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

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Accepted 26 November 2001

Abstract

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 jun-N-terminal-kinase (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. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Mechanotransduction; Skeletal muscle; Tension; Signal transduction; Mitogen-activated-protein-kinase

1. 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 and Eskin, 1997), airway smooth muscle (Smith et al., 1998), chondrocytes (Wright et al., 1997), bone cells (Mikuni-Takagaki, 1999), and cardiac (Sadoshima and 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

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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 remodeling, 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 and Gardiner, 1999), is a quantitative marker of mechanical stimulation in skeletal muscle and can be employed as an assay of the mechanotransduction process (Martineau and 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 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 mechanochemo 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 mechanostimulation 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.

2. 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 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 and 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 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.

Optimal length for muscle twitch tension development (Lo) to $< ±1$ 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 s while the muscle was slowly lengthened from a completely relaxed position. Following this determination, the muscle was subjected to a single tetanic contraction (100 Hz, 100 ms) 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 30 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 200g 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 and Gardiner, 1999). Stretch duration was set to 5min as this is sufficient time for the development of peak phosphorylation (Martineau and Gardiner, 1999). Muscles were excised and frozen in liquid nitrogen within 20 s 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 TTI and of the rate of change of tension (dT/dt) on tension-induced activation of p54-JNK MAPK are schematically summarized in Fig. 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-s 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 s and was repeated at a frequency of 0.2 Hz. Thus, over a 5-s 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 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-s 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 and 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 g and the protein concentration of the supernatants was determined by Bradford protein assay. Samples containing 200μg total protein were prepared for SDSPAGE 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 1.5h at room temperature 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

Fig. 1. Diagrammatic representation of the four passive stretch protocols differing in displacement–time integral (DTI) and/or in average rate of stretch, but not in length excursion.
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.

3. 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 DTI 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 (Fig. 2A). The Mid-TTI Low Rate and Mid-TTI High Rate groups were different from both the High and the Low TTI group.

A significant effect of TTI on phosphorylation of p54-JNK was attested to by the difference between the two protocols employing the same rate of stretch but featuring a 10-fold difference in DTI (Low TTI vs. Mid TTI High Frequency) and by the absence of difference between the two groups with identical DTIs (Mid-TTI High Rate and Mid-TTI Low Rate) (Fig. 2A). A near-perfect ($R^2 = 0.99$) positive linear relationship was observed between group-average TTI (initial 5-s TTI normalized by muscle force) and group-average p54-JNK phosphorylation (Fig. 2B). This relationship held for the final 5-s 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 DTI but featuring a 10-fold difference in rate of stretch (Mid-TTI Low Rate vs. Mid-TTI High Rate) (Fig. 2A).

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<th>Table 1 Physiological measures</th>
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All data are 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.
4. 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 TTI and rate of change of tension (d\(T/dt\)) (Martineau and Gardiner, 2001). The current study was designed to parcel out the sensitivity of skeletal muscle to TTI and to d\(T/dt\); 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 and 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 in mechanocytes.

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The JNK MAPK cascade has traditionally been associated with various cellular stresses (Tibbles and Woodgett, 1999). However, numerous lines of evidence support that the mechanical sensitivity of this pathway is not related to cellular damage: (1) passive strain of 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 of 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 d\(T/dt\) while avoiding complications due to fatigue. The MG muscle, which was used in the original characterization of stretch-induced p54-JNK activation (Martineau and 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 mechanostimulation (Martineau and 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 \(\frac{dT}{dt}\), 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 mechanostimulation 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 and 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 \(\frac{dT}{dt}\) 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 \(\frac{dT}{dt}\). While the rate of stretch and the rate of relaxation vary together when using sinusoidal functions, the findings clearly indicate that \(\frac{dT}{dt}\) does not affect the measured signaling, and it is therefore not necessary to uncouple these variables. A quicker return to \(L_0\) would be expected to simply reduce TTI and reduce the resulting signal.

Clearly, both peak tension and TTI, but not \(\frac{dT}{dt}\), 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 \(\frac{dT}{dt}\) 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 mechanostimulation 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 \(\frac{dT}{dt}\), 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.

Acknowledgements

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

References


