater TTI. Therefore, like contraction-type (i.e. concentric, isometric, or eccentric) differences in resulting signaling can be explained by differences in peak tension (Martineau & Gardiner, 2001), stretch-type (i.e. cyclical vs static) differences can be explained by differences in TTI.

The stretch protocols employed were chosen to vary TTI and dT/dt over a wide physiological range. Sinusoidal functions were employed because they are more physiological than ramp functions and because they facilitate the control of TTI during the manipulation of dT/dt. While the rate of stretch and the rate of relaxation vary together when using sinusoidal functions, the findings clearly indicate that dT/dt does not affect the measured signaling, and it is therefore not necessary to uncouple these variables. A quicker return to Lo would be expected to simply reduce TTI and reduce the resulting signal.

Clearly, both peak tension and TTI, but not dT/dt, must be included in the modeling of the mechanical stimulus-response of skeletal muscle. The relative weight of these two parameters remains to be established, as do other issues concerning signaling kinetics. Such a model should be as applicable to contractile activity, within the constraints of fatigue, as to passive stretch, and thus may help increase the efficiency of resistance training protocols and may prove useful in the design of alternative disuse atrophy countermeasures.

The study of the sensitivity of skeletal muscle to mechanical forces is revelative of the biophysics of the mechanotransduction process. An understanding of mechanotransduction, in skeletal muscle or in other mechanocytes, may contribute to the development of new strategies based on

modulation of mechanosensitivity for the artificial control of myocyte cell volume or other mechanically-regulated cellular processes. The finding of a lack of contribution of dT/dt to tension-induced signaling may be interpreted to mean that the putative mechanosensor protein exhibits spring-like behavior described by Hooke's law, whereby its deformation or strain is proportional to the applied stress, regardless of rate. The positive linear relationship between TTI and p54-JNK phosphorylation and the finding that mechano-stimulation by static stretch is more effective than by cyclical stretch would suggest that the primary enzymatic activity associated with mechanotransduction is sustained for the duration of the strain applied to the mechanosensor. Thus, strain magnitude appears to determine the rate of activity, while duration of activity, and therefore duration of the signaling pulse, is a function of time-under-tension.

In summary, by demonstrating that in addition to peak tension, TTI, but not dT/dt, is an important factor in the activation of mechanically-sensitive intracellular signaling, this study provides insight into the sensitivity of skeletal muscle to mechanical stimulation which regulates its cellular volume, as well as into the poorly understood mechanism of mechanotransduction through which these forces initiate intracellular signaling ultimately regulating gene expression.

Acknowledgments

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Figure legends

Figure 1

Diagrammatic representation of the four passive stretch protocols differing in displacement-time integral and/or in average rate of stretch, but not in length excursion.

Figure 2

Figure 2A: Average content of phosphorylated p54-JNK measured immediately following one of four 5-minute passive stretch protocols. Baseline content is measured in a control group held at Lo for the same duration. Results are expressed relative to baseline levels normalized to 1. Bars identified by similar letters are not different, while all others are significantly different ($p<0.05$), as determined by ANOVA. Error bars show SEM.

Figure 2B: A positive linear relationship is apparent between group average phospho-JNK content and tension-time integral normalized to muscle force. Error bars show SEM.

Table 1: Physiological Measures

Group	Body Weight (g)	Twitch Tension (g)	Tetanic Tension (g)	Initial Peak Passive Tension (g)	% loss over 5-min	Initial Resting Tension (g)	% loss over 5-min	Initial 5-sec TTI (g*s)	% loss over 5-min	Initial Peak dT/dt (g/s)	% loss over 5-min
Low TTI	197 ± 5	121 ± 5	531 ± 19	302 ± 42 ab	24 ± 2% a	13.5 ± 1.3	37 ± 5% a	43 ± 7 a	36 ± 3% a	3172 ± 354 a	21 ± 2% a
Mid TTI Low Rate	199 ± 4	130 ± 13	545 ± 43	261 ± 25 ac	26 ± 2% a	13.0 ± 0.7	54 ± 3% b	362 ± 36 b	43 ± 2% bc	307 ± 30 b	24 ± 2% a
Mid TTI High Rate	198 ± 3	119 ± 12	554 ± 17	324 ± 32 b	38 ± 4% b	13.7 ± 1.0	68 ± 4% c	381 ± 36 b	44 ± 6% b	3283 ± 255 a	32 ± 4% b
High TTI	197 ± 2	125 ± 11	547 ± 42	229 ± 25 c	37 ± 2% b	13.7 ± 1.7	n/a	1024 ± 109 c	37 ± 2% ac	n/a	n/a

All data is presented as mean ± SEM for n=6. Within any column, numbers identified by similar letters are not different, while all others are significantly different ($p<0.05$), as determined by ANOVA.

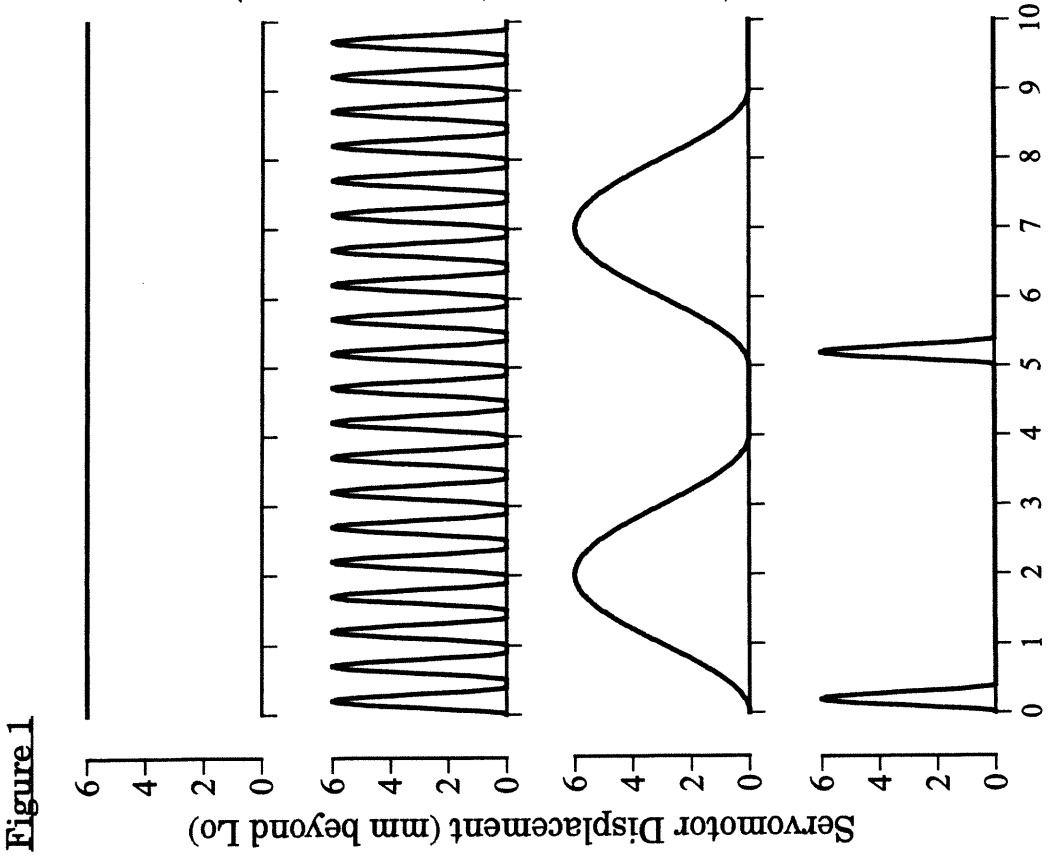
High TTI protocol
Average rate of stretch: 0 mm/sec
Frequency of stretch: 0 Hz
Area under illustrated curve: 60 mm*s
Mid TTI High Rate protocol
Average rate of stretch: 30 mm/sec
Frequency of stretch: 2 Hz
Area under illustrated curve: 24 mm*s
Mid TTI Low Rate protocol
Average rate of stretch: 3 mm/sec
Frequency of stretch: 0.2 Hz
Area under illustrated curve: 24 mm*s
Low TTI protocol
Average rate of stretch: 30 mm/sec
Frequency of stretch: 0.2 Hz
Area under illustrated curve: 2.4 mm*s
Servomotor Displacement (mm beyond Lo)
Time (s)

Figure 1

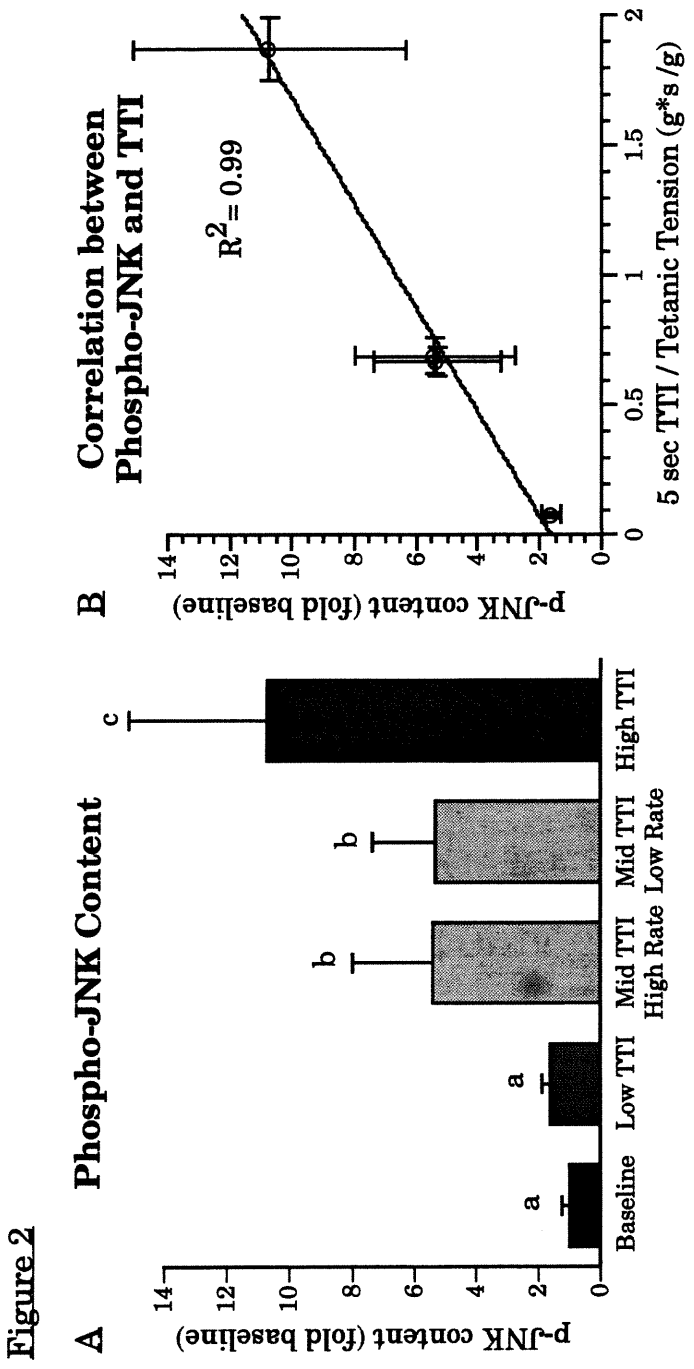
A
Phospho-JNK Content
p-JNK content (fold baseline)
0
2
4
6
8
10
12
14
a
a
b
b
c
Baseline
Low TTI
Mid TTI
High Rate
Mid TTI
Low Rate
High TTI
B
Correlation between
Phospho-JNK and TTI
R^2= 0.99
p-JNK content (fold baseline)
0
0.5
1
1.5
2
5 sec TTI / Tetanic Tension (g*s /g)

Figure 2

Chapter 4

Manuscript 3

Title

Contraction-stimulated p54 JNK activation is attenuated in δ-sarcoglycan-deficient skeletal muscle

Authors

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Journal

Muscle and Nerve

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Summary

To assess the functionality of mechanically-induced signaling in dystrophic muscle and the involvement of the dystrophin-glycoprotein complex (DGC) in this signaling, activation of p54 JNK mitogen-activated-protein-kinase, a marker of mechanical stimulation in muscle, was measured in normal and δ-sarcoglycan-deficient BIO 14.6 hamsters in response to contractile activity in-situ. Contraction-stimulated activation of p54 JNK was found to be substantially impaired in dystrophic BIO 14.6 muscle, suggesting an involvement of the DGC in mechanosignaling and possibly having implications for the regulation of gene expression.

Sommaire (Translated summary)

Pour évaluer le fonctionnement de la signalisation induite par stimulation mécanique dans le muscle dystrophique ainsi que le rôle potentiel du complexe dystrophine-protéines associées à la dystrophine (CDPA) dans cette signalisation, l'activation de la kinase p54 JNK, un marqueur établi de la stimulation mécanique du muscle squelettique, a été mesurée suite à un protocole de contractions musculaires in-situ chez des hamsters normaux et des hamsters BIO 14.6 déficients en δ-sarcoglycan. Les résultats ont démontré que l'activation de la p54 JNK induite par contractions musculaires est réduite de façon importante dans les muscles dystrophiques. Une telle réduction peut avoir des effets sur la régulation de l'expression génétique dans la dystrophie musculaire et suggère une participation du CDPA dans la signalisation déclenchée par la stimulation mécanique.

Introduction

Significant progress has been made towards elucidating the architecture of the dystrophin-glycoprotein complex (DGC). However, the function of this complex and of its various components still remains unclear. The DGC appears to serve a structural role by conferring mechanical stability [23, 24] and by contributing to cellular adhesion and force transmission [4, 7, 21, 22, 36]. In addition, the DGC may also participate in intracellular signaling. In support of this are the observations that a number of signaling proteins associate with the DGC [1, 6, 10, 15, 20, 31, 32, 35], and that the DGC interacts with the focal adhesion complex [2, 12, 19, 29, 36]. Also, the finding that some models of muscular dystrophy result from a molecular defect of the DGC which does not compromise sarcolemmal integrity [8] supports an etiology other than mechanical instability.

The purpose of this study was to test for an involvement of the DGC in mechanically-induced signaling. This was achieved by tetanically stimulating muscles of wild-type and δ-sarcoglycan-deficient BIO 14.6 hamsters [9, 11, 26] in situ, and measuring the resulting activation of the p54 JNK mitogen-activated-protein-kinase (MAPK), a mechanically-responsive kinase in muscle whose activation is quantitatively related to imposed or developed tension [17, 18]. Results demonstrate impaired mechano-signaling in this model of muscular dystrophy and suggest an involvement of the DGC in this signaling.

Materials and methods

Twelve 90 day-old male δ-sarcoglycan-deficient UMX-7.1 hamsters (Université de Montréal), a subline of the BIO 14.6 strain [9, 11, 26], and twelve age-matched male wild-type golden syrian hamsters (Lake View Golden hamsters; Charles-River Laboratories) were anesthetized (ketamine/xylazine, 160/8 mg/kg), and prepared surgically for indirect electrical stimulation of the left gastrocnemius in-situ. Following the experiment, animals were killed by anaesthetic overdose. Treatment of animals was certified by the animal ethics committee of the Université de Montréal. The preparation was similar to the rat nerve-muscle preparation described in detail elsewhere [17], with the exception that the entire gastrocnemius complex was isolated instead of the plantaris. Isolated muscles were set at optimal length for twitch tension development (Lo) and allowed to recover for 5 min. Experimental muscles were then stimulated indirectly to contract tetanically at 100 Hz for 150 ms per second for 5 min either isometrically at Lo (n=6 BIO 14.6 and n=6 LVG), or eccentrically by imposing 2 mm of lengthening between Lo and Lo + 2 mm over the 150 ms stimulation period (n=3 BIO 14.6 and n=3 LVG). Control muscles were held at Lo for 5 min (baseline condition; n=3 BIO 14.6 and n=3 LVG). Immediately following the experimental period, muscles were excised and frozen in liquid nitrogen. Tissue processing, electrophoretic separation of proteins, and analysis of JNK content by immunoblotting with anti-phospho-JNK and anti-JNK2 antibodies were performed as described in detail elsewhere [17].

Results

The gastrocnemius muscles of dystrophic BIO 14.6 hamsters exhibited significantly lower p54 JNK MAPK phosphorylation in response to isometric or eccentric contractions than control muscles of LVG wild-type hamsters, despite no difference in baseline p54 JNK phosphorylation or total content of p54 JNK (Figure 1). The p54 JNK response of LVG wild-type hamsters to isometric contractions was similar to that observed in rats [17]. BIO 14.6 muscles were confirmed to exhibit a clear dystrophic phenotype by standard histological techniques. Gastrocnemius specific tetanic tension was only slightly reduced in the dystrophic animals (1533 g/g wet muscle weight BIO 14.6; 1616 g/g LVG) and thus similar levels of mechanical stimulation could be expected to result from the isometric stimulation, even in the presence of an 11% difference in muscle weight and a substantial difference in body weight (109 ± 8 g BIO 14.6; 159 ± 3 g LVG). Rate of fatigue was identical over the first 30 seconds of stimulation. After this time, BIO 14.6 muscles exhibited a greater rate of fatigue, as previously observed [13], and thus a greater relative loss of peak tension. However, the average peak tension over the first minute, the best predictor of p54 JNK activation under these experimental conditions [17], was not significantly different.

Discussion

Reduced contraction-stimulated activation of p54 JNK in BIO 14.6 hamsters is evidence of blunted mechano-signaling related to a defect of the DGC and suggests a mechanosensory role for the DGC, as previously hypothesized [8, 9], or a role in the transduction of mechano-signaling. It has recently been observed that another mechanically-induced event quantitatively related to tension, namely increased association of β1 integrin to the cytoskeleton [16], is unaffected in BIO 14.6 muscle. This supports that any mechanosensory function of the DGC would have to be complementary to the DGC-independent mechanosensory function of the focal adhesion. A role in transduction of mechano-signaling is perhaps more likely; by binding the adapter protein Grb-2 [20, 35], which plays a critical role in the activation of JNK by integrins in response to mechanical stimulation in endothelial cells [3, 14], as well as at least one member of the MAPK family [10], the DGC may contribute to the local availability of these proteins to a mechano-signaling pathway initiated by the focal adhesion, and this availability may be compromised by a defects of the DGC. Alternatively, it is possible that the DGC is not involved in mechano-signaling and that impaired signaling is due to secondary consequences of a DGC defect, such as the constitutively elevated protein-phosphatase-1 activity reported in muscles of the dystrophin-deficient mdx mouse [34].

The outcome of impaired mechano-signaling is likely altered gene expression. The reduced expression of the muscle-specific splice variant of IGF-1 in response to muscle stretch observed in dystrophin- or merosin-deficient murine models of muscular dystrophy [5] may be related to such defective signaling. Furthermore, as there is a link between mechanical stimulation and cell survival in mechanocytes [27, 30], deficient mechano-signaling may impair the transduction of survival cues and result

in reduced inhibition of apoptosis. This is of particular interest in light of the growing recognition of apoptotic cell death as an etiological factor in the muscular dystrophies [25, 28, 33].

Acknowledgments

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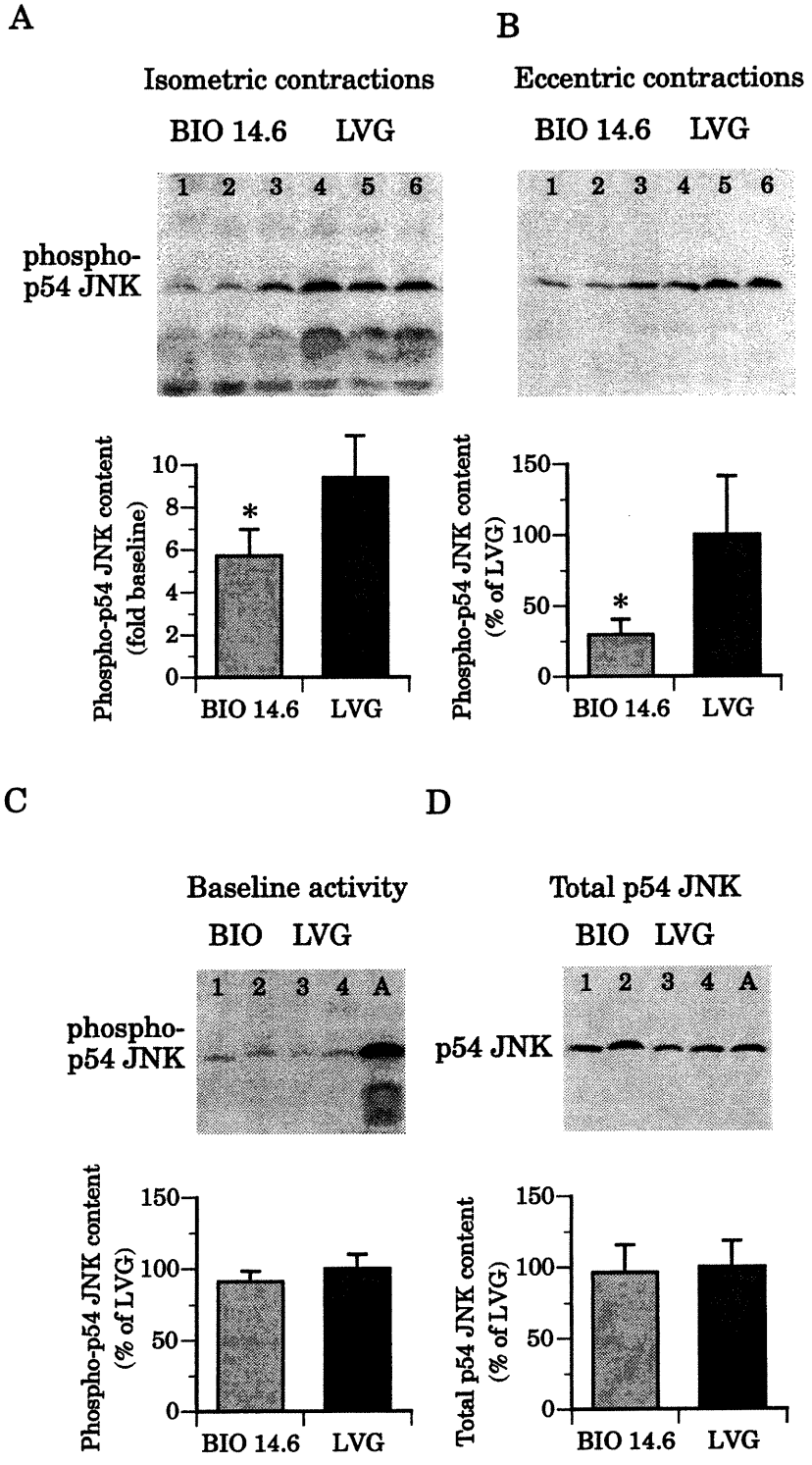
Figure legends

Figure 1

Muscles of the δ-sarcoglycan-deficient BIO 14.6 hamster exhibit substantially reduced contraction-stimulated p54 JNK MAPK phosphorylation, as compared to muscles of LVG (Lake View Golden) wild-type golden syrian hamsters (**Figure 1A and B**), despite similar levels of baseline p54 JNK phosphorylation (**Figure 1C**), similar total content of p54 JNK (**Figure 1D**), and only a 5% deficit in specific tension. Response was measured in the gastrocnemius complex immediately following 5 min of in-situ isometric contractions (**A**) or eccentric contractions (**B**). All samples were analyzed simultaneously on a single blot and all lanes contain the same amount of protein. In **D**, the same blot used in **C** was stripped and reprobed with an anti-JNK2 antibody in order to assess total p54 JNK content. Lane A contains a sample of an eccentrically-stimulated muscle in order to illustrate the extremely low levels of baseline p54 JNK phosphorylation (**C**) as well as the efficiency of the stripping procedure (**D**). In **A**, **B**, and **C**, the differing intensity of the background as well as the intensity of lower bands, presumably corresponding to p46 JNK, are indicative of length of exposure used for each set of measurements. The graphs in **A**, **B**, and **C** illustrate mean + SEM phospho-JNK content in isometrically-contracted (n=6), eccentrically-contracted (n=3), and unstimulated muscles (n=3). The graph in **D** illustrates mean + SEM total JNK content (n=12). * denotes a significant difference ($p<0.05$).

FIGURE 1


A
B
Isometric contractions
Eccentric contractions
BIO 14.6
LVG
BIO 14.6
LVG
1 2 3 4 5 6
1 2 3 4 5 6
phospho-p54 JNK
Phospho-p54 JNK content (fold baseline)
10
8
6
4
2
0
*
BIO 14.6
LVG
Phospho-p54 JNK content (% of LVG)
150
100
50
0
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BIO 14.6
LVG
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Baseline activity
Total p54 JNK
BIO
LVG
BIO
LVG
1 2 3 4 A
1 2 3 4 A
phospho-p54 JNK
p54 JNK
Phospho-p54 JNK content (% of LVG)
150
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Total p54 JNK content (% of LVG)
150
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BIO 14.6
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Chapter 5

Manuscript 4

Title

Acute strengthening of β1 integrin association to the cytoskeleton in response to tension in skeletal muscle in vivo

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Journal

Journal of Biological Chemistry

Submitted, April 2002

Summary

Cellular adhesion and transmission of forces between the cytoskeleton and the basement membrane are mediated by integrins and the protein scaffold which anchors them to the cortical cytoskeleton within structures called focal adhesions. These structures are sensitive to the forces that act through them, initiating intracellular signaling and exhibiting local stiffening in response to mechanical stimulation. This study was designed to assess changes in solubility of integrins, indicative of altered association with the cytoskeleton, in response to precisely measured forces applied to skeletal muscle in-vivo. Muscle was observed to contain RIPA-soluble and -insoluble sub-populations of β1 integrin. Intermittent tetanic contractions or constant passive stretch of the rat plantaris muscle in-situ induced a rapid and short-lived solubility shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction, which was linearly related (R^2= 0.96 to 0.99) to peak developed tension. Vinculin also exhibited a similar solubility shift. In dystrophic muscle from the δ-sarcoglycan-deficient BIO 14.6 hamster, the β1 integrin solubility shift was not affected, suggesting that the dystrophin-glycoprotein complex is not involved in this response. A tension-dependent increase in strongly cytoskeleton-associated β1 integrins, possibly occurring through a recruitment of integrins and their protein scaffold to existing focal adhesions, may serve a cytoprotective function and have implications for force transmission in muscle fibers and other mechanocytes.

Sommaire (Translated summary)

L'adhésion cellulaire et la transmission des forces mécaniques entre le cytosquelette et la lame basale sont effectuées par les intégrines et les protéines qui les attachent au cytosquelette sous-membranaire à l'intérieur de structures nommées adhésions focales. Ces structures sont sensibles aux forces qu'elles transmettent, déclenchant des cascades de signalisation intracellulaire et augmentant aussi leur rigidité en réponse à la stimulation mécanique. Le but de cette étude était de vérifier si les intégrines subissent un changement de solubilité, indiquant une augmentation de la force d'adhésion au cytosquelette, en réponse à des tensions précises appliquées in-vivo au muscle squelettique. Il a été observé que le muscle contient deux sous-populations d'intégrine β1 qui peuvent être différenciées d'après leur solubilité dans une solution tampon RIPA. En réponse à des contractions intermittentes ou à un étirement maintenu du muscle plantaire du rat in-situ, une diminution rapide et de faible durée de la solubilité de l'intégrine β1 a été observée. Ce changement de solubilité était fortement corrélé (R^2 = 0.96 à 0.99) à la tension développée par le muscle. La vinculine, une autre protéine de l'adhésion focale, a aussi démontré un tel changement de solubilité. Dans le muscle dystrophique du hamster BIO 14.6, caractérisé par une déficience en δ-sarcoglycan, le changement de solubilité de l'intégrine β1 n'était pas affecté, suggérant que le complexe dystrophine-protéines- associées ne participe pas à cette réponse. Une augmentation linéaire de la concentration d'intégrines β1 fortement liées au cytosquelette en réponse à la tension, probablement induite par un recrutement d'intégrines et de leurs protéines de liaison cytosquelettiques aux adhésions focales établies, pourrait servir d'une part, de mécanisme cytoprotecteur, et pourrait avoir d'autre part, un impact un niveau de la transmission des forces dans les fibres musculaires et autres mécanocytes.

Introduction

Focal adhesions are specialized cytoskeletal structures which tether adherent cells to their surrounding extracellular matrix (ECM)(1). Through integrins, the integral membrane proteins which function as receptors for ECM proteins, and the scaffold of structural proteins which anchor integrins to the cortical cytoskeleton, mechanical forces are transmitted bidirectionally between the inside and the outside of the cell (1, 2).

Focal adhesions not only channel mechanical forces but also exhibit responsiveness to these forces, as studied in various cell types in culture. Intracellular signaling events are initiated by focal adhesions in response to mechanical stimulation (3-9) and to changes in integrin binding state (10, 11). Furthermore, focal adhesion structure is modified in response to mechanical cues. For example, increased association between integrins and the cytoskeleton results from cross-linking of integrins in cultured myoblasts (12) and hepatocytes (13), while increased stiffness of the integrin-cytoskeleton linkage in fibroblasts (2, 14-17) as well as focal adhesion assembly in fibroblasts and cardiomyocytes (18, 19) result from the application of mechanical forces directly to focal adhesions. Increases in association of integrins to the cytoskeleton or in stiffness of the integrin-cytoskeleton linkage are likely manifestations of a common mechanism for increasing strength of adhesion, which can be rapidly adjusted by regulating integrin-ECM interactions (20, 21) or interactions between integrin, its protein scaffold, and the cytoskeleton (22-25).

Skeletal muscle is especially well-suited to the study of the effects of mechanical stimulation. Muscle in-vivo can be subjected to a wide range of well-defined tensile forces in the form of contractions or passive stretch, allowing to establish quantitative relationships between force and

mechanically-responsive events (26, 27). Skeletal myocytes are large mechanically-resistant cells that sustain high mechanical forces. The mechanical stability of myocytes is conferred by an extensive cytoskeleton and a high density of focal adhesions anchoring the muscle fiber from all sides to its investing basement membrane and specialized to withstand large stresses (23, 28-32). Focal adhesions are found throughout the length of the fiber, in perfect register with Z-discs as costameres, and in very high density at the myotendinous junctions (28, 29, 31). All adult myocyte integrin heterodimers are composed of the skeletal- and cardiac-muscle-specific β1D integrin (30), which exhibits higher ECM binding and higher association with the cytoskeleton, as compared to the β1A isoform (23). Adhesion mediated by the dystrophin-glycoprotein complex also contributes to the specialization of myocyte focal adhesions (33, 34). While the structure of muscle focal adhesions is relatively well-characterized, motivated in part by the devastating outcome of the disorders which target proteins of the dystrophin-glycoprotein complex or of the basement membrane, the responsiveness of muscle focal adhesions to mechanical stimulation has not yet been addressed.

The purpose of the present study was to investigate the impact of increased tension on focal adhesions using the skeletal muscle in-vivo model. Specifically, we examined tension-induced changes in β1 integrin association to the cytoskeleton. This study reports a rapidly developing, tension-dependent β1 integrin solubility shift. These findings represent the first report of increased association between integrins and the cytoskeleton induced by mechanical stimulation and the first report of acute focal adhesion responsiveness to mechanical stimulation in-vivo. This response may confer a cytoprotective effect and have implications for force transmission.

Materials and methods

Animals: Forty-five female Sprague-Dawley rats (Charles River Laboratories), weighing 195 ± 5 g, underwent an in-situ preparation of the sciatic nerve and plantaris muscle under ketamine/xylazine anesthesia (61.5 and 7.7 mg/kg, respectively). Nine 90 day-old male UM-X7.1 Syrian hamsters (Université de Montréal), a sub-line of the δ-sarcoglycan-deficient BIO 14.6 strain, weighing 109 ± 8 g and nine age-matched male wild-type LVG golden Syrian hamsters (Charles-River Laboratories) weighing 159 ± 3 g, underwent an in-situ preparation of the sciatic nerve and gastrocnemius complex under ketamine/xylazine anesthesia (160 and 8 mg /kg, respectively). Following the experiment, animals were killed by anaesthetic overdose. Treatment of animals was certified by the animal ethics committee of the Université de Montréal and conformed to the regulations of the Canadian Council of Animal Care.

In situ nerve-muscle preparation: The rat in situ model used here has been previously described in detail (26). Briefly, the left plantaris muscle and the common tendon of the ankle extensors were isolated from the other extensors. The calcaneous was clipped, leaving a bone chip attached to the common tendon, and a silk ligature was firmly placed around the bone-tendon interface. The animal was secured in the prone position within a stereotaxic frame with the left foot clamped and the left knee pinned in a slightly flexed position. The silk ligature was attached to the lever arm of a muscle puller servomotor (305B-LR; Aurora Scientific), without putting the isolated muscle under tension. The skin of the hindlimb was pulled into a bath which was filled with heated mineral oil and the muscle temperature was maintained at 36-37° C while core temperature was maintained at 35-36° C. Optimal length for muscle twitch tension development (Lo) was determined by stimulating the sciatic nerve with a supramaximal square

pulse 0.05 ms in duration, every 3 s while the muscle was slowly lengthened from a completely slack position. Following this determination, electrical stimulation was ceased and the muscle held at this length for 5 min before the onset of the experimental protocol. Hamsters underwent a similar preparation with the exception that the entire gastrocnemius complex was isolated. The use of this muscle required a minimum of modifications to the experimental protocol as it produced comparable contractile tensions and quantities of tissue as the rat plantaris.

Contractile and passive stretch stimulation: The isolated plantaris was either maintained at Lo for an additional 5 min period (baseline condition; n=4), or indirectly stimulated via the sciatic nerve to contract tetanically at 100 Hz for 150 ms per second for 5 min either isometrically at Lo (n=4), concentrically by allowing 3 mm of shortening between Lo + 3 mm and Lo over the 150 ms stimulation period (n=4), or eccentrically by imposing 3 mm of lengthening between Lo and Lo + 3 mm over the stimulation period (n=4). These different models of contractile activity, described in detail elsewhere (26), produce peak tensions on the order of 4-fold twitch-tension for concentric, 6-fold twitch-tension for isometric, and 10-fold twitch-tension for eccentric. In addition to contractile activity, constant passive stretch to a length excursion of Lo + 8 mm, corresponding to approximately a 25% strain, was also employed as a form of mechanical stimulation for an additional group (n=4). The isolated hamster gastrocnemius complex, comparable in size and force to the rat plantaris, was either maintained at Lo (baseline; n=3 BIO 14.6 and n=3 LVG), or stimulated to contract isometrically for 5 min at Lo (n=6 BIO 14.6 and n=6 LVG) according to the same stimulation protocol used for rat muscle. For all groups and conditions described above, muscles were immediately excised and frozen in liquid nitrogen at the termination of the 5 min experimental period. For time course experiments, additional groups of isometrically-stimulated rat plantaris muscles were generated which differed

in the duration of stimulation or in time of sampling. For three groups (n=3), muscles were stimulated 30 s, 1 min, and 2.5 min, respectively, and held at Lo until excision and freezing at 5 min following the onset of stimulation. For two groups (n=3), muscles stimulated for 5 min were allowed an additional 5 min and 15 min of rest, respectively, before excision and freezing. All frozen muscles were stored at -80° C for subsequent biochemical analysis.

Sample preparation, electrophoretic separation, immunoblotting and analysis: Frozen muscles were powdered under liquid nitrogen and approx. 100 mg of muscle powder was solubilized by frequent vortexing over a period of 1 h in 10 vol. of ice-cold RIPA buffer (50 mM HEPES, 150 mM NaCl, 5% glycerol, 5 mM EGTA, 2 mM $MgCl_2$, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors (Boehringer Mini-Protease and 2 mM PMSF) and phosphatase inhibitors (100 µM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride). Homogenates were centrifuged 1 h at 4500 g, 4° C, and the RIPA-soluble fraction was decanted. The RIPA-insoluble pellets were resuspended in reducing SDS-PAGE sample buffer (60 mM Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, pH 6.8) with mechanical disruption of packed material and frequent vortexing, followed by a 10 min immersion in boiling water and additional vortexing. These homogenates were centrifuged 10 min at 600 g, the supernatant decanted, and the second pellet discarded. Protein concentration of both fractions was determined by Bradford protein assay (Bio-Rad) of aliquots diluted 2000 x in water. Samples containing 200 µg of protein were prepared for SDS-PAGE by dilution and boiling in reducing sample buffer. Samples were separated on 8% acrylamide gels and electro-transferred to PVDF membrane. Successful transfer and equal loading were confirmed by Ponceau stain. In addition to the fractionation procedure, total muscle protein was also obtained from a number of control and experimental muscles by directly solubilizing powder in SDS-PAGE reducing

sample buffer, as described for RIPA-insoluble pellets.

β1 integrin protein content was assessed by immunoblot using an anti-β1 antibody (Santa Cruz Biotechnology). Identification of β1 integrin on immunoblots was confirmed using an anti-β1 integrin antibody from a second source (Chemicon). Vinculin content was also assessed using an anti-vinculin antibody (Upstate Biotechnology). All membranes were blocked with BSA, bathed overnight at 4° C in primary antibody solution, and bathed 1.5 h at room temp. in secondary antibody solution. Revelation was performed by chemiluminescence with film exposure times ranging from 2 to 20 min. Films were scanned with a flatbed scanner and densitometry of results within the linear range of the technique was performed with NIH Image software.

Assuming that all β1 integrin was recovered by the fractionation procedure, the total β1 integrin content of a sample can be calculated as the sum of the RIPA-soluble and the RIPA-insoluble β1 integrin contents of that sample, and RIPA-soluble and -insoluble concentrations of β1 integrin can be expressed as proportions of that total. However, because RIPA-soluble and -insoluble concentrations were measured separately using different relative arbitrary units, it was necessary to normalize these units before calculating total β1 integrin concentration from the 2 fractions. Since any change of β1 integrin content in one fraction must be mirrored by an equal and opposite change of β1 integrin content in the other fraction, normalization was performed on the basis of average magnitude of change between control and stimulated samples: RIPA-insoluble β1 integrin concentration units were multiplied by the constant which resulted in a nearly equal average algebraic sum of individual sample RIPA-soluble and -insoluble β1 integrin concentrations across all 4 experimental conditions. This constant algebraic sum was considered the total concentration of β1 integrin, and RIPA-soluble and -

insoluble concentrations were expressed as proportions of this total.

One-way ANOVA analysis with Fisher post-hoc was used to assess between-group differences in untransformed densitometry data.

Alkaline phosphatase treatment: Samples of baseline and eccentrically-contracted muscles were homogenized in RIPA buffer containing no phosphatase inhibitors. Following the 1 h extraction period, 1000 U of bovine intestine alkaline phosphatase (Sigma) was added to the homogenates and samples were incubated at 37° C for 1 h. Homogenates were then fractionated and analyzed as described above. As a positive control, blots of these samples were probed with an anti-phosphotyrosine antibody (PY99; Santa Cruz Biotechnology).

Lipid raft analysis for β1 integrin: Lipid rafts were isolated from the RIPA-insoluble pellets of baseline and eccentrically-contracted muscles by a procedure adapted from Dermine et al. (35). The pellets were resuspended in RIPA buffer with a Dounce homogenizer (final volume about 2 ml), and the resulting suspension was adjusted to 40% (w/v) sucrose by 1:1 dilution with 80 % (w/v) sucrose in RIPA buffer, poured at the bottom of an Ultraclear centrifuge tube (Beckman) and overlaid successively with 4 ml of 35% sucrose and 3.5 ml of 5 % sucrose in RIPA buffer. After a 19 h centrifugation at 38,000 rpm (SW41 rotor), the lipid rafts, identified as a light scattering band floating at the 5%/35% sucrose interface, were removed by collecting 1 ml at this interface. The proteins in this fraction were precipitated with a methanol/chloroform mixture (36) and resuspended in reducing sample buffer for anti-β1 integrin immunoblotting, as described above. The resulting pellets were also collected and processed for anti-β1 integrin immunoblotting.

Results

Contractions or passive stretch decrease the concentration of RIPA-soluble β1 integrin and reciprocally increase the concentration of RIPA-insoluble β1 integrin: Western blot analysis of rat plantaris skeletal muscle separated into a RIPA-buffer-soluble and -insoluble fraction revealed the presence of β1 integrin in both fractions. Rat skeletal muscle β1 integrin was identified at approximately 97 kDa using anti-β1 integrin antibodies from 2 different sources. Application of tension to muscle for 5 min by either intermittent contractions or constant passive stretch induced a solubility shift in β1 integrin observed as a marked decrease in concentration of RIPA-soluble β1 integrin and a reciprocal increase in concentration of RIPA-insoluble β1 integrin (Figure 1). Total concentration of β1 integrin was unaffected by the application of tension to muscles, as revealed by western blot analysis of muscles solubilized directly in SDS-PAGE reducing sample buffer (not shown).

Tension-dependency of the β1 integrin solubility shift: The relationship between tension and shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction was investigated by quantifying the concentrations of RIPA-soluble and -insoluble β1 integrin after muscles had been subjected to one of three protocols of contractile activity, each generating different precisely measurable levels of peak tension. Muscles were indirectly stimulated to contract tetanically for 150 ms every second for 5 min. During each contraction, length was either simultaneously shortened by 3 mm (concentric contractions), kept constant (isometric contractions), or lengthened by 3 mm (eccentric contractions). The concentric, isometric, and eccentric contractile protocols produced maximal peak tensions of 241 ± 6 g, 398 ± 18 g, and 630 ± 13 g, respectively. Concentration of RIPA-soluble β1 integrin was 76 ± 5 %, 59 ± 5 %, and 24 ± 3 % of unstimulated muscle

concentration, whereas concentration of RIPA-insoluble β1 integrin was 119 ± 8 %, 162 ± 11 %, and 195 ± 6 % of unstimulated muscle concentration, in concentrically-, isometrically- and eccentrically-contracted muscles, respectively. When expressed as a % of total (RIPA-soluble + RIPA-insoluble) concentration of β1 integrin, the concentration of RIPA-soluble β1 integrin in unstimulated muscle was 56 ± 5 % (Figure 2A), and this proportion was reduced to 42 ± 3 %, 33 ± 3 %, and 13 ± 1 % in concentrically-, isometrically- and eccentrically-contracted muscles, respectively (Figure 2A). Correlation analysis between fractional concentrations of β1 integrin and peak tension measured in the four conditions revealed a highly-significant (R^2=0.99) negative linear relationship for RIPA-soluble concentration of β1 integrin (Figure 2B) and an equally significant (R^2=0.96) positive linear relationship for RIPA-insoluble concentration of β1 integrin (Figure 2B).

Dependency on duration of stimulation and time-course of the contraction-induced β1 integrin solubility shift: The dependency of the contraction-induced shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction on duration of stimulation was investigated by measuring the concentration of RIPA-soluble β1 integrin in muscles stimulated to contract isometrically for 30 s, 1 min, 2.5 min, or 5 min and excised at 5 min following onset of stimulation (Figure 3). Comparison with unstimulated muscles showed that 30 s of stimulation was insufficient to alter the concentration of RIPA-soluble β1 integrin. In contrast, significant reduction in concentration was observed when stimulation lasted 1, 2.5 and 5 min, and this effect was not different between these three stimulation durations. These results indicate that, under the stimulation conditions of this study, the shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction required more than 30 s to develop and required at least 30 to 60 s of stimulation to be induced. Receding of the shift following cessation of stimulation was assessed by measuring the concentration of RIPA-soluble

β1 integrin in muscles stimulated to contract isometrically for 5 min, and excised immediately, 5 min later, and 15 min later (Figure 3). Results indicate that the half-life of the solubility shift induced by 5 min of stimulation was between 5 and 15 min.

Absence of β1 integrin reactivity in the lipid raft fraction: To assess whether stimulation-induced solubility changes resulted from β1 integrin incorporation into low-density detergent-insoluble glycolipid-rich complexes, RIPA-insoluble pellets from stimulated and unstimulated muscles were subjected to sucrose gradient equilibrium density centrifugation to isolate the lipid raft fraction and analyze its β1 integrin content. While the lipid raft fraction contained protein (results not shown), no β1 integrin reactivity was observed within this fraction in samples from baseline or eccentrically-contracted muscles, nor was the stimulation-induced increase in RIPA-insoluble β1 integrin content affected by removal of the lipid raft fraction (Figure 4).

Alkaline phosphatase treatment: Experiments were performed to assess whether the contraction-induced β1 integrin fractional shift is mediated by a tyrosine phosphorylation of β1 integrin and/or of other proteins involved in the linking of β1 integrin to the cytoskeleton. RIPA homogenates from control and eccentrically-contracted muscles were treated for 1 h with alkaline phosphatase prior to fractionation (Figure 5A), a procedure which efficiently eliminated tyrosine phosphorylation, as confirmed by anti-phosphotyrosine immunoblot (Figure 5B). The alkaline phosphatase treatment did not abolish the contraction-induced increase in concentration of RIPA-insoluble β1 integrin.

Contractions or passive stretch decrease the concentration of RIPA-soluble vinculin and reciprocally increase the concentration of RIPA-insoluble vinculin: The hypothesis that the β1 integrin solubility shift is mirrored by a fractional shift of other proteins involved in linking β1 integrin to the cytoskeleton was tested by measuring the RIPA-soluble and RIPA-insoluble concentrations of vinculin in unstimulated muscles and muscles stimulated by contractile activity or passive stretch. Like β1 integrin, vinculin reactivity was observed in RIPA-soluble and -insoluble fractions of unstimulated muscle, and the application of tension for 5 min in the form of contractions or stretch induced a solubility shift, observed as a decrease in concentration of RIPA-soluble vinculin and a reciprocal increase in concentration of RIPA-insoluble vinculin (Figure 6).

Contraction-induced β1 integrin solubility shift is not impaired in dystrophic δ-sarcoglycan deficient muscle: To test whether a defect in the dystrophin-glycoprotein complex would impair the contraction-induced shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction, the RIPA-soluble concentration of β1 integrin was measured in unstimulated and isometrically-contracted gastrocnemius muscles of δ-sarcoglycan-deficient BIO 14.6 hamsters and of age-matched wild-type LVG golden Syrian hamsters. BIO 14.6 hamsters exhibited a clear dystrophic phenotype, as confirmed histologically (results not shown). In both dystrophic and control muscles, 5 min of intermittent tetanic contractions induced a significant decrease in RIPA-soluble β1 integrin concentration (Figure 7), on the same order as that observed in rat muscle subjected to the same stimulation protocol. Concentrations of RIPA-soluble β1 integrin in dystrophic muscle were not significantly different from control. The relative capacity for generating tension (specific tension) was only slightly reduced in dystrophic gastrocnemius muscle (1533 g / g wet muscle weight in BIO 14.6 and 1616 g / g in LVG hamsters). Therefore, it can be assumed that a protocol of maximal

tetanic contractions resulted in comparable relative levels of tensile stress, even in the presence of an 11% difference in muscle weight and a substantial difference in body weight (109 ± 8 g BIO 14.6 and 159 ± 3 g LVG).

Discussion

Understanding how the force-transmitting structures of adherent cells respond to mechanical stimulation may help to unravel the mechanisms through which these structures sense mechanical forces and initiate signaling events which have a profound impact on gene expression (4, 37). This study employed an in-situ skeletal muscle model to examine the tension-induced responses of focal adhesion components, such as β1 integrin, following the application of precisely quantified tensile forces. The findings include a rapidly-developing, short-lived, and tension-dependent solubility shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction in response to contractions or passive stretch of skeletal muscle. This is the first report of increased association between integrins and the cytoskeleton induced by mechanical stimulation, and the first demonstration of acute focal adhesion responsiveness to mechanical stimulation in-vivo.

The skeletal-muscle-specific β1D integrin isoform, the only β-isoform present in mature muscle (30), differs from the widely-distributed β1A integrin in that it is insoluble under mild nonionic detergent conditions and requires the more stringent conditions of a RIPA-buffer, containing sodium deoxycholate and sodium dodecyl sulfate in addition to nonionic Triton X-100, in order to be solubilized (23). The observation that treatment with the actin-disrupting agent cytochalasin D restores solubility of β1D integrin under nonionic detergent conditions indicates that β1D integrin is more strongly-associated to the cortical cytoskeleton than β1A (23). This property is likely conferred by the increased affinity of β1D for the cytoskeletal linker protein talin (23).

In light of the constitutively stronger association of β1D integrin to the cytoskeleton of skeletal muscle, the present study assessed protein strength of association to the cytoskeleton by solubility in RIPA buffer following an approach already used for other focal adhesion proteins (38, 39), rather than by solubility under mild nonionic detergent conditions, as is commonly performed (7, 23, 40-44). The fractionation procedure employed here revealed that β1 integrin exists in skeletal muscle as two cytoskeleton-associated sub-populations differing in strength of association to the cytoskeleton. In unstimulated muscle, the proportion of strongly cytoskeleton-associated β1 integrin represents slightly less than half of the total β1 integrin concentration, whereas, in response to tension, this proportion can increase to more than 85% of the total concentration. β1 integrin solubility shifts, observed by immunofluorescence as decreased extraction of β1 integrin by nonionic detergent, have previously been reported to result from integrin cross-linking with antibodies (12, 13, 45, 46). The disparity in the solubility of the β1 integrin sub-populations observed between these previous reports and the present study can probably be attributed to constitutively enhanced association to the cytoskeleton (23) rendering all muscle integrins more resistant to detergent extraction.

An alternate hypothesis to explain the increase in resistance to detergent extraction would be β1 integrin association with plasma membrane microdomains or "lipid rafts", as there exists evidence of β1 integrin incorporation into low-density detergent-insoluble glycolipid-rich complexes (DIG) (47, 48). This hypothesis was ruled out in the present study as β1 integrin was not detected in DIG isolated from control or stimulated muscles.

The mechanism by which the concentration of integrins strongly associated to

the cytoskeleton is increased may involve the recruitment of high mobility, loosely-associated integrins to existing focal adhesions in a manner similar to integrin clustering during focal adhesion formation (1). This is supported by reports that integrin localization to focal adhesions is associated with decreased detergent-solubility (49) and reduced mobility (50). The finding that the β1 integrin solubility shift is mirrored by a solubility shift of vinculin supports that putative recruitable integrins exist as part of simple actin-binding complexes and that the decrease in β1 integrin solubility is not due to increased affinity of β1 integrin for one of its binding proteins, but rather to increased association of β1 integrin containing complexes to the cortical cytoskeleton. Therefore, entire isolated integrin-cytoskeleton linkages may be recruited to focal adhesions by a remodelling of the actin to which the linkages are bound. Such remodelling would likely be regulated by the Rho-family of small GTPases (1, 19, 51-54), some members of which appear to be mechanically-responsive (55, 56). Integration into the highly ordered and protein-dense structure of the focal adhesion and the underlying cortical cytoskeleton would conceivably result in a greater degree of association of newly-recruited linkages to the cytoskeleton, observable as an increased concentration of RIPA-insoluble integrins and linker proteins.

Such a model of recruitment fits with the decrease in integrin solubility induced by the clustering effect of integrin cross-linking with antibodies (12, 13), as well as with the local accumulation of actin at mechanically-stimulated focal adhesions (16), and with the recently reported relationship between applied force and focal adhesion area (19). Furthermore, under such a model, the observed linear relationship between tension and increase in RIPA-insoluble β1 integrin could explain the linear increase in local stiffness of the cytoskeleton which results from the application of force to single focal adhesions (2, 14, 17). The finding that tyrosine-phosphatase treatment of tissue homogenates prior to fractionation did not inhibit the

change in solubility of β1 integrin indicates that the solubility shift is not the result of a conformational change of integrin or of its linker proteins induced by tyrosine phosphorylation. However, this treatment does not address whether a tyrosine phosphorylation may have initiated pathways responsible for cytoskeletal remodelling.

It was observed that the β1 integrin solubility shift is a rapid response, occurring within minutes of stimulation. However, this appears slower than mechanically-induced strengthening of the cytoskeleton in fibroblasts (15), or than the thrombin-induced association of β1 integrin to the cytoskeleton in endothelial cells (57), possibly suggesting the involvement of a different mechanism. Undoubtedly, the inherent complexity of an in-vivo system, as well as to the extensiveness of the cytoskeleton of skeletal muscle (28, 31, 32), complicate the comparison of kinetics between this study and in-vitro studies.

Force transmission between the basement membrane and the cortical cytoskeleton in myocytes occurs at well-defined focal adhesions located throughout the length of the muscle fiber (i.e. costameres) and at the fiber ends (i.e myotendinous junctions) (28, 31, 32). It can therefore be speculated that integrin-cytoskeleton linkages do not transmit forces unless they are strongly-associated to the cytoskeleton as part of one of these focal adhesions. The recruitment of isolated linkages to focal adhesions would then result in an increase of the number of force-transmitting linkages and could serve a cytoprotective function by spreading forces over more linkages, therefore reducing the magnitude of force channeled through each linkage and its constituent proteins. Initiating this cytoprotective response may be one of the functions of muscle stretching and warm-up which often precede physical activity in humans and animals. A cytoprotective increase in rigidity has previously been ascribed to the force-induced accumulation of actin at focal adhesions (16).

As integrins are believed to be central to the process of mechano-chemical coupling (3, 6-9, 58), it can also be speculated that any change in the number of integrins subjected to force within a focal adhesion or in the amount of force to which each integrin is subjected may affect the signaling response to mechanical stimulation. However, the tension-induced increase in concentration of RIPA-insoluble β1 integrins may not be rapid enough to acutely impact mechanotransduction since MAPK signaling can be induced by as little as 30 seconds of muscle stimulation (26), a stimulation duration insufficient to trigger the β1 integrin solubility shift.

Assessment of the β1 integrin response to contractions in δ-sarcoglycan-deficient muscle of the BIO 14.6 hamster, an animal model of limb-girdle muscular dystrophy (59, 60), indicated that this response is not different from that of wild-type hamster or rat muscle subjected to the same stimulation protocol. This implies that the mechanism regulating the β1 integrin solubility shift is unaffected in this strain, and suggests that a defect within the dystrophin-glycoprotein complex (DGC), presumably decreasing strength of adhesion and affecting force transmission, does not impair the sensitivity of myocytes to mechanical stimulation. In light of the functionality of the integrin response to mechanical stress in BIO 14.6 hamsters, the recent finding that mechanically-induced p54 JNK activation is attenuated in these same muscles (L.C. Martineau and P.F. Gardiner - submitted) would suggest that the mechanism responsible for the β1 integrin solubility shift is not regulated by the same signaling events which lead to activation of p54 JNK.

The study of the responsiveness of focal adhesions to mechanical stimulation in skeletal muscle in-vivo has revealed that the concentration of β1 integrin strongly associated with the cytoskeleton is acutely increased in response to

tension. This increase may occur through a recruitment of integrins and their linker proteins to existing focal adhesions. Such a response may serve a cytoprotective function by increasing the number of integrin-cytoskeleton linkages through which forces are channeled at focal adhesions.

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FIGURE LEGENDS

Figure 1

Shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction induced by muscle contraction or passive stretch. Rat plantaris muscles were stimulated in-situ by 5 min of intermittent contractile activity (**Figure 1A**), or 5 min of constant stretch (**Figure 1B**), and compared to unstimulated muscles held at optimal length for 5 min. On blots of RIPA-soluble proteins (left), lanes 1 to 4 contain unstimulated (Baseline) samples, while lanes 5 to 8 contain stimulated samples. On blots of RIPA-insoluble proteins (right), lanes 1 and 2 contain unstimulated samples, while lanes 3 and 4 contain stimulated samples. Each lane was loaded with 200 μg of protein. A blank lane was inserted between each sample during electrophoresis of RIPA-insoluble proteins in order to enhance resolution.

Figure 2

Reciprocal changes in concentration of RIPA-soluble and of RIPA-insoluble β1 integrin were related to the magnitude of tension developed by muscle.

Figure 2A: Proportions of RIPA-insoluble β1 integrin (open bars) and of RIPA-soluble β1 integrin (shaded bars) relative to total concentration of β1 integrin for unstimulated muscles (Baseline) and muscles stimulated to contract concentrically (shortening contractions), isometrically, or eccentrically (lengthening contractions). Stimulation parameters (duration, frequency, amplitude) were identical in all three protocols, and all muscle length excursions were performed between Lo and Lo + 3 mm. Data are presented as mean ± SEM for sample sizes of 4. RIPA-soluble fractions are all different ($p<0.05$), as assessed by ANOVA, while RIPA-insoluble fractions are all different with the exception of Baseline and Concentric.

Figure 2B: Concentrations of RIPA-soluble and -insoluble β1 integrin relative to total concentration of β1 integrin are plotted against peak tension developed by the contractile activity protocols described above. A highly-significant positive linear relationship between concentration of RIPA-insoluble β1 integrin and tension, and an equally significant negative linear relationship between concentration of RIPA-soluble β1 integrin and tension were observed. Points represent mean ± SEM.

Figure 3

Dependency on duration of stimulation and time course of the contraction-induced β1 integrin solubility shift. The shift of β1 integrin from the RIPA-soluble to the RIPA-insoluble fraction required between 30 and 60 s of stimulation to be induced. Return to unstimulated (Baseline) concentration of RIPA-soluble β1 integrin following 5 min of stimulation required between 5 and 15 min. Bars represent mean + SEM for samples sizes of 3 to 4. * identify significant ($p<0.05$) differences in concentration of RIPA-soluble β1 integrin compared to unstimulated concentration, as assessed by ANOVA.

Figure 4

Low-density detergent-insoluble glycolipid-rich complexes (lipid rafts), isolated from RIPA-insoluble fractions of unstimulated (Baseline) and contraction-stimulated muscles, did not show β1 integrin reactivity (lanes 3 and 4). Removal of this sub-fraction from the RIPA-insoluble fraction did not affect the stimulation-induced increase in β1 integrin (lanes 1 and 2).

Figure 5

Figure 5A: Alkaline phosphatase treatment of muscle homogenates prior to fractionation did not affect the stimulation-induced increase in β1 integrin in the RIPA-insoluble fraction. Lane 1 contains an unstimulated sample while lane 2 contains a contraction-stimulated sample.

Figure 5B: Anti-phosphotyrosine immunoblotting of the RIPA-soluble fraction of control and phosphatase-treated samples confirmed efficient tyrosine-dephosphorylation induced by the treatment. Lanes 1 and 2 contain untreated control and contraction-stimulated samples while lanes 3 and 4 contain phosphatase-treated control and stimulated samples.

Figure 6

Vinculin and muscle-specific meta-vinculin underwent a contraction-induced shift from the RIPA-soluble to the RIPA-insoluble fraction, similar to that observed for β1 integrin. Vinculin western blots were performed from same samples used for β1 integrin western blots. On blots of RIPA-soluble proteins (left), lanes 1 to 2 contain unstimulated (Baseline) samples, while lanes 3 to 4 contain contraction-stimulated samples. On blots of RIPA-insoluble proteins (right), lane 1 contains an unstimulated sample, while lane 2 contains a contraction-stimulated sample. Each lane was loaded with 200 μg of protein. A blank lane was inserted between each sample during electrophoresis of RIPA-insoluble proteins in order to enhance resolution.

Figure 7

Contraction-induced decrease in concentration of RIPA-soluble β1 integrin was similar in muscles of δ-sarcoglycan-deficient BIO 14.6 hamsters and age-matched wild-type LVG golden syrian hamsters. For dystrophic (left) and wild-type muscles (right), lanes 1 to 3 contain samples of the RIPA-soluble fraction of unstimulated (Baseline) gastrocnemius muscles, while lanes 4 to 6 contain samples of the RIPA-soluble fraction of isometrically-contracted gastrocnemius muscles. Each lane was loaded with 200 μg of protein. The graph illustrates mean + SEM RIPA-soluble β1 integrin concentration for 6 contraction-stimulated muscles and 3 unstimulated muscles in each group. * indicates significantly different ($p<0.05$) β1 integrin concentration compared to unstimulated muscles.

FIGURE 1

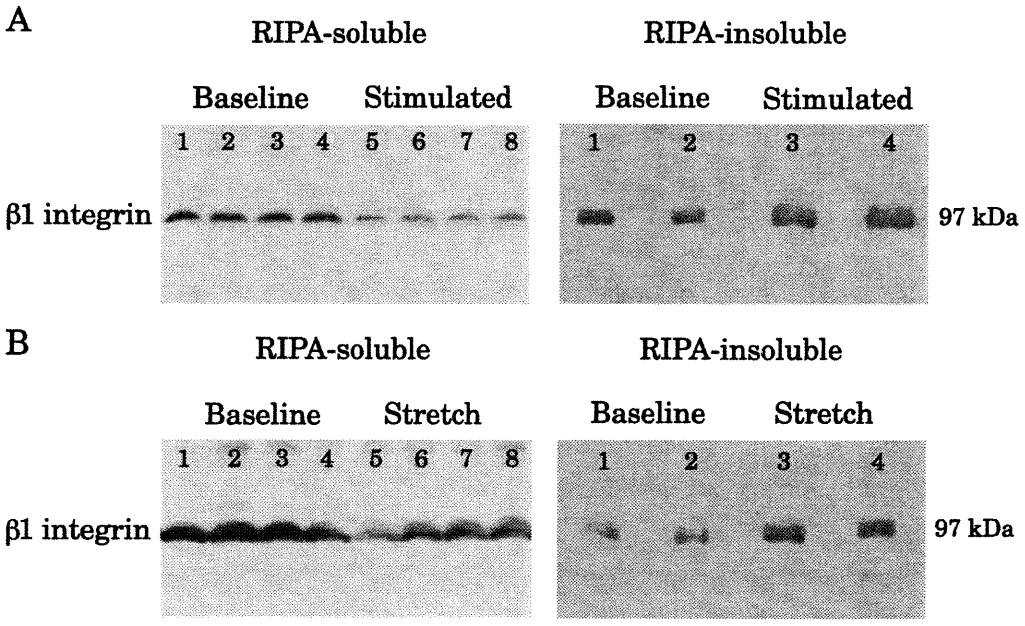
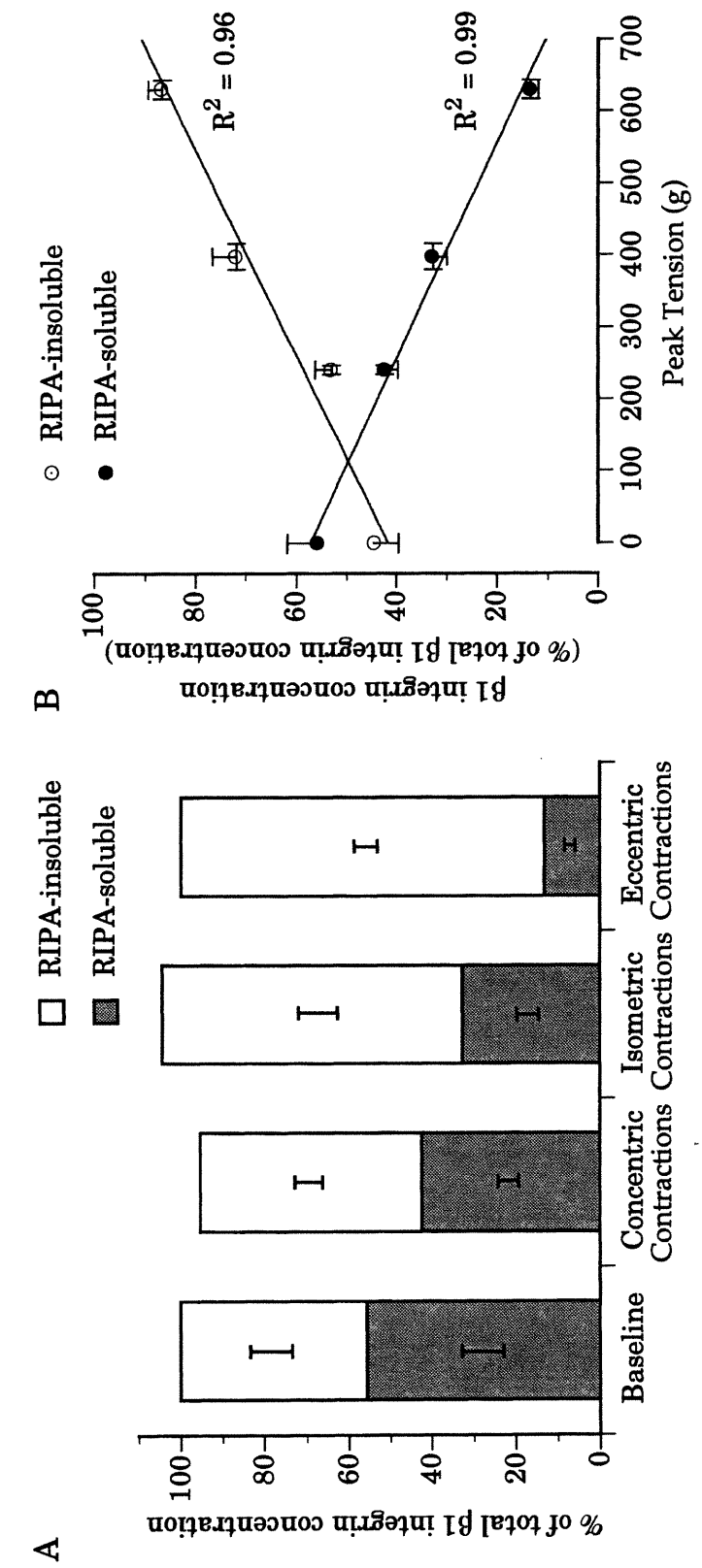

A
RIPA-soluble
RIPA-insoluble
Baseline
Stimulated
Baseline
Stimulated
1 2 3 4 5 6 7 8
1 2 3 4
β1 integrin
97 kDa
B
RIPA-soluble
RIPA-insoluble
Baseline
Stretch
Baseline
Stretch
1 2 3 4 5 6 7 8
1 2 3 4
β1 integrin
97 kDa

FIGURE 2


A
% of total β1 integrin concentration
0
20
40
60
80
100
Baseline
Concentric Contractions
Isometric Contractions
Eccentric Contractions
RIPA-insoluble
RIPA-soluble
B
β1 integrin concentration (% of total β1 integrin concentration)
RIPA-insoluble
RIPA-soluble
$R^2 = 0.96$
$R^2 = 0.99$
0
100
200
300
400
500
600
700
Peak Tension (g)

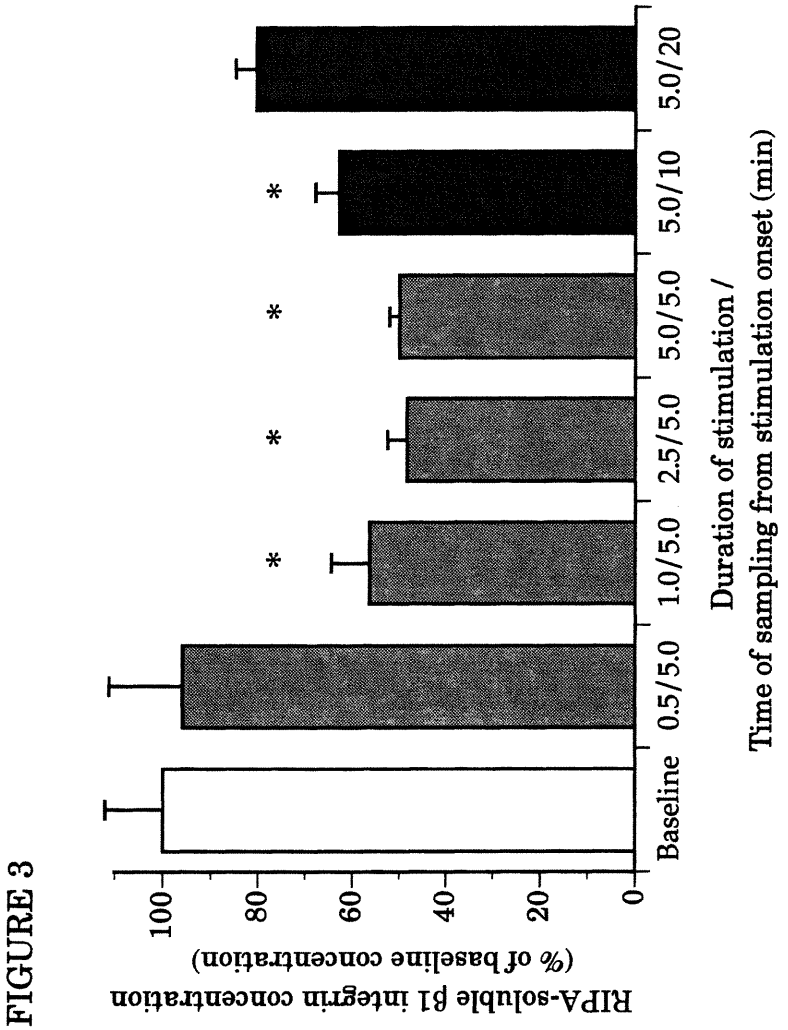

RIPA-soluble β1 integrin concentration
(% of baseline concentration)
0
20
40
60
80
100
*
*
*
*
Baseline
0.5/5.0
1.0/5.0
2.5/5.0
5.0/5.0
5.0/10
5.0/20
Duration of stimulation /
Time of sampling from stimulation onset (min)

FIGURE 3

FIGURE 4

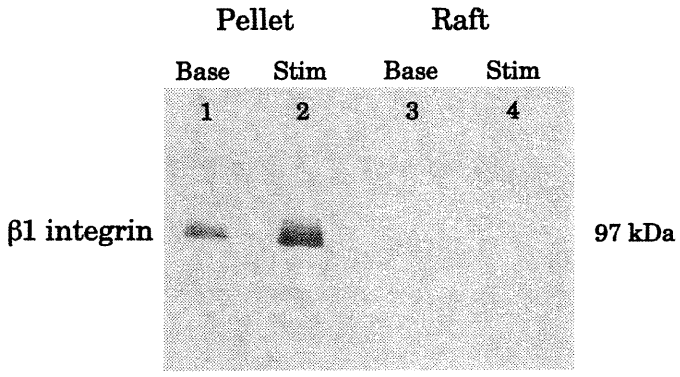


FIGURE 5

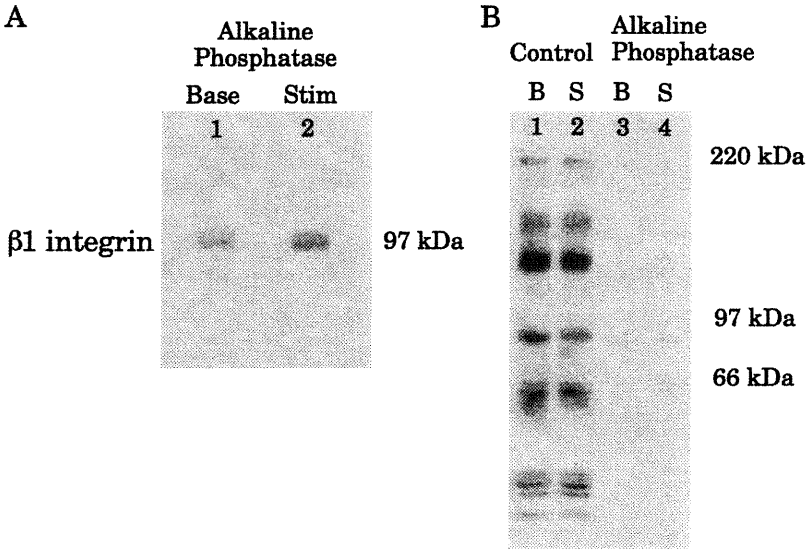

A
Alkaline Phosphatase
Base
Stim
1
2
β1 integrin
97 kDa
B
Control
Alkaline Phosphatase
B S B S
1 2 3 4
220 kDa
97 kDa
66 kDa

FIGURE 6

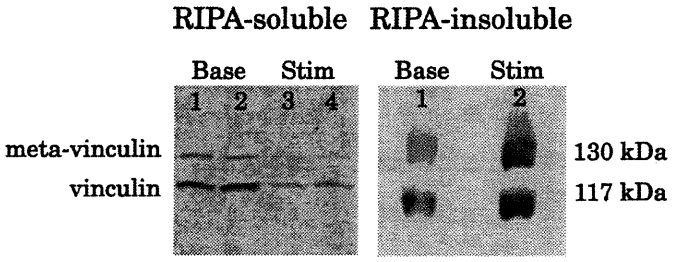
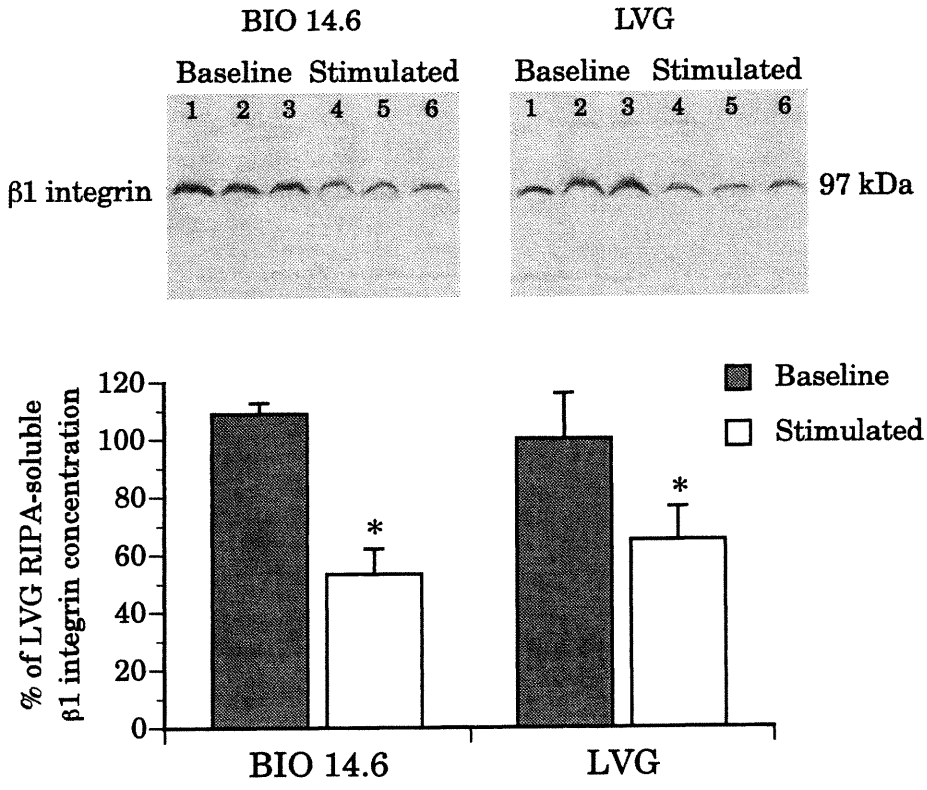

RIPA-soluble
RIPA-insoluble
Base
Stim
Base
Stim
1 2 3 4
1
2
meta-vinculin
vinculin
130 kDa
117 kDa

FIGURE 7


BIO 14.6
LVG
Baseline Stimulated
Baseline Stimulated
1 2 3 4 5 6
1 2 3 4 5 6
β1 integrin
97 kDa
% of LVG RIPA-soluble β1 integrin concentration
120
100
80
60
40
20
0
*
*
Baseline
Stimulated
BIO 14.6
LVG

Chapter 6

Manuscript 5

Title

Chronic modulation of mechanosensitivity and concentration of strongly cytoskeleton-associated β1 integrin in skeletal muscle in-vivo: Is mechanosensitivity dictated by the concentration of force-transmitting integrins?

Authors

Louis C. Martineau and Phillip F. Gardiner

Status

To be submitted upon acceptation of manuscript 4.

Summary

Tension has recently been shown to acutely increase the concentration of RIPA-insoluble, and presumably strongly cytoskeleton-associated, β1 integrins in skeletal muscle in vivo. In light of the mechanosensory function ascribed to integrins, a modulation of the concentration of integrins subjected to a given force, and hence of the magnitude of force applied to each integrin, may directly impact the mechanotransduction process. The present study tests the hypothesis that there exists a relationship between the magnitude of signaling initiated in response to a standardized mechanical stimulus, i.e. mechanosensitivity, and the concentration of strongly cytoskeleton-associated β1 integrins. Rat skeletal muscle was chronically unloaded or overloaded and it was observed that mechanosensitivity, defined as the activation of the mechanically-responsive p54 JNK mitogen-activated-protein-kinase in response to a protocol of maximal tetanic contractions, was inversely related to muscle loading state or resulting muscle size while the concentration of RIPA-insoluble β1 integrin was directly related to loading state or muscle size. A highly significant negative relation was observed between mechanosensitivity and concentration of RIPA-insoluble β1 integrin and an equally significant positive relationship was observed between mechanosensitivity and force per RIPA-insoluble β1 integrin. However, no relationship between mechanosensitivity and total integrin concentration was apparent. These findings support a model whereby mechanosensitivity is regulated by integrins. They also suggest that force transmission through integrins is dependent on the degree of integrin association to the cortical cytoskeleton and that the relationship between stimulation and mechanosignaling is non-linear. The novel observations of this study support the existence of a physiologically relevant and potentially manipulatable mechanism for modulating mechanosensitivity in vivo.

Sommaire (Translated summary)

Il a récemment été démontré que dans le muscle squelettique in vivo, la tension induit une augmentation aiguë de la concentration d'intégrines β1 insolubles dans une solution tampon RIPA, et donc fortement liées au cytosquelette. Comme il existe de nombreuses preuves que les intégrines exercent une fonction de mécano-détection, il est possible d'extrapoler qu'un changement de la concentration d'intégrines soumises à une stimulation mécanique donnée, et donc de la quantité de force subie par chaque intégrine, pourrait avoir un impact sur le processus de couplage mécano-chimique. Cette étude a amené l'hypothèse qu'il existe une relation entre l'amplitude de la signalisation intracellulaire déclenchée en réponse à un stress mécanique standardisé (i.e. la mécano-sensibilité), et la concentration d'intégrines β1 fortement liées au cytosquelette. En utilisant des modèles d'augmentation et de diminution chroniques de la stimulation musculaire chez le rat, il a été observé que la mécano-sensibilité, soit l'activation de la kinase p54 JNK en réponse à un protocole de contractions maximales intermittentes, était inversement reliée à l'état de charge musculaire ou à la taille musculaire résultant de cet état de charge chronique. Réciproquement, la concentration d'intégrines β1 insolubles en solution RIPA était directement reliée au niveau de charge ou à la taille musculaire. De plus, une relation significative fut observée entre la mécano-sensibilité et la concentration d'intégrines β1 insolubles en solution RIPA. Cependant, aucune corrélation entre la mécano-sensibilité et la concentration totale d'intégrines n'a été observée. Ces résultats soutiennent l'idée que la mécano-sensibilité puisse être dictée par les intégrines. Ils suggèrent de plus que la transmission de forces par les intégrines dépend du degré d'association entre celles-ci et le cytosquelette, et que la relation entre la stimulation et la mécano-signalisation qui en est induite est non-linéaire. Ces nouvelles observations supportent l'existence d'un mécanisme de modulation de la mécano-sensibilité in-vivo, ayant une importance physiologique et offrant la possibilité d'être régulé artificiellement.

Introduction

Cellular adhesion to the basement membrane is mediated by integrins [7, 9, 19]. Through integrins and the protein scaffold linking integrins to the cytoskeleton, forces are transmitted bidirectionally between the inside and outside of the cell [2]. In addition to mediating adhesion, there exists convincing evidence that integrins are also mechanosensors [3, 10, 12, 15-17]. In particular, the recent demonstration that turnover of integrin binding to extracellular matrix ligands is essential to mechanotransduction not only supports a mechanosensory function but also appears to reconcile the similarities of signaling in response to integrin ligation and signaling in response to mechanical stimulation [10]. Significant progress has also been made towards elucidating the details of the signaling cascades linking integrins to the mitogen-activated-protein-kinases (MAPK) and gene regulation [3, 10, 11]. The finding that Shc associates with integrin in response to mechanical stimulation is a breakthrough in the identification of the earliest signaling events initiated by integrins [3, 10].

Skeletal muscle in vivo appears to be a useful model for the study of mechanotransduction due in large part to the ease with which a wide range of precisely measurable tensions can be applied or generated and related to mechanically responsive cellular events. Using such a model, our laboratory has established a quantitative non-linear relationship between peak tension and activation of the p54 JNK MAPK [13]. More recently, we have demonstrated that β1 integrin exhibits acute responsiveness to mechanical stimulation in the form of intermittent contractions or constant passive stretch (Martineau et al. - submitted). This responsiveness was observed as a linear relationship between developed tension and increase in the concentration of a sub-population of β1 integrin exhibiting insolubility in a RIPA buffer. This β1 integrin solubility shift was interpreted to represent an

increase in the concentration of integrins bound to the cytoskeleton, and was proposed to serve a cytoprotective function by channeling forces over a greater number of bridges between the cytoskeleton and the basement membrane.

In light of the mechanosensory function of integrins, the concept of a variable concentration of integrins bound to the cytoskeleton and transmitting force may have implications for mechanotransduction; magnitude of force subjected to each integrin, presumably a determining factor of the magnitude of resulting mechanosignaling, would necessarily be altered as a result of a change in the concentration of force-transmitting integrins. This reasoning, in conjunction with our observations of a non-linear relationship between tension and mechanosignaling in muscle [13], leads to the hypothesis that the relationship between force per integrin and magnitude of signaling at the single integrin level is non-linear and therefore that mechanosensitivity, the magnitude of the aggregate signaling initiated in response to a given mechanical stimulus, can be modulated through a change in concentration of strongly cytoskeleton-associated and force-transmitting integrins.

The purpose of this study was twofold: to demonstrate that mechanosensitivity can be modulated and to demonstrate that such modulation is related to the concentration of force-transmitting integrins. This was addressed in skeletal muscle in-vivo whereby mechanosensitivity, defined as the increase in phosphorylation of p54 JNK in response to an acute protocol of maximal tetanic contractions, and concentration of RIPA-insoluble β1 integrins were both modulated using models of chronically-elevated and -reduced muscle usage which result in large adaptive changes in muscle cross-sectional area. In muscles atrophied as a result of paralysis by tetrodotoxin-induced nerve block, the p54 JNK response to maximal contractions was found to be heightened while the concentration

of RIPA-insoluble β1 integrin was decreased. Conversely, the p54 JNK response was found to be diminished while the concentration of RIPA-insoluble β1 integrin was increased in muscles overloaded by synergist tenotomy. The relationship between these 2 variables supports a model of mechanosensitivity regulated by concentration of force-transmitting integrins and suggest that modulation of mechanosensitivity is physiologically relevant.

Materials and methods

Thirty female Sprague-Dawley rats (Charles River) were employed for this study. Treatment of animals was certified by the animal ethics committee of the Université de Montréal and conformed to the regulations of the Canadian Council of Animal Care.

Chronic muscle unloading by tetrodotoxin-induced paralysis: Nine rats weighing 190 ± 5 g underwent 13 to 15 days of reversible paralysis of the left triceps surae induced by continuous superfusion of the sciatic nerve with tetrodotoxin (TTX). The delivery system and implantation procedures used were as previously described [4, 18] with minor modifications. Briefly, 400 µg / ml of citrate-free TTX (Alamone Labs) dissolved in a pH 4.8 salt solution (70 mM sodium chloride, 50 mM sodium acetate, 50 mM acetic acid, 3 mM potassium chloride, 2 mM calcium chloride) containing 200 IU / ml penicillin and 0.2 mg / ml streptomycin, was delivered to the sciatic nerve through a ~10 cm catheter (0.047" OD, 0.025" ID) and an 8-10 mm cuff (0.125" OD, 0.078" ID) made of Silastic tubing (Dow Corning) by an osmotic mini-pump (Alzet, model 2002) implanted subcutaneously on the back. The delivery system was implanted under sterile conditions and ketamine/xylazine anaesthesia (61.5 mg / kg and 7.7 mg / kg, respectively; i.p. injection). Following surgery, rats were warmed on a heating pad and given an i.p. bolus of saline.

Buprenorphine was administered by intramuscular injections over a period of 48 h post-op. To minimize automutilation, rats were fitted with an Elizabethan collar and the surgical limb was painted with a saturated solution of picric acid. Rats were housed individually in standard plastic cages for the duration of the protocol. Onset of paralysis within 24 ± 12 h of implantation was confirmed by visual verification of absence of the toe spreading reflex, extensor activity during locomotion, and response to toe pinching. Maintenance of paralysis throughout the experimental period was assessed daily. At day 13 of paralysis, the osmotic pump was removed under anaesthesia and the rat was allowed to regain function of the paralyzed limb over 24-48 h before undergoing the acute experiment. Rats which exhibited any degree of motor or sensory functionality of the left ankle or foot throughout the paralysis period or which did not regain function following pump removal were excluded from the experiment. Eight control rats of the same initial weight were housed individually for the same duration as the experimental rats.

Chronic muscle overloading by synergist tenotomy: Seven rats weighing 195 ± 5 g underwent 58 to 62 days of bilateral compensatory overload of the plantaris and soleus muscles. Overload was induced by tenotomy and reflection of the medial and lateral gastrocnemius (MG and LG) according to a procedure previously described [6]. Briefly, under ketamine/xylazine anaesthesia, a small longitudinal incision was made between the Achilles tendon and the knee on both legs. The hamstring muscle covering the triceps surae was partially ablated. The common extensor tendon was carefully separated. The MG and LG were separated, tenotomized, partially reflected, and their distal tendons sutured to the hamstring fascia just below the knee in order to prevent reattachment to the common tendon or ankle extensors. Post-operative care was administered as described above. Rats were individually housed for 7 days in standard plastic cages. Following this

period, elizabethan collars were removed and rats were individually housed for seven weeks in voluntary wheel cages. Rats were weighed regularly and monitored for signs of automutilation or running-induced damage to foot pads. Seven control rats of the same initial weight were also individually housed 7 d in standard cages followed by 7 wk in running wheels.

Acute in-situ contractile activity: Following the chronic manipulations described above, all animals underwent an in-situ nerve-muscle preparation of the left sciatic nerve and plantaris muscle, as previously described in detail elsewhere [13]. Briefly, under ketamine/xylazine anesthesia, the plantaris was isolated from the other extensors and the calcaneus was clipped leaving a bone-chip attached to the common extensor tendon. At this bone-tendon interface, a silk thread was firmly tied and the ligature prestressed. The rat was fixed in the prone position within a stereotaxic frame and its left foot clamped and left knee pinned in a slightly flexed position. The silk thread was attached to the arm of a muscle lever system (Aurora Scientific model 305B-LR) without putting the muscle under tension. The skin of the hindlimb was pulled into a bath which was filled with heated mineral oil and the muscle temperature was maintained at 36-37° C while core temperature was maintained at 35-36° C. Optimal length for muscle twitch tension development (Lo) was determined by stimulating the isolated sciatic nerve with a supramaximal square pulse 0.05 ms in duration, every 3 s through a bipolar platinum electrode while the muscle was slowly lengthened from a completely slack position. Following this determination, electrical stimulation was ceased and the muscle held at this length for 5 min before the onset of the experimental protocol.

Muscles (n= 6 Unloaded and n= 5 Control; n= 4 Overloaded and n= 4 Control) were indirectly stimulated to contract tetanically at 100 Hz for 150 ms every second for 5 min at Lo. Developed tension was recorded for offline analysis.

Unstimulated muscles (n= 3 Unloaded and n= 3 Control; n= 3 Overloaded and n= 3 Control) were maintained at Lo without stimulation for the same duration in order to provide baseline measurements. Immediately following the 5-min experimental protocol, the plantaris muscle was rapidly excised and frozen in liquid nitrogen. Muscles were stored at -80° C until biochemical analysis. Following muscle excision, animals were killed by anaesthetic overdose.

Tissue processing: Frozen muscles were processed as previously described in detail [13](Martineau et al, 2002 submitted). Briefly, muscles were powdered under liquid nitrogen and solubilized in ice-cold modified-RIPA buffer (50 mM HEPES, 150 mM NaCl, 5% glycerol, 5 mM EGTA, 2 mM $MgCl_2$, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), containing protease inhibitors (Boehringer Mini-Protease and 2 mM PMSF) and phosphatase inhibitors (100 µM sodium orthovanadate, 1 mM sodium pyrophosphate, 10 mM sodium fluoride), by frequent vortexing over a period of 1 h. Homogenates were fractionated into RIPA-soluble and -insoluble fractions by centrifugation at 4500 g. The RIPA-insoluble pellets were resuspended in SDS-PAGE sample buffer (60 mM Tris, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, pH 6.8), boiled 10 min, and centrifuged 10 min at 600 g to remove insoluble material. Protein concentration of both fractions was determined by Bradford protein assay (Bio-Rad) and samples containing 200 µg of protein were prepared for SDS-PAGE by dilution and boiling in reducing sample buffer.

Electrophoresis and immunoblotting: Samples of experimental and control muscles were separated simultaneously on 8 and 10% acrylamide gels and electrotransferred to a single PVDF membrane. All procedures were performed in duplicate. Successful transfer and equal loading were confirmed by Ponceau stain. Immunoblotting using a phospho-specific antibody against

p54 JNK was performed to assess content of activated p54 JNK in the RIPA-soluble fraction of the samples. Blots were stripped and reprobed with an anti-JNK2 (p54 JNK) antibody to assess total p54 JNK content. Content of β1 integrin in RIPA-soluble and in RIPA-insoluble fractions was assessed using an anti-β1 integrin antibody. All antibodies were supplied by Santa Cruz Biotechnology. Membranes were blocked with BSA, bathed overnight at 4° C in primary antibody solution, and bathed 1.5 h at room temp. in secondary antibody solution. Revelation was performed by chemiluminescence (Amersham) with film exposure times ranging from 2 to 30 min (longest exposure times were for revelation of phospho-p54 JNK in samples from unstimulated muscles). Results within the linear range of the technique were analyzed by densitometry using a flatbed scanner and NIH Image software. Calculation of the total concentration of β1 integrin from the measured relative concentrations of RIPA-soluble and -insoluble β1 integrin was performed as previously described (Martineau et al., 2002 submitted). Since a change in β1 integrin content in one fraction must be mirrored by an equal and opposite change of β1 integrin content in the other fraction, relative concentration units of RIPA-soluble and -insoluble β1 integrin were normalized on the basis of average magnitude of change between control and stimulated samples. Following normalization, total β1 integrin concentration was calculated as the average of the algebraic sums of the RIPA-soluble and -insoluble β1 integrin concentrations for each sample, and fractional concentrations were expressed as proportions of this total.

Statistics: Between-group comparisons of untransformed densitometry data and physiological variables were performed separately for unloading and overloading experiments using the t-test and a significance level of 0.05; unloading and overloading experiments were conducted separately and featured different durations and therefore different terminal ages and weights. Relationships between variables were assessed simultaneously for

unloading and overloading experiments, and strength of relationships was determined by correlation analysis of group averages.

Results

Physiology of chronically unloaded and overloaded muscles: Chronic superfusion of the left sciatic nerve with TTX over a period of 2 wk resulted in a 40 ± 4 % loss of wet muscle weight of the plantaris, as compared to contralateral muscle, or a 48 ± 3 % reduction as compared to plantaris of age-matched controls (Table 1). The magnitude of atrophy induced in this muscle and in other ankle extensors by this model of disuse is consistent with previous reports [14, 18]. The paralysis protocol resulted in a 15 ± 2 % deficit in body weight at the time of the acute procedure as well as a 39 ± 19 % increase in twitch specific tension and a 51 ± 6 % decrease in tetanic specific tension (Table 1), all of which are also consistent with previous reports. Unloaded muscles also exhibited decreased fatigue resistance during the tetanic contractile protocol and thus a greater relative loss of peak tension over the stimulation period. However, the average peak tension over the first minute, the best predictor of p54 JNK activation under these experimental conditions [13], was not significantly different (Table 1).

Compensatory overload of the plantaris and soleus muscles over a period of 8 wk resulted in 94 ± 17 % increase in plantaris wet muscle weight as compared to age-matched controls (Table 2). This magnitude of hypertrophy is consistent with previous reports [6, 8]. Over the last 4 wk of wheel-cage confinement, there was no difference in daily running distance between groups. Overloaded resulted in a 30 ± 7 % deficit in twitch specific tension and a 24 ± 7 % deficit in tetanic specific tension (Table 2). Overloaded muscles exhibited increased fatigue resistance during the tetanic contractile protocol and average peak tension over the first minute of stimulation was 8 ± 3 %

greater than in controls (Table 2).

Acute p54 JNK activation in chronically unloaded and overloaded muscles: In response to a 5-min tetanic contractile challenge, concentration of phosphorylated p54 JNK was increased 8.8 ± 1.1 fold above baseline levels in control muscles (Figure 1B). This level of activation is consistent with previous reports using the same stimulation protocol [13]. Following stimulation, concentration of phosphorylated p54 JNK in unloaded muscles was more than two-fold greater than in control, or 18.2 ± 3.0 -fold above control unstimulated levels (Figure 1B). In an attempt to account for the deficit in relative maximal peak tension in unloaded muscles, p54 JNK phosphorylation in stimulated muscles was also expressed normalized by specific tetanic tension. Normalized concentration of phosphorylated p54 JNK in unloaded muscles was 4.5 ± 1.6 -fold greater than in control muscles (Figure 1A). Baseline phosphorylation of p54 JNK, measured in unstimulated muscles, was significantly elevated in unloaded muscles (Figure 1C). Total (phosphorylated + unphosphorylated) concentration of p54 JNK concentration was not different between groups (Figure 1D).

In response to a 5-min tetanic contractile challenge, concentration of phosphorylated p54 JNK was increased 9.0 ± 0.7 -fold above baseline levels in control muscles (Figure 2B), a response consistent with the control group for the unloading experiments (Figure 1B). Following stimulation, concentration of phosphorylated p54 JNK in overloaded muscles was less than half that in controls, or 4.2 ± 1.2 -fold above control unstimulated levels (Figure 2B). To account for the deficit in relative maximal peak tension in overloaded muscles, p54 JNK phosphorylation in stimulated muscles was also expressed normalized by specific tetanic tension. Normalized concentration of phosphorylated p54 JNK in overloaded muscles was 45 ± 13 % smaller than in controls (Figure 2A). Baseline phosphorylation of p54 JNK was significantly

elevated in overloaded muscles (Figure 2C). Total p54 JNK concentration in overloaded muscles was reduced by 16 ± 5 % (Figure 2D).

β1 integrin concentrations in chronically unloaded and overloaded muscles: Total (RIPA-soluble + RIPA-insoluble) concentration of β1 integrin was not significantly different between unloaded and control muscles (Figure 3). In unstimulated control muscles, the proportion of RIPA-soluble to RIPA-insoluble β1 integrin was 59 / 41 ± 12 %, which is consistent with previously reported proportions (Martineau et al. - submitted). This proportion shifted to 23 / 77 ± 4% in response to a 5-min tetanic contractile challenge, representing an increase in insoluble β1 integrin concentration of 90 % (Figure 3). In unloaded muscles, the unstimulated proportion of soluble to insoluble β1 integrin was 86 / 14 ± 8 %, and this proportion shifted to 34 / 66 ± 5 % in response to stimulation, representing an increase in insoluble β1 integrin concentration of 366 % (Figure 3).

Total (RIPA-soluble + RIPA-insoluble) concentration of β1 integrin in the overloaded group was 38 ± 20 % greater than in the control group (Figure 4). In unstimulated control muscles, the proportion of RIPA-soluble to RIPA-insoluble β1 integrin was 58 / 42 ± 12 %. This proportion shifted to 21 / 79 ± 2 % in response to a 5-min tetanic contractile challenge, representing an increase in insoluble β1 integrin concentration of 89 % (Figure 4). In overloaded muscles, the unstimulated proportion of soluble to insoluble β1 integrin was 33 / 67 ± 3 %, and this proportion shifted to 20 / 80 ± 1 % in response to stimulation, representing an increase in insoluble β1 integrin concentration of 19 % (Figure 4).

Relationships between p54 JNK activation, β1 integrin responsiveness, β1 integrin concentration, and muscle size: Relationships between a number of variables were explored by correlation analysis of group averages. The

variables examined were defined as follows:

muscle size: muscle wet weight expressed as % of control
p54 JNK activation: increase in concentration of phospho-p54 JNK in response to tetanic stimulation expressed as -fold control baseline phosphorylation
β1 integrin total concentration: RIPA-soluble plus -insoluble concentration of β1 integrin expressed as arbitrary densitometry units per unit protein
baseline β1 integrin insoluble concentration: total concentration of β1 integrin multiplied by proportion of RIPA-insoluble β1 integrin in unstimulated muscle
β1 integrin responsiveness: % increase in concentration of insoluble β1 integrin in response to tetanic stimulation

1) Relationship between baseline β1 integrin insoluble concentration and muscle size or mechanosensitivity: Baseline β1 integrin RIPA-insoluble concentration was reduced in unloaded muscles and elevated in overloaded muscles, indicating a positive relationship to muscle loading state or resulting muscle size. This relationship was strong, as indicated by a high degree of correlation ($R^2 = 0.99$) (Figure 5).

To test the hypothesis that magnitude of mechanically-induced signaling in response to a standardized mechanical stimulation (i.e. mechanosensitivity) is inversely related to the concentration of β1 integrins through which the stimulation is transmitted, the relationship between p54 JNK activation and baseline β1 integrin insoluble concentration was assessed. These two variables were found to be negatively related. Assuming a linear relationship, correlation analysis resulted in an R^2 of 0.80. This correlation was improved to R^2 of 0.95 when p54 JNK activation was log-transformed. The strength of

the relationship was found to be maximal when the variables were related by a power function (R^2 of 0.99) (Figure 6).

Because a deficit in specific tension resulted from the models of chronically altered loading, thereby adding variability to the standardized mechanical stimulation, a corollary of the above-mentioned hypothesis was tested. Specifically, the hypothesis that magnitude of signaling resulting from mechanical stimulation (i.e. mechanosensitivity) is related to the magnitude of force applied per molecule of force-transmitting integrin was tested by assessing the relationship between p54 JNK activation and specific tension per unit baseline insoluble β1 integrin concentration. These 2 variables were found to be positively related, and, assuming a linear relationship, correlation analysis resulted in an R^2 of 0.82. This correlation was improved to R^2 of 0.96, when p54 JNK activation was log-transformed. The strength of the relationship was found to be maximal when the variables were related by a power function (R^2 of 0.99).

Taken together, the relationships between magnitude of p54 activation and baseline β1 integrin RIPA-insoluble concentration or specific tension per unit baseline β1 integrin insoluble concentration both support that RIPA-insoluble β1 integrins are force-transmitting integrins, that integrins function as mechanosensors, and that mechanosensitivity is dictated by the concentration of RIPA-insoluble β1 integrins.

2) Absence of relationship between total integrin concentration and muscle size or mechanosensitivity: Total concentration of β1 integrin in unloaded muscles was not significantly different from control, while it was elevated in overloaded muscle. There was therefore no relationship between loading state or muscle size and total β1 integrin concentration. Similarly, there was no relationship between p54 JNK activation and total β1 integrin

concentration or between integrin responsiveness and total β1 integrin concentration.

3) Relationship between measures of p54 activation and β1 integrin responsiveness: p54 JNK activation and β1 integrin responsiveness are both events quantitatively related to tension. The positive relationship between these two markers was strong, as determined by a high degree of correlation ($R^2 = 0.97$).

4) Relationship between mechanosensitivity and muscle size: p54 JNK activation in response to tetanic contractile activity was heightened in unloaded muscles and diminished in overloaded muscles, indicating a negative relationship between activation and loading state or muscle size. While the mechanical stimulation resulting from tetanic contractile activity was not constant across groups, specific tetanic tension cannot account for the altered p54 JNK response since a deficit in specific tension was observed in both models of altered loading. Magnitude of p54 JNK activation was appreciably correlated to muscle size ($R^2 = 0.81$). Similarly, β1 integrin responsiveness was heightened in unloaded muscle and diminished in overloaded muscle, indicating a negative relationship to loading state or muscle size. β1 integrin responsiveness was modestly correlated to muscle size ($R^2 = 0.61$).

Discussion

There is considerable evidence to support that integrins are central to the process of mechanotransduction [3, 10, 12, 15-17]. The recent demonstration by our laboratory that, in skeletal muscle in vivo, a sub-population of β1 integrin undergoes an acute decrease in solubility in response to mechanical stimulation in the form of tension (Martineau et al. - submitted) raises the

prospect of a novel mechanism for regulating the sensitivity of mechanotransduction at the cellular level. The present study demonstrates that the magnitude of signaling initiated in response to a given magnitude of mechanical stimulation (i.e. mechanosensitivity) can be modulated physiologically, and is related to the concentration of RIPA-insoluble β1 integrins. These findings support that integrins are mechanosensors, that force-transmission through integrins is dependent on the degree of integrin association to the cortical cytoskeleton, and that the relationship between mechanical stimulation and resulting signaling is non-linear at the molecular level.

The tension-induced increase in concentration of RIPA-insoluble β1 integrin, mirrored by a similar change in vinculin, has been interpreted to be the manifestation of an increase in strongly cytoskeleton-associated integrins occuring through a recruitment of mobile and less tightly-associated integrins to existing focal adhesions. It has been proposed that an increase in strongly-associated integrins represents an increase in force-transmitting linkages between the cytoskeleton and the basement membrane, serving a cytoprotective function by increasing the number of molecules through which forces are transmitted, and therefore decreasing the magnitude of force subjected to each molecule. The concept of a variable concentration of force-transmitting integrins, in conjunction with the emerging central function of integrins in mechanotransduction [3, 10, 12, 15-17], has led to the hypothesis that mechanosensitivity may be modulated by a chronic change in the concentration of RIPA-insoluble force-transmitting β1 integrins; for any given mechanical stimulation, the force channelled through each integrin would be dependent on the concentration of force-transmitting integrins, and force per integrin would determine the magnitude of mechanosignaling.

The purpose of this study was therefore to probe the possible relationship

between concentration of RIPA-insoluble β1 integrin and mechanosensitivity. In order to do this, disruption of the normal balance between these 2 variables was attempted in our skeletal muscle system using a physiological approach whereby muscles were chronically overloaded or unloaded. Since acute mechanical stimulation of muscle induces a temporary increase in concentration of RIPA-insoluble β1, and since this increase is tension-dependent, it was hypothesized that increasing the mechanical stimulation to which a muscle is chronically subjected would result in a chronically elevated concentration of RIPA-insoluble β1 integrin. Conversely, chronically reduced mechanical stimulation would result in chronically decreased concentration of RIPA-insoluble β1 integrin. Muscle overload was achieved by eliminating the contribution of synergistic muscles, thereby resulting in a substantial augmentation of fiber area and muscle wet weight [6, 8], while unloading was achieved by reversible nerve-block-induced paralysis, a model of disuse which results in rapid and important reductions in fibers cross-sectional area and muscle wet weight [18]. As depicted in Figure 5, concentration of RIPA-insoluble β1 integrin was found to tightly parallel muscle weight wet.

Following the chronic change in muscle loading, mechanosensitivity was measured as the increase in activation of the mechanically-responsive p54 JNK MAPK in response to a protocol of maximal tetanic contractions, theoretically producing a constant magnitude of tension per muscle cross-sectional area or wet weight. It was found that mechanosensitivity is inversely related to muscle wet weight ($R^2 = 0.81$), and inversely related to concentration of RIPA-insoluble β1 integrin ($R^2 = 0.99$; Figure 6), but not to the total concentration of β1 integrin. These findings therefore fit the model that in muscles with a low concentration of presumably force-transmitting integrins, standardized stimulation results in more force being applied to each functional integrin and the generation of more mechanosignaling than

in muscles with a high-concentration of force-transmitting integrins.

The baseline level of p54 JNK activation in unstimulated muscles was observed to be elevated in both unloaded and overloaded muscle. Chronic mechanical stimulation may explain this elevated level of baseline signaling in both models since, in the hours preceding the acute stimulation procedure, the unloaded muscles may have been overloaded by the recovery from paralysis which is necessary to ensure that no damage has resulted from the nerve block. As the divergent response to acute, high-tension stimulation was not masked despite elevated baseline activation in both unloaded and overloaded muscles, chronic elevation of baseline p54 JNK activation most likely does not affect the magnitude of response to an acute challenge either through a desensitization or a cumulative effect.

Similarly, both models of altered muscle loading state also resulted in decreased specific tension. However, such a deficit does not invalidate the use of maximal tetanic contractile activity as standardized mechanical stimulation; the model of mechanosensitivity regulated by concentration of RIPA-insoluble β1 integrins is supported whether standardized stimulation is assumed and the relationship between mechanosensitivity and concentration is addressed directly or whether a calculation of force per RIPA-insoluble β1 integrin is performed. While the p54 JNK response used for establishing these relationships was not normalized to force, linear normalization by specific tension does not alter the conclusion that mechanosensitivity is modulated in models of chronically-altered muscle loading. Finally, the effects of a change in fatigue resistance on stimulation over time were considered negligible since they would be expected to reduce the change in mechanosensitivity observed in both models, rather than add to it.

While the magnitude of signaling generated in response to mechanical

stimulation must be dependent on the magnitude of stimulation applied to a cell, there is little data available to address whether the relationship between stimulation and signaling is linear or not. Our work on the relation between tension and p54 JNK signaling suggests a non-linear relationship [13] and similar relationships can also be seen in the results of other studies of mechanically-induced signaling in skeletal muscle [1, 5]. By showing an inverse relationship between concentration of RIPA-insoluble β1 integrins and signaling, the present work suggests that mechanosensitivity, at the molecular level, must be non-linear since increase in p54 JNK activation in response to a standardized stimulation is variable despite a constant product of force/integrin and integrin content. The finding that the relationships between p54 JNK response and concentration of RIPA-insoluble β1 integrin or force per RIPA-insoluble β1 integrin are best fit by power functions further support non-linearity.

In a model of mechanosensitivity regulated by the concentration of strongly cytoskeleton-associated β1 integrins, it may be important to take into account not only the baseline concentration at the time of stimulation, but also the acute increase in concentration which is induced by the stimulation. However, because of the significant delay between initiation of stimulation and induction of the increase in concentration, the contribution of this phenomenon to rapidly-initiated mechanosignaling may be negligible. Interestingly, while the vast majority of β1 integrins in unloaded muscle are not tightly-associated to the cytoskeleton, there is nevertheless a large potential for acutely increasing the concentration of RIPA-insoluble β1 integrins. This suggests that the localization of unbound integrins, which is likely important for recruitment to focal adhesions, is tightly regulated and that these "spare" integrins are not simply targeted for degradation.

The model proposed here raises questions about limits and regulation of

mechanosensitivity that are beyond the available data. For example, it can only be speculated that while a four-fold range in sensitivity can be accounted for by this model, a lower limit to sensitivity must be imposed by the total concentration of β1 integrins and an upper limit must be dictated by the maximum rate of the mechanotransduction process, if force transmission and cellular adhesion are not first compromised. Furthermore, it is unclear whether the stimulus for change in concentration of RIPA-insoluble β1 integrins observed here is altered muscle loading, activity pattern, or muscle size. Additional experiments examining developing muscle of the same wet weight as the unloaded muscles reveal heightened mechanosensitivity and decreased concentration of RIPA-insoluble β1 integrin, as compared to mature muscles (results not shown). Since developing muscles are more akin to overloaded muscles than to unloaded muscle in terms of muscle loading state and pattern or activity, this would suggest that muscle size is a more important determinant of concentration of RIPA-insoluble β1 integrin and mechanosensitivity. Finally, it is unknown whether the concentration of RIPA-insoluble β1 integrins returns to control values once muscle has completely adapted to a change in its loading state and a steady state is once again reached. It is likely that such a steady state cannot be reached with the models of unloading and overloading used here, and thus the use of less severe models, in conjunction with time-course analysis, may prove instructive.

The data presented here conform to a model of variable mechanosensitivity regulated through the concentration of force-transmitting integrins. The demonstration that mechanosensitivity can be altered physiologically in-vivo strongly suggests that this mechanism is physiologically relevant in muscle and perhaps in other mechanocytes. These findings introduce the possibility of artificial modulation of mechanosensitivity. The applications of such modulation, even if they are limited to muscle, are important. Increasing

mechanosensitivity as a countermeasure for disuse atrophy could greatly reduce the social costs associated with aging-induced loss of autonomy and rehabilitation from musculoskeletal injuries. Decreasing mechanosensitivity in the hypertrophying heart, and possibly attenuating the detrimental effects of this condition, could similarly have a large social impact. The potential for such artificial modulations makes this subject worthy of further investigation.

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Figure legends

Figure 1

The increase in p54 JNK phosphorylation in response to a 5 min protocol of maximal contractions is significantly greater in plantaris muscles unloaded for 2 wk by paralysis than in age-matched controls.

Figure 2

The increase in p54 JNK phosphorylation in response to a 5 min protocol of maximal contractions is significantly lower in plantaris muscles overloaded for 8 wk by synergist tenotomy than in age-matched controls.

Figures 1 and 2

A: Results of phospho-p54 JNK western blot of contraction-stimulated samples normalized by specific tension.
B: Results of phospho-p54 JNK western blot of contraction-stimulated samples.
C: Results of phospho-p54 JNK western blot of unstimulated samples.
D: Results of pan-p54 JNK western blot of all samples.

Results are expressed as mean ± SEM, relative to control group set to 100%, except in **B** where they are expressed relative to p54 mean JNK phosphorylation in unstimulated control samples. * identifies a significant ($p<0.05$) difference from control, as assessed by t-test. Statistical analysis was performed on untransformed densitometry data, except for **A** where densitometry data were individually normalized by specific tension prior to analysis. Concentration is defined as arbitrary densitometry units per 200 μg of RIPA-soluble protein. Untransformed densitometry data and sample size are indicated within each bar in **B**, **C**, and **D**.

Figure 3

Proportions of RIPA-insoluble and -soluble β1 integrin in unstimulated muscles, as well as the magnitude of contraction-stimulated increase in concentration of RIPA-insoluble β1 integrin, are altered in chronically unloaded muscle.

Figure 4

Proportions of RIPA-insoluble and -soluble β1 integrin in unstimulated muscles, the magnitude of contraction-stimulated increase in concentration of RIPA-insoluble β1 integrin, and the total concentration of β1 integrin are altered in chronically overloaded muscle.

Figures 3 and 4

Concentration of RIPA-insoluble β1 integrin relative to total concentration is superimposed onto concentration of RIPA-soluble β1 integrin relative to total concentration for baseline unstimulated muscles (n=3) and muscles subjected to a 5 min protocol of maximal contractile activity (n=4). Total concentration of β1 integrin is calculated from baseline and contraction-stimulated muscles (n=7). Data are expressed as mean ± SEM. The calculated increase in concentration of RIPA-insoluble β1 integrin induced by the contractile protocol is indicated as a %. * between bars identifies a significant ($p<0.05$) difference in concentration of RIPA-insoluble or -soluble β1 integrin between baseline and stimulated muscles, as assessed by t-test. * above bars identifies a significant ($p<0.05$) difference in total concentration of β1 integrin, as assessed by t-test. NS identifies non-significant differences. Statistical analysis was performed on untransformed densitometry data.

Figure 5

Linear relation between the changes in baseline concentration of RIPA-insoluble β1 integrin and the changes in muscle size induced by models of chronically altered muscle loading. The concentration of RIPA-insoluble β1 integrin in unstimulated muscles is obtained by multiplying the total (RIPA-soluble + RIPA-insoluble) concentration of β1 integrin, which is unrelated to muscle size, by the proportion of RIPA-insoluble β1 integrin in unstimulated muscles. Muscle size is expressed as % of control (above) or expressed as g of wet weight (not shown).

Figure 6

The baseline concentration of RIPA-insoluble β1 integrin and the increase in p54 JNK phosphorylation in response to a 5 min protocol of maximal contractions are negatively related by a power function.

TABLE 1: Physiological measures for Unloaded and Control groups

Group	Body weight (g)	Plantaris wet weight (mg)	Resting tension at Lo (g)	Maximal twitch tension (g)	Maximal tetanic tension (g)	Resting specific tension (g/g)	Twitch specific tension (g/g)	Tetanic specific tension (g/g)	Avg peak tension over 1st min (% of max)
Control	235 ± 1 n=8	244 ± 12 n=8	12.0 ± 1 n=5	74 ± 3 n=5	450 ± 17 n=5	48 ± 3 n=5	296 ± 13 n=5	1788 ± 68 n=5	78 ± 1 % n=5
Unloaded Muscle	199 ± 5 * n=9	128 ± 5 * (right 213 ± 13) n=9	6.0 ± 0 * n=6	53 ± 6 * n=6	114 ± 11 * n=6	46 ± 3 n=6	410 ± 55 n=6	883 ± 99 * n=6	73 ± 4 % n=6

All data presented as mean ± SEM.
Within any column, * denotes a significant difference ($p<0.05$), as determined by t-test.
(g/g): gram tension per gram muscle wet weight.

TABLE 2: Physiological measures for Overloaded and Control groups

Group	Body weight (g)	Plantaris wet weight (mg)	Resting tension at Lo (g)	Maximal twitch tension (g)	Maximal tetanic tension (g)	Resting specific tension (g/g)	Twitch specific tension (g/g)	Tetanic specific tension (g/g)	Avg peak tension over 1st min (% of max)
Control	264 ± 12 n=7	315 ± 12 n=7	13.7 ± 1 n=4	96 ± 6 n=4	554 ± 41 n=4	44 ± 4 n=4	308 ± 22 n=4	1706 ± 57 n=4	75 ± 2 % n=4
Overloaded Muscle	255 ± 9 n=7	612 ± 49 * n=7	31.6 ± 3 * n=4	128 ± 6 * n=4	778 ± 34 * n=4	53 ± 7 n=4	215 ± 15 * n=4	1299 ± 109 * n=4	81 ± 1 % * n=4

All data presented as mean ± SEM
Within any column, * denotes a significant difference ($p<0.05$), as determined by t-test.
(g/g): gram tension per gram muscle wet weight.

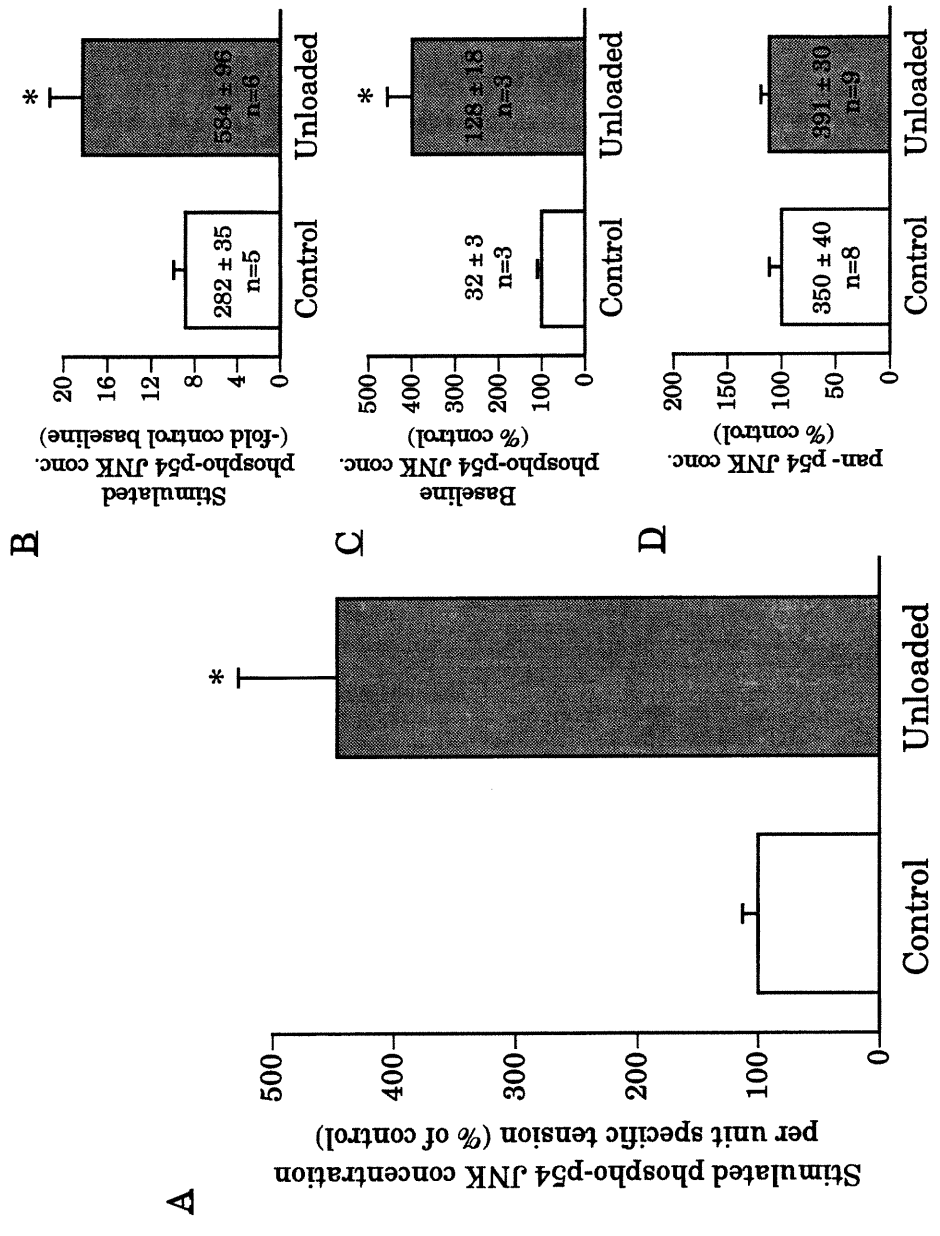

A
Stimulated phospho-p54 JNK concentration
per unit specific tension (% of control)
500
400
300
200
100
0
*
Control
Unloaded
B
Stimulated
phospho-p54 JNK conc.
(-fold control baseline)
20
16
12
8
4
0
282 ± 35
n=5
534 ± 96
n=6
*
Control
Unloaded
C
Baseline
phospho-p54 JNK conc.
(% control)
500
400
300
200
100
0
32 ± 3
n=3
128 ± 18
n=3
*
Control
Unloaded
D
pan- p54 JNK conc.
(% control)
200
150
100
50
0
350 ± 40
n=8
391 ± 30
n=9
Control
Unloaded

FIGURE 1

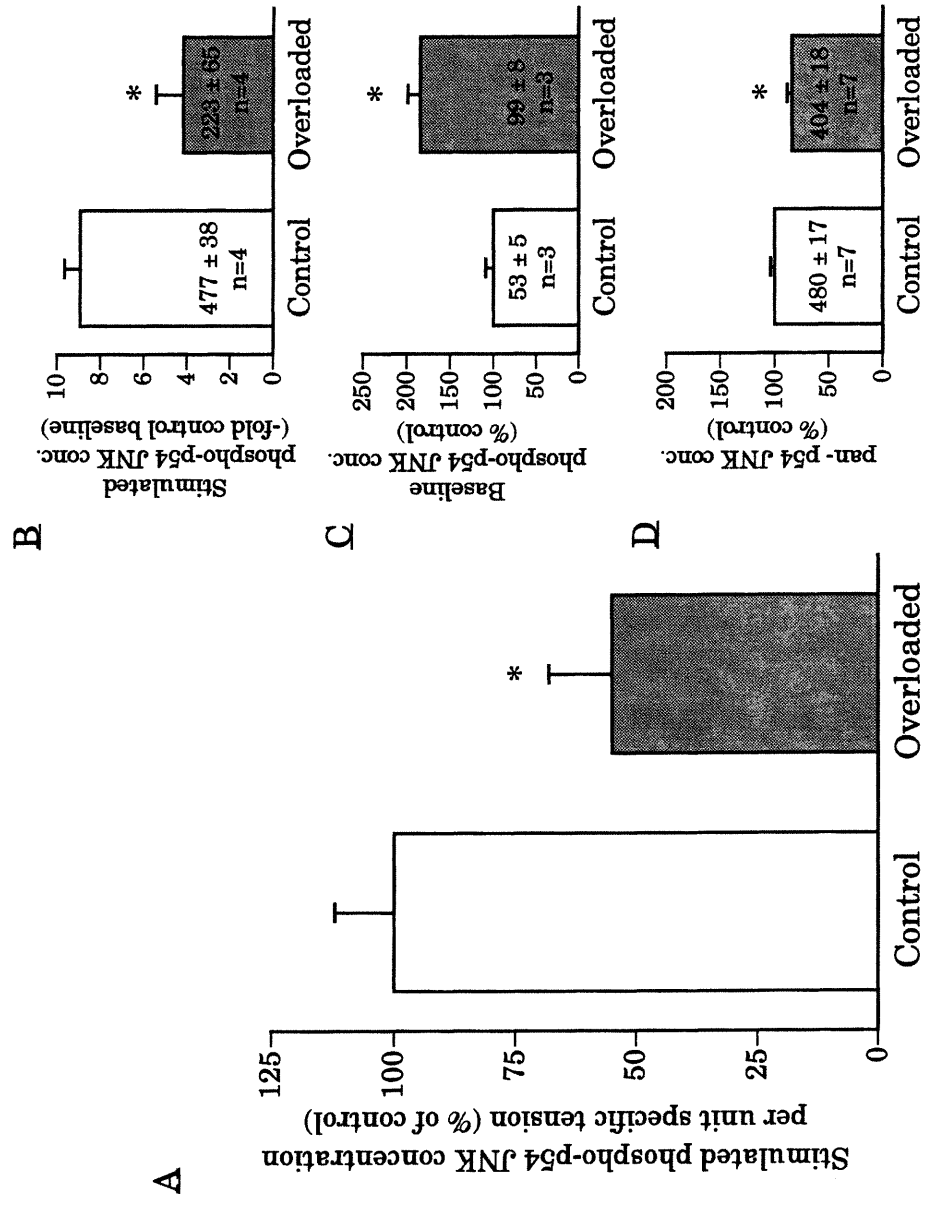

A
Stimulated phospho-p54 JNK concentration
per unit specific tension (% of control)
125
100
75
50
25
0
Control
Overloaded
*
B
Stimulated
phospho-p54 JNK conc.
(-fold control baseline)
10
8
6
4
2
0
477 ± 38
n=4
223 ± 65
n=4
Control
Overloaded
*
C
Baseline
phospho-p54 JNK conc.
(% control)
250
200
150
100
50
0
53 ± 5
n=3
99 ± 8
n=3
Control
Overloaded
*
D
pan- p54 JNK conc.
(% control)
200
150
100
50
0
480 ± 17
n=7
404 ± 18
n=7
Control
Overloaded
*

FIGURE 2

FIGURE 3

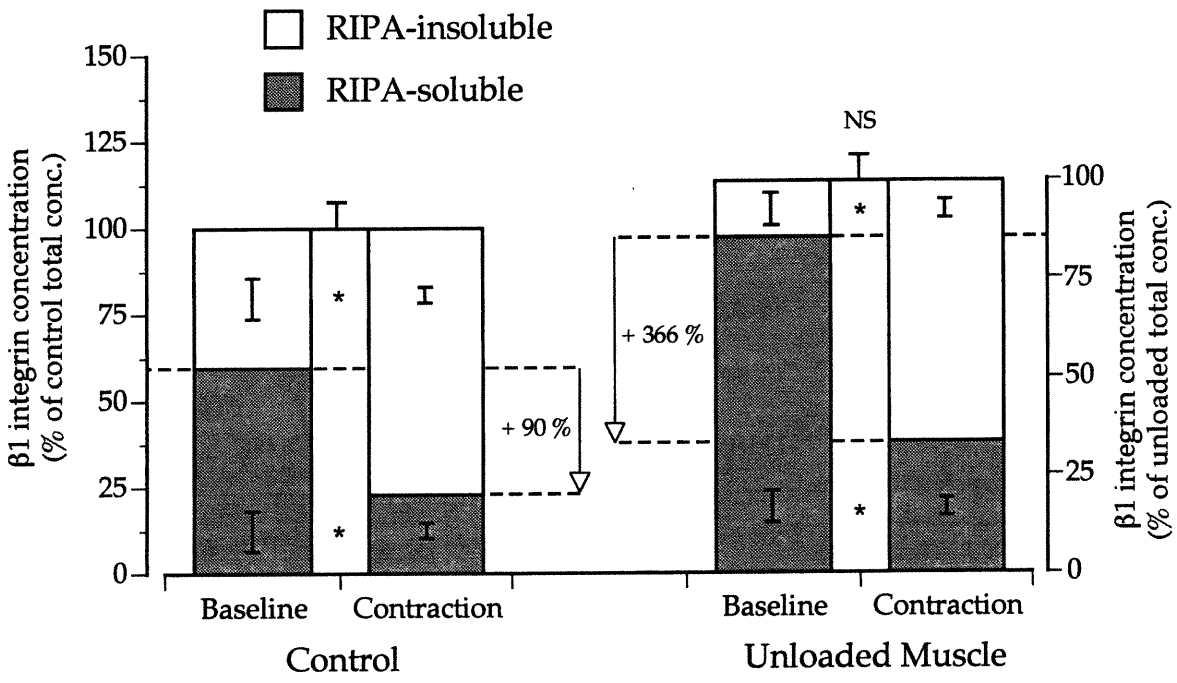

RIPA-insoluble
RIPA-soluble
β1 integrin concentration (% of control total conc.)
150
125
100
75
50
25
0
NS
+ 366 %
+ 90 %
β1 integrin concentration (% of unloaded total conc.)
100
75
50
25
0
Baseline
Contraction
Baseline
Contraction
Control
Unloaded Muscle

FIGURE 4

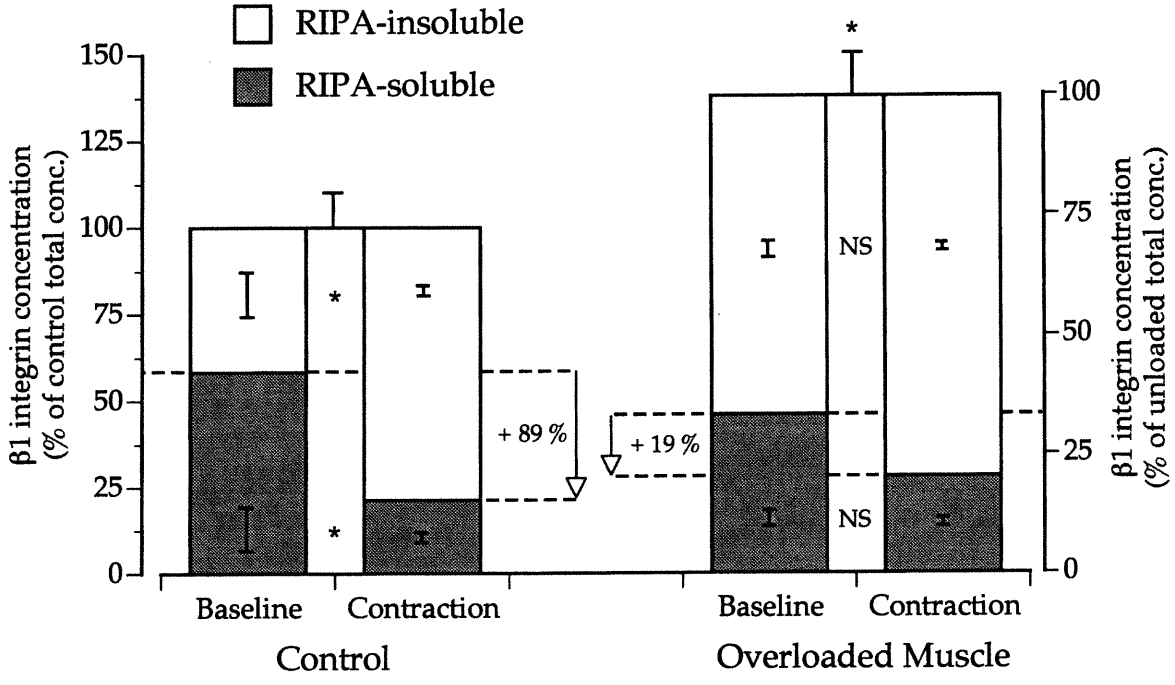

RIPA-insoluble
RIPA-soluble
β1 integrin concentration (% of control total conc.)
β1 integrin concentration (% of unloaded total conc.)
150
125
100
75
50
25
0
100
75
50
25
0
*
*
I
I
+ 89 %
+ 19 %
NS
Baseline
Contraction
Baseline
Contraction
Control
Overloaded Muscle

FIGURE 5

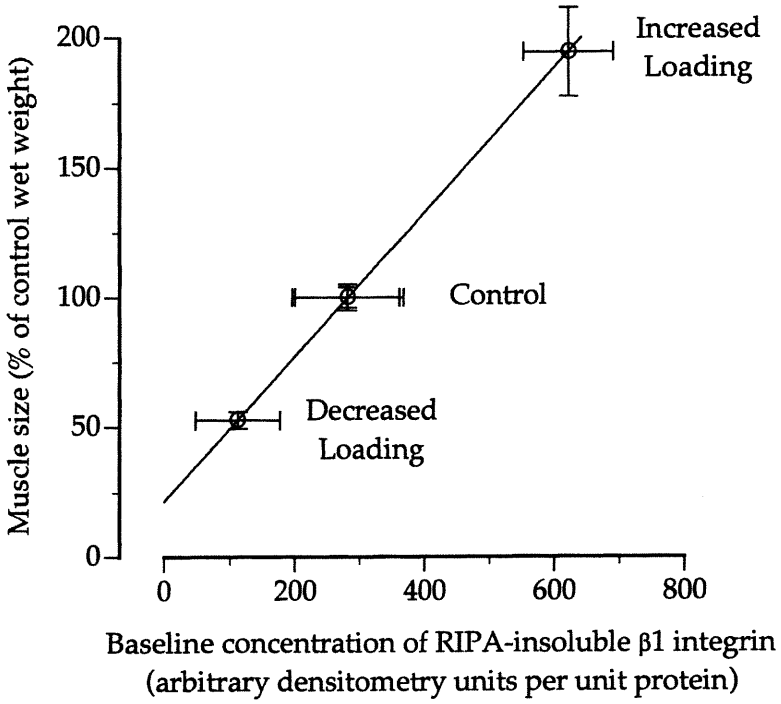
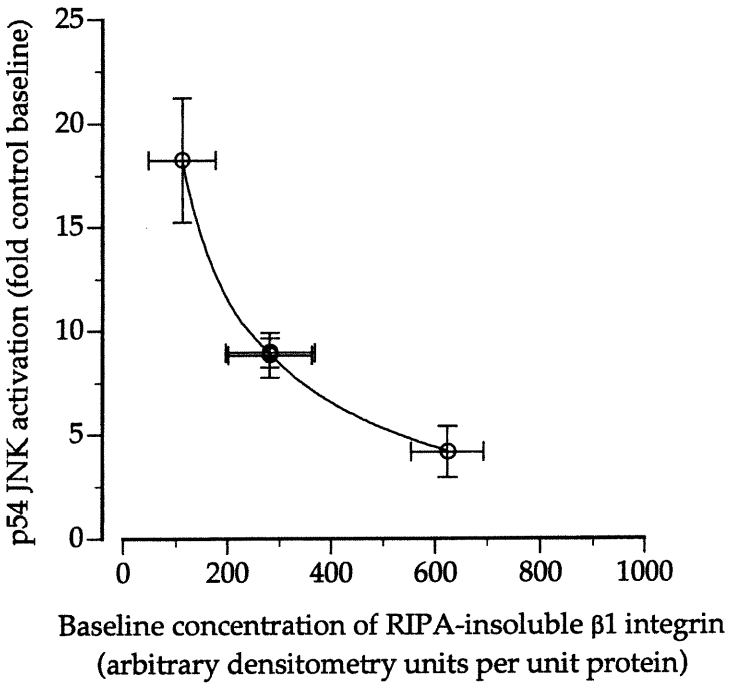

Increased
Loading
Control
Decreased
Loading
Muscle size (% of control wet weight)
200
150
100
50
0
0
200
400
600
800
Baseline concentration of RIPA-insoluble β1 integrin
(arbitrary densitometry units per unit protein)

FIGURE 6


p54 JNK activation (fold control baseline)
25
20
15
10
5
0
0
200
400
600
800
1000
Baseline concentration of RIPA-insoluble β1 integrin
(arbitrary densitometry units per unit protein)

Chapter 7

Discussion

The five studies presented in this thesis share the common goal of furthering our understanding of the mechanism through which mechanical forces are detected and initiate intracellular signaling in skeletal muscle. Research into mechano-chemical coupling, in skeletal muscle fibers or in other adherent cell types, has progressed relatively slowly in spite of the growing recognition that virtually every aspect of cellular function is impacted by mechanical forces. In light of this, the results of these five studies as a whole, should contribute significantly to advancing the current state of knowledge regarding mechanotransduction.

Activation of p54 JNK MAPK is a valuable marker of mechanical stimulation in muscle, serving throughout this thesis as a tool for probing mechanotransduction and for identifying the characteristics of mechanical stimulation to which muscle is most sensitive. p54 JNK appears to be part of an important mechano-signaling pathway, as suggested by evidence of mechanical responsiveness of this kinase in a variety of cell types, a quantitative relationship between peak developed tension and its activation in muscle, and a very large response amplitude resulting from physiological levels of developed tension in muscle. The elucidation of the events upstream of p54 JNK mechanical activation will be necessary to evaluate the relationship of this pathway to mechanotransduction. The potential role of p54 JNK in regulating muscle hypertrophy will also need to be assessed, perhaps by blocking its activation with newly identified pharmacological inhibitors, or by identifying all of its downstream substrates regulating transcriptional and translational events.

While ERK 1/2 did not exhibit a quantitative relationship to tension over the range of peak tension tested, and featured a much smaller response amplitude than p54 JNK, the mechanical responsiveness of these kinases

nevertheless suggests that the ERK pathway plays an important role in the transduction of mechanically-induced signals in skeletal muscle. As MAPKs are thought to produce a wide range of effects through combinatorial activation, it may be instructive to study the interplay between p54 JNK and ERK 1/2. It may also be useful to study p38 MAPK within this context, as others have reported mechanical activation of this family of MAPKs in muscle in response to longer stimulation protocols than used here.

Establishing the kinetics of mechanically-induced MAPK activation may reveal important information for the determination of the optimal duration and frequency parameters of disuse atrophy countermeasure and strength training protocols. By optimizing these parameters, it may be possible to increase the efficacy of existing countermeasures, thereby accelerating rehabilitation from musculo-skeletal injury, attenuating atrophy induced by disease states, minimizing loss of human performance during extended spaceflight or upon return from a microgravity environment, and attenuating aging-related loss of autonomy. Similarly, it may be possible to increase the efficacy of strength training protocols in order to improve athletic or work performance, or to minimize the time invested in training.

Mechanical activation of p54 JNK was used in the first two studies featured in this thesis as a tool to identify the characteristics of mechanical stimulation to which muscle fibers are most responsive. Knowledge of these characteristics and of their relative importance to mechanical stimulation can also be used to refine existing disuse atrophy countermeasures and methods of strength training. The finding that peak tension and TTI are determining factors of muscle stimulation is in accord with current practices of strength training. However, muscle insensitivity to dT/dt under conditions of constant peak tension and TTI is a novel finding which merits further investigation in light of its potential to affect current training methods. More work will be needed

to establish the importance of TTI relative to peak tension and to develop a model of the interplay of the parameters contributing to mechanical stimulation.

In addition to identifying the characteristics of tensile stimulation to which muscle is most sensitive, activation of p54 JNK can be employed as a reporter to assess the role of structural components or upstream signaling pathways in mechanically-induced signaling following selective targeting of such components or pathways. This strategy was employed in the third study whereby assessment of contraction-induced p54 JNK activation in muscles of dystrophic animals revealed an attenuated response, indicative of an involvement of the DGC in mechanotransduction or mechanically-induced signaling. This novel finding supports the current opinion that impaired signaling may be an important etiological factor in the dystrophinopathies. It will be important to repeat this work in other animal models of muscular dystrophy, such as the mdx mouse, and with other mechanically-regulated signaling pathways, and to continue to study the signaling functions of the DGC. Continued exploitation of p54 JNK activation as a reporter in combination with pharmacological or molecular biological targeting of cytoskeletal elements and of pathways involved in cytoskeleton remodelling will surely be instructive as to the role of the cytoskeleton in mechanotransduction and mechanically-induced signaling.

In the fifth study, p54 JNK activation was employed to demonstrate that the signaling response to a standardized mechanical stimulus (i.e. mechanosensitivity) is physiologically variable and is related to muscle loading state or its resulting muscle size. This novel and exciting finding raises more questions than it answers: Is mechanosensitivity constant among different muscles featuring different fiber-type composition, fiber lengths and areas, and fiber architecture? Is mechanosensitivity affected by development

and aging? Can mechanosensitivity be modulated artificially? This last question carries important implications as it introduces the concept of increasing mechanosensitivity to enhance the hypertrophying effect of mechanical stimulation as a molecular countermeasure for disuse atrophy. A similar approach could also prove useful for correcting potential signaling impairments in the muscular dystrophies. If mechanosensitivity is equally plastic in other cell types, then a decrease in the mechanosensitivity of cardiomyocytes could potentially serve to treat heart disease by braking the hypertrophy of cardiomyocytes before it becomes maladaptive. In all of these cases, targeting the mechanism responsible for signal initiation may be a simpler and less dangerous alternative to targeting signaling cascades or targeting a large number of genes.

The potential for artificial modulation of mechanosensitivity is an impetus for elucidating the details of mechanotransduction. The observation that mechanosensitivity varies as a result of chronically altered muscle loading has provided the opportunity to identify elements which may have contributed to this modulation, and thus to better understand mechanotransduction. One such element was found to be β1 integrin, the focal adhesion protein believed to function as a mechanosensor in adherent cells. β1 integrin was observed to acutely and chronically exhibit a solubility shift, indicative of increased association to the cytoskeleton, in response to mechanical stimulation. The proportion of tightly cytoskeleton-associated β1 integrins was found to be well correlated to the change in mechanosensitivity in unloaded and overloaded skeletal muscle. In light of the function of integrins, this finding led to the proposal of a model whereby the signal generated in response to standardized mechanical stimulation is dependent on the concentration of integrins which are subjected to this stimulation. Additional studies will be needed to test this hypothesis, to elucidate the cytoskeletal mechanisms through which the integrin solubility shift occurs,

and to determine whether it is feasible to exert control over this process. Another application of manipulating the proportion of tightly cytoskeleton-associated β1 integrins involves increasing strength of adhesion as a treatment for deficiencies of the DGC in muscular dystrophy.

In conclusion, this thesis has contributed significantly to the understanding of mechanotransduction in skeletal muscle fibers by identifying a marker of mechanical stimulation which can be used to study mechanotransduction, by identifying characteristics of tensile stimulation to which muscle is most sensitive, by describing impaired mechano-signaling in dystrophic muscle, by developing the concept of mechanosensitivity and proposing a mechanism for the regulation of mechanosensitivity based on novel observations of cytoskeletal plasticity. It is to be hoped that the results of the studies which comprise this thesis will stimulate more research into mechanotransduction, and that these results may serve as a foundation from which to develop new approaches for exerting control over the regulation of muscle size and force generation capacity, as well as over other cellular functions dependent on mechanical forces in muscle fibers and other mechanocytes.

Chapter 8

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Appendix 1

Limitations of this thesis

A degree of caution must be applied to the interpretation and extrapolation of the results presented in this thesis due to limitations which apply to this work. A few of the more important limitations are addressed here.

A number of limitations arise from the use of an animal model in the five studies presented here. While the rat was studied because of the established similarities of its neuromuscular system to the human neuromuscular system, it is important to consider that, despite these similarities, the results obtained may be model-specific and not transferable to humans. Furthermore, while the in-situ nerve-muscle preparation employed throughout the thesis is highly physiological, such a reduced preparation nevertheless differs from the true in-vivo situation in many ways. For example, under deep anaesthesia, reflexes and systemic responses are blunted. Moreover, as muscles are stimulated to contract by imposing supramaximal stimulation through the sciatic nerve at a fixed stimulation frequency, the resulting pattern of muscle activation differs greatly from the physiological recruitment of muscle fibers.

Other limitations stem from the experimental design employed in the studies. For example, while correlational analysis is well suited to identifying potential relationships between variables, it cannot establish causality. The causal nature of newly-identified relationships must therefore be formally tested in follow-up experiments.

Appendix 2

Abstracts of co-authored manuscripts

Title

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

Authors

Kristina J. Csukly, Louis C. Martineau, and Phillip F. Gardiner

Journal

accepted in final form to European Journal of Physiology, June 2002

Abstract

"Muscle phenotype is regulated by mechanical forces. However, it is not well understood how these forces are translated into intracellular signaling which impacts gene expression. The purpose of this study was to test the hypothesis that muscles displaying a wide range of metabolic profiles and fiber-type composition exhibit differences in the detection and transmission of mechanical stimuli. A mechanical challenge in the form of passive stretch normalized to 3 N/g muscle weight was applied to the rat extensor digitorum longus (EDL), soleus (SOL), and plantaris (PLN) in situ for 5 minutes following which, activities of the mechanically-responsive p54 c-jun NH_2-terminal kinase (JNK) and extracellular-regulated kinase (ERK) 1/2 were measured. EDL, SOL, and PLN were not different in their stretch-induced JNK (4.5, 5.2, 6-fold baseline, respectively) and ERK (2.2, 2.2, 1.9-fold baseline, respectively) responses in spite of differing fiber-type compositions. Medial gastrocnemius (MG), a compartmentalized muscle with red (MGr) and white (MGw) regions was subjected to the same normalized mechanical stretch protocol and resulting JNK and ERK activities were observed to be significantly higher in the MGr (13, 4.5-fold baseline, respectively) compared to MGw (5, 1.2-fold baseline, respectively) and all other muscles. In contrast to stimulation by passive stretch, stimulation of the MG by isometric contractile activity did not result in a heterogenous response between compartments. This study demonstrates an absence of difference among muscles of varying phenotype in their ability to transmit mechanical stimuli to the mitogen-activated protein kinase signaling pathways and hence in their mechanosensitivity. Furthermore, the results highlight the importance of considering aspects of the functional organization of different muscles, such as compartmentalization and architecture, when studying mechanical signaling in vivo."

Appendix 3

Published abstracts of presentations given at international conferences

Title

Static stretch induces MAPK activation in skeletal muscle

Authors

Louis C. Martineau and Phillip F. Gardiner

Journal

FASEB Journal, 13(4): A410, 1999

Conference

Experimental Biology 1999, Washington D.C.

Abstract

"Mitogen-activated protein kinase (MAPK) signalling cascades are activated by static stretch in cardiomyocytes and by shear stress or cyclical stretching in smooth muscle cells. MAPKs are involved in the activation of gene regulatory proteins. Static stretch-induced c-jun and c-fos transcription has been demonstrated in skeletal muscle, but upstream signalling events have yet to be characterized. Likewise, contractile activity has been shown to activate the JNK and ERK MAPK cascades, but the mechanism of mechanotransduction is unknown. Using an in-situ nerve/muscle preparation of the rat medial gastrocnemius (MG), a stretch of 4, 6 or 8 mm beyond the optimal muscle twitch length (L_o) was applied continuously for 5 to 45 min. Following the perturbation, the MG was snap-frozen and analyzed by western blot for content of phosphorylated JNK and ERK. For all stretch durations, a positive relationship between muscle length and activation of JNK was observed over the range tested. Content of p-JNK attained more than tenfold control levels for all L_o+8 mm groups. No significant relationship was observed between stretch duration and activation. ERK activation was of lower magnitude, reaching less than twofold control levels for all stretch conditions, and was neither length nor duration dependent. Cyclical stretch did not increase MAPK activation as compared to continuous stretch. Similar experiments on mice suggest that the ERK response is quantitatively more important in this species than in the rat. The observed pattern of stretch-induced MAPK activation is similar to, and may partially explain, that induced by contractile activity. Supported by NSERC Canada."

Title
Static stretch, but not repetitive twitches, activates MAPK signalling in rat medial gastrocnemius in-situ

Authors
Louis C. Martineau and Phillip F. Gardiner

Journal
Canadian Journal of Applied Physiology, 24(5): 466, 1999

Conference
Canadian Society for Exercise Physiology 1999, Toronto, ON

Abstract
"Mitogen-activated protein kinase (MAPK) signalling cascades constitute important pathways of communication to the nucleus and may be involved in relaying hypertrophic stimuli in skeletal muscle. The Jun N-terminal kinase (JNK) and, to a lesser extent, the extracellular- regulated kinase (ERK) have been found by our lab to be activated in response to short periods of static muscle stretch, a perturbation known to increase transcription of c-jun and c-fos and to promote hypertrophy. A similar activation has been reported in response to high-frequency contractile activity. In an initial attempt to parcel out the contributions of tension, length, and metabolic activity to activation of these pathways, in-situ low-frequency indirect stimulation of the rat medial gastrocnemius muscle (5 twitches/sec for 5 min) was compared to static stretch of 25% above Lo held for the same duration. Following the perturbation, muscles were snap-frozen and analyzed by western blot. While the two protocols generate tensions of similar magnitude (~150g), MAPK activation was only slightly above control in response to contractile activity, whereas stretch resulted in a 5- to 80-fold activation. This difference may be due to the cyclical nature of the tension developed during contractions. Alternatively, length changes and/or deformation of the extracellular matrix, which may not be present during low- frequency activation, may be necessary to activate these pathways and promote changes in gene expression.
Supported by NSERC Canada."

Title
Eccentric contractions cause rapid and massive activation of MAPK in rat skeletal muscle in-situ

Authors
Louis C. Martineau and Phillip F. Gardiner

Journal
FASEB Journal, 14(4): A318, 2000

Conference
Experimental Biology 2000, San Diego CA

Abstract
"Activation of the JNK and ERK mitogen-activated protein kinases (MAPK), which phosphorylate a number of transcription factors, has previously been shown to be sensitive to static passive muscle stretch in a length-dependent manner (Martineau et al., FASEB J. 13(4): A410, 1999). The goal of the present study was to determine the extent to which MAPK activation could be induced at a physiological muscle length by active stretch. By using a servomotor to control muscle length in an in-situ nerve/muscle preparation of the rat hindlimb, tetanic eccentric contractions lasting 150 msec and having a 3 mm excursion were imposed every second for 5 min on the plantaris set to Lo. Control conditions included passive cyclical stretch at the same amplitude and frequency, isometric contractions at Lo using identical stimulation parameters, and an unstretched-unstimulated condition. Immediately following the perturbation, muscles were excised and frozen in liquid nitrogen. Activation was assessed by western blot using antibodies against phosphorylated JNK, ERK, and p38 MAPK. Eccentric contractions resulted in an 80-fold activation over unstretched-unstimulated controls, a 40-fold activation over cyclically-stretched controls, and in a 10-fold activation over electrically-stimulated controls. ERK activation was qualitatively similar to JNK activation, but smaller in magnitude. p38 activation was not sensitive to stretch and/or contraction. These results support the hypothesis that activation of the JNK and ERK signalling pathways is sensitive to total muscle tension, developed either passively or actively. Supported by NSERC Canada."

Title
Mechanosensitivity of rat skeletal muscle is inversely related to muscle size

Authors
Louis C. Martineau and Phillip F. Gardiner

Journal
Canadian Journal of Applied Physiology, 26(5): 496, 2001

Conference
Canadian Society for Exercise Physiology 2001, Montréal, QC

Abstract
"As muscle fibers adapt to altered levels of mechanical loading by modulating their cross-sectional area, it is unknown if their sensitivity to mechanical stress is also modulated. This study tested the hypothesis that mechanosensitivity is inversely related to muscle size. Activation of p54 JNK mitogen-activated- protein-kinase, a quantitative marker of mechanical stimulation, was assessed in normal plantaris muscles, in plantaris muscles atrophied by 2 wk of tetrodotoxin-induced paralysis, and in plantaris muscles surgically-overloaded for 8 wk, in response to a 5 min in-situ contractile protocol (100Hz, 150ms /s). JNK activation per unit total muscle protein immediately following the contractile protocol was 2-fold greater in atrophied muscles than in age-matched control muscles. When normalized by specific tension, the response was 4-fold greater than in controls. JNK activation in hypertrophied muscles following the protocol was ~50% that of age-matched controls. As mechanotransduction is believed to involve the cortical cytoskeleton, the simple change in surface-to-volume ratio resulting from trophic remodelling may result in altered mechano-sensitivity. Such modulation secondary to remodelling may partially explain the small range of fiber cross-sectional areas observed across mammalian muscle. This work was supported by NSERC Canada."

Title

Heterogeneous p54 JNK MAPK activation in compartments of the rat medial gastrocnemius muscle in response to contractile activity in-situ

Authors

Kristina J. Csukly, Louis C. Martineau, and Phillip F. Gardiner

Journal

Canadian Journal of Applied Physiology, 26(5): 472, 2001

Conference

Canadian Society for Exercise Physiology 2001, Montréal, QC

Abstract

"Contractile activity results in rapid activation of mitogen-activated protein kinases (MAPK), including p54 JNK whose activation has recently been shown to be quantitatively related to tension. Fiber type differences have been alluded to in the literature to explain inter-muscle differences in JNK response to mechanical stimulation. The purpose of this study was to compare the JNK response to contractile activity of two metabolically different compartments of the same muscle. The medial gastrocnemius (MG) muscle of the rat was subjected to an in-situ protocol of tetanic stimulation (100 Hz, 150 ms /s, 5 min), following which the muscle was excised, frozen and separated into red and white compartments. Western immunoblot analysis revealed that content of phosphorylated (activated) p54 JNK was significantly higher in the red MG than in the white, despite similar contents of total JNK. Thus, heterogeneity in MAPK mechano-signalling occurs in various regions of the same contracting muscle, which may reflect intramuscular variations in fiber type composition and/or tension excursions. This work was supported by NSERC Canada."

Title

Absence of a refractory period for mechanical activation of p54 JNK mitogen-activated protein kinase in rat skeletal muscle in situ

Authors

Peter Tzavaris, Louis C. Martineau, and Phillip F. Gardiner

Journal

Canadian Journal of Applied Physiology, 26(5): 520, 2001

Conference

Canadian Society for Exercise Physiology 2001, Montréal, QC

Abstract

"Activation of the p54 JNK mitogen-activated-protein kinase (MAPK) occurs in a tension-dependent manner within seconds of mechanical stimulation of skeletal muscle, and may represent a very early event in muscle hypertrophy. Inactivation of MAPKs is tightly regulated by a family of MAPK phosphatases activated by MAPKs through a negative feedback mechanism. Such regulation could result in a refractory period for MAPK reactivation and have implications for the optimization of training protocols and disuse atrophy countermeasures. This hypothesis was tested by restimulating the rat plantaris muscle in situ 2h following an initial bout of mechanical stimulation, at a time when JNK activation has returned to 25% of its peak. The stimulation protocol consisted of 5 min of eccentric contractions (100Hz, 150 ms /s, 3 mm length excursion). It was observed by western immunoblot that restimulation resulted in similar levels of activation of p54 JNK as the initial mechanical stimulation, thereby indicating an absence of a refractory period. Thus, mechanotransduction and signalling events upstream of JNK do not appear to be rate-limiting to the maximization of mechanically induced mitogenic signalling in skeletal muscle. This work was supported by NSERC Canada."

Title
Contractile activity rapidly increases the association of rat skeletal muscle β1 integrins to the cytoskeleton

Authors
Louis C. Martineau and Phillip F. Gardiner

Journal
FASEB Journal, 16(5), A761, 2002

Conference
Experimental Biology 2002, New Orleans, LA

Abstract
"Integrins mediate cellular adhesion to extracellular matrix proteins. The strength of adhesion is regulated by modulation of receptor affinity, receptor avidity, or association between receptors and the cytoskeleton. Little is known about regulation of integrin function in skeletal muscle. This study demonstrates that the strength of adhesion of muscle β1 integrins is regulated during contractile activity. Within seconds following the onset of isometric contractile activity of the rat plantaris muscle in situ, association between β1 integrins and the cytoskeleton was enhanced as evidenced by loss of β1 integrin immunoreactivity in the detergent-soluble cell fraction and a corresponding increase in the cytoskeletal fraction. Eccentric contractions potentiated this effect, suggesting a tension-dependent response. However, passive stretch was not as effective at promoting association, indicating that increased cytoplasmic calcium or a change in energy state may be required. It is unknown whether modulation of receptor affinity or avidity also contribute to increased adhesion. Enhanced association suggests that cytoskeletal stiffening can occur in skeletal myocytes, and may have implications for mechanotransduction or mechanosensitivity. Supported by NSERC Canada."

Title
JNK MAPK activation in rat hindlimb muscles differing in fiber type and size in response to a standardized mechanical stress

Authors
Kristina J. Csukly, Louis C. Martineau, and Phillip F. Gardiner

Journal
FASEB Journal, 16(5), A760, 2002

Conference
Experimental Biology 2002, New Orleans, LA

Abstract
"Skeletal muscle is sensitive to mechanical loading. Variation in early mechanically-induced signaling in different muscle fiber types may underlie differences in sensitivity to hypertrophic stimuli. This study compared the p54 JNK mitogen-activated-protein-kinase (JNK MAPK) activation response to a standardized mechanical stress of muscles of rat hindlimb which differ in their fiber sizes and fiber-type compositions. Constant, normalized passive stretch was applied to the rat extensor digitorum longus (EDL), soleus (SOL), plantaris (PLN), and medial gastrocnemius (MG) in situ for 5 minutes, followed immediately by measurement of the activation of JNK MAPK, a mechanically-sensitive kinase rapidly activated in a dose-response manner by active or passive tension. Rat hindlimb muscles EDL, SOL, PLN and superficial MG were surprisingly similar in their JNK MAPK response (3-to 5-fold baseline values), in spite of differing fiber-type compositions and mean fiber sizes. The only notable variation observed among muscles was a significantly higher JNK MAPK activation in deep MG (13-fold) compared to all other muscles. Differences among MG compartments may be attributable to more complex architectural and/or fiber cross-sectional area factors. Supported by NSERC Canada."