TACE release of TNF-α mediates mechanotransduction-induced activation of p38 MAPK and myogenesis

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Summary

Skeletal muscle responds to mechanical stimulation by activating p38 MAPK, a key signal for myogenesis. However, the mechanotransduction mechanism that activates p38 is unknown. Here we show that mechanical stimulation of myoblasts activates p38 and myogenesis through stimulating TNF-α release by TNF-α converting enzyme (TACE). In C2C12 or mouse primary myoblasts cultured in growth medium, static stretch activated p38 along with ERK1/2, JNK and AKT. Disrupting TNF-α signaling by TNF-α-neutralizing antibody or knocking out TNF-α receptors blocked stretch activation of p38, but not ERK1/2, JNK or AKT. Stretch also activated differentiation markers MEF2C, myogenin, p21 and myosin heavy chain in a TNF-α- and p38-dependent manner. Stretch stimulated the cleavage activity of TACE. Conversely, TACE inhibitor TAPI or TACE siRNA abolished stretch activation of p38. In addition, conditioned medium from stretched myoblast cultures activated p38 in unstretched myoblasts, which required TACE activity in the donor myoblasts, and TNF-α receptors in the recipient myoblasts. These results indicate that posttranscriptional activation of TACE mediates the mechanotransduction that activates p38-dependent myogenesis via the release of TNF-α.

Key words: Stretch, Myogenesis, p38 MAPK, TACE, TNF-α

Introduction

It is well established that mechanical stimulation induces an adaptive response in skeletal muscle by activation of quiescent satellite cells, initiation of myogenic differentiation, and promotion of protein synthesis and muscle fiber growth (Hornberger and Esser, 2004; Tidball, 2005; Wozniak et al., 2005). This process involves mechanotransduction which relays mechanical stimulation received on the cell surface to the nucleus via various intracellular signaling pathways, which results in the concerted expression of muscle-specific genes for muscle growth. Studies of various load-sensitive cells indicate that the major cellular components that mediate mechanotransduction to induce cell-type-specific responses include the integrins, cytoskeleton, G proteins, tyrosine kinases, mitogen-activated protein kinases (MAPKs) and stretch-activated ion channels (Wang and Thampatty, 2006; Ingber, 2006). Notwithstanding, the signaling mechanism of mechanotransduction that regulates muscle adaptation remains unclear. One of the key missing mechanisms is the conversion of mechanical simulation to biochemical activation of the myogenic program.

It is now clear that the p38 MAPK plays an indispensable role in the activation of the myogenic program. Activation of p38 is an early and essential event in myogenic differentiation in embryos and myoblasts (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000; Puri et al., 2000; Cabane et al., 2003; Penn et al., 2004; de Angelis et al., 2005). p38 activates myogenesis through multiple actions on some sequential steps critical to myogenic differentiation. The MEF2 family of transcription factors, which bind to promoters of the majority of muscle-specific genes and interact with members of the MyoD family of proteins to activate skeletal muscle differentiation (McKinsey et al., 2002), are activated by p38-mediated phosphorylation of their transactivation domain (de Angelis et al., 2005). In addition, p38 stimulates MyoD transactivation activity (Puri et al., 2000) by promoting the association of E47 with MyoD via the phosphorylation of E47 (Lluis et al., 2005). Moreover, p38 mediates phosphorylation of the BAF60 subunit of the Brg1 SWI/SNF enzyme, which is required for chromosome remodeling. This remodeling allows the access of MyoD, E proteins and MEF2 to their binding sites in the myogenin promoter and, hence, the transcription of myogenin (Simone et al., 2004; de la Serna et al., 2005; Lluis et al., 2005). Furthermore, p38 promotes cell-cycle exit by induction of the expression of the Cdk inhibitor, p21 (Schafer, 1998; Wu et al., 2000; Cabane et al., 2003), so that terminal differentiation can proceed. In fact, p38 activation is considered a necessary and sufficient switch for the activation of myogenesis.

It has been shown that mechanical stimulation activates p38 in muscle (Boppart et al., 2001; Hornberger et al., 2005) and in myoblasts that have undergone 2 days of differentiation (Rauch and Loughna, 2005). However, the mechanism whereby mechanical stimulation activates p38 in muscle cells

remains undefined, which is a significant gap in our understanding of mechanotransduction that initiates myogenesis. The response of skeletal muscle to mechanical stimulation involves the release of a number of growth factors as autocrine/paracrine factors, including hepatocyte growth factor (HGF) (Tatsumi et al., 2001; Tatsumi et al., 2002), fibroblast growth factor (FGF) (Clarke and Feeback, 1996) and insulin-like growth factor I (IGF-I) (Perrone et al., 1995), to promote satellite cell activity or hypertrophic growth of muscle fibers. These growth factors are all capable of stimulating satellite cell activation or proliferation, with IGF-I also being capable of stimulating myogenic differentiation of satellite cells (Hawke and Garry, 2001). However, IGF-I can neither activate p38, nor induce myogenesis when p38 is inhibited (Wu et al., 2000).

The proinflammatory cytokine TNF-α, a potent activator of p38 in muscle cells (Li et al., 2005), has recently been shown to play a physiological role in muscle repair (Warren et al., 2002; Chen et al., 2005) and myogenesis (Li and Schwartz, 2001), in additional to its long-recognized pathological role in mediation of such disorders as cachectic muscle wasting, inflammatory myopathies, and insulin resistance (Reid and Li, 2001; Lundberg and Dastmalchi, 2002). Although, as a regulator of immune and inflammatory response, TNF-α is primarily synthesized by macrophages and other immune cells (Tracey and Cerami, 1993), it is also synthesized by skeletal muscle in a highly regulated manner. Myoblasts constitutively synthesize TNF-α (Saghizadeh et al., 1996), and this activity increases transiently upon differentiation (Li and Schwartz, 2001). Myofibers respond to various types of injury with dramatically increased expression of TNF-α and its receptors (Tews and Goebel, 1996; De Bleecker et al., 1999; Collins and Grounds, 2001; Zador et al., 2001; Warren et al., 2002). In addition, strenuous exercise elevates the level of circulating TNF-α (Ostrowski et al., 1999; Starkie et al., 2001), which originates from skeletal muscle (Starkie et al., 2001; Nieman et al., 2005). Although there are reports describing an inhibitory effect of exogenously added TNF-α on myoblast differentiation (Guttridge et al., 2000; Langen et al., 2001), it is difficult to reconcile such an inhibitory effect of TNF-α on myogenesis with the fact that muscle increases TNF-α production during adaptation response and regenerates well in the high TNF-α environment. Accumulating evidence indicates a regulatory role of TNF-α in muscle adaptation. TNF-α stimulates chemotactic response in mouse myogenic cells, which promotes myogenesis (Torrente et al., 2003). We observed previously that TNF-α promotes myogenesis during the early stages of C2C12 myoblast differentiation as an autocrine factor (Li and Schwartz, 2001). Warren et al. (Warren et al., 2002) reported an attenuation of muscle force recovery in the hindlimb of TNF-α receptor double knockout mice ($p55^{-/-}p75^{-/-}$) injured by freezing, which indicates a physiological role for TNF-α in muscle repair. More recently, we demonstrated in cardiotoxin-injured soleus muscle of $p55^{-/-}p75^{-/-}$ mice that p38 activation during muscle regeneration is dependent on TNF-α-receptor-mediated signaling; and in the absence of TNF-α-receptor-mediated signaling myogenesis and regeneration are impaired (Chen et al., 2005).

Based these data, we hypothesize that TNF-α is an autocrine mediator critical for the activation of p38 by various stimuli of muscle adaptation, including mechanical stimulation. Given that mechanical stimulation rapidly activates p38 in whole muscle within minutes (Boppart et al., 2001), we further hypothesize that a regulatory mechanism that rapidly increases the release of membrane-bound TNF-α, rather than the synthesis of TNF-α, is directly responsible for p38 activation by mechanical stimulation. TNF-α is synthesized as a 26 kDa pro-TNF-α, which is then anchored to the plasma membrane. The membrane-bound pro-TNF-α is cleaved and released as a 17 kDa secreted form of TNF-α by TNF-α converting enzyme (TACE). TACE, also known as ADAM17, is a ubiquitous enzyme that belongs to the ADAM family of disintegrin metalloproteinases (Blobel, 1997; Black, 2002). TACE activity can be acutely regulated posttranscriptionally by cellular signaling events (Zhang et al., 2000; Zhang et al., 2001; Fan et al., 2003); thus, it is of interest to determine whether TACE plays an active and rate-limiting role in mediating p38 activation by mechanical stimulation. The current study was devised to evaluate whether TNF-α is a key mediator of mechanical stimulation-induced p38 activation and myogenesis in myoblasts, and the potential role of TACE as the key regulator of the availability of autocrine TNF-α in mechanotransduction. Our analysis of myoblast response to mechanical stretch demonstrated that: (1) TNF-α is crucial to p38 activation and p38-dependent myogenic gene expression in myoblasts provoked by mechanical stimulation, and (2) this response is mediated by an activation of TACE causing release of TNF-α from myoblasts.

Results

Stretch activates MAPKs, AKT and myogenesis in myoblasts

Among the various stretch methods previously used in the study of mechanotransduction, static stretch is a simple and useful approach (Vandenburgh and Kaufman, 1979; Perrone et al., 1995). We adopted a static stretch device designed by Rauch and Loughna (Rauch and Loughna, 2005), which globally stretches cells grown on an elastic silicon membrane by approximately 8-9%. Using this apparatus, these investigators showed that stretch stimulates p38 activity in C2C12 myoblasts that have been incubated in low serum differentiation medium for 2 days, and enhances myogenesis (Rauch and Loughna, 2005). Given that p38 activation is an early event in myoblast differentiation, we chose to test our hypothesis in undifferentiated myoblasts cultured in serum-rich growth medium, rather than in low-serum differentiation medium, so that the early stages of differentiation could be monitored. This approach allows us to exclusively evaluate stretch effect on the initiation of myogenesis, without the interference of the effect of serum restriction which is a different type of differentiation stimulus. Stretch of C2C12 myoblasts was initiated when cells reached approximately 85% confluence. It has been previously reported that stretch activates the p38, ERK1/2 and JNK MAPKs, as well as AKT, in whole muscle (Martineau and Gardiner, 2001; Boppart et al., 2001; Sakamoto et al., 2003). Using western blot analysis with antibodies specific to their respective phosphorylated forms, we ascertained whether static stretch activated these kinases in our myoblast culture system. We observed that p38, ERK1/2 and AKT were activated within 1 hour of stretch, whereas, JNK was activated within 2 hours of stretch. Activation of p38 peaked at 2 hours, activation of AKT and

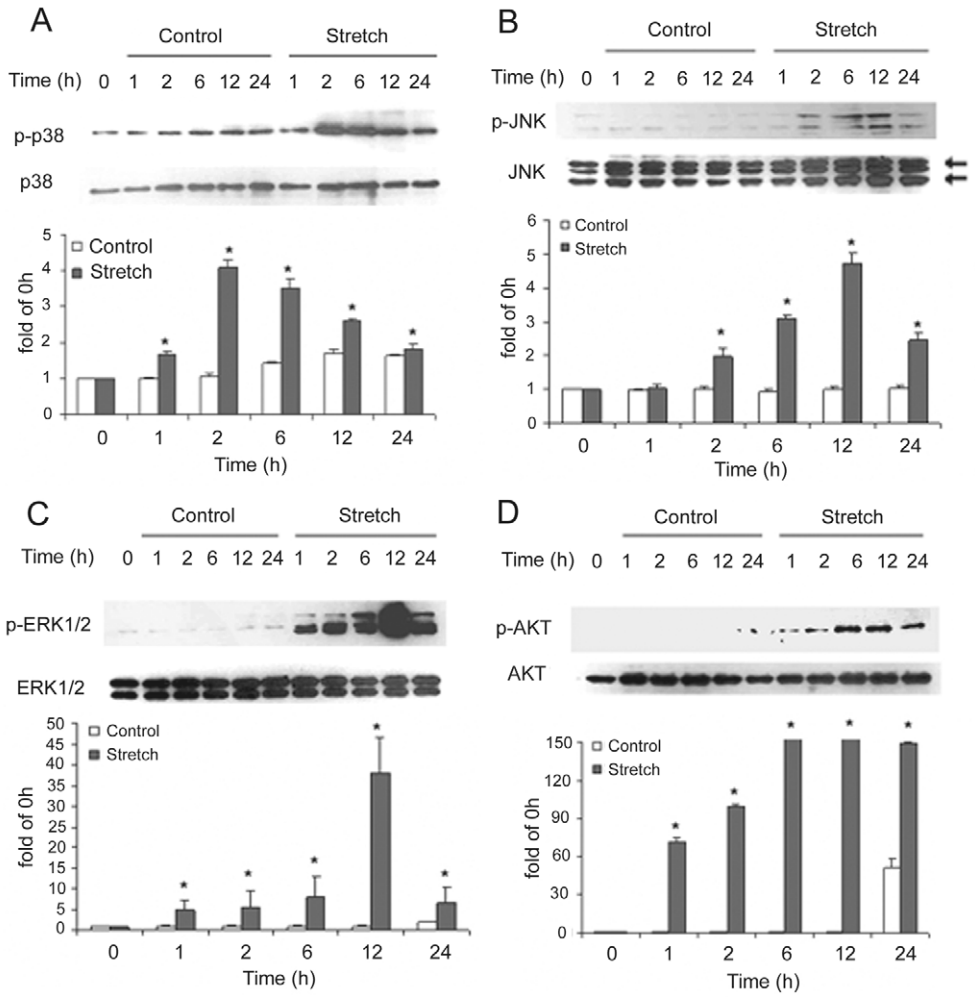

Fig. 1. Static stretch activates the p38, ERK1/2 and JNK MAPKs, as well as AKT. C2C12 myoblasts were seeded in six-well Bioflex® plates and allowed to proliferate to ~85% confluence. Stretch was conducted in fresh growth medium for the indicated time periods. Time-matched, non-stretched myoblasts were used as controls. Whole cell extracts of myoblasts were prepared and analyzed by western blotting using antibodies against the phosphorylated form of (A) p38 (43 kDa), (C) ERK1/2 (42/44 kDa), (B) JNK (46/54 kDa) and (D) AKT (60 kDa). Total levels of the kinases were also monitored by western blot analysis. Representative blots from three independent experiments are shown. Arrows indicate bands of phosphorylated JNK. The optical density (OD) of corresponding bands, detected by western blot, was measured and analyzed with the paired *t*-test, by comparison of stretched samples with time-matched controls ($n \geqslant 3$). * indicates a statistical significance at $P<0.01$.

ERK1/2 peaked at 6 hours, and JNK activation peaked at 12 hours (Fig. 1). Western blot analysis was also employed to monitor total protein levels of the kinases examined, and there were no significant changes over the 24 hours of stretch (Fig. 1). These data indicate that all the four kinases are activated by mechanical stimulation, yet, their rate and magnitude of response to mechanical stimulation varied.

To assess whether static stretch was capable of inducing myogenesis, we analyzed several differentiation markers. MEF2C, a transcription factor that stimulates expression of the majority of muscle-specific genes upon phosphorylation by p38 (McKinsey et al., 2002), was found to be activated within 1 hour of stretch, and reached its peak activation at 6 hours; while total MEF2C levels remained constant (Fig. 2). The expression of myogenin, which plays a key role in the execution of the myogenic differentiation program (Molkentin and Olson, 1996), and p21, which mediates cell cycle exit (Schafer, 1998), were also activated by stretch (Fig. 2). By contrast, in non-stretched control myoblasts, a slow and progressive activation of the three differentiation markers occurred over the 24 hour period, and similar unprovoked activation of MAPKs and AKT were observed (Fig. 1), which indicates that spontaneous differentiation was taking place while myoblasts maintained proliferation in growth medium and became highly confluent. These results indicate that stretch activates p38 and the myogenic differentiation program in myoblasts.

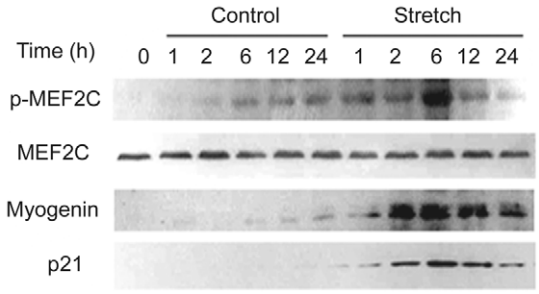

Fig. 2. Static stretch activates myogenic differentiation markers. C2C12 myoblasts were stretched for the indicated time periods as described in Fig. 1. Whole cell extracts were prepared and subjected to western blot analysis for phosphorylated MEF2C and total MEF2C (46 kDa), myogenin (37 kDa) and p21 (21 kDa). Total MEF2C also serves as a control for the sample loading quality of myogenin and p21 blotting.

TNF-α mediates stretch activation of p38 and myogenesis as an autocrine factor

Among the four protein kinases activated by stretch shown

above, AKT is also known to be critical for myogenesis (Li et al., 2000), in addition to its stimulation of hypertrophy via promoting protein synthesis (Lai et al., 2004) and inhibition of protein breakdown (Sandri et al., 2004; Stitt et al., 2004). By contrast, ERK1/2 mediates growth factor stimulation of satellite cell proliferation (Coolican et al., 1997; Jones et al., 2001; Halevy and Cantley, 2004). JNK also appears to have an influence on myogenesis; however, both inhibitory and stimulatory effects have been reported (Meriane et al., 2000; Rousse et al., 2001; Khurana and Dey, 2004). We have previously shown that serum-restriction-induced C2C12 myoblast differentiation is mediated by TNF-α as an autocrine factor (Li and Schwartz, 2001). Because TNF-α is capable of activating the above four protein kinases (O'toole et al., 2001; Li et al., 2005), we investigated the relationship between TNF-α and stretch activation of the kinases. C2C12 myoblasts were stretched in growth medium supplied with a TNF-α-neutralizing antibody. This antibody blocked p38 activation in myoblasts that were stretched for 2 hours, and attenuated p38 activation in myoblasts that were stretched for 6 hours, whereas pre-immune IgG did not affect p38 activation (Fig. 3A). By contrast, stretch activation of ERK1/2, JNK and AKT was not altered by the TNF-α neutralizing antibody. We further observed that the TNF-α-neutralizing antibody blocked stretch-induced activation of MEF2C, as well as expression of myogenin and p21 (Fig. 3B). These results indicate that stretch activation of p38 and the myogenic program is mediated by TNF-α as an autocrine factor.

To evaluate whether p38 mediates TNF-α activation of the differentiation markers, we tested the effect of SB203580, a selective p38 inhibitor, on this process. Pretreatment of myoblasts with SB203580 blocked the activation of MEF2C and the expression of myogenin and p21 in myoblasts that were stretched for 2 or 6 hours (Fig. 4). To determine whether stretch activation of the above differentiation markers results in myogenic gene expression, we analyzed the effect of 6 hours of stretch on the expression of myosin heavy chain (MHC) in C2C12 myoblasts. MHC was detected in myoblasts within 48 hours after the stretch, but not in the unstretched controls (Fig. 5A). Both the inclusion of TNF-α-neutralizing antibody in the growth medium and the pretreatment of myoblasts with SB203580 blocked stretch-induced expression of MHC (Fig. 5B). These results indicate that stretch stimulates myogenic gene expression and this action is mediated by TNF-α activation of p38.

To verify whether stretch activation of p38 and myogenesis is mediated by TNF-α receptor-mediated signaling, we compared the effect of stretch on primary myoblasts isolated from wild-type (WT) and TNF-α receptor double-knockout ($p55^{-/-}$ $p75^{-/-}$) mice. Owing to the strong capacity of primary myoblasts to differentiate spontaneously in growth medium once they are about 70% confluent, we initiated stretch at 65% confluence instead of 85% as in the case of C2C12 myoblasts, to minimize the effect of spontaneous differentiation. In preliminary studies, we observed that p38 activation by stretch is also a function of the degree of cell confluence. Therefore, we

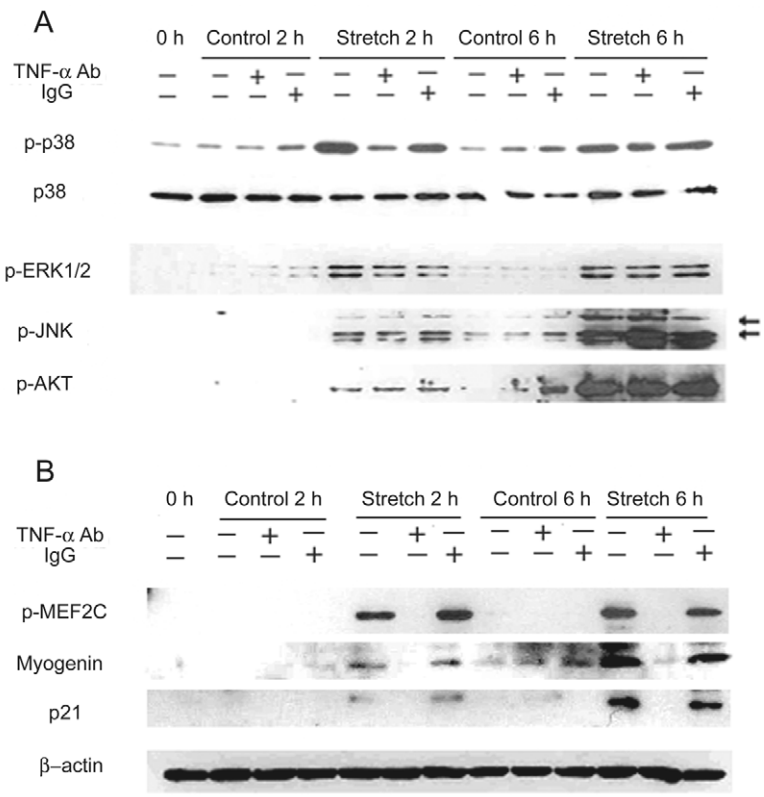

Fig. 3. Stretch activation of p38 and myogenic differentiation markers are blocked by a TNF-α-neutralizing antibody. C2C12 myoblasts were stretched for the indicated periods in growth medium that contained an antibody that neutralizes TNF-α or pre-immune IgG (5 μg/ml). Western blot analysis was performed to determine phosphorylation of p38, ERK1/2, JNK and AKT (A), as well as MEF2C phosphorylation and the expression of myogenin and p21 (B). Levels of total p38 or β-actin are shown as the loading control.

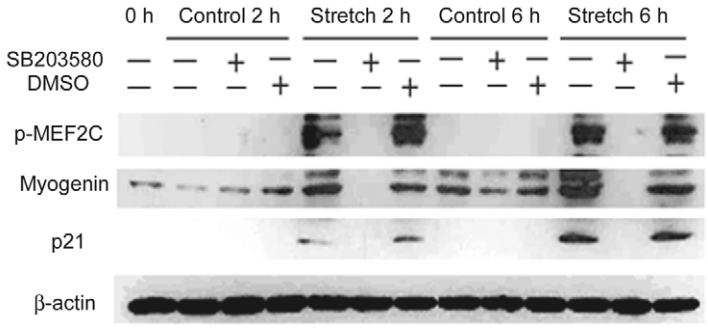

Fig. 4. Stretch activation of myogenic differentiation markers is mediated by p38. C2C12 myoblasts were pre-incubated (30 minutes) and stretched for the indicated periods in growth medium that contained the p38 inhibitor SB203580 (5 μM) or the same volume of DMSO (1 μl/ml), which was used as vehicle for SB203580. Western blot analysis was conducted to determine MEF2C phosphorylation and the expression level of myogenin and p21. β-actin was monitored as the loading control.

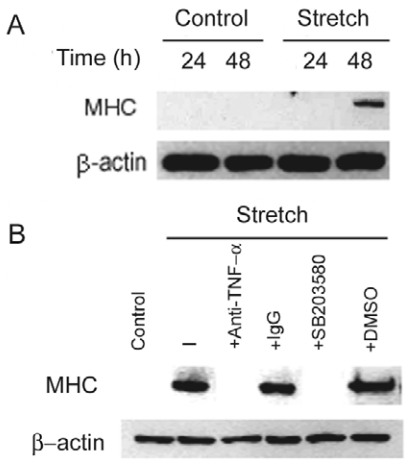

Fig. 5. Stretch stimulates MHC expression in a TNF-α- and p38-dependent manner. (A) Stretch stimulates MHC expression. C2C12 myoblasts were stretched for 6 hours and then incubated for an additional 24 or 48 hours without stretch. MHC (200 kDa) expression in myoblasts was compared with that of non-stretched controls by western blot analysis. (B) Stretch stimulation of MHC expression requires TNF-α and p38. C2C12 myoblasts were stretched for 6 hours in culture medium that contained the TNF-α neutralizing antibody (5 μg/ml), pre-immune IgG (5 μg/ml), SB-203580 (5 μM), or the DMSO vehicle (1 μl/ml), following a 30-minute pre-incubation period. Myoblasts were then incubated for an additional 48 hours without stretch. Western blot analysis was performed to evaluate MHC expression.

stretched primary myoblasts for 6 hours rather than 2 hours to compensate for the lower confluence, in order to achieve similar level of p38 activation. Whereas stretch strongly activated p38 in wild-type (WT) myoblasts, it had little effect on p38 activity in myoblasts prepared from $p55^{-/-}$ $p75^{-/-}$mice. On the other hand, stretch activated ERK1/2, JNK and AKT to essentially the same extent in WT and $p55^{-/-}$ $p75^{-/-}$ myoblasts. Stretch did not alter the total levels of the kinases (Fig. 6). In addition, we observed that stretch stimulated the expression of myogenin in WT primary myoblasts, but not in $p55^{-/-}$ $p75^{-/-}$ myoblasts (Fig. 7). These results indicate that TNF-α receptor-mediated signaling is required for stretch activation of p38 and myogenesis.

Stretch activation of p38 is mediated by TACE release of TNF-α

The blockade of stretch activation of p38 by the TNF-α neutralizing antibody added to the culture medium indicated that this response was dependent on TNF-α released into the medium by myoblasts. To assess whether TACE release of TNF-α is regulated by stretch, we measured the cleavage activity of TACE by using a peptide substrate that contained the TACE cleavage site present in pro-TNF-α in an in vitro assay, and observed a strong activation of TACE by 30 minutes of stretch (Fig. 8A). Next, we tested the effect of the selective TACE inhibitor, TAPI (Mohler et al., 1994), on the stretch activation of p38. Pretreatment of C2C12 myoblasts with TAPI eliminated the stretch activation of p38 (Fig. 8B). To further verify the critical role of TACE in stretch activation of p38, we used TACE siRNA to knock down TACE expression. Fig. 8C demonstrates that transfection of C2C12 myoblasts with TACE siRNA suppressed TACE expression; and lowered TACE expression hampered stretch activation of p38. These results indicate that TACE indeed mediates stretch activation of p38. The quick activation of TACE in response to stretch suggests that this reaction is regulated by posttranscriptional mechanisms.

To further verify whether stretch activates p38 via stimulation of TACE-mediated release of TNF-α, we transferred cell culture medium from C2C12 myoblast cultures that had been stretched for 2 hours to unstretched myoblast cultures, and

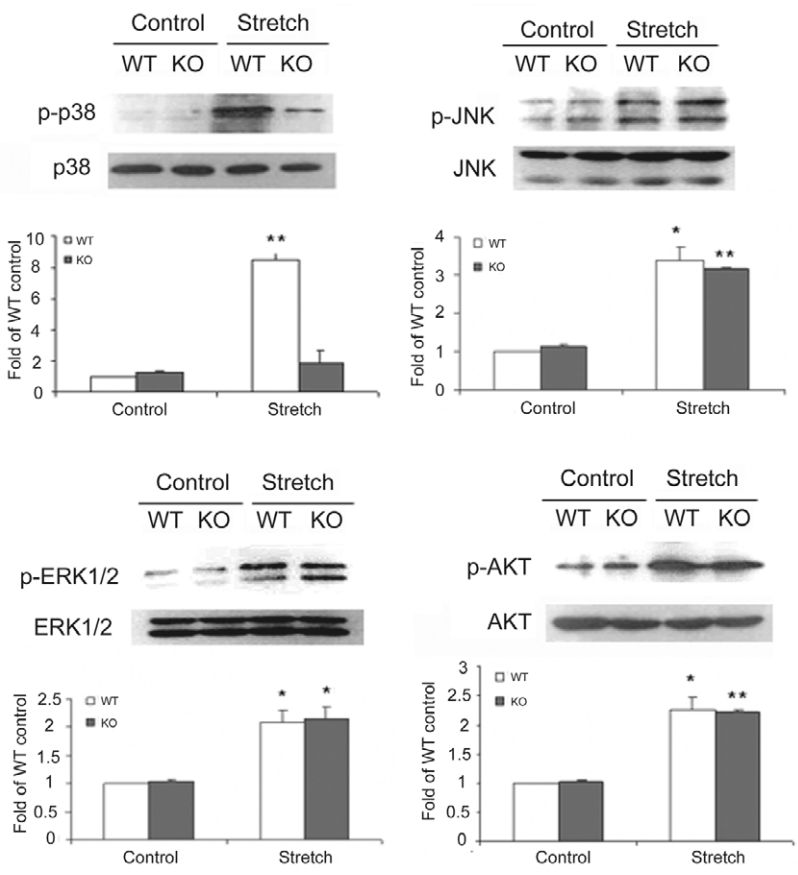

Fig. 6. Stretch activation of p38 requires TNF-α receptor-mediated signaling. Primary myoblast cultures were prepared from wild-type (WT) or $p55^{-/-}$ $p75^{-/-}$ mice (KO). Myoblasts were seeded in six-well Bioflex® plates and allowed to proliferate to ~65% confluence. Stretch was conducted in fresh growth medium for 6 hours. Time-matched, non-stretched myoblasts were used as controls. Whole cell extracts of myoblasts were prepared and analyzed by western blotting for levels of the phosphorylated form and the total levels of the kinases tested. The optical density of the phosphorylated form of each kinase detected western blotting was normalized to the respective total level and analyzed by paired *t*-test to compare control and stretched myoblasts for each type of myoblasts (n=3). *P<0.05; **P<0.01.

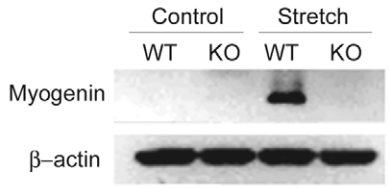

Fig. 7. Stretch activation of myogenin expression requires TNF-α receptor-mediated signaling. Primary myoblasts were stretched as described in Fig. 6. Myogenin expression was determined by western blot analysis.

observed p38 activation within 15 minutes of treatment with the stretch-conditioned medium (Fig. 9A). In addition, preincubation of the donor myoblasts with TAPI abolished the ability of stretch-conditioned medium to activate p38 (Fig. 9B). Given that TACE is responsible for the release of multiple proteins (Blobel, 1997; Black, 2002; Seals and Courtneidge, 2003), we double checked whether TNF-α released into the stretch-conditioned medium is responsible for the activation of p38 by including the TNF-α neutralizing antibody in stretch-conditioned medium, and observe a blockade of p38 activation by this medium (Fig. 9C). Furthermore, to verify whether stretch-conditioned medium activates p38 via TNF-α receptors, we used $p55^{-/-}\,p75^{-/-}$ myoblasts as the recipient of the conditioned medium. We observed that stretch-conditioned medium activated p38 in WT primary myoblasts, but not in $p55^{-/-}\,p75^{-/-}$ myoblasts (Fig. 9D). These data confirm that TACE-released TNF-α is responsible for p38 activation by stretch.

Discussion

The current study demonstrates, for the first time, that mechanical stimulation activates p38 and myogenesis in myoblasts via stimulation of TACE-mediated release of TNF-α. These findings fill a gap in our understanding of the signaling mechanism that mediates muscle adaptation provoked by mechanical stimulation.

Mechanical strain influences muscle physiology at the level of mononucleated satellite cells, which drives cell proliferation and differentiation (Darr and Schultz, 1987; Darr and Schultz, 1989). Mechanical stretch of cultured myoblasts provides an in vitro model for muscle strain that allows analysis of strain-induced intracellular signaling. Previous studies in cultured myoblasts have demonstrated that mechanical stretch stimulates myoblast proliferation (Tatsumi et al., 2001; Tatsumi et al., 2002) and differentiation (Rauch and Loughna, 2005) via specific signaling pathways. The results of these in vitro studies are consistent with the physiological effect of mechanical strain observed in whole muscle (Carson and Alway, 1996; Carson and Booth, 1998), and provided further analysis of the signaling mechanism. In general, 8-9% stretch of muscle cells, which causes cell deformation without significant injury, is considered to be a model for mechanical activity; whereas stretch of 17% or greater is considered an injury model (Vandenburgh et al., 1991; Schultz and McCormick, 1994). We chose to stretch myocytes by 8-9% as a model for mechanical activity.

The p38 MAPK is a crucial early signal for myogenic differentiation (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000; Puri et al., 2000; Cabane et al., 2003; Penn et al., 2004; de Angelis et al., 2005). Thus, the upstream signal that is responsible for myogenic activation of p38 should act at the initiation stage of myogenesis. To observe the biological events taking place at the initiation stage of myoblast differentiation, we conducted stretch experiments with undifferentiated myoblasts that were incubated in serum-rich growth medium. This approach allows us to catch the very early moments of myogenic differentiation with stretch as the

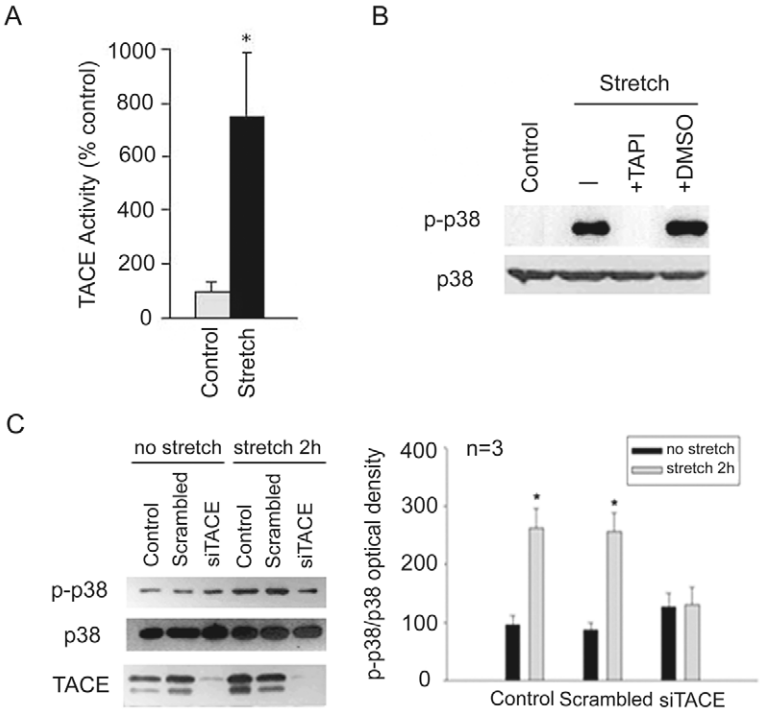

Fig. 8. TACE mediates stretch activation of p38. (A) Stretch activates TACE. C2C12 myoblasts were stretched for 30 minutes and lysed in RIPA buffer. The cleavage rate of a peptide that contained the TACE cleavage site found in pro-TNF-α by 5 μg of the cell lysate was determined as described in Materials and Methods. Data from three independent experiments were analyzed by paired *t*-test, and * indicates a difference at $P<0.01$. (B) Stretch activation of p38 is dependent on TACE activity. C2C12 myoblasts were pre-incubated for 30 minutes and stretched for 2 hours in growth medium that contained TAPI (5 μM) or an equal volume of DMSO (1 μl/ml) used as vehicle for TAPI. Myoblasts were then processed for western blot analysis of p38 activation. (C) Stretch activation of p38 is dependent on TACE expression. C2C12 myoblasts were transfected with a TACE siRNA duplex or a scrambled siRNA duplex as control. After 40 hours incubation, myoblasts were stretched for 2 hours, and p38 activation and TACE expression were evaluated by western blot analysis. Both the pro-TACE (upper band, 110 kDa) and processed TACE (lower band, 90 kDa) are shown. Results from three independent experiments were expressed in arbitrary unit and analyzed using a paired *t*-test comparing stretched samples with non-stretched samples in each group (control, scrambled siRNA and TACE siRNA). *Significant difference ($P<0.01$).

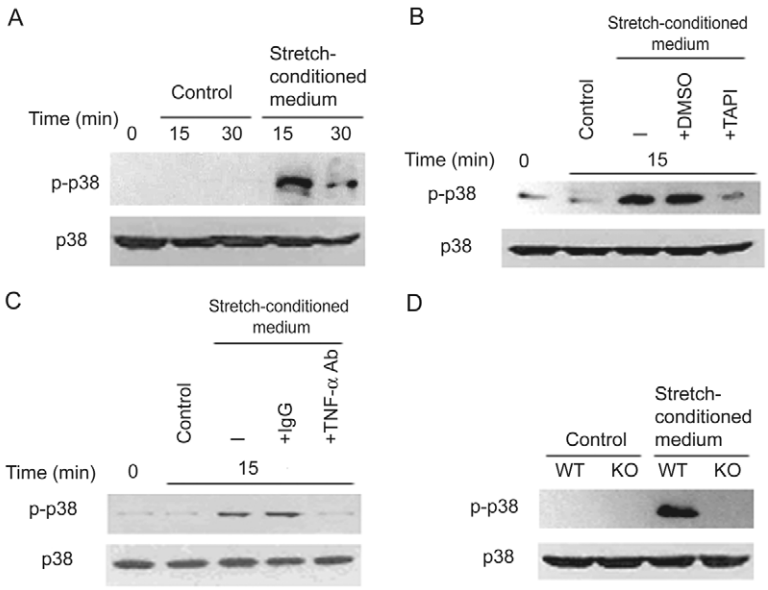

Fig. 9. Stretch-conditioned medium activates p38 via TACE-released TNF-α. (A) Stretch-conditioned medium activates p38. C2C12 myoblasts were stretched for 2 hours. The medium in the stretched myoblast culture was collected, cleared of floating cells by centrifugation, and transferred into plates that contained unstretched C2C12 myoblasts for a 15 or 30 minutes incubation. Medium transferred from unstretched myoblasts was used as control. Activation of p38 in the myoblasts was then evaluated by western blot analysis. (B) TACE activity is responsible for p38 activation by stretch-conditioned medium. C2C12 myoblasts were preincubated with or without TAPI (5 μM) or an equal volume of DMSO (1 μl/ml) and stretched for 2 hours. The media were then transferred into plates that contained unstretched C2C12 myoblast cultures and incubated for 15 minutes. Western blot analysis was carried out to evaluate p38 activation. (C) TNF-α released into stretch-conditioned medium is responsible for p38 activation. C2C12 myoblasts were stretched for 2 hours in the presence of TNF-α-neutralizing antibody or pre-immune IgG (5 μg/ml). The media were then transferred to unstretched C2C12 myoblast cultures and incubated for 15 minutes. Western blot analysis was carried out to evaluate p38 activation. (D) Stretch-conditioned medium activates p38 via TNF-α receptor-mediated signaling. Culture medium from C2C12 myoblasts that were stretched for 2 hours was transferred to WT or $p55^{-/-}$ $p75^{-/-}$ (KO) primary myoblasts and incubated for 15 minutes. Activation of p38 in the myoblasts was then evaluated by western blot analysis

only stimulus of differentiation. Under this condition, we observed that stretch stimulation of myoblasts resulted in a rapid TNF-α-dependent activation of p38, which was followed by p38-dependent differentiation. These results indicate that stretch activates myogenesis, and this action is mediated by the activation of p38 via autocrine TNF-α. The complementary use of TNF-α neutralizing antibody and TNF-α receptor double knockout myoblasts to block TNF-α signaling ensured that the TNF-α dependency of p38 activation observed was not due to the potential non-specificity of the antibody or the genetic background of the knockout mice. These observations are consistent with our previous findings in cardiotoxin-injured soleus that TNF-α-receptor-mediated signaling is critical for p38 activation and myogenesis in regenerating muscle (Chen et al., 2005) and, therefore, supports our hypothesis that TNF-α is an autocrine mediator critical for the activation of p38 by various stimuli of muscle adaptation.

We observed that stretch activates ERK, JNK and AKT in a TNF-α-independent manner. Although TNF-α has the capacity to activate ERK, JNK and AKT (O'toole et al., 2001; Li et al., 2005), these kinases are also activated by the growth factors released by myoblasts (e.g. IGF-I, HGF or FGF) in response to stretch (Halevy and Cantley, 2004; Tidball, 2005). However, p38 is known not to be activated by growth factors. The observations that the peak activation of these three kinases and the onset of JNK activation took a longer time to reach than that of p38 indicate that p38 is more responsive to mechanical stimulation. It is noteworthy that when TNF-α/p38 signal transduction is inhibited, stretch-induced myogenesis is blocked even though AKT, another essential mediator of myogenesis (Li et al., 2000), is activated normally, which is consistent with previous observations in different settings where AKT phosphorylation alone is not sufficient to induce myogenesis (Wu et al., 2000; Tiffin et al., 2004).

The dependence of stretch activation of p38 on TNF-α and its receptors suggests that mechanical activation of p38 is mediated by TNF-α released into the medium from myoblasts, a process controlled by TACE (Blobel, 1997; Black, 2002). We show for the first time that TACE is dramatically activated by mechanical stimulation. Although another member of the ADAM family, ADAM12, has been shown to be involved in myogenesis (Lafuste et al., 2005), TACE has never been shown before to play a role in myogenesis, much less in mechanotransduction. The observations that stretch rapidly activates TACE, and TACE inhibition or TACE gene knockdown blocked stretch activation of p38 indicate that stretch activates p38 via stimulation of TACE-mediated release of TNF-α. Unfortunately, because of the very low basal level release of TNF-α by C2C12 myoblasts in growth medium, and the larger volume of medium required by the stretch apparatus, which further diluted TNF-α content, we were not able to accurately measure stretch-induced change in the level of TNF-α by using ELISA with concentrated medium. However, the observations that stretch-conditioned medium activates p38 in a TACE- and TNF-α receptor-dependent manner confirms that TACE-mediated release of TNF-α is responsible for stretch activation of p38. The quick increase of TACE activity in response to stretch within 30 minutes is in line with the activation of p38 within 1 hour, and hints that stretch activates TACE via a posttranscriptional mechanism. The present study provides the first evidence that TACE plays a significant role in myogenesis as the mediator of the mechanotransduction that activates p38. These findings also provide an explanation, at the cellular level, for previous observations that exercise stimulates TNF-α release from skeletal muscle (Nieman et al., 2005), which results in elevated circulating TNF-α levels (Ostrowski et al., 1999; Starkie et al., 2001).

There have been reports that describe an inhibitory effect of exogenously added TNF-α on myogenesis at late stages of myoblast differentiation (Guttridge et al., 2000; Langen et al., 2001), which appears contradictory to our findings in this study. The discrepancy is attributable to the differences in experimental

conditions. We evaluated the effect of endogenous TNF-α on myogenesis, rather than the effect of exogenously added TNF-α. We focused on the effect of TNF-α as an autocrine factor on the early stages of myoblast differentiation when the early differentiation signal p38 is activated. We have shown previously that the effect of TNF-α on myogenesis is dependent on differentiation stage. It simulates MHC expression during the early stages of differentiation, but attenuates MHC accumulation at late stages of differentiation (Li and Schwartz, 2001). Similarly, p38 has temporal divergence in its effect on myogenesis. Although p38 activation is required for the early stages of myogenesis, at late stages of myogenesis p38-mediated phosphorylation inhibits myogenesis (Weston et al., 2003; Suelves et al., 2004). The above referenced studies, which observed an inhibitory effect of exogenous TNF-α on myoblast differentiation, only looked at late stages of myogenesis and therefore the effect of TNF-α on the initiation of myogenesis was not observed. Another factor that contributes to the discrepancy is that TNF-α has bimodal effects on myogenesis, which depends on concentration. The concentration of exogenously added TNF-α used in the above referenced studies ranged from 10 to 20 ng/ml in culture medium, at least a thousand-fold greater than the physiological concentration of TNF-α observed in normal serum. We have observed that the addition of 0.05 ng/ml of recombinant TNF-α to differentiation medium, which simulates the raised TNF-α level in injured muscle, stimulates myogenesis in myoblasts. However, at 0.5 or 5 ng/ml [levels seen in inflammatory diseases (Vreugdenhil et al., 1992; Nakashima et al., 1995)] TNF-α inhibits myogenesis (Chen et al., 2007). As a pleiotropic cytokine, TNF-α is known to exert divergent actions depending on concentration. Opposing effects of TNF-α at different concentrations have been observed previously in skeletal (Alvarez et al., 2001) and cardiac muscle (Mann, 2003). The 10-20 ng/ml of TNF-α used in the above referenced studies exceed even the pathological levels seen in severe inflammatory diseases (Vreugdenhil et al., 1992; Nakashima et al., 1995). Effect of TNF-α at these levels cannot be considered physiological.

In non-muscle cells, TNF-α activation of p38 involves interactions of TNF receptor-associated factor 2 (TRAF2) with such proteins as germinal center kinase (GCK) and receptor interacting protein (RIP) (Yuasa et al., 1998), leading to the activation of MKK3/MKK6, which directly phosphorylates p38 (Brancho et al., 2003). The detailed mechanism of TNF-α activation of p38 in muscle cells is a very interesting topic, and we intend to address this issue in our future study.

Taken together, the current study reveals that autocrine TNF-α is a key mediator of p38 activation in myoblasts subjected to mechanical stimulation. Furthermore, TACE is the rate-limiting controller of the availability of TNF-α; thereby, bridging a gap in our understanding of mechanotransduction in skeletal muscle. These findings, along with our previous observation that TNF-α mediates p38 activation in skeletal muscle cells in response to muscle injury (Chen et al., 2005), delineate a unique and critical role for TNF-α in muscle adaptation in response to diverse stimuli. However, our data indicate that caution must be taken with regards to the clinical use of anti-TNF-α reagents to combat various inflammatory diseases. For example, the long-term blockade of TNF-α signaling may have a negative impact on muscle maintenance and adaptation.

Materials and Methods

Myogenic cell culture

Murine C2C12 myoblasts [American Type Culture Collection (ATCC), Rockville, MD] were cultured, as previously described (Li et al., 1998). Cells of passage 2-10 were used. Primary myoblast cultures were prepared from hindlimb muscles of 2- to 4-day-old TNF-α receptor double-knockout ($p55^{-/-}$ $p75^{-/-}$) mice (B6;129S-*Tnfrsf1a*^{tm1Imx} *Tnfrsf1b*^{tm1Imx}) and wild-type mice (B6;129SF2/J) purchased from Jackson Laboratory (Bar Harbor, ME), as previously described (Li, 2003), with a minor modification, in that myoblasts were replated twice instead of preplating of myoblasts. Protocols for animal use were approved in advance by the Institutional Animal Care and Use Committee at Baylor College of Medicine. Immunostaining of primary cultures with an antibody against desmin (D3; Developmental Studies Hybridoma Bank at the University of Iowa) was employed to confirm that at least 90% of the cells in the primary cultures were myoblasts.

Static stretch of myoblasts

A static stretch device designed by Rauch and Loughna (Rauch and Loughna, 2005) was adopted to stretch myoblasts in six-well Bioflex® plates (Flexcell International, Hillsborough, NC) that have an elastic silicon membrane bottom coated with collagen I. This stretch device enables a constant ~8–9% global equibiaxial strain of cells grown on the silicon membrane. Myoblasts were plated at approximately 1×10^5 cells/well, and allowed to proliferate to approximately 85% (C2C12) or 65% (primary myoblasts) confluence in growth medium (DMEM supplemented with 10% fetal bovine serum and gentamicin for C2C12, or 20% fetal bovine serum for primary myoblasts). Stretch was then initiated in fresh growth medium. Parallel sets of non-stretched myoblasts cultured in fresh growth medium were used as controls. Stretch was maintained for a designated period of time under normal cell culture conditions (37°C with 5% CO_2 in a humidified incubator). A TNF-α neutralizing antibody (R&D Systems, Minneapolis, MN) or goat pre-immune IgG (Sigma-Aldrich, St Louis, MO) was included in the growth medium during stretch, as indicated. SB203580 (Calbiochem/EMD Biosciences, San Diego, CA) or TAPI (Calbiochem) was dissolved in DMSO and added directly into the medium 30 minutes before stretch, and remained in the medium during stretch as indicated. Control samples were treated with the same volume of vehicle DMSO. After the completion of stretch, myoblasts were collected by trypsinization for western blotting or were lysed in RIPA buffer for TACE activity assay.

TACE siRNA transfection

A siRNA duplex (the sense sequence: 5′-GAG AAG CUU GAU UCU UUG C dTdT-3′) derived from the mouse TACE mRNA was prepared by Dharmacon (Lafayette, CO). C2C12 myoblasts, at approximately 50% confluence, were transfected with the siRNAs at 50 nM each by using the siPORT Lipid transfection agent (Dharmacon) or the Lipofectamine 2000 reagent (Invitrogen). TACE protein levels in cells 40 hours after transfection were examined by western blot analysis.

Western blot analysis

Whole cell extracts and western blot analysis were performed, as previously described (Li and Schwartz, 2001). Antibodies for pan and phosphorylated p38 (T181/Y182), JNK (T183/Y185), ERK1/2 (T202/Y204) and AKT (S473) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for pan MEF2C or phosphorylated MEF2C (S387), and p21 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for myogenin (F5D) and MHC (MF20) were purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Antibody against TACE was purchased from QED Bioscience. Horseradish peroxidase-conjugated secondary antibodies were used to detect primary antibodies. Antibodies were visualized by the enhanced chemiluminescence method (Pierce Biotechnology, Rockford, IL), which detects horseradish peroxidase-conjugated secondary antibodies. The detected bands on X-ray film were quantified using ImageQuant densitometry software (Molecular Dynamics/Amersham Biosciences/GE Healthcare, Piscataway, NJ). Protein concentrations of cell extracts were determined with the Bio-Rad protein assay. All western blot experiments were repeated at least once, and representative blots are shown.

TACE activity assay

TACE activity was determined as the rate of the cleavage a 12-residue peptide which spans the Ala-76 to Val-77 residues in pro-TNF-α (ADAM17 Substrate IV). Lysates of myoblasts in the RIPA buffer (5 μg of protein), were incubated in an assay buffer (pH 7.4) that contained 50 mM Tris, 25 mM NaCl, 4% glycerol, and the fluorophoric peptide substrate Abz-LAQAVRSSSR-Dpa (Calbiochem, San Diego, CA) at a final concentration of 10 μM, for 20 minutes in the dark at 37°C in a black 96-well plate. The fluorescence of the cleavage product was measured in a fluorescence microplate reader (SpectraMAX Gemini) with excitation at 320 nm and emission at 420 nm.

Statistical analysis

All data are expressed as mean ± s.e.m. Statistical analysis was performed using ANOVA or a paired *t*-test, which compared time-matched controls with stretched samples. A P value <0.05 was considered to be statistically significant.

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