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Mechanical Stimuli and Nutrients Regulate Rapamycin-Sensitive Signaling Through Distinct Mechanisms in Skeletal Muscle

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Abstract The mammalian target of rapamycin (mTOR) has been identified as a growth factor and nutrient-sensitive molecule that controls the translational machinery and cell growth. Rapamycin-sensitive (RS) signaling events have also been shown to be necessary for mechanical load-induced growth of skeletal muscle, but the mechanisms involved in the mechanical activation of RS signaling are not known. The finding that mechanical stimuli induce nutrient uptake in skeletal muscle raises the possibility that mechanically induced RS signaling is mediated via a nutrient-dependent mechanism. To investigate this hypothesis, skeletal muscles (ex vivo) were stimulated with nutrients or intermittent mechanical stretch and the phosphorylation of p70S6k [P-p70(389)], PKB [P-PKB], mTOR [P-mTOR(2481)], and p38 [P-p38] was assessed. In comparison to vehicle-treated controls, both nutrient and mechanical stimuli induced P-p70(389), neither stimulus altered P-PKB or P-mTOR(2481), and only mechanical stimuli induced P-p38. The nutrient and mechanically induced increase in P-p70(389) was blocked by rapamycin, but only nutrient-induced signaling to P-p70(389) was blocked by wortmannin. Furthermore, the mechanically induced increase in P-p70(389) was not impaired by the removal of exogenous nutrients. Taken together, these results indicate that exogenous nutrients are not required for mechanically induced RS signaling and that nutrient and mechanical stimuli activate RS signaling through distinct upstream mechanisms. J. Cell. Biochem. 97: 1207–1216, 2006.

Key words: amino acids; growth; mTOR; p70S6k; stretch

Mechanical stimuli play a major role in the regulation of muscle mass and the maintenance of muscle mass contributes significantly to disease prevention and quality of life. Although the link between mechanical signals and the control of muscle mass has been recognized for decades, the mechanisms involved in converting mechanical signals into the molecular events that control this process are not known [Goldspink, 1977; Palmer et al., 1983; Vandenburgh, 1987; Bolster et al., 2004].

Studies with nutrients and growth factors have identified the mammalian target of rapamycin (mTOR) and its downstream substrate, ribosomal S6 kinase (p70S6k), as central components of a signaling pathway that controls cell growth [Thomas and Hall, 1997; Fingar et al., 2002; Harris and Lawrence, 2003]. Furthermore, a rapamycin-sensitive (RS) mechanism (presumably involving mTOR) has been shown to be necessary for mechanically induced growth [Bodine et al., 2001; Hornberger et al., 2003]. These observations indicate that the RS pathway serves to integrate several types of growth-promoting stimuli.

In a recent study on the molecular pathways involved in mechanically induced RS signaling, it was determined that a wortmannin-sensitive [i.e., phosphotidylinositol-3-kinase (PI3K)-dependent] mechanism was not involved [Hornberger et al., 2004]. However, growth factors such as insulin activate RS signaling events via a wortmannin-sensitive mechanism [Dufner and Thomas, 1999; Harris and Lawrence, 2003]. These results suggest that there are at least two core pathways that can activate RS signaling,

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one is PI3K-dependent (wortmannin-sensitive) and the other is PI3K-independent (wortmannin-insensitive) [Hornberger et al., 2004].

As mentioned above, both nutrients and growth factors have been implicated in the regulation of RS signaling, but nutrients, unlike growth factors, do not produce an increase in protein kinase B (PKB) phosphorylation which is a marker of PI3K activity [Kimball et al., 1999; Armstrong et al., 2001]. Based on this observation, it has been suggested that nutrients utilize a PI3K-independent mechanism to regulate RS signaling. This raises the possibility that nutrients may be part of the PI3K-independent mechanism involved in mechanically induced RS signaling. This possibility is further supported by the finding that mechanical stimuli can induce the uptake of nutrients such as amino acids and glucose [Vandenburgh and Kaufman, 1981; Mitsumoto et al., 1992; Ihlemann et al., 1999]. Therefore, the primary goal of this study was to determine the role of nutrients in mechanically induced RS signaling.

MATERIALS AND METHODS

Materials

Peroxidase-conjugated anti-rabbit antibody was purchased from Vector Laboratories (Burlingame, CA). All other antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) detection reagent was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). DC protein assay kit was purchased from Bio-Rad (Hercules, CA). Wortmannin and rapamycin were purchased from Sigma (St. Louis, MO). A 300-BLR force transducer/dynamometer (Aurora Scientific, Aurora, Ontario) was used to record force measurements and produce stretch movements. Data from the force transducer was integrated with a PCI-MIO A/D board (National Instruments, Austin, TX) and interfaced with Labview software for data analysis (National Instruments).

Animal Care and Use

All experimental procedures were approved by the University of California San Diego Animal Care and Use Committee. Male C57BL6 mice (Jackson Laboratories, Bar Harbor, MA and Harlan, Indianapolis, IN), 10.4 ± 1.9 weeks of age, were randomly assigned to different experimental groups. All animals were allowed free access to food and water. On the day of the experiment, the animal was anaesthetized with sodium pentobarbital (40 mg/kg) and the extensor digitorum longus (EDL) muscle of the hind limb was exposed. Sutures (4-0 silk) were tied at the proximal and distal myotendinous junctions of the EDL. The suture at the distal end of the EDL was tied to a lever arm for attachment to the force transducer/dynamometer and the suture at the proximal end of the EDL was tied to a lever arm for attachment to a micromanipulator. When the sutures had been secured, the EDL was excised and immediately placed in an organ culture bath. Mice were euthanasized by cervical dislocation following muscle extraction.

Organ Culture

The organ culture bath system (mechanical stimulator) consisted of a refined myograph (Kent Scientific, Torrington, CT) with Krebs Henseleit Buffer (120 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 2 mM $MgSO_4$, 5 mM HEPES) supplemented with $1 \times$ MEM amino acid mixture (Invitrogen, Carlsbad, CA) and 25 mM glucose unless otherwise noted. The organ culture bath was maintained at 37°C with continuous 95% O_2 and 5% CO_2 gassing as previously described [Hornberger et al., 2004]. It should be noted that the EDL muscle in this system remains viable for at least 2 h as determined by the stability of [ATP], [phosphocreatine]/[total creatine], twitch tension, and rates of protein synthesis [Hornberger et al., 2004].

By moving the lever arms attached to the force transducer and micromanipulator, the EDL was adjusted to its optimal length which had been initially determined in pilot experiments by stimulating the muscle with a 0.5 ms, 10 V pulse, and measuring twitch tension. The length of the muscle was adjusted until a peak twitch tension was observed and the passive tension at this length was recorded. The results from these experiments indicated that the passive tension on the muscle at Lo was 8.25 ± 1.25 mN ($n = 4$). Therefore, in all subsequent experiments, muscle length was adjusted until a passive tension of 8.25 mN was obtained, and this length was assumed to be

approximately the Lo. The average length of the EDL muscles used in these experiments was 12.2 ± 0.6 mm. Fresh media were added to the bath at 30 min intervals.

Stretch Paradigm

Following a 30 min pre-incubation at Lo, the muscle preparations were subjected to 90 min of stretch (STR+) or static (STR−) conditions as previously described [Hornberger et al., 2004]. Briefly, muscles were subjected to 15% stretch using 50 ms ascending ramp, held static for 100 ms and then returned to Lo with a 50 ms descending ramp. This pattern of stretch was repeated once every 3 s for 90 min while control muscles were held static at Lo for 90 min. It should be noted that 90 min of stretch was previously determined to be optimal for measuring mechanically induced RS signaling events [Hornberger et al., 2004].

Amino Acid Stimulation

Stimulation with a MEM amino acid mixture has previously been shown to induce phosphorylation on the RS site of p70S6k (P-p70(389)) [Armstrong et al., 2001]. Thus, following a 30 min pre-incubation with wortmannin, rapamycin, or vehicle, EDL muscles were stimulated with KHB media containing a threefold concentration of MEM amino acids (3XAA) for 30 min. The concentration of amino acids in the 3XAA media was 1.2 mM L-Arginine, 0.6 mM L-Cystine, 0.6 mM L-Histidine, 2.4 mM L-Isoleucine, 2.4 mM L-Leucine, 2.4 mM L-Lysine, 0.6 mM L-Methionine, 1.2 mM L-Phenylalanine, 2.4 mM L-Threonine, 0.24 mM L-Trytophan, 1.2 mM L-Tyrosine, and 2.4 mM L-Valine. It should be noted that preliminary studies indicated the increase in P-p70(389) phosphorylation, in response to 3XAA stimulation, was maximal at 30 min when compared with shorter and longer incubation periods.

Nutrient Deficient Media

In the nutrient-deficiency experiments, the exogenous nutrients in the KHB media were not added. Specifically, EDL muscles were incubated with KHB media from which the amino acid mixture (AA−) or glucose and amino acid mixture (AA/GLC−) had not been added. Muscles were pre-incubated in the nutrient-deficient media for 30 min followed by an additional 90 min of STR− or STR+ conditions as described above.

Western Blots

EDL muscles were removed from the organ culture bath and immediately frozen in liquid nitrogen. Frozen muscles were homogenized with a Polytron in a buffer containing 40 mM Tris (pH 7.5), 1 mM EDTA, 5 mM EGTA, 0.5% Triton X-100, 25 mM β-Glycerolphosphate, 25 mM NaF, 1 mM Na_3VO_4, 10 μg/ml Leupeptin, and 1 mM PMSF. Homogenates were centrifuged at 10,000*g* for 5 min and the supernatant was saved for analysis. Protein concentration of the supernatant was determined by the DC protein assay (Bio-Rad Laboratories). From the homogenate, samples containing 30 μg protein were dissolved in Laemmli buffer and subjected to electrophoretic separation by SDS–PAGE on 7.5% acrylamide gels. Following electrophoretic separation, proteins were transferred to a PVDF membrane, blocked with 5% powdered milk in TBST (Tris-buffered saline, 1% Tween 20) for 3 h followed by an overnight incubation at 4°C with primary antibody. After overnight incubation, the membranes were washed for 30 min in TBST and then probed with an anti-rabbit antibody for 45 min at room temperature. Following 30 min of washing in TBST, the blots were developed using ECL. Once the appropriate image was captured, the membranes were stained with Coomassie Blue to verify equal loading in all lanes. Densitometric measurements were carried out using the public domain NIH Image program (ImageJ) developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

Pharmacological Inhibitors

Vehicle (0.2% DMSO), 500 nM wortmannin (WORT) in DMSO, or 150 nM rapamycin (RAP) in DMSO were present in the media throughout the entire incubation period. The concentrations employed for each of these inhibitors was based on previous studies in which the minimal effective dose was determined [Hornberger et al., 2004]. It should also be noted that WORT and RAP did not alter the peak passive tension that was produced during the stretch paradigm (Vehicle 276 ± 17 mN, WORT 272 ± 7 mN, and RAP 280 mN $\pm$ 9 mN, n = 4/group).

Statistical Analysis

All values are expressed as means + SEM. Statistical significance was determined using

ANOVA, followed by Student Newman-Kuels post hoc analysis. Differences between groups were considered significant if $P < 0.05$.

RESULTS

Mechanical Stimuli and Exogenous Amino Acids Induce Rapamycin-Sensitive Signaling Through Distinct Mechanisms

Intermittent passive mechanical stretching of the EDL muscle induced a significant increase in P-p70(389) (Fig. 1A). Wortmannin caused a decrease in P-p70(389) under control static condition, but did not block the mechanically induced increase in P-p70(389). Rapamycin abolished the basal level of P-p70(389) as well as its induction by mechanical stimulation. These results indicate that mechanical stimuli activate RS signaling via a PI3K-independent mechanism.

Stimulating the EDL muscle with a threefold concentration of exogenous amino acids (3XAA) also induced a significant increase in P-p70 (389). This 3XAA-induced increase in P-p70 (389) was blocked by both wortmannin and rapamycin (Fig. 1B). These results indicate that unlike mechanical stimuli, 3XAA activate RS signaling through a wortmannin-sensitive mechanism. It should be noted that in the presence of rapamycin, a small amount of P-p70(389) was detectable in the 3XAA experiments, while there was no P-p70(389) detected in the stretch experiments. This is attributable to the fact that the muscles in the stretch experiment were exposed to rapamycin for a longer duration (120 min, i.e., 30 min pre-incubation and 90 min ± stretch) than those in the 3XAA experiments (60 min, i.e., 30 min pre-incubation and 30 min ± 3XAA).

Exogenous Nutrients Are not Required for Mechanically-Induced Rapamycin-Sensitive Signaling

Depriving cells of exogenous nutrients has been reported to inhibit growth factor-induced RS signaling [Hara et al., 1998; Proud et al., 2001]. To determine whether exogenous nutrients are required for mechanically induced RS signaling, muscles were subjected to mechanical stimulation in amino acid free (AA−) or amino acid and glucose free media (AA/GLC−). Consistent with a role for nutrients in the regulation of the RS signaling, incubation with AA−, as well as AA/GLC− media, caused a significant reduction in basal P-p70(389), but the mechanically induced increase in P-p70(389)

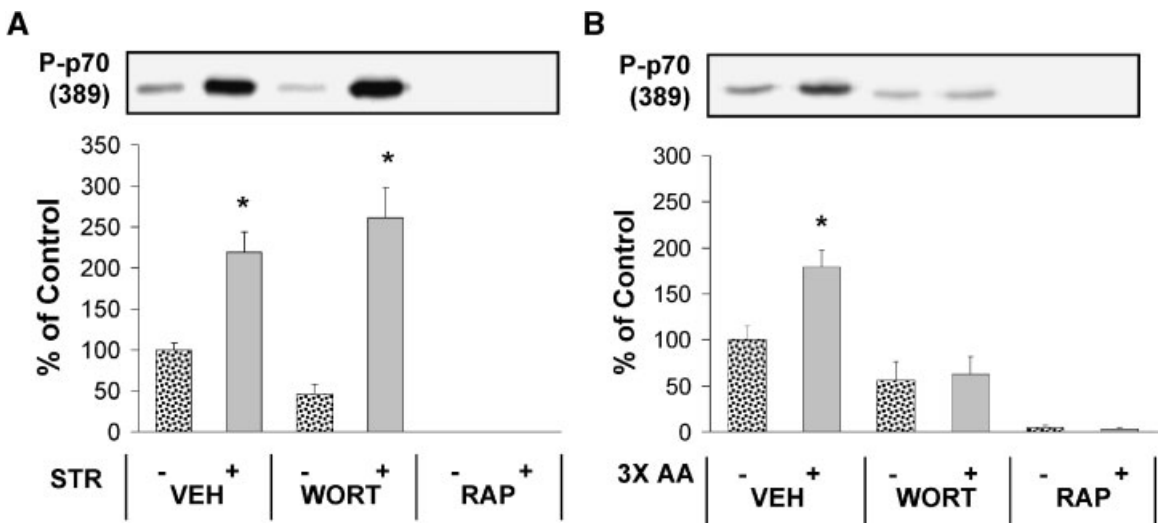

Fig. 1. Mechanical stimuli and exogenous amino acids induce rapamycin-sensitive signaling via distinct mechanisms. Muscles were held at optimal length (Lo) for a 30 min pre-incubation in KHB media containing a vehicle (0.2% DMSO) (VEH), 500 nM wortmannin (WORT), or 150 nM rapamycin (RAP). Following the pre-incubation period, muscles were subjected to 90 min of mechanical stimulation (STR+) or held static at Lo (STR−) (**A**). In a separate series of experiments, muscles were pre-incubated as described above and then subjected to a 30 min incubation with KHB media containing a threefold concentration of an MEM amino acid mixture (3XAA+) or control KHB media (3XAA−) (**B**) VEH, WORT, and RAP were present throughout the entire experimental period. Samples were subjected to Western blot analysis of p70S6k phosphorylated on threonine 389 (P-p70(389)). Graphs represent the mean values + SEM for each group expressed as a percentage of the value obtained in vehicle-treated, static control samples (VEH STR− or VEH 3XAA−), (n = 4–7/group). * Significantly different from treatment static control (i.e., VEH, WORT, or RAP), ($P < 0.05$).

was still present (Fig. 2A,B). These results demonstrate that exogenous nutrients are not required for mechanically induced RS signaling. However, it should be noted that exogenous nutrients were required for a maximal P-p70 (389) phosphorylation (Fig. 2A,B).

Mechanical Stimuli and Exogenous Amino Acids do not Induce PKB Phosphorylation

Changes in P-PKB(473) phosphorylation were used to assess whether the mechanically and 3XAA-induced RS signaling involved changes in PI3K activity. Neither mechanical stimulation nor 3XAA stimulation altered the amount of P-PKB(473) at the time point measured (Fig. 3A,B). These results indicate that neither 3XAA nor mechanical stimulation induce PI3K activity.

Mechanical Stimuli and Exogenous Nutrients do not Alter mTOR(2481) Phosphorylation

The phosphorylation of mTOR on serine 2481 [P-mTOR(2481)] was used as a marker of mTOR kinase activity. To confirm that this antibody could detect changes in P-mTOR(2481) under conditions that have been reported to stimulate mTOR kinase activity (i.e., insulin stimulation [Scott et al., 1998]), muscles were stimulated with 100 nM insulin for 30 min. Insulin stimulation promoted a significant increase in P-mTOR(2481) while mechanical stimulation and 3XAA had no effect (Fig. 4). These results suggest that mechanical stimuli and 3XAA do not alter the kinase activity of mTOR. Furthermore, wortmannin, rapamycin, and nutrient deprivation did not modify the amount of P-mTOR(2481) suggesting that these conditions do not affect the kinase activity of mTOR (Figs. 4 and 5); however; measurements of mTOR kinase activity will be required to verify these conclusions.

Exogenous Nutrients are not Necessary for Mechanically-Induced Signaling to p38

The stress-activated protein kinase p38 has been shown to be highly responsive to mechanical stimuli, but whether exogenous nutrients are required for mechanically induced signaling to p38 has not been addressed. Our results indicate that the mechanically induced increase in P-p38 was not prevented in AA− or AA/GLC− media (Fig. 6A,B). It should be noted that P-p38 was elevated in the absence of AA and GLC, suggesting that this condition induces a stress response in the muscle. In summary, the results in Figure 6 demonstrate that exogenous

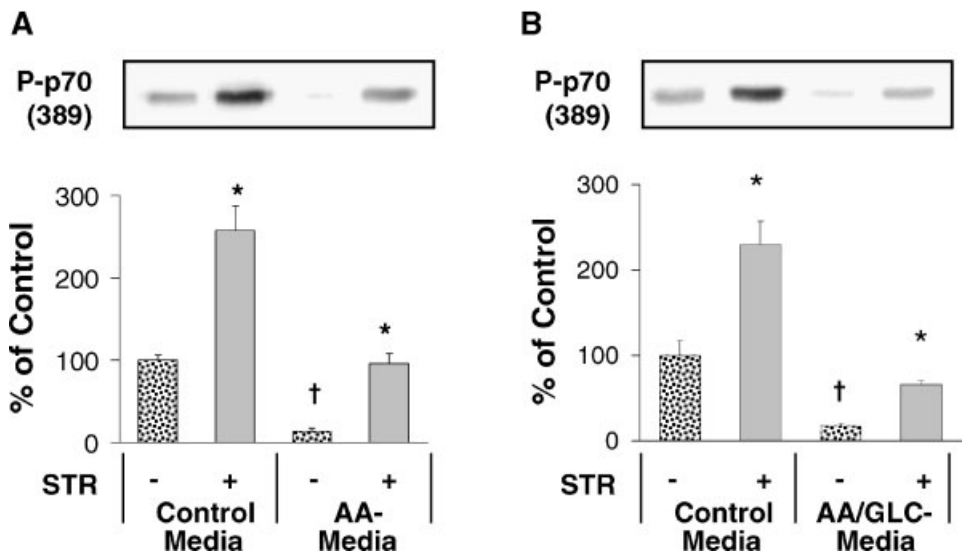

Fig. 2. Mechanically stimuli activate rapamycin-sensitive signaling in the absence of exogenous amino acids and glucose. Muscles were held at optimal length (Lo) for a 30 min preincubation in KHB media (Control), amino acid free (AA−) (**A**), or amino acid and glucose free KHB media (AA/GLC−) (**B**). The preincubation period was followed by an additional 90 min of static (STR−) or intermittent stretch (STR+) conditions. Samples were subjected to Western blot analysis for p70S6k phosphorylated on threonine 389 (P-p70(389)). Graphs represent the mean values + SEM for each group expressed as a percentage of the value obtained in static control media samples (Control Media, STR−), (n = 4–6/group). * Significantly different from static treatment control (i.e., Control Media STR−, AA− Media STR−, or AA/GLC− Media STR−), † Significantly different from Control Media STR−, ($P < 0.05$).

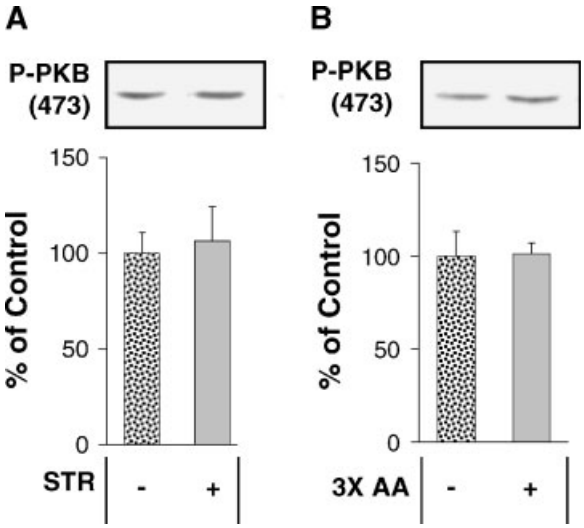

Fig. 3. Mechanical stimuli and exogenous amino acids do not induce PKB phosphorylation. Muscles were held at optimal length (Lo) for a 30 min pre-incubation in KHB media. Following the pre-incubation period, muscles were subjected to 90 min of mechanical stimulation (STR+) or held static at Lo (STR−) (**A**). In a separate series of experiments, muscles were pre-incubated as described above and then incubated for an additional 30 min with KHB media containing a threefold concentration of an MEM amino acid mixture (3XAA+) or control KHB media (3XAA−) (**B**). Samples were subjected to Western blot analysis of PKB phosphorylated on serine 473 [P-PKB(473)]. Graphs represent the mean values + SEM for each group expressed as a percentage of the value obtained in static control samples (VEH STR− or VEH 3XAA−), (n = 4–7/group).

nutrients are not required for the mechanically induced signaling to p38.

DISCUSSION

Studies with nutrients and growth factors have identified mTOR and p70S6k as central components of a rapamycin-sensitive (RS) pathway that controls cell growth [Thomas and Hall, 1997; Fingar et al., 2002; Harris and Lawrence, 2003; Fingar and Blenis, 2004]. Furthermore, RS signaling events have also been shown to be necessary for mechanically induced growth of cardiac and skeletal muscle [Bodine et al., 2001; Hornberger et al., 2003; Shioi et al., 2003]. Taken together, it appears that the RS pathway serves to integrate several types of growth-promoting stimuli; however, the molecular

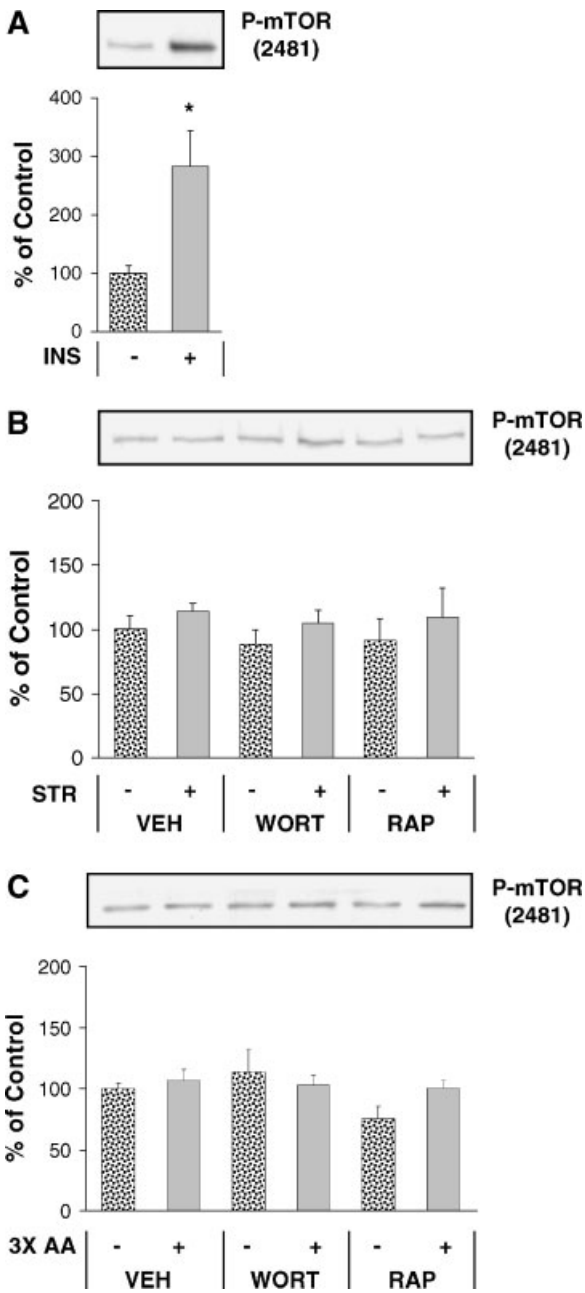

Fig. 4. Mechanical stimuli and exogenous nutrients do not alter mTOR(2481) phosphorylation. Muscles were held at optimal length (Lo) and pre-incubated in KHB media and then subjected to a 30 min incubation in KHB media containing 100 nM insulin (INS+) or control KHB media (INS−) (**A**). Muscles were held at optimal length (Lo) for a 30 min pre-incubation in KHB media containing a vehicle (0.2% DMSO) (VEH), 500 nM wortmannin (WORT), or 150 nM rapamycin (RAP). Following the pre-incubation period, muscles were subjected to 90 min of mechanical stimulation (STR+) or held static at Lo (STR−) (**B**). In a separate series of experiments, muscles were pre-incubated as described in (B) and then subjected to a 30 min incubation with KHB media containing a threefold concentration of an MEM amino acid mixture (3XAA+) or control KHB media (3XAA−) (**C**). VEH, WORT, and RAP were present throughout the entire experimental period. Samples were subjected to Western blot analysis of mTOR phosphorylated on serine 2481 [P-mTOR (2481)]. Graphs represent the mean values + SEM for each group expressed as a percentage of the value obtained in static control samples (VEH STR− or VEH 3XAA−), (n = 3–7/group). * Significantly different from control $P < 0.05$.

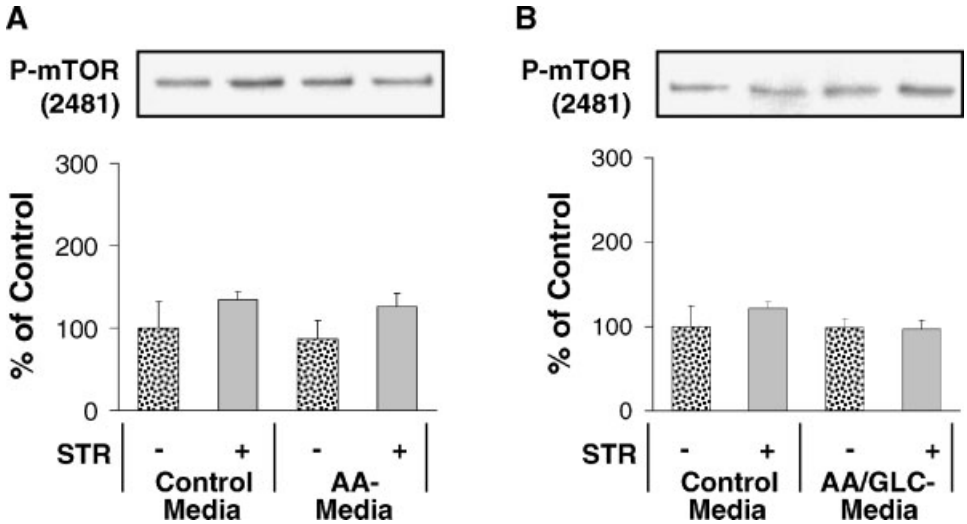

Fig. 5. Nutrient deprivation does not alter mTOR(2481) phosphorylation. Muscles were held at optimal length (Lo) for a 30 min pre-incubation in control KHB media, amino acid free (AA−) (**A**), or amino acid and glucose free KHB media (AA/GLC−) (**B**). Following the pre-incubation period, muscles were subjected to 90 min of mechanical stimulation (STR+) or held static at Lo (STR−). Samples were analyzed by Western blot analysis of mTOR phosphorylated on serine 2481 (P-mTOR (2481). Graphs represent the mean values + SEM for each group expressed as a percentage of the value obtained in control media STR− samples, (n = 4–7/group).

mechanisms which convert the initial growth stimulus (i.e., nutrient, growth factors or mechanical stimuli) into RS signaling events are only beginning to be understood.

Recent efforts aimed at understanding the mechanisms involved in RS signaling have primarily focused on growth factor and nutrient-induced events. For example, it has been demonstrated that insulin-like growth factors bind the extracellular domain of their receptor, which promotes the phosphorylation of the insulin receptor substrate (IRS) proteins [Harris and Lawrence, 2003]. Phosphorylated IRS recruits and activates PI3K with a concomitant phosphorylation of p70S6k(389) and activation of its kinase activity [Harris and Lawrence, 2003]. The insulin-induced increases in p70S6k kinase activity and p70S6k(389) phosphorylation are inhibited by wortmaninn [Dennis et al., 1996; Hornberger et al., 2004]. Over-expressing a dominant negative isoform of PI3K also inhibits insulin-induced p70S6k kinase activity [Ueki et al., 2000]. Furthermore, rapamycin inhibits p70S6k kinase activity and p70S6k (389) phosphorylation but has no effect on PI3K-dependent signaling events, such as induction of PKB phosphorylation [Dennis et al., 1996; Hornberger et al., 2004]. Taken together, these findings indicate that PI3K-dependent signals are necessary for insulin-like growth factor-induced signaling to p70S6K and that PI3K lies upstream or parallel to the RS pathway.

In this study, we demonstrate that ex vivo stimulation of skeletal muscle with nutrients such as amino acids also induces signaling to p70S6k (Fig. 1B). The amino acid-induced effects on p70S6k(389) phosphorylation were blocked by rapamycin, demonstrating that this event is part of the RS pathway (Fig. 1B). However, unlike growth factors, amino acid stimulation did not produce an increase in PKB phosphorylation (Fig. 3B). This observation is consistent with the conclusion that amino acid-induced RS signaling is independent of PI3K activation [Shah et al., 2000; Greiwe et al., 2001]. However, inhibition of PI3K with wortmannin markedly reduced the basal level of p70S6k(389) phosphorylation and blocked the amino acid-induced signaling to p70S6k(389) (Fig. 1B). Based on these observations, it would appear that the basal PI3K activity (which is inhibited by wortmannin), rather than an amino acid-induced stimulation of PI3K activity, is required for amino-acid induced RS signaling. Such a scenario would occur if, for example, basal levels of PI3K activity provided a priming event that was required for subsequent amino acid-induced RS signaling. An alternative explanation for this observation is that, as reported by [Brunn et al., 1996], wortmannin

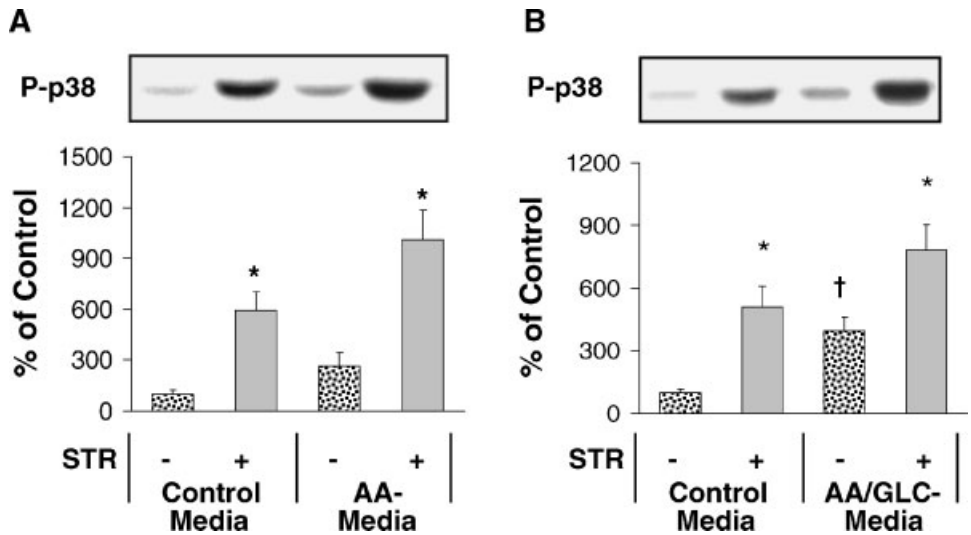

Fig. 6. Exogenous nutrients are not necessary for mechanically induced signaling to p38. Muscles were held at optimal length (Lo) for a 30 min pre-incubation in KHB media (Control), amino acid free (AA−) (**A**), or amino acid and glucose free KHB media (AA/GLC−) (**B**). The pre-incubation period was followed by an additional 90 min of static (STR−) or intermittent stretch (STR+) conditions. Samples were subjected to Western blot analysis for phosphorylated p38 (P-p38). Graphs represent the mean values + SEM for each group expressed as a percentage of the value obtained in static control media samples (Control Media, STR−), (n = 4–6/group). * Significantly different compared to static treatment control (i.e., Control Media STR−, AA− Media STR−, or AA/GLC Media STR−), † Significantly different from control media, STR−, ($P < 0.05$).

can inhibit mTOR kinase activity. However, the mTOR autophosphorylation data presented in Figure 4 suggests that mTOR kinase activity was not affected by the concentration of Wortmannin employed in this study. Another possible explanation is that amino acids cause a very transient activation of PI3K and PKB phosphorylation, an effect that would not have been detected at the time point measured in this study (30 min). It should be noted that a transient increase in PI3K activity after 2 min of amino acid stimulation has been reported in one study [Peyrollier et al., 2000], but not others [Armstrong et al., 2001].

Mechanically stimulating skeletal muscle with intermittent passive stretch induced an increase in p70S6k(389) phosphorylation (Fig. 1A). The mechanically induced increase in p70(389) phosphorylation was blocked by rapamycin, demonstrating that this event is part of the RS pathway (Fig. 1A). However, unlike amino acids and growth factors, the mechanically induced increase in p70S6k(389) phosphorylation was not inhibited by wortmannin (Fig. 1B). These results indicate that mechanical stimuli utilize a distinct mechanism from growth factors and nutrients to regulate RS signaling. Furthermore, these results support the hypothesis that there are at least two core pathways that promote RS signaling. One core pathway is utilized by growth factors and nutrients and is PI3K-dependent (wortmannin-sensitive). The other core pathway is utilized by mechanical stimuli and involves unknown PI3K-independent (wortmannin-insensitive) event(s) (see schematic in Fig. 7).

The presence of exogenous nutrients has previously been reported to be necessary for growth factor-induced RS signaling [Hara et al.,

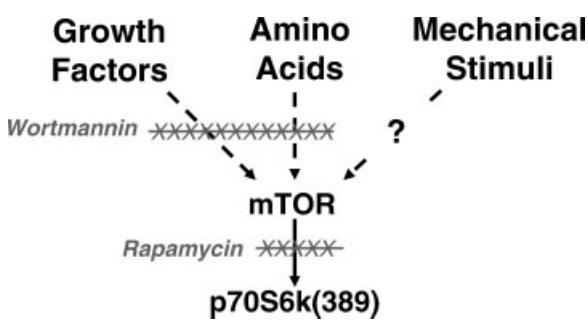

Fig. 7. Schematic of the core pathways involved in growth factor, amino acid, and mechanically induced rapamycin-sensitive signaling. Growth factors, amino acids, and mechanical stimuli induce p70S6K(389) phosphorylation through a rapamycin-sensitive (RS) pathway. Growth factor- and amino acid-induced RS signaling involve a wortmannin-sensitive mechanism, while mechanically induced RS signaling involves a wortmannin-independent mechanism. The molecular mechanisms involved in mechanically induced RS signaling remain to be determined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1998; Campbell et al., 1999; Proud et al., 2001]. Our initial finding that mechanical stimuli activate RS signaling via mechanisms distinct from those for nutrients and growth factors did not address whether exogenous nutrients were necessary for the mechanically induced RS signaling. To address this question, muscles were subjected to mechanical stimulation in nutrient-deficient media. The results from these experiments indicate that the absence of exogenous amino acids, or amino acids and glucose, did not prevent the mechanically induced signaling to both RS (p70S6k) and non-RS (p38) molecules (Figs. 2 and 6). These results provide further evidence that mechanical stimuli regulate RS signaling via a mechanism that is distinct from the core pathway utilized by nutrients and growth factors. However, it should be noted that exogenous nutrients were required for a maximal activation of P-p70(389) phosphorylation.

To further address the potential mechanisms involved in nutrient and mechanically induced RS signaling, the phosphorylation of mTOR on serine 2481 phosphorylation [P-mTOR(2481)] was assessed. Previous studies have demonstrated that wild-type mTOR, but not a kinase-dead mutant, can autophosphorylate itself on serine 2481 [Peterson et al., 2000]; hence, measurements of P-mTOR(2481) can serve as an indirect reporter for mTOR kinase activity in vivo. Consistent with previous reports that insulin activates mTOR kinase activity, P-mTOR(2481) was enhanced by the presence of insulin while nutrients and mechanical stimuli had no effect (Fig. 4) [Scott et al., 1998]. The findings with nutrients are also consistent with other studies which have demonstrated that mTOR autophosphorylation in-vitro (kinase activity) is not altered by nutrient stimulation [Hara et al., 2002]. Thus, based on the measurements of P-mTOR(2481), it appears that mechanical stimuli and nutrients both activate RS signaling through a mechanism that does not involve changes in mTOR kinase activity.

In conclusion, mechanical stimuli, nutrients, and growth factors are all capable of activating RS signaling events. However, unlike nutrients and growth factors, mechanical stimuli activate RS signaling through a PI3K-independent mechanism. Furthermore, the presence of exogenous nutrients is not required for mechanically induced RS signaling. These results indicate that mechanical stimuli regulate RS signaling via a unique mechanism that is distinct from those mediated by growth factors and nutrients. In light of recent observations which have shown that mechanically induced growth of cardiac and skeletal muscles is dependent on RS signaling [Bodine et al., 2001; Shioi et al., 2003], the results from this study help to elucidate the mechanisms by which mechanical stimuli regulate the RS pathway and overall muscle growth.

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