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High-frequency electrically stimulated skeletal muscle contractions increase p70^{s6k} phosphorylation independent of known IGF-I sensitive signaling pathways

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ABSTRACT

Insulin-like growth factor (IGF-I) is hypothesized to be a critical upstream regulator of mammalian target of rapamycin (mTOR)-regulated protein synthesis with muscle contraction. We utilized a mouse model that expresses a skeletal muscle specific dominant-negative IGF-I receptor to investigate the role of IGF-I signaling of protein synthesis in response to unilateral lengthening contractions (10 sets, 6 repetitions, 100 Hz) at 0 and 3 h following the stimulus. Our results indicate that one session of high frequency muscle contractions can activate mTOR signaling independent of signaling components directly downstream of the receptor.

1. Introduction

The initial phases of skeletal muscle hypertrophy are characterized by significant increases in protein synthesis rates followed by increases in total protein content that are not matched by changes in total RNA content [1]. This suggests that during the initial phases of mechanical loading of the muscle, mechanisms that regulate protein synthesis are critical for induction of muscle hypertrophy. It is still largely unclear how a load stimulus is converted into the activation of signaling pathways that enhance muscle mass. Recent evidence has suggested that the mammalian target of rapamycin (mTOR) signaling pathway is a critical component for muscle to translate increased load into growth, but the means by which load activates mTOR signaling has remained elusive. The most common hypothesis is that mechanical load of muscle increases autocrine/paracrine production of insulin-like growth factor-I (IGF-I) expression, which leads to phosphatidylinosoltol 3 kinase (PI3K) and phosphatidylinosoltol regulated protein kinase (Akt) mediated activation of mTOR. p70^{S6k} phosphorylation at threonine residue 389 is a direct substrate of mTOR signaling, and thus is regarded as an indicator of active mTOR [2,3]. This has similarly been shown in muscle as the change in p70^{S6k} (Thr-389) with mechanical load is inhibited with rapamycin (mTOR inhibitor) treatment [4]. Genetic ablation of p70^{S6k} resulted in significantly decreased muscle fiber size that was similar to decreases with inhibition of mTOR via rapamycin [5], indicating that p70^{S6k} is indeed important for muscle hypertrophy. Thus, ablation of downstream signaling substrates of mTOR leads to inhibition of mechanical load-induced muscle hypertrophy.

IGF-I has been proposed as a mechanism by which mechanical load (i.e. resistance exercise) activates muscle protein synthesis via the PI3K/Akt/mTOR/p70^{S6k} pathway. This hypothesis is based on the fact that skeletal muscle IGF-I expression is increased with mechanical load [6] and that exogenous IGF-I has hypertrophic effects on cultured myotubes [7] and skeletal muscle in rodents [8]. The purpose of this study was to further investigate the ability of lengthening contractions to induce activation of known IGF-I-sensitive signaling proteins that are upstream of mTOR and proposed to contribute to the regulation of mechanisms specific for mTOR activation.

2. Materials and methods

2.1. Animals and genotyping

All procedures were approved by the University of Maryland Animal Care and Use Committee. Adult (21-24 weeks) male

Abbreviations: Akt, phosphatidylinosoltol regulated protein kinase; IGF-I, insulin-like growth factor-I; IOD, integrated optical densities; IRS-1, insulin related substrate-1; MKR, IGF-I receptor mutant mouse; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinosoltol 3 kinase; PRAS40, proline-rich Akt substrate 40; TA, tibialis anterior; WT, wild-type

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wild-type (WT) and IGF receptor mutant (MKR) mice were utilized in this study [9,10]. MKR mice possess a skeletal muscle specific dominant-negative kinase-inactive IGF-I receptor. Mice were maintained under a 12 h light-dark cycle with free access to a standard chow diet. All food was removed 4–5 h prior to testing.

2.2. Muscle stimulation protocol

Mice were anesthetized, weighed and placed prone onto a platform with the left hindlimb hanging freely over the side. The sciatic nerve was stimulated posterior to the knee via subcutaneous needle electrodes (Harvard Apparatus 723742, Cambridge, MA). The stimulating electrode was positioned proximal to the bifurcation of the sciatic nerve, thus contractions occurred in all compartments of the leg. Proper electrode position was confirmed by palpating the tibialis anterior (TA) during a series of 1 m twitches and by observing plantarflexion at the ankle joint. This protocol [11] elicits an overall effect of plantar flexion due to the overriding force of the gastrocnemius, soleus, and plantaris. The net result is a lengthening contraction of the TA muscle, the primary dorisiflexor. The right limb was not stimulated and used as an internal control limb. Muscle contractions were elicited by stimulating the nerve at 100 Hz for 3 s followed by 10 s rest for 6 repetitions. Following the 6th repetition was a 30 s rest. The foot was returned passively to the resting position at the end of each contraction in order to ensure plantarflexion was occurring throughout the protocol. The total protocol consisted of 10 sets for a total exercise time of 22 min [11].

2.3. Muscle collection and processing

Mice were sacrificed either directly following (0 h) or 3 h after the completion of the stimulation protocol. For the 3 h cohort, following the completion of the contraction protocol, the animals were returned to their cage and after 3 h animals were anesthetized again for removal of the tissue. TA muscles were quickly removed from anesthetized animals, blotted, weighed, and immediately frozen in liquid nitrogen. TA muscles were homogenized on ice in buffer containing 50 mM HEPES (pH 7.4), 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7{\cdot}H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, protease inhibitor cocktail (Roche) and 5 mM Na_3VO_4 as previously described [12,13]. Protein concentration was determined by BCA protein assay (Pierce, Rockford, IL).

2.4. Immunoprecipitation

Five hundred micrograms of total protein was incubated with insulin related substrate-1 (IRS-1) antibody (1:50) with gentle rocking at 4 °C overnight. Protein A-Sepharose gel beads (Sigma) were used to precipitate IRS-1 complexes by incubating the washed beads with the IRS-1 complexes for 3 h at 4 °C. Samples were centrifuged, re-suspended in loading buffer, boiled for 5 min and processed as described below.

2.5. SDS–PAGE, Western blotting, and immunodetection

Samples were solubilized in loading buffer and boiled for 5 min. Hundred microgram of total protein was loaded into SDS–PAGE gels and transferred onto PVDF membranes (Millipore, Billerica, MA). To confirm transfer of proteins and equal loading of lanes, membranes were stained with Ponceau S. After successful transfer of proteins, membranes were blocked for 1 h at room temperature and incubated with primary antibody in dilution buffer (BSA in TBS-T) overnight at 4 °C. Membranes were then incubated with a secondary antibody, washed and then incubated with enhanced chemiluminescence reagent (Pierce, Rockford, IL). Exposures were attained to keep the integrated optical densities (IODs) within the linear and non-saturated range. IODs were quantified using Image J software (NIH, Version 1.42).

2.6. Antibodies

The primary antibodies Ser^{473} – Akt, Akt, Thr^{389} –p70S6 kinase ($p70^{S6K}$, 1:500), $p70^{S6K}$ (1:500), Thr^{246} -PRAS40, PRAS40, Ser^{307} -IRS-1, $Ser^{636/639}$ -IRS-1, IRS-1 (1:500), PI3 Kinase p85 were purchased from Cell Signaling Technologies Inc. (Beverly, MA). All were used at 1:1000 unless indicated. Antibody specificity was verified by molecular weight and positive controls where possible.

2.7. Statistics

Data are means ± S.E.M. All protein analyses are expressed relative to the unstimulated (right) control limb from the WT animals. *T*-tests were used to analyze differences between body and skeletal muscle weight between WT and MKR mice. One way analysis of variance with Dunnett's post hoc tests was used to determine differences from the control. A *P*-value of <0.05 was considered statistically significant.

3. Results

To determine if a functional IGF-I receptor is obligatory for the activation of the Akt/mTOR signaling pathway, we utilized a transgenic mouse that expresses a mutated IGF-I receptor specifically in skeletal muscle. Demonstration of the dysfunctional IGF-I and insulin receptors in these mice via recombinant IGF-I and insulin injections has been previously described [13].

Total body weight was not significantly different between WT and MKR age-matched mice for the 3 h time point ($P > 0.05$), however, body weight and TA weight were greater in the WT compared to the MKR mice for the 0h time point (32.2 ± 0.6 g vs. 28.6 ± 1.1 g, $P < 0.05$ and 0.051 ± 0.001 g vs. 0.035 ± 0.002 g, $P < 0.05$, respectively). In addition, at the 3 h time point, WT TA weight was significantly greater compared to the MKR mice (0.052 ± 0.005 g vs 0.039 ± 0.002 g, $P < 0.05$, respectively). These findings are similar to previous reports showing slightly decreased muscle and body weights in adult MKR mice [9,13].

Directly following the stimulation protocol (0 h), we observed a small but significant increase in $p70^{S6k}$ phosphorylation (Thr-389) in the WT stimulated limb compared with the control limb. At 3 h following the stimulation protocol, there was a robust increase in $p70^{S6k}$ phosphorylation (Thr-389) in the WT stimulated limb compared with the control limb and a significant increase in $p70^{S6k}$ phosphorylation (Thr-389) in the stimulated TA from MKR (Fig. 1A and B).

Immunoprecipitation experiments were conducted to investigate contraction-induced activation of the hypothesized downstream components of the IGF-I signaling pathway. Despite detection of insulin receptor substrate (IRS-1) in all of the conditions, there was no detectable co-immunoprecipitation of IRS-1 with p85 (regulatory subunit of PI3-K), and no IRS-1 phosphorylation at Ser-307 or Ser-636/639 (Fig. 2).

Increased Akt phosphorylation (Ser-437) was observed at 0 h in the stimulated limb of MKR mice relative to the MKR control muscle. By 3 h this difference in Akt phosphorylation in the stimulated limb of MKR mice was not apparent (Fig. 3A and B). Interestingly, no changes in Akt phosphorylation were detected in WT mice with the exercise protocol at either time point.

PRAS40 has been identified as a protein that inhibits downstream mTOR signaling [14]. Akt can alleviate the inhibition of

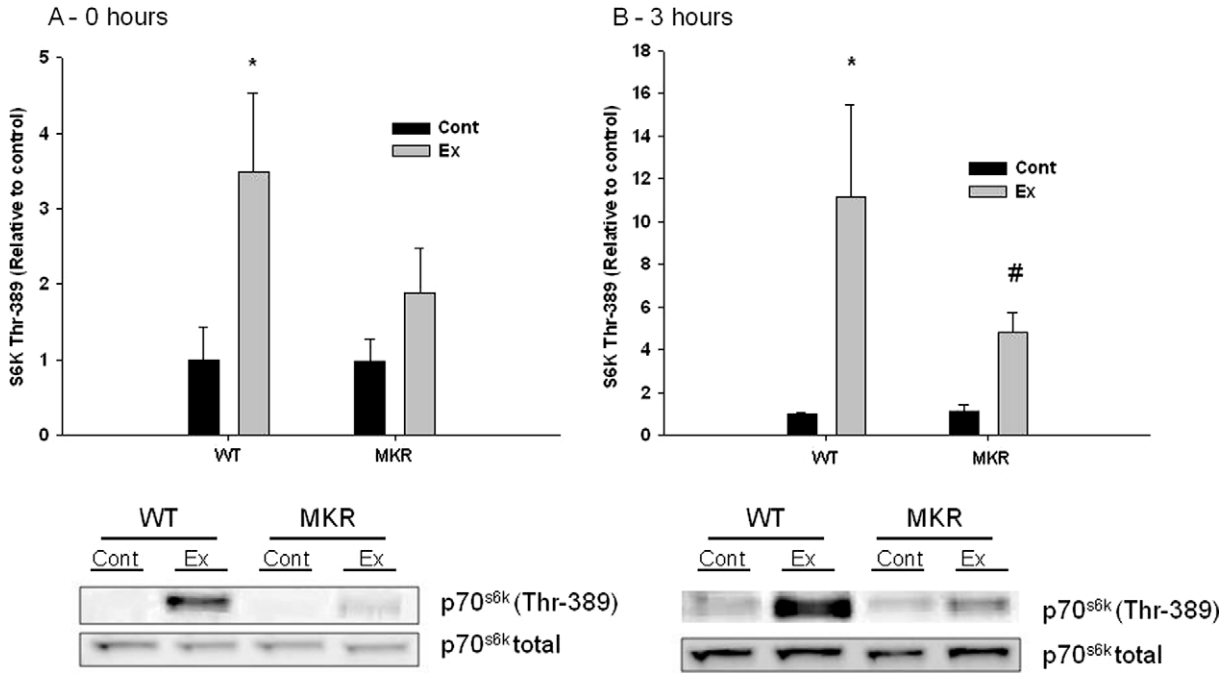

Fig. 1. (A and B). Effects of an acute bout of stimulated muscle contractions in WT and MKR mice on $p70^{S6k}$ phosphorylation (Thr-389) immediately after (A) and 3 h following (B) muscle contractions. Immediately following (0 h) muscle contractions, a significant increase in $p70^{S6k}$ phosphorylation was observed in the exercised (Ex) tibialis anterior (TA) compared to the contralateral control (Cont) TA of WT mice. Three hour following muscle contractions, significant increases were observed in $p70^{S6k}$ phosphorylation in the Ex TA of both WT and MKR mice. Data expressed as fold differences from unstimulated control limb. (* different from WT-Cont, # different from MKR-Cont, $P < 0.05$).

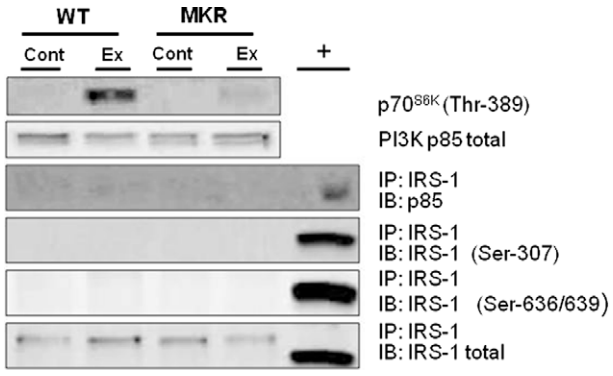

Fig. 2. IGF-I receptor signaling in WT and MKR mice with mechanical stimulation. There were no differences in co-localization of p85 with IRS-1 for WT or MKR mice in the Ex or Cont limb and no differences in the phosphorylation of IRS-1 (Ser-307, Ser-636/639), despite activation of $p70^{S6k}$. No quantification is presented due to a lack of detection of any activation of any of the targets. Data collected in samples 3 h following stimulated muscle contractions. (+) = refers to protein lysates from parameterial adipose tissue in WT mice.

mTOR via phosphorylation of PRAS40 on Thr-246 [15]. Therefore, we investigated PRAS40 phosphorylation (Thr-246) as a potential contributor to mTOR signaling with acute muscle stimulation. Phosphorylation of PRAS40 (Thr-246) was decreased in the WT stimulated limb compared to control at 0 h. At 3 h there were no differences between PRAS40 phosphorylation in WT exercise and control limb (Fig. 4A and B). No changes in PRAS40 were observed in MKR mice.

4. Discussion

Our primary finding is that one bout of high frequency muscle contractions increased $p70^{S6k}$ phosphorylation without evidence of activation of specific IGF-I-sensitive signaling proteins. In addition, we observed this result despite the presence or absence of a functional IGF-I receptor.

Activation of $p70^{S6k}$ has been related to hypertrophy in cardiac myocytes [16,17], and increases in protein synthesis in cultured fibroblasts [18]. In skeletal muscle, increased $p70^{S6k}$ phosphorylation occurs in rats following an exercise protocol identical to the current study and is significantly correlated to increases in muscle mass [11]. Significant increases in $p70^{S6k}$ phosphorylation have also been shown after seven days of chronic loading via functional overload with no differences in $p70^{S6k}$ phosphorylation between WT and MKR mice [13]. In the present study, we demonstrated that $p70^{S6k}$ phosphorylation occurs in WT and MKR mice with acute exercise however, $p70^{S6k}$ phosphorylation was not observed at 0 h in MKR mice and the increase in MKR mice was attenuated compared with WT mice at 3 h. Therefore, the ability to increase cellular signals that initiate protein synthesis is present in mice without a functional IGF-I receptor, albeit the response was delayed and attenuated. It is not clear why the attenuation occurred, but it is possible that it is a result of the known metabolic defects that occur in the MKR. For example, the MKR mice exhibit elevations in resting blood glucose and serum free fatty acids [9]. The animals also respond poorly to glucose challenges and are proposed to be a model of type 2 diabetes [9], thus it is possible that reduced levels of $p70^{s6k}$ phosphorylation in response to the mechanical load is a result of the metabolic dysfunction. Indeed, recent findings indicate that contraction-induced $p70^{s6k}$ phosphorylation is attenuated in rodent models that develop obesity and/or insulin resistance [19]. MKR mice exhibit elevations in circulating free fatty acids and thus it is possible that the elevation in FFA exposure is affecting mTOR signaling by altering substrate utilization. Nonetheless, contractions were still able to induce significant increases in $p70^{s6k}$ phosphorylation even when IGF-I signaling is ablated in muscle.

Despite the presence or absence of a functional IGF-I receptor, the acute stimulation protocol did not elicit activation of the immediate signaling proteins downstream of the IGF-I receptor.

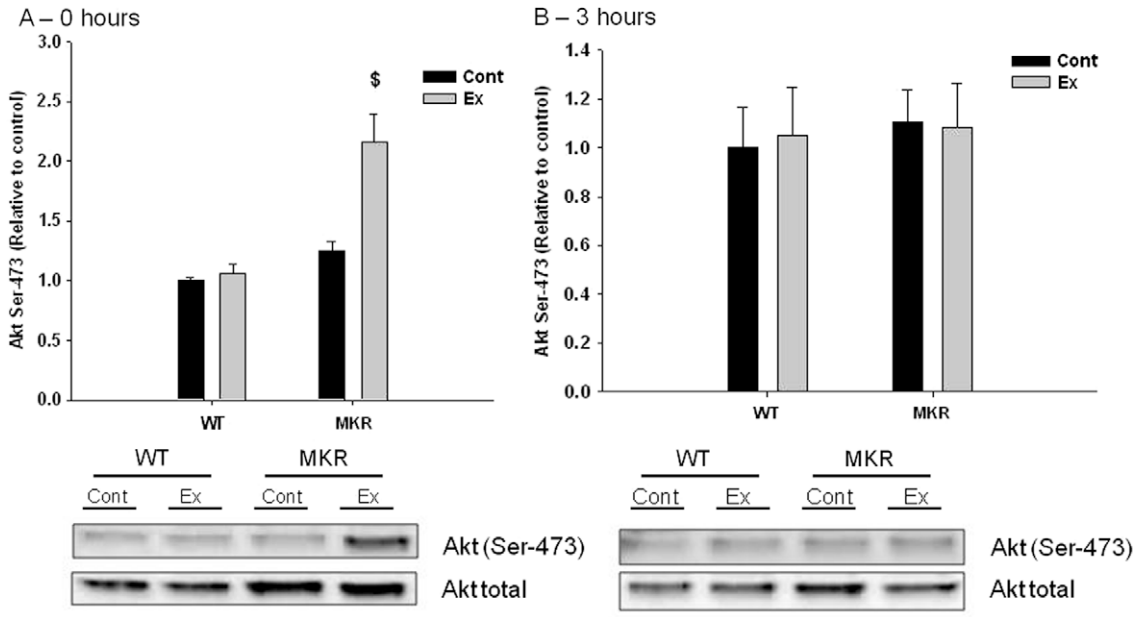

Fig. 3. (A and B). Effects of an acute bout of stimulated muscle contractions in WT and MKR mice on Akt phosphorylation (Ser-473) immediately after (A) and 3 h following (B) muscle contractions. Immediately following (0 h) muscle contractions, a significant increase in Akt phosphorylation was observed in the exercised (Ex) tibialis anterior (TA) of MKR mice only. No differences were observed 3 h following muscle contractions. Data expressed as fold differences from unstimulated control limb (WT Cont). ($ different from all groups, $P < 0.05$).

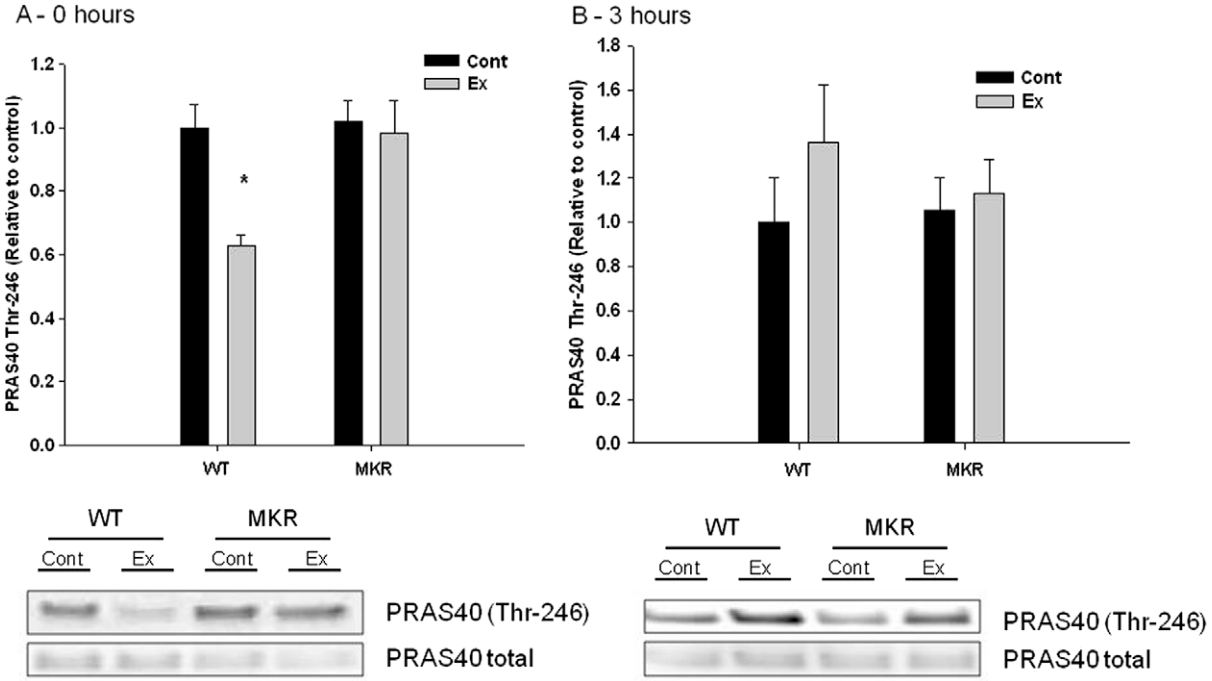

Fig. 4. (A and B). Effects of an acute bout of stimulated muscle contractions in WT and MKR mice on PRAS40 phosphorylation (Thr-246) immediately after (A) and 3 h following (B) muscle contractions. Immediately following (0 h) muscle contractions, a significant decrease in PRAS40 phosphorylation was observed in the exercised (Ex) tibialis anterior (TA) of WT mice. No differences were observed 3 h following muscle contractions. Data expressed as fold differences from unstimulated control limb (WT Cont). (* different from WT control, $P < 0.05$).

There is considerable controversy regarding the importance of endogenous IGF-I for load-induced muscle hypertrophy [1]. Many of the current hypotheses over the role of endogenous IGF-I are based upon the ability of exogenous IGF-I to induce hypertrophy [20]. Furthermore, exogenous IGF-I increases protein synthesis and content in muscle through activation of PI3-K-Akt mediated signaling [7]. Exposure of muscle cells to PI3K inhibitors, wortmannin or LY294002, prevents any effect of IGF-I on Akt signaling [21]. Thus, if IGF-I is critical for load-induced hypertrophy, we should see evidence of PI3-K-Akt activation in response to mechanical load. In order for PI3-K to be activated by IGF-I, it should functionally interact with IRS-1, however using immunoprecipitation, we found no evidence of PI3-K interaction with IRS-1 in response to load. This is in agreement with previous evidence, which indicated

that inhibition of PI3-K did not prevent stretch-induced activation of mTOR signaling [22]. We attempted to measure PLD1 phosphorylation at both time points and were unable to reliably detect any PLD1 phosphorylation under any condition. It is possible that the endogenous forms of phosphorylated PLD are expressed at such low levels that it is difficult to detect through standard Western blotting applications (Troy Hornberger, Ph.D. personal communication). In addition, recent data suggest that PLD1 may be regulated through direct interaction with Rheb [23]. Thus, it is clear that translation of mechanical load to mTOR signaling is complex and remains undefined.

Notably, we observed evidence of mTOR activation despite either an inability to activate the IGF-I receptor or an indication that IRS-1 is bound to the p85 subunit of PI3-K. Measuring Thr-389 phosphorylation of $p70^{s6k}$ is the most reliable indicator of mTOR activation in that mTOR activity does not appear to be regulated by alterations in phosphorylation status [1]. In addition, IGF-I is a known activator of serine 307 phosphorylation of IRS-1 in muscle [24], yet we found no evidence of any serine phosphorylation of IRS-1 at any time point after stimulation. Together with previous data showing that MKR exhibited a normal hypertrophic response to chronic functional overload [13], this suggests that activation IGF-I receptor signaling is not obligatory for load-induced activation of mTOR signaling. In fact, other groups have recently found evidence in humans that increases in exogenous in IGF-I do not contribute to significant increases in fractional protein synthesis in skeletal muscle [25,26].

Proline-rich Akt substrate 40 (PRAS40) has been identified as a target of Akt [27] that can modulate the activity of mTOR via mTOR complex 1 (mTORC1). Sancak et al. showed that PRAS40 binds with raptor and mTORC1 to suppress $p70^{S6k}$ phosphorylation, whereas insulin-stimulated Akt-induced phosphorylation of PRAS40 (Thr-246) released the inhibition of PRAS40 on mTORC1 to increase $p70^{S6k}$ phosphorylation [15]. Surprisingly, we found a decrease in PRAS40 phosphorylation (Thr-246) in the stimulated TA of WT mice immediately following the protocol which corresponded to an increase in $p70^{S6k}$ phosphorylation (Thr-389) indicating that mechanical stimulation may involve a PRAS40 influence on mTOR in manner that is different than seen with insulin. However, more studies need to be conducted to confirm or examine the role of PRAS40 in load-induced hypertrophy in a more detailed fashion.

In summary, our results indicate that one session of high frequency muscle contractions in mice can activate cellular signaling that initiates the protein synthesis independent of traditionally described IGF-I signaling. Future studies are required to further elucidate the mechanisms by which cells sense and translate the various signals elicited by exercise stimuli into functional adaptations.

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