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Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension

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Martineau, Louis C., and Phillip F. Gardiner. Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. *J Appl Physiol* 91: 693–702, 2001.—The mechanism by which mechanical forces acting through skeletal muscle cells generate intracellular signaling, known as mechanotransduction, and the details of how gene expression and cell size are regulated by this signaling are poorly understood. Mitogen-activated protein kinases (MAPKs) are known to be involved in mechanically induced signaling in various cell types, including skeletal muscle where MAPK activation has been reported in response to contraction and passive stretch. Therefore, the investigation of MAPK activation in response to mechanical stress in skeletal muscle may yield important information about the mechanotransduction process. With the use of a rat plantaris in situ preparation, a wide range of peak tensions was generated through passive stretch and concentric, isometric, and eccentric contractile protocols, and the resulting phosphorylation of c-Jun NH_2-terminal kinase (JNK), extracellular regulated kinase (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 rate of tension development. Differences in maximal response amplitude and sensitivity between JNK and ERK MAPKs suggest different roles for these two kinase families in mechanically induced signaling. 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 the fact 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.

intracellular signaling; mitogen-activated protein kinases; c-Jun NH_2-terminal kinase; eccentric contraction

MECHANOTRANSDUCTION IS THE fundamental mechanism by which mechanical stress acting through a cell initiates intracellular signaling. Through this mechanism, forces promote cellular growth and survival (10, 27), influence metabolic responses (17), and govern tissue architecture in various cell types (5, 32, 34, 39). Whereas all adhesion-dependent cells appear to be sensitive to mechanical forces (27, 35), 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 (16, 37), osteocytes (24), cardiomyocytes (29, 32, 38), and skeletal muscle cells (13, 34). Striated muscle cells are particularly responsive to mechanical stress, as evidenced by the fact that cellular size is in large part dictated by physical forces: loading elicits hypertrophy whereas unloading elicits atrophy (26).

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

Whereas numerous pathways have been implicated, the involvement of the mitogen-activated protein kinase (MAPK) cascades in mechanically induced signaling from the cytosol to the nucleus is consistent across various cell types (3, 15, 16, 18–20, 29–31, 38, 40). This function is compatible with the well-known role of the MAPKs as points of convergence for various signaling cascades regulating gene expression (11), including signaling 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 NH_2-terminal kinase (JNK) family (also known as stress-activated protein kinases), the extracellular-regulated kinase (ERK) family, and the p38 family, translocate to the nucleus on 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 adapt-

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ability 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. The events involved in the initiation of muscle hypertrophy are not known. 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 which was 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 that 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 (TTI), were analyzed. Whereas speed appears to have little influence on MAPK activation, time 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).

METHODS

Physiology

In situ nerve-muscle preparation. 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 anesthetized with an intraperitoneal injection of ketamine and xylazine (61.5 mg/kg ketamine and 7.7 mg/kg xylazine) and maintained under anesthesia by hourly injections of 25% of the initial dose. After the experiment, animals were killed by anesthetic 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 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 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.

After 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 (Cambridge LR 350), without putting the isolated muscle under tension. The skin of the hindlimb was pulled into a bath that 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.

To determine optimal length for muscle twitch tension development (L_o), 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 (5-V) single square pulses of 0.05 ms in duration were delivered once every 3 s by a Grass S88 stimulator. L_o determination to less than ±1 mm required, on average, 60 twitches. After determination of L_o, electrical stimulation was ceased, and the muscle was held at this length for 5 min 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 1 s for exactly 5 min (i.e., 300 tetanic contractions). Muscle length was either kept constant at L_o (isometric contraction) or varied from L_o to L_o + 3 mm (eccentric contraction with passive return to L_o) or L_o + 3 mm to L_o (concentric contraction with passive stretch return to L_o + 3 mm) over 150 ms synchronously with the electrical stimulation, by using a computer-controlled servomotor. A 3-mm length excursion corresponds to ~10% of muscle length. A ramp function was employed to keep velocity constant at 20 mm/s or ~⅔ L_o/s. Time spent at L_o was identical to time spent at L_o + 3 mm for both eccentric and concentric groups. This was accomplished by using a 50:50 duty cycle. Thus, after the 15-ms length excursion, muscles were held at the new length for 350 ms before being returned to starting length over 150 ms. Refer to Fig. 1 for a schematic representation of the various contractile protocols.

To parcel out the effects of activation, stretch, movement, and contraction type on MAPK phosphorylation, contractile groups were compared with three control groups: an unstimulated group maintained at L_o for the same duration as the contractile protocols (Static L_o); an unstimulated group maintained at L_o + 3 mm (Static L_o + 3 mm); and an unstimulated group that passively underwent the same length excursion as the eccentric and concentric groups (Passive Stretch L_o to L_o + 3 mm). Analysis was performed by ANOVA with a Fisher post hoc test.

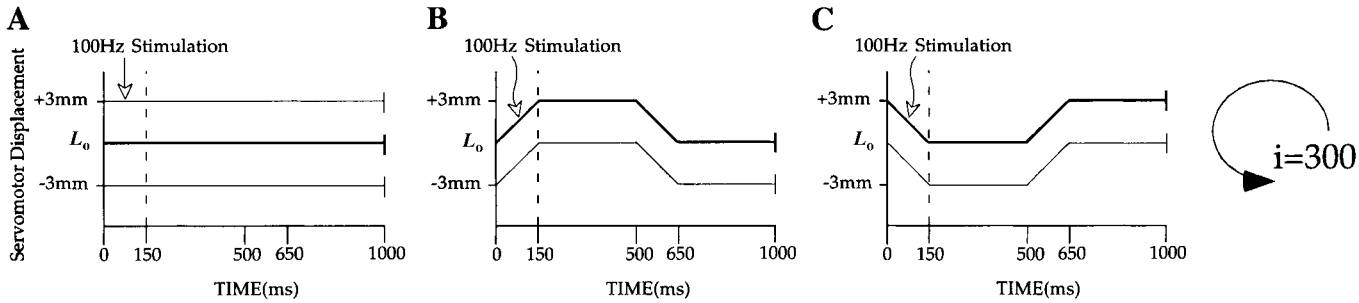

Fig. 1. Graphical representation of 1,000-ms contractile protocols repeated 300 times. *A*: isometric contractions; *B*: eccentric contractions; *C*: concentric contractions. Thick lines represent the 3 protocols employed in the study of the effect of different types of contractions on mitogen-activated protein kinase (MAPK) phosphorylation (*study A*), whereas the thin lines represent the additional 4 protocols employed in the study of the relationship between tension and MAPK phosphorylation (*study B*). Electrical stimulation is delivered between 0 and 150 ms, coinciding with the 3-mm ramps of the eccentric and concentric protocols. Static control groups are identical to the isometric optimal length for muscle twitch tension development (L_o) and isometric L_o + 3 mm protocols without electrical stimulation, whereas 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 L_o, regardless of initial length.

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

RELATIONSHIP BETWEEN TENSION AND MAPK PHOSPHORYLATION. To investigate the relationship between tension-related parameters and MAPK phosphorylation, a wide range of tension (15-fold range) was generated. However, to keep stimulation parameters constant and, in the case of anisometric contractions, to 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 L_o described above, isometric contractions were performed at L_o − 3 mm and at L_o + 3 mm. In addition to the eccentric contractions performed between L_o and +3 mm, eccentric contractions were performed between L_o − 3 mm and L_o. In addition to the concentric contractions performed between L_o + 3 mm and L_o, concentric contractions were performed between L_o and L_o − 3 mm. All groups conformed to the 50:50 duty cycle design described in the preceding section. This symmetry allowed time spent reaching L_o and at L_o to be kept constant across all groups, regardless of starting length. Refer to Fig. 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 eight groups or 32 points for regression analysis. Refer to Fig. 2 for representative tension tracings for the eight 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 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 immediately after the contractile protocol. Excision was performed in <10 s, 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 measurements and kinase assays has been demonstrated.

Homogenization and sample preparation. Frozen muscles were powdered in liquid nitrogen. Approximately 90 mg of muscle powder were 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 for 30 s and placed on ice for 1 h. Throughout this 1-h period, samples were frequently vortexed. Samples were then centrifuged at 4,500 *g*, 4°C, for 1 h 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 min.

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 electrotransferred to a single piece of polyvinylidene difluoride 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 for 1.5 h at room temperature in secondary antibody solution. All washes were done in TBST. Monoclonal antibodies directed against phosphorylated (p) 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 1:2,000 in TBST plus 1% BSA and 0.5% NaN_3, whereas horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (Jackson Immunoresearch) were prepared at 1:10,000 in TBST plus BSA. Two hundred micrograms of whole cell lysate of ultraviolet-treated HeLa cells or heat-shocked NIH/3T3 cells (Santa Cruz Biotechnology) were 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 5–20 min. Membranes were stripped, reblocked, and reprobed 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 (~16-fold resolution), short and long exposures of the same blots were quantified. A ratio was calculated from the results of samples that 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 after the three 5-min contractile protocols that were compared: eccentric (L_o to L_o + 3 mm), isometric (L_o), and concentric (L_o + 3 mm to L_o) (Fig. 3). Refer to Table 1 for group descriptive statistics. 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, whereas p54 phosphorylation was observed over a wide range of tension, p46 phosphorylation was only observed in conjunction with a saturating p-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 (ERK1) isoform of the ERK family was observed to be phosphorylated in response to tension, and this phosphorylation was quantified. The eccentric protocol resulted in ERK1 phosphorylation of fivefold control levels, the isometric protocol in fourfold control levels, and the concentric protocol in threefold 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 L_o + 3 mm group or the Passive Stretch L_o to L_o + 3 mm control group did not result in more JNK phosphorylation than did the Static L_o 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 that was twofold greater than 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 after the 5-min protocol.

See Fig. 4 for representative p-JNK, p-ERK, and p-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 greater than twitch tension (Table 1). An unstimulated cyclic stretch group, the Passive Stretch L_o to L_o + 3 mm group, generated on average peak tensions of 44 g or 0.8-fold greater than twitch tension. When taken together, the eight groups produced a 15-fold range in peak tension. Group descriptive data are summarized in Table 1. Representative tension tracings are illustrated in Fig. 2.

Phosphorylation values for p54 JNK (p-JNK densitometry values linearized by log10 transformation) and p44 ERK (p-ERK raw densitometry values) were subjected to regression and correlational analyses against the parameters of peak tension, TTI, and dT/dt. Raw ERK densitometry values and JNK densitometry values linearized by log10 transformation were used for this analysis. 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 s had the highest correlation coefficient ($r^2 = 0.89$). When group average raw densitometry data were plotted against average peak tension over the

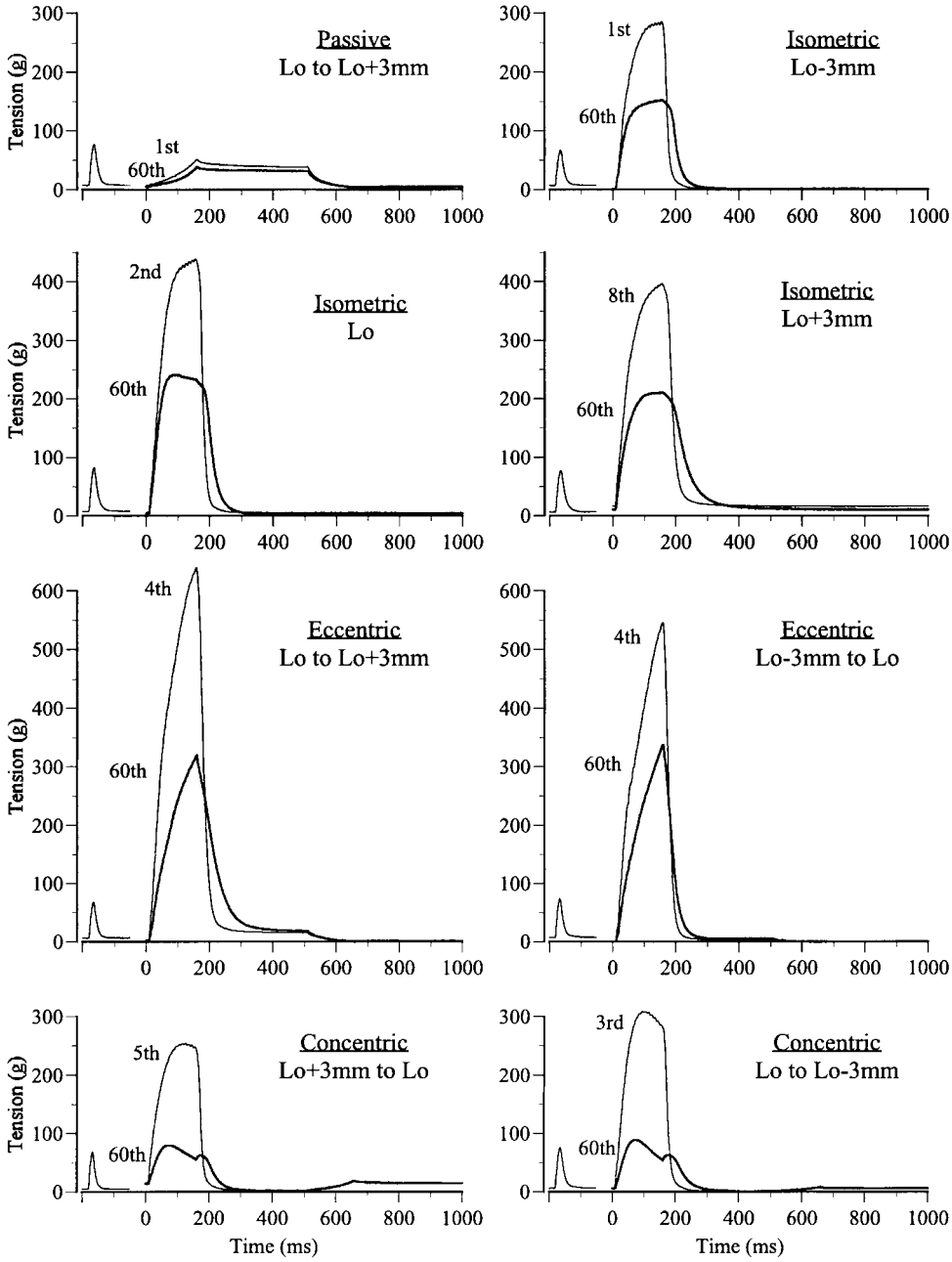

Fig. 2. Representative tension tracings from the 7 contractile protocols and 1 cyclic passive stretch protocol employed in *studies A* and *B*. For each of the experiments depicted, a tracing of a twitch at L_o (*left* trace) is followed by a tracing of the iteration at which maximal peak tension is achieved (iteration no. indicated beside tracing). The 60th iteration (thick line) is superimposed onto the maximal peak tension tracings (thin line). Note that the rate of fatigue resulting from the 2 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.

first 60 s, a simple power function ($y = 0.3\ x^{4.67}$) produced a near-perfect fit ($r^2 = 0.998$). These relationships are illustrated in Fig. 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 Fig. 5. This supports the fact that MAPK phosphorylation is independent of contraction type.

DISCUSSION

The present study suggests that MAPKs may be involved in mechanically induced signaling 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 a quantitative marker of the magnitude of mechanical stress applied to a muscle.

Table 1. *Physiological Measures*

Group	Sample Size	Body Weight, g	Preparatory Parameters		Experimental Parameters		
			L_o twitch tension, g	L_o passive tension, g	Maximal baseline passive tension, g	Maximal developed tension, g	Developed tension normalized by twitch tension
Static L_o	4	190 ± 2^{a}	$62 \pm 3^{a,b}$	7 ± 1^{a}	7 ± 1^{a}	n/a	n/a
Static $L_o + 3$ mm	4	191 ± 2^{a}	$63 \pm 5^{a,b}$	6 ± 1^{a}	32 ± 5^{b}	n/a	n/a
Passive L_o to $L_o + 3$ mm	4	196 ± 3^{a}	58 ± 5^{a}	7 ± 1^{a}	7 ± 1^{a}	44 ± 5^{a}	0.8 ± 0.1^{a}
Isometric $L_o - 3$ mm	4	199 ± 3^{a}	60 ± 6^{a}	7 ± 0^{a}	2 ± 1^{a}	$283 \pm 3^{b,c}$	4.8 ± 0.2^{c}
Isometric L_o	4	193 ± 3^{a}	$71 \pm 4^{b,c,d}$	6 ± 1^{a}	7 ± 1^{a}	398 ± 18^{e}	5.6 ± 0.2^{d}
Isometric $L_o + 3$ mm	4	195 ± 3^{a}	$65 \pm 2^{a,b,c}$	6 ± 1^{a}	31 ± 3^{b}	337 ± 16^{d}	$5.2 \pm 0.1^{c,d}$
Eccentric L_o to $L_o + 3$ mm	4	193 ± 4^{a}	$60 \pm 3^{a,b}$	6 ± 1^{a}	7 ± 1^{a}	630 ± 13^{g}	10.5 ± 0.3^{f}
Eccentric $L_o - 3$ mm to L_o	4	195 ± 2^{a}	59 ± 4^{a}	7 ± 0^{a}	1 ± 0^{a}	508 ± 22^{f}	8.6 ± 0.3^{e}
Concentric $L_o + 3$ mm to L_o	4	194 ± 2^{a}	$64 \pm 2^{a,b,c}$	5 ± 1^{a}	25 ± 3^{b}	241 ± 6^{b}	3.8 ± 0.2^{b}
Concentric L_o to $L_o - 3$ mm	4	195 ± 2^{a}	$74 \pm 4^{c,d}$	6 ± 0^{a}	7 ± 0^{a}	$299 \pm 7^{c,d}$	4.1 ± 0.3^{b}

Values are means ± SE. L_o, optimal length for muscle twitch tension development. Different letters denote significant differences ($P < 0.05$) between groups.

This study also supports the fact 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, independent of contraction type. Finally, the results of this study indicate that peak tension is a better predictor of MAPK phosphorylation than are other tension-related parameters, such as TTI and peak d*T*/d*t*.

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 the fact 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 mechanochemi-

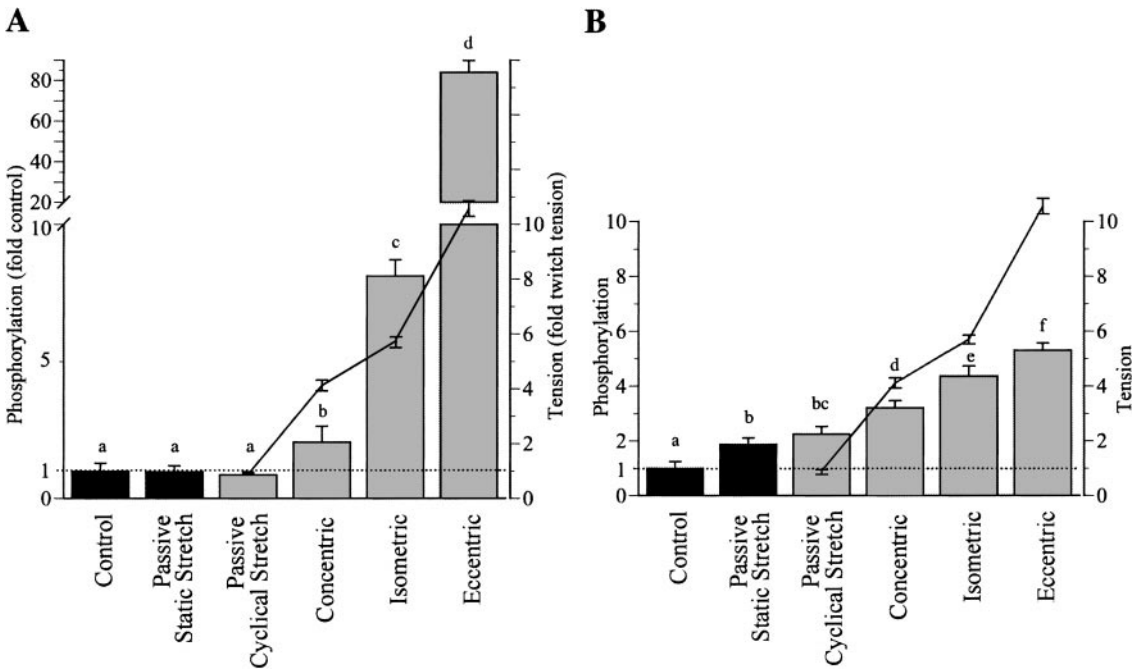

Fig. 3. Effect of contraction type on p54 c-Jun NH_2-terminal kinase (JNK; *A*) and p44 extracellular regulated kinase (ERK) MAPK (*B*) phosphorylation, as measured by Western blot. Data are expressed as fold increase over static L_o unstimulated control levels. Additional unstimulated control groups are used to parcel out the effects of a length excursion and of the resulting tension at $L_o + 3$ mm from contraction type. All groups have a sample size of 4. Different letters (a–f) identify significant ($P < 0.05$) between-group differences, as determined by ANOVA. Normalized maximal peak tensions generated during the anisometric protocols are overlaid (solid line) 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.

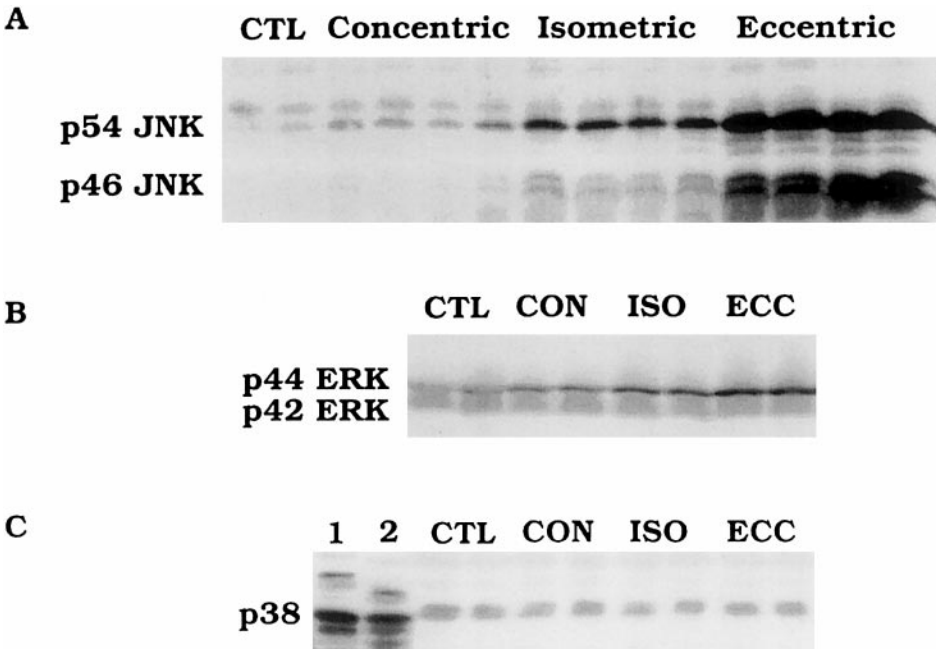

Fig. 4. Representative films of a phospho-JNK (*A*), phospho-ERK (*B*), and phospho-p38 (*C*) Western blot. *A*: *lanes 1* and *2*, unstretched, unstimulated controls (CTL) at L_o; *lanes 3–6*, concentric contractile protocol; *lanes 7–10*, isometric contractile protocol; *lanes 11–14*, eccentric contractile protocol. This film is overexposed to visualize control levels. *B*: *lanes 1* and *2*, controls; *lanes 3* and *4*, concentric (CON); *lanes 5* and *6*, isometric (ISO); *lanes 7* and *8*, eccentric (ECC). *C*: *lane 1* contains whole cell lysate of ultraviolet-treated HeLa cells, whereas *lane 2* contains whole cell lysate of heat-shocked NIH/3T3 cells. Both of 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.

cal 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 mechanosensitive 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 signaling pathways suspected of participating in mechanotransduction and mechanical regulation of gene expression.

Table 2. *Assessment of linear regressions between tension-related variables and p-JNK and p-ERK densitometry values*

	Log-transformed p-JNK Values (R^2)	p-ERK Values (R^2)
Maximal peak tension/maximal twitch tension	0.86	0.64
Average peak tension/maximal 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 1000-ms TTI/maximal twitch tension	0.75	0.53
Aggregate TTI/maximal 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

p-JNK, phosphorylated c-Jun NH_2-terminal kinase; p-ERK, phosphorylated extracellular regulated kinase; TTI, tension-time integral; dT/dt, rate of tension development. *Highest r^2 values.

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 signaling 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 signaling. 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 mechano-

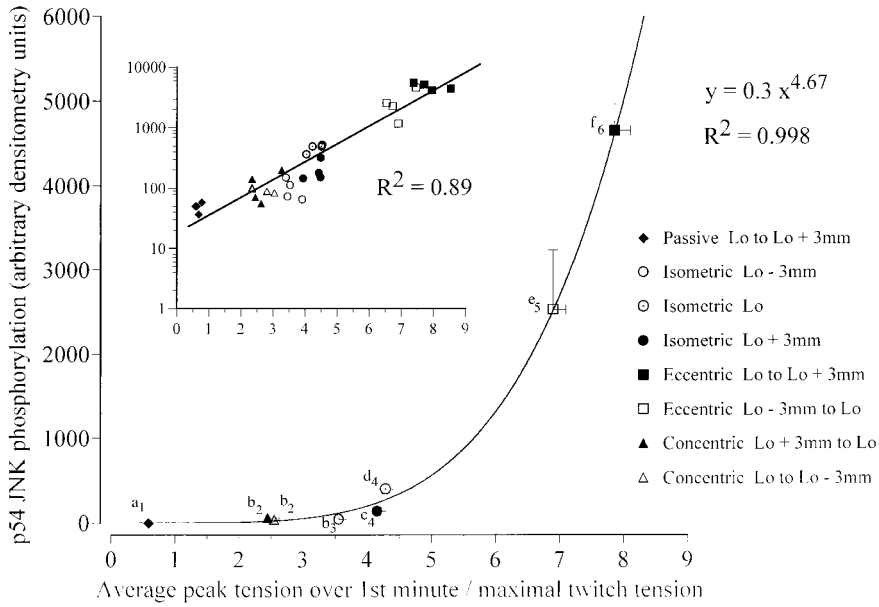

Fig. 5. p54 JNK phosphorylation can be reliably predicted from peak tension, as demonstrated by regression analysis. The average peak tension generated over the first minute normalized by the twitch tension is plotted against arbitrary densitometry data. *Inset graph*: densitometry data are log transformed, 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 (a–f) for phosphorylation and by different nos. (1–6) for tension. Note that the positive tension-phosphorylation relationship holds within the 2 eccentric protocols and within the 3 isometric protocols.

transduction mechanism based on elastic deformation. The relative importance of these parameters in the modeling 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 that need to be addressed to fully model the sensitivity of muscle to mechanical stimulation. Although 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 s, 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 (Fig. 6), suggests that phosphorylation measured immediately after the 5-min protocols is determined by more than 30 s of stimulation.

Although 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 have been reported in smooth muscle cells (15).

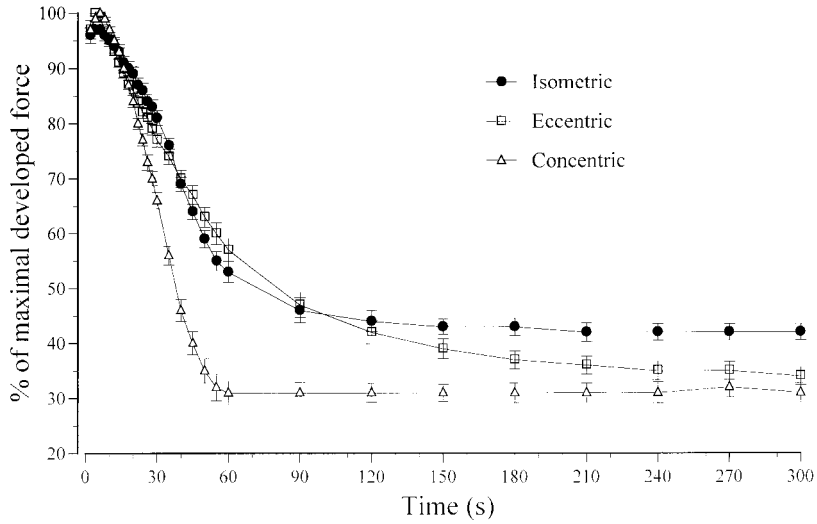

Fig. 6. Rate of fatigue, expressed as %decrease from peak tension over a given time interval, is greater in experiments employing a concentric contractile protocol (n = 8) than in experiments employing an isometric (n = 12) or an eccentric protocol (n = 8). This is especially evident between the 30th 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 min, is determined by >30 s of mechanical stimulation.

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, whereas 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 that is on the order of two- to fourfold greater than 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 fivefold. 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 because of 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 does 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 min 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 p38 activation resulting from 5 min of contractile activity can be observed at later time points (unpublished data). This is in accord with other reports of contractile activity-induced MAPK activation. Goodyear et al. (14) showed very low p38 activation (less than twofold greater than control values) occurring significantly later than JNK activation in exercised rat muscle, and Widegren et al. (36) showed p38 activation to be at least an order of magnitude lower than ERK activation in exercised human muscle.

In summary, the present study demonstrates a possible involvement of MAPKs in mechanically induced signaling in skeletal muscle. A quantitative relationship between p54 JNK phosphorylation and peak tension supports the fact 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 signaling events involved in mechanically induced muscle hypertrophy and for the understanding of the nature of the mechanical stimuli to which muscle is sensitive.

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