

mTOR is the rapamycin-sensitive kinase that confers mechanically-induced phosphorylation of the hydrophobic motif site Thr(389) in p70^{S6k}

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Abstract Mechanical stretch induces phosphorylation of the hydrophobic motif site Thr(389) in p70^{S6k} through a rapamycin-sensitive (RS) pathway that involves a unique PI3K-independent mechanism. Rapamycin is considered to be a highly specific inhibitor of the protein kinase mTOR; however, mTOR is also considered to be a PI3K-dependent signaling molecule. Thus, questions remain as to whether mTOR is the RS element that confers mechanically-induced signaling to p70^{S6k}(389). In this study, rapamycin-resistant mutants of mTOR along with mechanical stretch were used to address this question. The results indicate that mTOR is the RS element and reveal that mTOR signaling can be activated through a PI3K-independent mechanism.

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1. Introduction

Over 30 years ago, it was proposed that mechanically-induced tension was the critical event for initiating compensatory growth of skeletal muscle [1]. Today, this hypothesis is still widely accepted, and current studies are now focused on identifying the molecular events that are involved in this process. These studies have revealed that mechanically-induced growth is inhibited by the macrolide antibiotic, rapamycin [2,3]. Rapamycin is a highly specific inhibitor of a protein kinase called the mammalian target of rapamycin (mTOR) and based on this specificity, many have concluded that mechanical stimuli induce skeletal muscle growth through the activation of mTOR signaling [4,5].

The activation of mTOR signaling is often evaluated by measuring changes in the phosphorylation of the Thr389 resi-

due in the hydrophobic motif of the ribosomal S6 kinase 1 (p70^{S6k}(389)). Consistent with a role for mTOR in mechanically-induced growth, several studies have shown that mechanical stimuli can induce p70^{S6k}(389) phosphorylation, and the magnitude of this activation correlates with the extent of the concomitant growth response [6–8]. Furthermore, the mechanical activation of p70^{S6k}(389) phosphorylation occurs through a rapamycin-sensitive mechanism [2,9]. Combined, these observations indicate that mechanical stimulation is sufficient for the activation of mTOR signaling. However, mechanical stimuli have been reported to activate p70^{S6k}(389) phosphorylation through a phosphatidylinositol-3-kinase (PI3K)-independent mechanism [9,10]. Since signaling by mTOR is considered to be dependent on the PI3K, this point raises questions about whether mechanically-induced signaling to p70^{S6k}(389) is indeed an mTOR-dependent signaling event [11–13].

To determine if mTOR is the rapamycin-sensitive element responsible for mechanically-induced signaling to p70^{S6k}(389), we have combined the use of various rapamycin-resistant mutants of mTOR along with an in vitro stretch model for mechanically stimulating skeletal muscle myoblasts. Consistent with other models of mechanical stimulation, it was determined that, in vitro, the mechanical activation of p70^{S6k}(389) phosphorylation occurs through a PI3K-independent mechanism. Furthermore, the rapamycin-resistant mutants of mTOR allowed us to demonstrate that mTOR is the rapamycin-sensitive element that confers mechanically-induced signaling to p70^{S6k}(389), and that this event requires mTOR kinase activity. Taken together, this study demonstrates that p70^{S6k}(389) phosphorylation is a valid marker of mechanically-induced mTOR signaling, and that mTOR signaling can be activated through a PI3K-independent mechanism.

2. Materials and methods

2.1. Materials

Rapamycin, wortmannin and fibronectin were purchased from Sigma–Aldrich (St. Louis, MO). 1-Butanol was purchased from Fischer Scientific. [3H] Arachidonic acid (62.5 Ci/mmol) was purchased from New England Nuclear (Chicago, IL). PVDF membranes were purchased from Millipore (Bedford, MA). All antibodies were purchased from Cell Signaling Technologies (Danvers, MA). Peroxidase-conjugated anti-rabbit goat secondary antibody was purchased from Vector Laboratories (Burlingame, CA). Enhanced Chemiluminescence detection reagents (ECL and ECL Plus) were purchased from Amersham Pharmacia Biotech (Pittsburgh, PA).

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Abbreviations: JNK2, jun N-terminal kinase 2; mTOR, mammalian target of rapamycin; PA, phosphatidic acid; PLD, phospholipase D; PI3K, phosphatidylinositol-3-kinase; PKB, protein kinase B; RR-mTOR, rapamycin-resistant mTOR; RRKD-mTOR, rapamycin-resistant kinase-dead mTOR; p70^{S6k}, ribosomal S6 kinase 1; Ser, serine; Thr, threonine

2.2. Cell culture

Mouse C2C12 myoblasts were cultured in growth media consisting of high glucose DMEM supplemented with antibiotics (streptomycin 100 µg/ml, penicillin 100 U/ml and 0.25 µg/ml Fungizone (Gibco)) and 10% fetal bovine serum (FBS). Stable C2C12 cell lines expressing rapamycin-resistant mTOR (RR-mTOR) and rapamycin-resistant kinase-dead mTOR (RRKD-mTOR) have been previously described [14] and were kindly provided by Dr. Jie Chen (Department of Cell and Developmental Biology, University of Illinois, Urbana IL). These cells were maintained in growth media containing 0.2 mg/ml G418 (Calbiochem). G418 was not included in the media after the cells had been plated for mechanical stimulation experiments. All cell culture and stretch experiments were performed in a humidified 95% air, 5% CO₂ incubator at 37 °C.

2.3. Mechanical stimulation

C2C12 myoblasts were subjected to 10–30 min of cyclic 15% biaxial stretch at a frequency of 1 Hz using the system previously described by Sotoudeh et al. [15]. In this system, silicone membranes were coated with 2 µg/cm² fibronectin dissolved in phosphate buffered saline (PBS) for 1 h followed by treatment with UV light for 1 h. The membranes were rinsed with fresh PBS and then plated with C2C12 myoblasts at approximately 70% confluence. The myoblasts were allowed to grow on the membranes for 48 h at which point they were highly confluent. At 2 h prior to the initiation of mechanical stimulation, myoblasts were switched to serum-free high-glucose DMEM that did not contain any antibiotics. Myoblasts were subjected to 10–30 min of stretch and then collected in lysis buffer as described below. For the control condition, culture plates were placed on the running stretch device, but were not subjected to the stretch condition.

2.4. Sample preparation and Western blot analysis

Cells were collected in lysis buffer as previously described [10]. Cell lysates were centrifuged at 500 × g for 5 min and the supernatant was saved for analysis. Protein concentration was determined with DC protein assay kit (Bio-Rad, Hercules, CA) and equivalent amounts of protein from each sample were subjected to Western blot analysis as previously described [10].

2.5. Immunoprecipitation

Immunoprecipitations were carried out with EZview red ANTI-FLAG M2 agarose affinity gel beads (Sigma–Aldrich) according to the manufacturer's protocol. Briefly, 300 µg of sample protein was dissolved in 1 ml of lysis buffer plus 40 µl of EZview beads, and allowed to incubate at 4 °C for 2 h with gentle rocking. The beads were pelleted and washed several times with lysis buffer. The pellets were dissolved in 2 × Laemmli buffer and subjected to Western blot analysis as described above.

2.6. Phospholipase D activity

Phospholipase D (PLD) activity was measured as previously described with the exception that C2C12 myoblasts were labeled in growth media containing 1 µCi/ml [3H] arachidonic acid for 24 h [16].

2.7. Statistical analysis

All values are expressed as means ± S.E.M. Statistical significance was determined by using ANOVA, followed by Student–Newman–Keuls post hoc analysis. Differences between groups were considered significant if $P \leq 0.05$.

3. Results and discussion

3.1. Time course of mechanically-induced signaling events

To begin characterizing the in vitro model of mechanical stimulation, we first evaluated the time dependent changes in the phosphorylation of p70^{S6k}(389), protein kinase B (PKB) and Jun N-terminal kinase 2 (JNK2). The results indicated that mechanical stimulation induced a progressive increase in p70^{S6k}(389) and PKB phosphorylation. The increase in p70^{S6k}(389) and PKB phosphorylation reached a maximum

within 20 min and remained at this level for at least 30 min. Mechanical stimulation also induced an increase in JNK2 phosphorylation which reached a maximum within 10 min and was sustained at this level for at least 30 min (Fig. 1A).

3.2. The role of PI3K in the mechanical activation of p70^{S6k}(389) phosphorylation

The PI3K inhibitor wortmannin was used to address the role of PI3K in the mechanical activation of p70^{S6k}(389) phosphorylation. Incubating control cells with wortmannin promoted a reduction in the basal level of p70^{S6k}(389) phosphorylation, however, wortmannin did not block the mechanically-induced increase in p70^{S6k}(389) or JNK2 phosphorylation (Fig. 1B). On the other hand, wortmannin completely eliminated P-PKB phosphorylation in both control and mechanically stimulated cells.

In order to confirm that wortmannin fully inhibited PI3K activity, myoblasts were stimulated with insulin in the presence or absence of wortmannin and the changes in p70^{S6k}(389) phosphorylation were compared. The results indicated that stimulation with insulin promoted a robust increase in p70^{S6k}(389) phosphorylation, and this effect was completely blocked by wortmannin, thus, confirming that wortmannin did inhibit PI3K activity (Fig. 1C). Taken together, these experimental results indicate that mechanical stimulation of skeletal muscle myoblasts, in vitro, induces p70^{S6k}(389) phosphorylation through a PI3K-independent mechanism.

3.3. The role of PLD in the mechanical activation of p70^{S6k}(389) phosphorylation

Using an ex vivo model for mechanical stimulation of whole skeletal muscle explants, it was recently determined that a PLD-dependent synthesis of phosphatidic acid (PA) was required for the mechanical activation of p70^{S6k}(389) phosphorylation [16]. Consistent with these ex vivo observations, we have shown in the current in vitro experiments that mechanical stimulation of skeletal muscle myoblasts promotes an increase in PLD activity (Fig. 1D). Furthermore, inhibiting the PLD-dependent synthesis of PA with 1-butanol resulted in a dose-dependent inhibition in the mechanical activation of p70^{S6k}(389) phosphorylation. At 0.8%, 1-butanol completely inhibited mechanically-induced signaling to p70^{S6k}(389), but it did not have any significant effect on the magnitude of the mechanical activation of PKB or JNK2 phosphorylation (Fig. 1E).

In an effort to confirm that the effects of 1-butanol were specific to the inhibition of PLD, myoblasts were stimulated with insulin in the presence or absence of 0.8% 1-butanol. The results indicated that 1-butanol did not inhibit the insulin-induced increase in p70^{S6k}(389) phosphorylation. This observation demonstrates that signaling to p70^{S6k}(389) can occur in the presence of inhibitory doses of 1-butanol, and also further reveals that mechanical stimuli and insulin utilize distinct mechanisms to activate p70^{S6k}(389) phosphorylation.

3.4. The roles of rapamycin and mTOR in the mechanical activation of p70^{S6k}(389) phosphorylation

First, we used rapamycin to address the potential role of mTOR in the mechanical activation of p70^{S6k}(389) phosphorylation. As shown in Fig. 2A, rapamycin completely abolished p70^{S6k}(389) phosphorylation in both control and mechani-

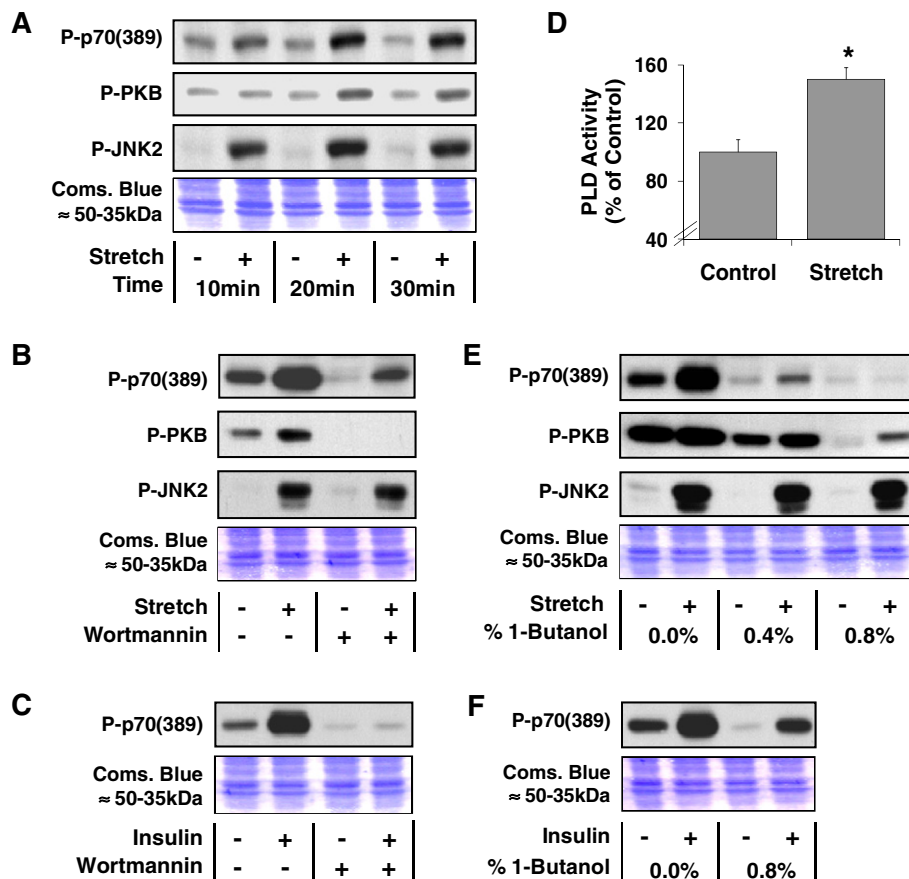


Fig. 1. Mechanically-induced p70^{S6k}(389) phosphorylation requires PLD but not PI3K. (A) Western blot analysis of phosphorylated p70^{S6k} (Thr389) [P-p70(389)], PKB(Ser473) [P-PKB], JNK2 (Thr183/Tyr185) [P-JNK2] in C2C12 myoblasts following 10–30 min of control or 15% cyclic (1 Hz) biaxial stretch [Stretch +] conditions. (B and C) C2C12 myoblasts were pre-incubated with 500 nM wortmannin or the solvent vehicle (0.2% DMSO) for 15 min before being subjected to 20 min of control or stretch conditions (B) or 10 min of 100 nM insulin stimulation [Insulin +] (C). (D) PLD activity during 0–20 min of control or stretch conditions. (E and F) C2C12 myoblasts were pre-incubated with 0.0–0.8% 1-butanol for 5 min before being subjected to 20 min of control or stretch conditions (E) or 10 min of 100 nM Insulin stimulation (F). At the completion of Western blotting, equal loading of protein in all lanes was verified with Coomassie Blue (Coms. Blue) staining of the transfer membranes (images of the region containing the ≈50–35 kDa proteins are shown). All Western blots are representative of at least three experiments. *Significantly different from treatment control ($P \leq 0.05$) ($n = 3$ –6/group).

cally-stimulated cells, while having no effect on JNK2 phosphorylation.

Numerous studies have shown that nutrients and growth factors activate p70^{S6k}(389) phosphorylation through a rapamycin-sensitive mechanism, and that mTOR is the rapamycin-sensitive element in this pathway [11,12,17,18]. Furthermore, it has been well established that PI3K activity is required for nutrient and growth factor-induced signaling through mTOR [10,11,18]. These observations have led to the general conclusion that PI3K activity is required for mTOR signaling, and we are not aware of any studies that have directly shown otherwise. Thus, it is intriguing that mechanical stimuli can induce p70^{S6k}(389) phosphorylation through a rapamycin-sensitive but PI3K-independent mechanism, and this observation raises questions as to whether mTOR is the rapamycin-sensitive element in this pathway.

Rapamycin inhibits mTOR signaling by forming a complex with the immunophilin FKBP12 which binds to mTOR in a region termed the FRB domain [19]. Although rapamycin is considered to be a highly specific inhibitor of mTOR, it remains possible that rapamycin can exert non-specific actions through the sequestration of the FKBP12 protein. For example, it has

been shown that FKBP12 plays an important role in the function of the ryanodine receptor and signaling by the TGF-beta superfamily [20,21].

To determine whether mTOR is the rapamycin-sensitive element that confers mechanically-induced signaling to p70^{S6k}(389), we employed C2C12 myoblasts that stably express a FLAG-tagged rapamycin-resistant variant of mTOR (RR-mTOR) (Fig. 2B). When subjected to mechanical stimulation, the RR-mTOR myoblasts showed changes in p70^{S6k}(389) and JNK2 phosphorylation that are similar to those observed in wild type myoblasts (Fig. 2A and C). However, unlike wild type myoblasts, p70^{S6k}(389) phosphorylation could easily be detected in RR-mTOR myoblasts incubated in the presence of rapamycin. Furthermore, RR-mTOR myoblasts incubated in the presence of rapamycin displayed a mechanically-induced increase in p70^{S6k}(389) phosphorylation (Fig. 2C). These observations provide genetic evidence that mTOR is the rapamycin-sensitive element that confers mechanically-induced signaling to p70^{S6k}(389) Fig. 3.

To determine whether mTOR kinase activity was required for mechanically-induced signaling to p70^{S6k}(389), we employed C2C12 myoblasts that stably express a FLAG-tagged

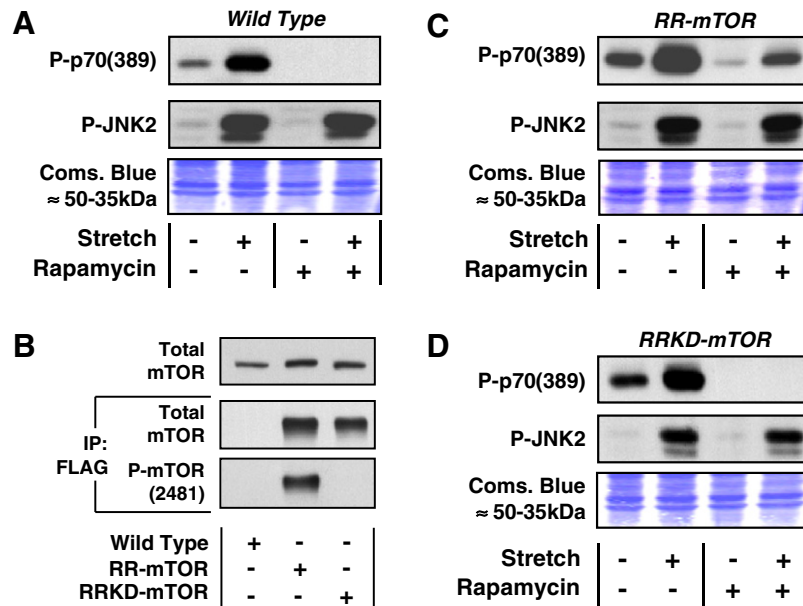


Fig. 2. mTOR is the rapamycin-sensitive kinase that confers mechanically-induced phosphorylation of p70^{S6k}(389). (A) C2C12 myoblasts were pre-incubated with 50 nM rapamycin or the solvent vehicle (0.2% DMSO) for 30 min before being subjected to 20 min of control or 15% cyclic (1 Hz) biaxial stretch [Stretch +] conditions. Samples were subjected to Western blot analysis of phosphorylated p70^{S6k} (Thr389) [P-p70(389)] and phosphorylated JNK2 (Thr183/Tyr185) [P-JNK2]. (B) Lysates from C2C12 myoblasts [Wild Type] and C2C12 myoblasts stably expressing either a FLAG-tagged rapamycin-resistant mutant of mTOR [RR-mTOR] or a FLAG-tagged rapamycin-resistant kinase-dead mutant of mTOR [RRKD-mTOR] were subjected to Western blot analysis of total mTOR (top inset) or immunoprecipitated for the FLAG tag [IP: FLAG] followed by western blot analysis of total mTOR or phosphorylated mTOR (Ser2481) [P-mTOR(2481)]. (C and D) C2C12 myoblasts stably expressing RR-mTOR (C) or RRKD-mTOR (D) were pre-incubated with 50 nM Rapamycin or the solvent vehicle (0.2% DMSO) for 30 min before being subjected to 20 min of control or stretch conditions. Samples were subjected to Western blot analysis as described in A. At the completion of Western blotting, equal loading of protein in all lanes was verified with Coomassie Blue (Coms. Blue) staining of the transfer membranes (images of the region containing the ≈50–35 kDa proteins are shown). All Western blots are representative of at least three experiments.

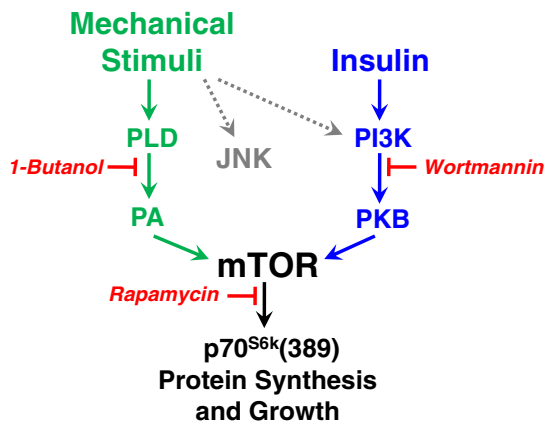


Fig. 3. Schematic of the different mechanisms involved in the activation of mTOR signaling by insulin and mechanical stimuli. Insulin and mechanical stimuli both induce phosphorylation of the hydrophobic motif Thr(389) in p70^{S6k} through a rapamycin-sensitive mechanism, and mTOR is the kinase that confers this event. Insulin activates mTOR signaling to p70^{S6k}(389) through a PLD-independent but PI3K-dependent mechanism. On the other hand, mechanical stimuli activate mTOR signaling to p70^{S6k}(389) through a mechanism that is dependent on PLD but does not require PI3K activity. Additionally, inhibiting PLD activity with 1-butanol does not block the mechanical activation of JNK or PKB phosphorylation implying that mechanical stimuli activate additional signaling pathways upstream of PLD.

rapamycin-resistant kinase-dead variant of mTOR (RRKD-mTOR). As shown in Fig. 2B, this mTOR construct does not contain detectable Ser2481 phosphorylation. This observa-

tion is consistent with the hypothesis that Ser2481 is an auto-phosphorylation site on mTOR, and that the RRKD-mTOR construct does not contain kinase activity [22]. In these RRKD-mTOR myoblasts, we found that rapamycin completely abolished p70^{S6k}(389) phosphorylation in both control and mechanically-stimulated conditions. This result indicates that the kinase activity of mTOR is necessary for both basal and mechanically-induced signaling to p70^{S6k}(389).

In summary, this study first describes an in vitro model that can be used to study the mechanisms involved in the mechanical activation of p70^{S6k}(389) phosphorylation in skeletal muscle. Similar to what has been reported with ex vivo and in vivo models of mechanical stimulation, the in vitro model of mechanical stimulation induces p70^{S6k}(389) phosphorylation through a PI3K-independent and PLD/PA-dependent mechanism. This model was then used to demonstrate that mTOR is the rapamycin-sensitive kinase that confers mechanically-induced signaling to p70^{S6k}(389) phosphorylation. Thus, measuring changes in p70^{S6k}(389) phosphorylation appears to be a valid marker for measuring mechanically-induced mTOR signaling. The results of this study also demonstrate for the first time that mTOR signaling can be activated through a PI3K-independent mechanism.

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