Mol. Cells *30*, 513-518, December 31, 2010
DOI/10.1007/s10059-010-0147-3

Molecules and Cells

Mechanical Stretch Activates Signaling Events for Protein Translation Initiation and Elongation in C2C12 Myoblasts

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It has been proposed that mechanically induced tension is the critical factor in the induction of muscle hypertrophy. However, the molecular mechanisms involved in this process are still under investigation. In the present study, the effect of mechanical stretch on intracellular signaling for protein translation initiation and elongation was studied in C2C12 myoblasts. Cells were grown on a silicone elastomer chamber and subjected to 30-min of 5 or 15% constant static or cyclic (60 cycles/min) uniaxial stretch. Western blot analyses revealed that p70 S6 kinase (p70S6K) and eukaryotic elongation factor 2 (eEF2), which are the markers for translation initiation and peptide chain elongation, respectively, were activated by both static and cyclic stretch. The magnitude of activation was greater in response to the 15% cyclic stretch. Cyclic stretch also increased the phosphorylation of MAP kinases (p38 MAPK, ERK1/2 and JNK). However, the pharmacological inhibition of MAP kinases did not block the stretch-induced activation of p70S6K and eEF2. An inhibitor of the mammalian target of rapamycin (mTOR) blocked the stretch-induced phosphorylation of p70S6K but did not affect the eEF2 activation. A broad-range tyrosine kinase inhibitor, genistein, blocked the stretch-induced activation of p70S6K and eEF2, whereas Src tyrosine kinase and Janus kinase (JAK) inhibitors did not. These results suggest that the stretch-induced activation of protein translation initiation and elongation in mouse myoblast cell lines is mediated by tyrosine kinase(s), except for Src kinase or JAK.

INTRODUCTION

Skeletal muscle is a highly plastic tissue. In response to repeated bouts of resistance exercise and overload, skeletal muscle is liable to undergo hypertrophy (Matoba and Gollnick, 1984; Timson, 1990). It has been proposed that mechanical stimuli play a major role in the regulation of skeletal muscle mass (Goldberg et al., 1975). In fact, passive stretch of muscle leads to an increase in muscle tension and promotes skeletal muscle growth by stimulating protein synthesis (Barnett et al., 1980).

Recently, the mammalian target of the rapamycin (mTOR) pathway has been reported to be of importance in the control of skeletal muscle mass in response to mechanical stimulation (Hornberger and Chien, 2006; Hornberger et al., 2006; 2007; Zanchi and Lancha, 2008). Activation of mTOR stimulates downstream targets for protein synthesis, such as p70 S6 kinase (p70S6K) and eukaryotic elongation factor 2 (eEF2). Phosphorylation of p70S6K at threonine 389 residue, by mTOR kinase, activates the ribosomal protein S6 and upregulates a subclass of mRNA encoding the translation apparatus (Dennis et al., 1996; Kimball et al., 1999). eEF2 is another potentially important signaling protein in the control of translation, which mediates the translocation step of elongation (Redpath et al., 1996). eEF2 is activated by dephosphorylation at threonine 56 in response to insulin or serum, and this correlates with the activation of elongation (Redpath et al., 1996; Wang et al., 2000).

There are potential candidates for the upstream signaling pathway of mTOR in response to mechanical stimulation. Phosphatidylinositol-3 kinase (PI3K)/Akt (Bodine et al., 2001), phosphatidic acid generated by phospholipase D (PLD) (Hornberger et al., 2006), integrin/FAK (focal adhesion kinase) (Marin et al., 2008), and mitogen-activated protein kinases (MAPKs) (Martineau and Gardiner, 2001) have been reported to be involved in mechanotransduction. However, the mechanism underlying the intracellular signaling upstream of the mTOR response to mechanical stimulation remains to be clarified. To investigate a wide variety of biological regulation, a cell culture model would be an invaluable tool. In the present study, mechanical stimulation was applied to myoblasts cultured in a silicon elastomer chamber using a stretching apparatus. Another advantage to using the cell culture model instead of intact tissue *in vivo* is the ability to apply various pharmacological inhibitors to block specific signaling pathways.

The aim of the present study was to investigate the effect of

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Received June 1, 2010; revised September 1, 2010; accepted September 2, 2010; published online October 14, 2010

Keywords: C2C12 myoblasts, eukaryotic elongation factor 2, mechanical stretch, p70 S6 kinase, tyrosine phosphorylation

mechanical stretch on intracellular signaling for protein translation initiation and elongation in C2C12 myoblasts. Here we present the evidence that mechanical stretch activates signaling events for protein translation initiation and elongation, while tyrosine phosphorylation is an indispensable process in this regulation.

MATERIALS AND METHODS

Materials

Culture medium, fetal bovine albumin (FBS), and antibiotics were purchased from Sigma-Aldrich (USA). Pharmacological inhibitors were purchased from Sigma-Aldrich, Cell Signaling Technology (USA) or Santa Cruz Biotechnology (USA) and dissolved in dimethyl sulfoxide (DMSO), methanol or phosphate-buffered saline (PBS), as was stated in the figure legends. Antibodies against phospho-p70S6K, phospho-eEF2, total-eEF2, phospho-extracellular-regulated kinase 1/2 (ERK1/2), phospho-p38 MAPK and phospho-c-Jun N-terminal kinase (JNK) were obtained from Cell Signaling Technology. Antibody against total-p70S6K was obtained from Santa Cruz Biotechnology.

C2C12 culture

C2C12 cells were plated on a fibronectin-coated silicone elastomer chamber (32 mm long, 32 mm wide, and 10 mm deep) in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin sulfate in a 5% CO_2-humidified chamber at 37°C. Cells were grown to approximately 90% confluence and used for experiments. Cells were subjected to 30-min of uniaxial mechanical static or cyclic stretch using a stretch apparatus (ST-150; Strex, Japan). The chamber was uniaxially stretched at 5 or 15% of the initial length. Cyclic stretch was accomplished by subjecting cells to the indicated length at 60 stretch-relaxation cycles/min. Static stretch was induced at a constant indicated length without relaxation. In the experiments using the inhibitors, the cells were pre-incubated with the inhibitor for an indicated time prior to stretch.

SDS-PAGE and immunoblotting

After stretching for 30 min, cells were rinsed with cold PBS and lysed in RIPA lysis buffer (Santa Cruz Biotechnology). Control cells were equally treated except that they were not stretched. Equal amounts of protein were subjected to SDS-PAGE and immunoblotting. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Nonspecific binding to the membrane was blocked with Tris-buffered saline (pH 7.4), containing 0.05% (v/v) Tween-20 and 5% bovine serum albumin (BSA) or 5% skim milk. Following an overnight incubation with indicated primary antibody, the membranes were washed and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as appropriate. Immunoreactive protein bands were visualized by ECL-plus (GE Healthcare UK Limited, UK) with an ECL mini camera (GE Healthcare UK Limited). The band intensity was quantified by a computer analysis package (NIH ImageJ).

Statistics

Data were presented as means ± SD. Statistical analysis for multiple comparisons was performed using one-way or two-way analysis of variance (ANOVA) followed by the Scheffe's post hoc test. Differences were considered statistically significant at $p < 0.05$.

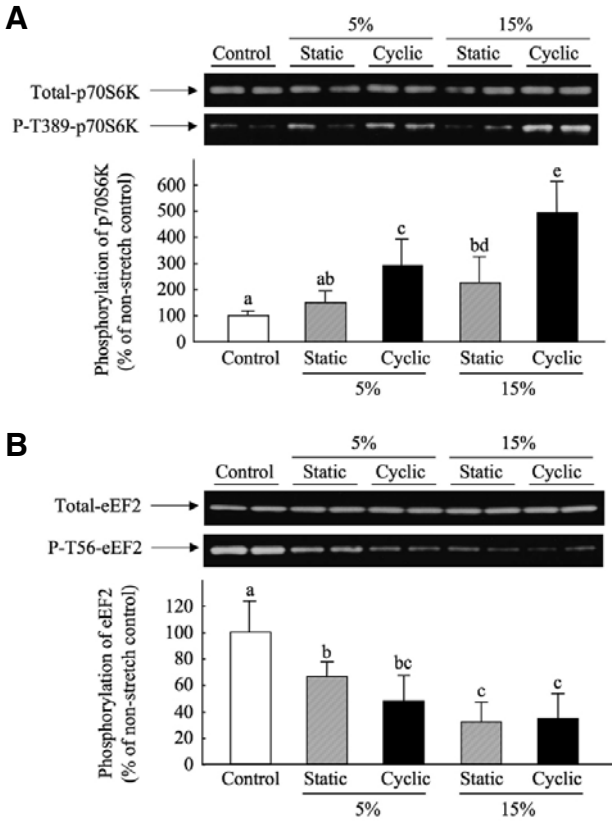

Fig. 1. Effects of static and cyclic stretch on the phosphorylation of p70 S6 kinase (p70S6K) (A) and the dephosphorylation of eukaryotic elongation factor 2 (eEF2) (B). Cells were subjected to 30-min of uniaxial mechanical static or cyclic stretch at 5 or 15% of the initial length. Photographs of representative Western blots of total-p70S6K and phospho (P)-T389-p70S6K are shown at the top (A). Photographs of representative Western blots of total-eEF2 and phospho (P)-T56-eEF2 are shown at the top (B). Data for P-T389-p70S6K (A) and P-T56-eEF2 (B) are expressed as the percentage relative to the non-stretched control cells (100%). Values are means ± SD from 4-6 independent experiments. Values with different letters are significantly different ($P < 0.05$).

RESULTS

Effects of static and cyclic stretch on phosphorylation of p70S6K and eEF2

Phosphorylation of p70S6K at the threonine 389 residue results in the maximal kinase activity (Dufner and Thomas, 1999). Western blot analysis revealed that the total amount of p70S6K protein was identical among the groups (Fig. 1A). Static and cyclic stretch significantly increased p70S6K (T389) phosphorylation, but the increase in the 5% static stretch group did not reach significance. The increment of p70S6K phosphorylation was greater in the cyclic stretch than in the static stretch group. The greatest increase was seen in the 15% cyclic stretch group (~5-fold vs. nonstretched control).

It has been shown that dephosphorylation of eEF2 at threonine 56 activates the translocation step of elongation (Redpath et al., 1993). Although the amount of total eEF2 was not different among the groups, both static and cyclic stretch significantly decreased eEF2 (T56) phosphorylation (Fig. 1B). Dephosphorylation of eEF2 was induced most markedly following static

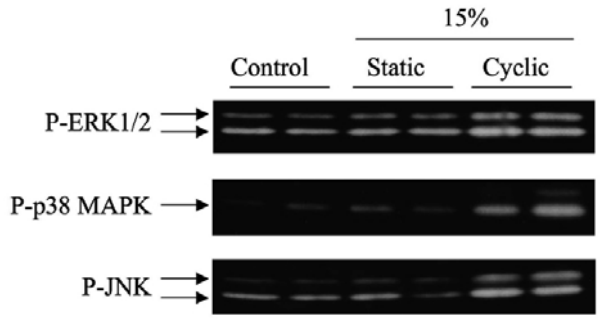

Fig. 2. Effects of static and cyclic stretch on the phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun N-terminal kinase (JNK). Cells were subjected to 30-min of uniaxial mechanical static or cyclic stretch at 15% of the initial length. Representative patterns of phosphorylated (P)-ERK1/2, p38 MAPK and JNK, determined by immunoblotting, are shown.

(32% vs. nonstretched control) and cyclic (35% vs. non-stretched control) stretch at 15% of elongation.

Effects of static and cyclic stretch on phosphorylation of MAPKs

The MAPKs are serine/threonine kinases that become activated upon tyrosine/threonine phosphorylation (Robinson and Cobb, 1997). The MAPK superfamily is a widely distributed group of enzymes that can be divided into several subfamilies. The three best-characterized MAPK cascades are: (i) ERKs, (ii) JNKs, and (iii) p38 MAPK cascade. The latter two are stress-activated protein kinases (SAPKs) (Robinson and Cobb, 1997). In the present study, the cyclic stretch, but not the static stretch, increased the phosphorylation of all three MAPKs (Fig. 2).

Effects of MAPK inhibitors on the stretch-induced activation of p70S6k and eEF2

The roles of the stretch-activated signaling pathways on the activation of p70S6K and eEF2 were evaluated using pharmacological inhibitors for MAPKs. For this purpose, inhibitors for ERK1/2, p38 MAPK, and JNK (PD98059, SB203580, and SP600125, respectively) were administered to the cells prior to the stretch. As shown in Figs. 3A, 3B, and 3C, none of the inhibitors for MAPKs blocked the stretch-induced activation of p70S6K and eEF2.

Effects of mTOR inhibitor, rapamycin, phospholipase D inhibitor, 1-butanol, and phosphatidylinositol-3 kinase inhibitor, LY294002, on the stretch-induced activation of p70S6k and eEF2

It is well known that mTOR catalyzes the phosphorylation of p70S6K (Pearson et al., 1995; Weng et al., 1998). The stretch-induced activation of p70S6K was completely diminished by pre-treatment with an mTOR inhibitor, rapamycin (Fig. 4A). Pre-treatment with rapamycin also inhibited the basal phosphorylation of p70S6K, suggesting that the mTOR pathway is essential for the basal and stretch-induced activation of p70S6K. On the other hand, dephosphorylation of eEF2 was not inhibited by rapamycin, suggesting that the mTOR pathway is not involved in the stretch-induced activation of eEF2 (Fig. 4A).

The lipid second messenger phosphatidic acid has been found to mediate the mitogenic activation of mTOR signaling (Fang et al., 2001). Phospholipase D (PLD) is one of the enzymes responsible for the production of phosphatidic acid and

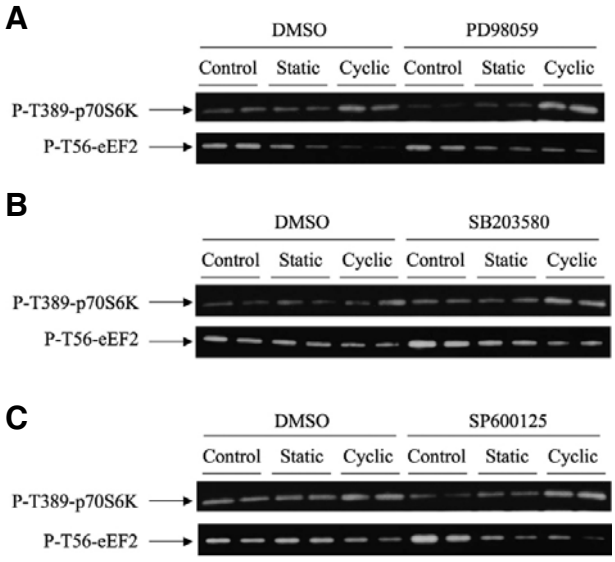

Fig. 3. Effects of MAPK inhibitors on the stretch-induced activation of p70S6K and eEF2. Cells were pre-treated with ERK1/2 inhibitor, PD98059 (50 μM in dimethyl sulfoxide, DMSO) (A), p38 MAPK inhibitor, SB203580 (10 μM in DMSO) (B), or JNK inhibitor, SP600125 (10 μM in DMSO) (C), for 30 min prior to the static or cyclic stretch. Cells were subjected to 30-min of uniaxial mechanical static or cyclic stretch at 15% of the initial length. Photographs of representative Western blots of phospho (P)-T389-p70S6K and phospho (P)-T56-eEF2 are shown.

is an upstream regulator in the mTOR pathway (Fang et al., 2003). In the present study, pre-treatment with a PLD inhibitor, 1-Butanol, partially blocked the stretch-induced activation of p70S6K (Fig. 4B). But 1-Butanol did not inhibit the stretch-induced activation of eEF2.

Phosphatidylinositol-3 kinase (PI3K) is another candidate for mTOR regulator (Bodine et al., 2001). The addition of PI3K inhibitor, LY294002, partially blocked the stretch-induced activation of p70S6K, but did not affect eEF2 activation (Fig. 4C).

Effects of genistein, PP2 and JAK inhibitor on the stretch-induced activation of p70S6k and eEF2

To investigate the involvement of tyrosine phosphorylation in the stretch-induced activation of p70S6K and eEF2, we studied the effects of tyrosine kinase inhibitors. Pre-treatment of the cells with genistein, a broad-range tyrosine kinase inhibitor, at a concentration of 50 μM did not affect the stretch-induced activation of p70S6K (Fig. 5A) or eEF2 (Fig. 5B). On the other hand, at 250 μM, genistein markedly blocked the stretch-induced activation of p70S6K and eEF2. Basal levels of p70S6K and eEF2 were also inhibited by pre-treatment with genistein. Pre-treatment of the cells with a more-specific tyrosine kinase inhibitor, PP2 (Src tyrosine kinase inhibitor) or Janus kinase (JAK) inhibitor (JAK inhibitor I, Santa Cruz Biotechnology), did not affect the stretch-induced activation of p70S6K (Fig. 6A) or eEF2 (Fig. 6B).

DISCUSSION

The effects of mechanical stretch on the activation of protein translation initiation and elongation were investigated in a mouse myoblast cell line. The data obtained in the present study showed that the mechanical stretch-induced activation of

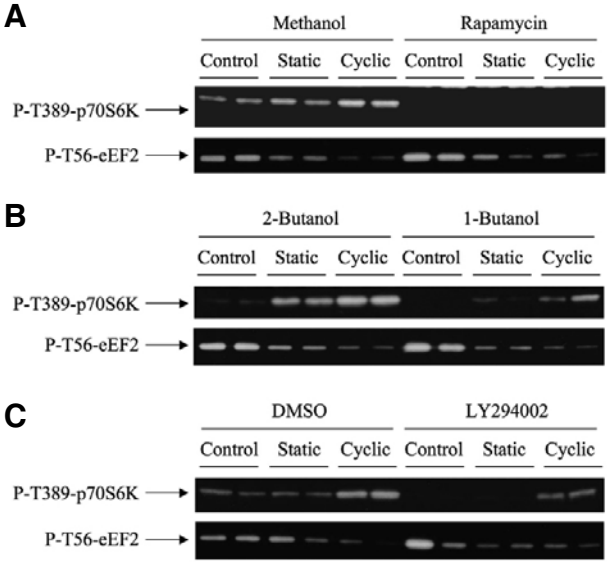

Fig. 4. Effects of mammalian target of rapamycin (mTOR) kinase (A), phospho lipase D (PLD) (B), and phosphatidylinositol-3 kinase (PI3K) (C) inhibitors on the stretch-induced activation of p70S6K and eEF2. Cells were pre-treated with mTOR kinase inhibitor, rapamycin (100 nM in methanol), PLD inhibitor, 1-Butanol (1% v/v), or PI3K inhibitor, LY294002 (10 μM in DMSO), for 30 min prior to the static or cyclic stretch. 2-Butanol dose not inhibit PLD and was used for control. Cells were subjected to 30-min of uniaxial mechanical static or cyclic stretch at 15% of the initial length. Photographs of representative Western blots of phospho (P)-T389-p70S6K and phospho (P)-T56-eEF2 are shown.

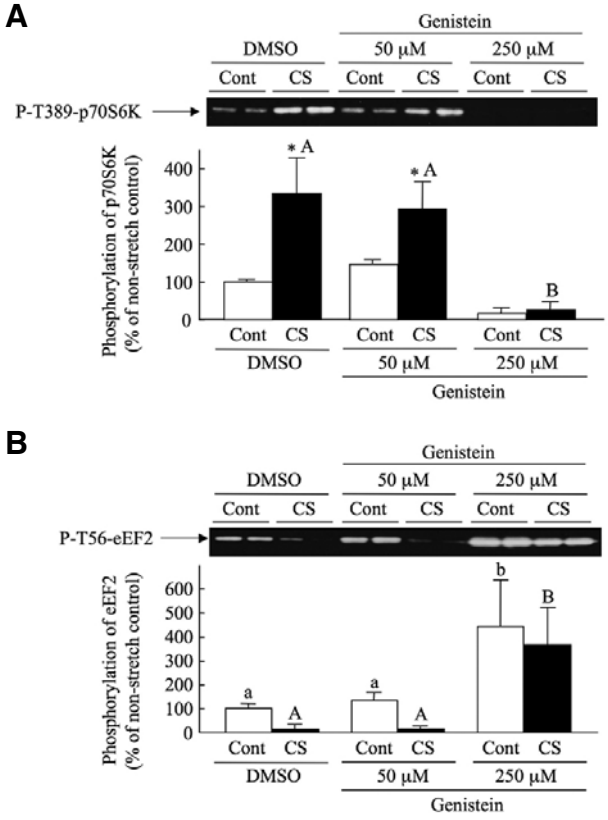

Fig. 5. Effects of a broad-range tyrosine kinase inhibitor on the stretch-induced activation of p70S6K (A) and eEF2 (B). Cells were pre-treated with tyrosine kinase inhibitor, genistein (50 or 250 μM in DMSO), for 45 min prior to the cyclic stretch. Cells were subjected to 30-min of uniaxial mechanical cyclic stretch at 15% of the initial length. Photographs of representative Western blots of phospho (P)-T389-p70S6K (A) and phospho (P)-T56-eEF2 (B) are shown at the top. Data for P-T389-p70S6K (A) and P-T56-eEF2 (B) are expressed as the percentage relative to the non-stretched control cells (100%). Cont; non-stretch control, CS; cyclic stretch. Values are means ± SD from 4-6 independent experiments. *, Significantly different from Cont in the same groups ($P < 0.05$). Values with different letters are significantly different ($P < 0.05$).

p70S6K and eEF2 was transmitted through different pathways, but tyrosine phosphorylation was an essential process in this regulation.

To examine the effects of mechanical stretch, we subjected cells to two different types of stretch, constant static and cyclic, at two different intensities (5 or 15% elongation above the initial length). Phosphorylation of p70S6K and dephosphorylation of eEF2 were highest in the 15% cyclic stretch group (Figs. 1A and 1B). Zheng et al. (2008) reported that the cyclic stretch induced a more robust vascular tube formation and branching than the static stretch in endothelial cells. Taken together, the previous and present results confirmed that the cyclic stretch is more effective than the static stretch for the induction of cellular responses. Alexander et al. (2004) reported that the cyclic stretch-induced arachidonic acid release was intensity-dependent (0% to 15% stretch) in rabbit proximal tubule cells. Our results showed the same trend, where the activation of p70S6K and eEF2 was greater in the 15% stretch group than in the 5% group.

To investigate the pathways involved in the stretch-induced activation of protein translation initiation and elongation, first the effect of stretch on MAPK phosphorylation was determined. Cyclic stretch increased the phosphorylation of three MAPKs: ERK1/2, p38 MAPK, and JNK (Fig. 2). However, the inhibition of MAPKs by pre-treatment of the cells with specific inhibitors did not block the stretch-induced activation of p70S6K or eEF2 (Figs. 3A, 3B, and 3C), suggesting that MAPKs may not be involved in this process.

It has been reported that eEF2 activation was regulated by eEF2 kinase, and eEF2 kinase activity was believed to be under the control of p70S6K (Wang et al., 2001). Interestingly, the stretch-induced dephosphorylation of eEF2 was not diminished, although pre-treatment with mTOR inhibitor rapamycin completely blocked the basal and stretch-induced phosphorylation of p70S6K (Fig. 4A). These results suggest that the stretch-induced activation of p70S6K is mTOR-dependent, but the activation of eEF2 is not transmitted through the mTOR/p70S6K pathway.

Another candidate for the upstream regulator of mTOR is phosphatidic acid generated by PLD and a pathway involving PI3K/Akt. Pre-treatment of the cells with PLD and PI3K inhibitors attenuated the stretch-induced activation of p70S6K (Figs. 4B and 4C). Our results confirmed a previous report that inhibition of PLD blocked the mechanically induced activation of mTOR/p70S6K signaling (Hornberger et al., 2006). However, the stretch-induced phosphorylation of p70S6K was not blocked completely, although we added the same concentration of PLD inhibitor, 1-Butanol (1%), as in their study (Hornberger et al., 2006). Differences in tissues (incubated muscle vs.

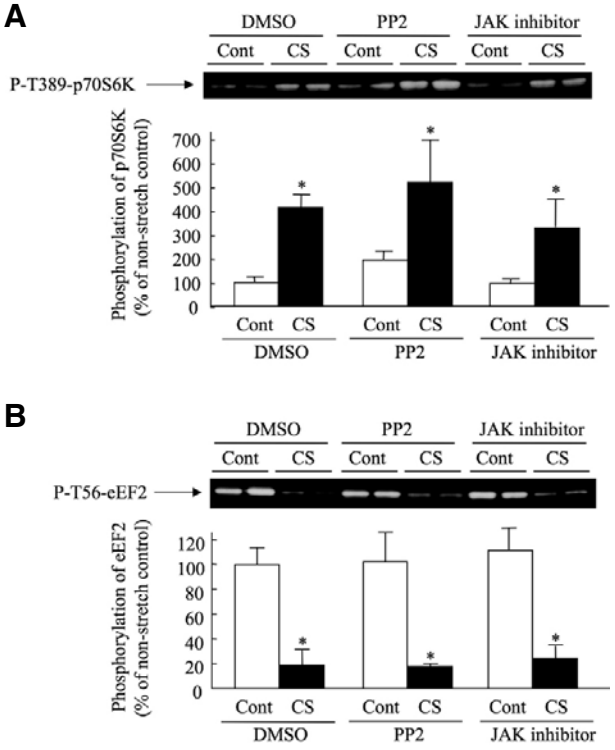

Fig. 6. Effect of Src family tyrosine kinase inhibitor, PP2, and Janus kinase (JAK) inhibitor on the stretch-induced activation of p70S6K (A) and eEF2 (B). Cells were treated with DMSO, PP2 (50 μM), or JAK inhibitor (20 μM) for 45 min prior to stretch. Cells were subjected to 30-min of uniaxial mechanical cyclic stretch at 15% of the initial length. Photographs of representative Western blots of phospho (P)-T389-p70S6K (A) and phospho (P)-T56-eEF2 (B) are shown at the top. Data for P-T389-p70S6K (A) and P-T56-eEF2 (B) are expressed as the percentage relative to the non-stretched control cells (100%). Cont; non-stretch control, CS; cyclic stretch. Values are means ± SD from 4-6 independent experiments. *, Significantly different from Cont in the same groups ($P < 0.05$).

cultured cells) and stretch protocols (90 min vs. 30 min) may be the reasons for these divergent results. On the other hand, results from the same laboratory have demonstrated that mechanical stimuli can activate the mTOR pathway, which is not directly related to PI3K (Hornberger and Chien, 2006). In the present study, the stretch-induced phosphorylation of p70S6K was diminished by ~50%, and remained partially even in the presence of the PI3K inhibitor (Fig. 4C). We stretched the cells in the culture medium containing 10% FBS, suggesting that a PI3K inhibitor, LY294002, may have blocked the growth-factor-dependent basal activation of p70S6K. In fact, Wu et al. (2004) reported that insulin-induced phosphorylation of p70S6K was completely blocked by LY294002. Further studies are needed to elucidate the involvement of PLD and PI3K in the stretch-induced activation of p70S6K. In contrast to the attenuation of the stretch-induced phosphorylation of p70S6K, dephosphorylation of eEF2 was not inhibited by pre-treatment with rapamycin (Fig. 4A), 1-Butanol (Fig. 4B), or LY294002 (Fig. 4C). Thus, mechanical stretch can activate eEF2 independent of the mTOR/p70S6K, PLD, and PI3K pathways.

The involvement of tyrosine kinases, especially focal adhesion kinase (FAK)/Src pathway (Crosara-Alberto et al., 2009; Marin et al., 2008; Zhang et al., 2007) and the janus kinase/signal transduction and activators of transcription (JAK/STAT) pathway (Pan et al., 1999; Wang et al., 2004) was reported to be involved in the regulation of stretch-induced molecular events. To investigate the involvement of tyrosine phosphorylation in the stretch-induced activation of p70S6K and eEF2, we performed pharmacological inhibition. First, we examined the effect of genistein, a broad-range tyrosine kinase inhibitor. The inhibition of tyrosine kinase by pre-treatment of the cells with genistein (250 μM) drastically blocked the stretch-induced activation of p70S6K (Fig. 5A). However, neither a more-specific tyrosine kinase inhibitor, PP2 (Src tyrosine kinase inhibitor), nor a JAK inhibitor blocked the stretch-induced p70S6K phosphorylation (Fig. 6A). The results of the present study are not consistent with those of Marin et al. (2008), who reported that inhibition of FAK/Src activity by PP2 or depletion of FAK by specific siRNA abolished the stretch-induced p70S6K phosphorylation. Their study was performed on primary cultured rat ventricular myocytes under starvation conditions, whereas we stretched the myoblast cell line under fed (10% FBS) conditions. Activation of the JAK/STAT pathway by mechanical stretch was reported in cardiomyocytes (Pan et al., 1999; Wang et al., 2004). Both studies suggested that stretch-induced activation of the JAK/STAT pathway was dependent on autocrine/paracrine-secreted angiotensin II and other cytokines. In the present study, the JAK inhibitor did not block the stretch-induced activation of p70S6K, although it did diminish the phosphorylation of STAT3 (data not shown), suggesting that p70S6K phosphorylation is not under the control of the JAK/STAT pathway. Therefore, unknown tyrosine kinase(s), excluding Src kinase and JAK, was involved in the stretch-induced activation of p70S6K in the C2C12 cell line.

Moreover, here we report for the first time that the inhibition of tyrosine kinase strongly blocked the basal and stretch-induced dephosphorylation of eEF2 (Fig. 5B). Stretch-induced activation of eEF2 has been demonstrated in muscle contraction in humans (Drummond et al., 2009) and rats (Thomson et al., 2008), and in a stretched cell model (Atherton et al., 2009). Recent reports provided evidence that AMP-activated protein kinase (AMPK) (Thomson et al., 2008) and/or Ca^{2+}-calmodulin kinase (Rose et al., 2009) may be involved in eEF2 regulation. In addition to these molecules, our results suggest that tyrosine kinase plays an essential role in the stretch-induced activation of translation initiation and elongation in C2C12 cells. However, further investigation is needed to determine which tyrosine kinase is involved in this regulation.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research C (20500577 to N.N.) from the Japan Society for the Promotion of Science (JSPS), Japan; by a the Grant-in-Aid for Scientific Research S (19100009 to Y.O.) from JSPS; and by a Grant-in-Aid for Young Scientists B (19700521 to F.K.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

Alexander, L.D., Alagarsamy, S., and Douglas, J.G. (2004). Cyclic stretch-induced cPLA2 mediates ERK 1/2 signaling in rabbit proximal tubule cells. Kidney Int. *65*, 551-563.

Atherton, P.J., Szewczyk, N.J., Selby, A., Rankin, D., Hillier, K., Smith, K., Rennie, M.J., and Loughna, P.T. (2009). Cyclic stretch reduces myofibrillar protein synthesis despite increases in FAK and anabolic signalling in L6 cells. J. Physiol. *587*, 3719-3727.

Barnett, J.G., Holly, R.G., and Ashmore, C.R. (1980). Stretch-induced growth in chicken wing muscles: biochemical and

morphological characterization. Am. J. Physiol. *239*, C39-46.
Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J., et al. (2001). Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy *in vivo*. Nat. Cell Biol. *3*, 1014-1019.
Crosara-Alberto, D.P., Inoue, R.Y., and Costa, C.R. (2009). FAK signalling mediates NF-kappaB activation by mechanical stress in cardiac myocytes. Clin. Chim. Acta *403*, 81-86.
Dennis, P.B., Pullen, N., Kozma, S.C., and Thomas, G. (1996). The principal rapamycin-sensitive $p70^{s6k}$ phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. Mol. Cell Biol. *16*, 6242-6251.
Drummond, M.J., Fry, C.S., Glynn, E.L., Dreyer, H.C., Dhanani, S., Timmerman, K.L., Volpi, E., and Rasmussen, B.B. (2009). Rapamycin administration in humans blocks the contraction-induced increase in skeletal muscle protein synthesis. J. Physiol. *587*, 1535-1546.
Dufner, A., and Thomas, G. (1999). Ribosomal S6 kinase signaling and the control of translation. Exp. Cell Res. *253*, 100-109.
Fang, Y., Vilella-Bach, M., Bachmann, R., Flanigan, A., and Chen, J. (2001). Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science *294*, 1942-1945.
Fang, Y., Park, I.H., Wu, A.L., Du, G., Huang, P., Frohman, M.A., Walker, S.J., Brown, H.A., and Chen, J. (2003). PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr. Biol. *13*, 2037-2044.
Goldberg, A.L., Etlinger, J.D., Goldspink, D.F., and Jablecki, C. (1975). Mechanism of work-induced hypertrophy of skeletal muscle. Med. Sci. Sports *7*, 185-198.
Hornberger, T.A., and Chien, S. (2006). Mechanical stimuli and nutrients regulate rapamycin-sensitive signaling through distinct mechanisms in skeletal muscle. J. Cell Biochem. *97*, 1207-1216.
Hornberger, T.A., Chu, W.K., Mak, Y.W., Hsiung, J.W., Huang, S.A., and Chien, S. (2006). The role of phospholipase D and phosphatidic acid in the mechanical activation of mTOR signaling in skeletal muscle. Proc. Natl. Acad. Sci. USA *103*, 4741-4746.
Hornberger, T.A., Sukhija, K.B., Wang, X.R., and Chien, S. (2007). mTOR is the rapamycin-sensitive kinase that confers mechanically-induced phosphorylation of the hydrophobic motif site Thr(389) in $p70^{s6k}$. FEBS Lett. *581*, 4562-4566.
Kimball, S.R., Shantz, L.M., Horetsky, R.L., and Jefferson, L.S. (1999). Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. J. Biol. Chem. *274*, 11647-11652.
Marin, T.M., Clemente, C.F., Santos, A.M., Picardi, P.K., Pascoal, V.D., Lopes-Cendes, I., Saad, M.J., and Franchini, K.G. (2008). Shp2 negatively regulates growth in cardiomyocytes by controlling focal adhesion kinase/Src and mTOR pathways. Circ. Res. *103*, 813-824.
Martineau, L.C., and Gardiner, P.F. (2001). Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. J. Appl. Physiol. *91*, 693-702.
Matoba, H., and Gollnick, P.D. (1984). Response of skeletal muscle to training. Sports Med. *1*, 240-251.
Pan, J., Fukuda, K., Saito, M., Matsuzaki, J., Kodama, H., Sano, M., Takahashi, T., Kato, T., and Ogawa, S. (1999). Mechanical stretch activates the JAK/STAT pathway in rat cardiomyocytes. Circ. Res. *84*, 1127-1136.
Pearson, R.B., Dennis, P.B., Han, J.W., Williamson, N.A., Kozma, S.C., Wettenhall, R.E., and Thomas, G. (1995). The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. EMBO J. *14*, 5279-5287.
Redpath, N.T., Price, N.T., Severinov, K.V., and Proud, C.G. (1993). Regulation of elongation factor-2 by multisite phosphorylation. Eur. J. Biochem. *213*, 689-699.
Redpath, N.T., Foulstone, E.J., and Proud, C.G. (1996). Regulation of translation elongation factor-2 by insulin via a rapamycin-sensitive signalling pathway. EMBO J. *15*, 2291-2297.
Robinson, M.J., and Cobb, M.H. (1997). Mitogen-activated protein kinase pathways. Curr. Opin. Cell Biol. *9*, 180-186.
Rose, A.J., Alsted, T.J., Jensen, T.E., Kobbero, J.B., Maarbjerg, S.J., Jensen, J., and Richter, E.A. (2009). A Ca^{2+}-calmodulin-eEF2K-eEF2 signalling cascade, but not AMPK, contributes to the suppression of skeletal muscle protein synthesis during contractions. J. Physiol. *587*, 1547-1563.
Thomson, D.M., Fick, C.A., and Gordon, S.E. (2008). AMPK activation attenuates S6K1, 4E-BP1, and eEF2 signaling responses to high-frequency electrically stimulated skeletal muscle contractions. J. Appl. Physiol. *104*, 625-632.
Timson, B.F. (1990). Evaluation of animal models for the study of exercise-induced muscle enlargement. J. Appl. Physiol. *69*, 1935-1945.
Wang, L., Wang, X., and Proud, C.G. (2000). Activation of mRNA translation in rat cardiac myocytes by insulin involves multiple rapamycin-sensitive steps. Am. J. Physiol. Heart Circ. Physiol. *278*, H1056-1068.
Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., and Proud, C.G. (2001). Regulation of elongation factor 2 kinase by $p90^{RSK1}$ and p70 S6 kinase. EMBO J. *20*, 4370-4379.
Wang, T.L., Yang, Y.H., Chang, H., and Hung, C.R. (2004). Angiotensin II signals mechanical stretch-induced cardiac matrix metalloproteinase expression via JAK-STAT pathway. J. Mol. Cell Cardiol. *37*, 785-794.
Weng, Q.P., Kozlowski, M., Belham, C., Zhang, A., Comb, M.J., and Avruch, J. (1998). Regulation of the p70 S6 kinase by phosphorylation *in vivo*. Analysis using site-specific anti-phosphopeptide antibodies. J. Biol. Chem. *273*, 16621-16629.
Wu, X., Reiter, C.E., Antonetti, D.A., Kimball, S.R., Jefferson, L.S., and Gardner, T.W. (2004). Insulin promotes rat retinal neuronal cell survival in a p70S6K-dependent manner. J. Biol. Chem. *279*, 9167-9175.
Zanchi, N.E., and Lancha, A.H., Jr. (2008). Mechanical stimuli of skeletal muscle: implications on mTOR/p70s6k and protein synthesis. Eur. J. Appl. Physiol. *102*, 253-263.
Zhang, S.J., Truskey, G.A., and Kraus, W.E. (2007). Effect of cyclic stretch on beta1D-integrin expression and activation of FAK and RhoA. Am. J. Physiol. Cell Physiol. *292*, C2057-2069.
Zheng, W., Christensen, L.P., and Tomanek, R.J. (2008). Differential effects of cyclic and static stretch on coronary microvascular endothelial cell receptors and vasculogenic/angiogenic responses. Am. J. Physiol. Heart Circ. Physiol. *295*, H794-800.