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## mTOR Pathway Activation Following Sciatic Stimulation in Wild-Type and Desmin Knockout Mice

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mTOR Pathway Activation Following Sciatic Stimulation in Wild-Type  
and Desmin Knockout Mice

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A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Master of Science

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## ABSTRACT

### mTOR Pathway Activation Following Sciatic Stimulation in Wild-Type and Desmin Knockout Mice

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The 52 kDa intermediate filament protein desmin plays an important role in force transmission in skeletal muscle by connecting myofibrils at Z-lines and to the sarcolemma. Desmin content in muscle adapts to contractile activity and may be involved in cellular signaling mechanisms responsible for muscle growth. Purpose: To compare signaling responses of the mTOR pathway in wild type (WT) vs desmin knock out (KO) mice. Methods: WT (n=12) and KO (n=12) mice were exposed to high frequency electric stimulation of the left hindlimb to elicit an acute response of the mTOR pathway. Non-stimulated right hindlimbs were used as a within animal control. Right and left TA and EDL muscles were dissected 30 min post-stimulation and examined for changes in mTOR, 4E-BP1 and p70S6K. Results: Relative to WT control samples, total mTOR and total 4E-BP1 content was higher in KO control samples. Electrical stimulation resulted in an increase p70S6K phosphorylation in WT and KO animals however there was no difference between the groups. 4E-BP1 phosphorylation was increased in WT but not KO following electrical stimulation. There was no change in mTOR phosphorylation in response to stimulation in WT or KO. Conclusion: The absence of desmin in skeletal muscle does not impair the phosphorylation of p70S6K demonstrating that a tensile load on the muscle will likely result in an increase in protein synthesis. Elevated levels of total mTOR and 4E-BP1 may imply an adaptation to increase sensitivity to growth stimuli in the muscle.

Keywords: desmin, knockout, mTOR, electrical stimulation, 4E-BP1, p70S6k

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## Introduction

Skeletal muscle contractile proteins are organized to effectively perform the function of producing tension and are associated with a highly organized cytoskeletal protein network inside the cell to facilitate the transmission of forces. The 52kD cytoskeletal protein desmin plays an important role in lateral force transmission (35). By connecting adjacent myofibrils at the Z-lines, desmin provides the structure necessary for laterally transmitting the forces produced by the contractile proteins out to the sarcolemma of the muscle fiber (7, 12, 13). The presence of desmin and its role in lateral transmission of force has been shown to influence force production in skeletal muscle (7, 35, 54) may play a role in cellular signaling mechanisms responsible for muscle growth and adaptation.

Desmin content in skeletal muscle altered loading conditions. In particular, high-tension contractions have been shown to elicit increases in desmin content in humans and animals (38, 41, 55, 56). This increase in desmin content occurs in conjunction with increases in the maximum force production of muscle tissue (55). In contrast, some have shown that mice lacking desmin in skeletal muscle have smaller muscles and altered muscle contractile characteristics (1, 7, 13, 26, 37, 40, 44, 47, 54).

Muscle mass is a function of the degree of chronic external loading (6). Cellular signaling pathways respond to external stimuli by increasing or decreasing rates of muscle protein synthesis (8, 9). The mammalian target of rapamycin (mTOR) pathway plays an important role in promoting this increase in protein synthesis (8, 9). Down stream targets of the mTOR pathway, p70S6k and 4E-BP1, can be measured to assess the acute hypertrophic response to a tensile overload (8, 9). The complete process of transmitting a tension signal to the

cytoplasm where mTOR is activated has not been completely elucidated. It is likely that the muscle cytoskeleton plays a role in this signaling process.

The purpose of this study is to determine if the structural protein desmin plays a role in transmitting the tension signal and activating cellular mechanisms associated with muscle growth. By measuring activation levels of the downstream components of the mTOR pathway (4E-BP1 and p70S6k) when desmin is absent then comparing them to the activity levels of muscles with desmin present, we will determine if desmin influences the hypertrophy response. We hypothesize that desmin does play a role in signaling muscle hypertrophy and that muscles lacking desmin will show decreased hypertrophic signaling responses to a given stimulus.

## **Methods**

### *Experimental Design*

This study exposed 12 wild type mice (mean body weight 19.9g SD  $\pm$ 2.57g) and 12 desmin knockout mice (mean body weight 21.8g SD  $\pm$ 1.96g) to high frequency electrical stimulation of the sciatic nerve to elicit a hypertrophy signal in the muscles of the hind limb. Following electrical stimulation the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles were removed together, homogenized, and probed using immunoblotting to determine phosphorylation levels of the following three proteins: mTOR, p70S6k, and 4E-BP1.

### *Animals*

24 young (3-4months) FVB albino strain mice from a mouse colony maintained at Brigham Young University were used in this study. The experimental groups included a knockout (KO) group comprised of 12 transgenically altered mice possessing an inoperable form

of the gene for the protein desmin, and a wild-type (WT) group of 12 control mice. All animals were bred and cared for in accordance with Brigham Young University Animal Care Facility and IACUC guidelines.

Ear punches were collected for the genotyping procedure and as an identification marker for individual animals. Prior to ear punches animals were anesthetized in an enclosed chamber using isoflurane dispensed at a rate of 2.5 l/min. After becoming unconscious the mice were transferred to the adjacent workbench surface with the animal muzzle placed inside an isoflurane dispensing breathing tube. A sterile ear punch instrument was then used to punch a circular hole in the pre-determined location on the mouse ear. Tissue collected by the ear punches was digested using DNA X-tract processing kit produced by Sigma Biochemical laboratories and processed according to the directions specified. Samples were first digested using the digestion mix then incubated for 3 minutes in a 95° C heat block to stop the digestion process. A Stop solution was then added, leaving the samples ready to refrigerate or immediately run through the PCR protocol. Samples were then prepared for PCR by adding the appropriate solution from the DNA X-tract mixture along with the following primers; (Invitrogen, Life Technologies, Grand Island, NY, USA) WT forward (TCCTTCGTGCTTACGGTATC), WT reverse (TGATGTCAGGAGGGCTACA), and KO reverse (TCCTTCGTGCTTTACGGTATC). Samples were then loaded into the thermal cycler and run at 95° C for 2:30, then 37 cycles of 0:35 @ 94° C, 1:00 @ 57° C, then 1:00 @ 72° C, to be followed by a single 6:00 cycle at 72° C. 16 µl of sample were then loaded into a 2% agarose (5 µl ethidium bromide added per 100 ml gel) horizontal electrophoresis gel and run at 100 volts for 60 minutes. Bands appearing at 720 base pairs (bp) indicated the presence of the functional form of the desmin gene, and bands appearing at 300 bp indicated the presence of the inoperable form of the desmin gene.

### *High Frequency Electrical Stimulation*

After determining the genotype of the mice, they were exposed to high frequency electric stimulation (HFES). The HFES protocol used was designed to create a sustained tetanic eccentric contraction (3 seconds) of the extensor muscles that has previously been shown to elicit a hypertrophy response (51). Mice were anesthetized before the sciatic nerve was surgically exposed. Total body weight of each animal was measured. The sciatic nerve was then exposed by first shaving the hair off of the upper limb of the left leg of the animal. A 1 cm incision running parallel to the femur was made in the skin just below the hip joint exposing the sciatic nerve, allowing the electrode tip to be inserted into the incision and contacting the sciatic nerve. The nerve was then stimulated causing tetanic contractions of all lower limb muscles using high frequency electric current (100 Hz; Grass Model S48 Stimulator, Quincy, MA) resulting in 10 sets of six maximal eccentric contractions of the extensor muscles (3 second duration with a 10 second rest between contractions) as utilized by Thomson *et al.* (51). The incision was then stapled shut until 30 minutes post stimulation when EDL and TA muscles were harvested.

### *Tissue Collection*

Dissected tissue was flash frozen using liquid nitrogen cooled tongs, then wrapped in foil and stored at -80°C until analysis. After samples were collected mice were euthanized by surgically opening the chest cavity and cutting open the heart.

### *Total Protein Analysis*

To increase sample volume, TA and EDL muscles were combined and homogenized together and will be referred to as muscle sample hereafter. Muscle sample homogenization was performed using cold round-glass homogenization tubes in a 19:1 homogenization buffer [50 mM Tris-HCl, (pH 7.4), 250 mM mannitol, 50 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM

EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM  $\beta$ -glycerophosphate, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM benzamidine, 0.1 mM phenylmethane sulfonyl fluoride, 5  $\mu$ g/ml soybean trypsin inhibitor] then cycled through three freeze-thaw cycles to break open the cells before being centrifuged at 4°C at 16,000 g for 10 minutes. The supernatant was then transferred to fresh tubes and frozen at -80°C to be stored until analysis. Total protein analysis was then performed using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Absorbance was measured at a wavelength of 750 nm using light spectroscopy (VICTOR3, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) and compared to known standards to determine the total protein content of each sample. Appropriate volumes of loading buffer were then added to each sample to obtain a final total protein concentration of 1  $\mu$ g/ $\mu$ l.

### *Western Blotting*

For immunoblot analysis a 20  $\mu$ g sample of protein was loaded along with an equal volume of loading buffer [125 mM Tris (pH 6.8), 4% SDS (sodium dodecyl polyacrylamide), 20% glycerol, 5%  $\beta$ -mercaptoethanol (BME), 0.01% bromophenol blue] into an SDS mini gel and run in an electrophoresis unit (Mini Protean II; Bio-Rad Laboratories, Hercules, CA) at 200 V held constant for 50 minutes. A molecular weight ladder (Precision Plus Protein Standard, Bio-Rad Laboratories, Hercules, CA) was also run with each gel to confirm the location of the protein of interest being detected on each blot. For each protein being detected a specific concentration of polyacrylamide mini gel was utilized to obtain optimal resolution as follows: mTOR 5%, p70S6k 7.5%, and 4E-BP1 15%. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane for 120 minutes at 350mA constant current. Membranes were then blocked in a 5% milk solution (diluted in Tris-Buffered saline with 0.05% Tween20, (TBST)) for 1 hour at room temperature with gentle rocking, then rinsed in TBST solution 4 times for 5

minutes at room temperature. The membranes were then incubated with primary antibody (Cell Signaling, Boston, MA, USA) diluted to the following concentrations with 5% bovine serum albumin (BSA) solution; total mTOR 1:1000 (#2983), phospho-mTOR (Ser2448) 1:500 (#2971), total p70S6k 1:1000 (#9202), phospho-p70S6k (Thr389) 1:500 (#9205), total 4E-BP1 1:1000 (#9644), phospho-4E-BP1 (Thr37/46) 1:500 (#9459) overnight with gentle rocking at 4°C. The following morning, membranes were rinsed 4 times for 5 minutes in TBST at room temperature followed by a 1-hour incubation in secondary antibody (goat anti-rabbit IgG @ 1:2,000 concentration, Cell Signaling, Boston, MA, USA) also at room temperature with gentle rocking. 4 more 5-minute rinses in BST followed at room temperature prior to being exposed to a chemiluminescent solution (Immun-Star WesternC Chemiluminescence, Bio-Rad, Hercules, CA, USA). After a 5-minute exposure, blots were placed inside an enclosed camera box and photographed with a digital CCD camera (ChemiDoc XRS) imaging system and analyzed with Quantity One 1-D Analysis Software (Version 4.6.5, Bio-Rad, Hercules, CA, USA).

### *Statistical Analyses*

Based on a power analysis, 12 animals per group was determined to be sufficient to detect significant differences groups. Means and standard deviations for this calculation were obtained from previously published data from our lab (17). The statistical model consisted of genotype, stimulation, and the genotype by stimulation interaction. Two measures of each dependent (mTOR, p70S6k, and 4E-BP1) were taken from each mouse, one from the stimulated muscle and one from the non-stimulated muscle. The other independent variable of interest was the genotype of the mouse, wild type or knockout. These data were analyzed using ANOVA. T-tests were performed to compare the body mass and muscle mass of animals in each group. An

alpha level of  $p \leq 0.05$  was used to indicate statistical significance. Data are presented as mean  $\pm$  SD.

## Results

### *Animals*

Mean body weight of the animals was  $19.9 \pm 2.7$ g for the WT group and  $21.8 \pm 2.0$ g for the KO group with no statistical difference between them ( $p > 0.05$ ). The excised muscle, which included the TA and EDL complex, had an average weight of  $42.2 \pm 7.4$ mg in the WT animals and  $42.3 \pm 10.8$ mg in the KO animals, also with no statistical difference between them ( $p > 0.05$ ).

### *Signaling*

Total mTOR content was  $80.0 \pm 26.4\%$  higher ( $p < 0.05$ ) in the KO animal control limbs than WT control limbs and  $50 \pm 20.8\%$  higher ( $p < 0.05$ ) in the stimulated limbs of the KO animals compared to the stimulated limbs of the WT group (Figure 1). No significant changes were detected in either the WT or KO mice with regard to phosphorylated levels of mTOR normalized to total mTOR between electrically stimulated limbs and control limbs (Figure 2). Non-normalized phosphorylated levels of mTOR were  $2.08 \pm 0.57$  fold ( $p < 0.001$ ) higher in the stimulated KO animal limbs compared to the stimulated WT limbs (Figure 3).

Total 4E-BP1 was also observed to be higher in KO mice than WT,  $78.5 \pm 12.0\%$  ( $p < 0.01$ ) higher in control limbs, and  $90.9 \pm 23.2\%$  higher in stimulated limbs (Figure 4). Phosphorylated levels of 4E-BP1 normalized to total 4E-BP1 content increased  $29.9 \pm 12.0\%$  ( $p < 0.05$ ) in WT animals with electrical stimulation but not in KO animals (Figure 5). Non-normalized phosphorylated 4E-BP1 was  $28.0 \pm 24.7\%$  greater ( $p < 0.05$ ) in the KO control limbs than WT control limbs (Figure 6).

No difference was observed in the total p70S6k content between WT and KO control limbs (Figure 7). The electrically stimulated limbs of WT and KO groups showed significant increases in phosphorylated p70S6k levels when normalized to total p70S6k; WT animals increased  $10.2 \pm 4.3$  fold ( $p < 0.05$ ) while the KO animals increased  $11.6 \pm 11.7$  fold ( $p < 0.01$ ). No significant difference was detected between WT and KO groups (Figure 8). Non-normalized p70S6k phosphorylation levels increased  $12.3 \pm 10.1$  fold ( $p < 0.01$ ) in WT animals, and  $12.8 \pm 14.0$  fold ( $p < 0.05$ ) in KO animals when stimulated. No significant differences existed between the controlled baseline levels and stimulated levels in WT and KO (Figure 9).

## Discussion

Activation of mTOR in skeletal muscle cells increases phosphorylation of downstream targets 4E-BP1 and p70S6K resulting in increased protein synthesis (3, 5, 9, 10). mTOR pathway activation occurs in response to increased mechanical loading, mitogenic growth factors, and feeding (19, 25, 31, 43). Desmin is a structural protein found in the cytoskeleton of skeletal muscle and plays a role in lateral force transmission and may contribute to force production (7, 37, 44, 54) and is mechanically associated with mitochondrial structures and nuclei (50). Because of desmin's role in the transmission of forces in the muscle we hypothesized that desmin may play a role in activation of the mTOR pathway (32, 42). Increased tension in a muscle fiber results in elevated protein synthesis rates (30). Evidence suggests that mechanical transmission of the tension may influence signaling pathways and protein synthesis responses in skeletal muscle (34). Desmin is important in the transmission of mechanical loads through the cytoskeleton (46) and therefore is a likely candidate in the process of mechanotransduction and adaptation.

In the current study we used high frequency electrical stimulation (HFES) to elicit an acute mechanical overload response in the extensor muscles of the lower hind limbs in mice to determine if the absence of desmin influenced the activation of the mTOR signaling pathway. We report that in muscle lacking desmin there is an increase in mTOR and 4E-BP1 total protein content and that electrically stimulated muscle contractions result in elevations in p70S6K phosphorylation similar to that of normal muscles.

p70S6k is involved in the initiation of mRNA translation (27). Resistance exercise increases p70S6k phosphorylation levels (5), followed by increased protein synthesis due to greater ribosomal efficiency (33). Blocking p70S6k phosphorylation through the inhibition of the mTOR complex (9) or genetic disruption of the p70S6k protein (49) results in decreased rates of protein synthesis and muscle size.

In the current study HFES resulted in increased p70S6k phosphorylation, which is consistent with previous research (3, 5, 9, 51). Phosphorylation of p70S6k increased in both WT and KO mice. When phosphorylated, p70S6k can act on its target, ribosomal protein S6 (rpS6), a component of the 40S subunit of the ribosome, which promotes translation initiation, resulting in increased protein synthesis (27). Since there was no difference in the magnitude of p70S6k phosphorylation levels between WT and KO, we conclude that p70S6k activation is not effected by the absence of desmin.

Hornberger *et al.* has shown that different types of mechanical forces activate different pathways in skeletal muscle fibers (30). Specifically, only multi-axial stretch will elicit a response and activate p70S6k phosphorylation, while both uniaxial and multi-axial stretch devices have been shown to result in the phosphorylation of the signaling protein Akt (29). In addition, administration of cytochalasin D, which disrupts the actin cytoskeleton, leads to

decreases in phosphorylation of p70S6k, but not Akt. This suggests that activation of protein synthesis may have other routes of stimulation, such as insulin or other mitogenic growth factors, which do not depend upon mechanical linkages or the Akt pathway. These other paths may compensate for or even obviate the need for mechanical signal transmission through the desmin network and explain the similar p70S6k phosphorylation response in KO and WT.

4E-BP1 is an inhibitor of the translational phase of protein synthesis (18, 23, 27). When phosphorylated, 4E-BP1 vacates its inhibitory binding position on eukaryotic initiation factor 4E (eIF4E), allowing the cap dependent initiation factor to function as an initiating component of a protein synthesizing ribosome (27). Due to its ability to regulate post-transcriptional activity, 4E-BP1 has been a well-studied target of mTOR and demonstrated itself to be a valid indicator of protein synthesis activation (9, 21-23).

In WT animals, significant increases were observed in 4E-BP1 phosphorylation while phosphorylation levels did not increase in KO animals. The observed increase in WT animals was anticipated as HFES has been shown to elicit an increase in the phosphorylation of mTOR and its downstream components (3, 51). 4E-BP1 has previously been shown to be phosphorylated by passive stretch protocol that generated tension in the cytoskeleton (4) which supports the idea that mechanotransduction may play a role in 4E-BP1 phosphorylation. Thus without desmin, an integral constituent of the cytoskeletal architecture and transmitter of lateral force transmission, the absence of 4E-BP1 phosphorylation following acute contractions suggest some influence of desmin but is in conflict with the increase in non-normalized phosphor-mTOR.

The mTOR pathway mediates cellular growth and proliferation and has been the target of multiple studies (8, 9, 15, 18, 20, 21, 28, 36, 45). mTOR became a primary target for cellular

growth research after cellular growth was attenuated in the presence of rapamycin, a known inhibitor of mTOR. When phosphorylated, mTOR and its downstream targets have been shown to up-regulate initiation factors that increase protein synthesis in a cell (9, 11, 14, 19).

In the present study mTOR phosphorylation was unchanged at 30 minutes following electrical stimulation. However, downstream factors 4E-BP1 and p70S6k were phosphorylated, indicating the current HFES protocol was effective in generating a hypertrophic signal in the muscle. Despite being an integral signaling protein in regulating hypertrophy, there are some discrepancies in the literature as to whether mTOR itself becomes detectably phosphorylated in response to hypertrophic stimuli. Shavlakadze *et al.* used an amino acid diet to increase phosphorylation levels of the upstream regulatory protein Akt as well as downstream p70S6k without observing any changes in phospho-mTOR (48). These findings are similar to ours and suggest that we may have utilized a muscle stimulation protocol that achieved detectable activation of p70S6k and 4E-BP1, but not mTOR.

Interestingly, total mTOR protein levels were found to be 80% higher in KO versus WT. While many cellular feedback systems exist to regulate the expression of specific proteins involved in cellular mechanisms, the exact components of the regulatory system governing mTOR content have not been elucidated. One study that examined prolonged rapamycin treatment in podocytes did report a decrease in total mTOR protein content (53) and another found LKB1 knock-out mice to have lower levels of total mTOR content (52), indicating that mTOR protein expression is influenced by cellular stresses. In the present study the absence of desmin may result in a muted tension signal in the muscle. To compensate for the reduced tension signal and to maintain normal downstream signaling activity and protein synthesis rates,

total mTOR may be up-regulated in the cell to increase overall sensitivity to any and all hypertrophy signals.

Similar to total mTOR content, we observed a 78.5% increase the total 4E-BP1 content of KO compared to WT mice. Total 4E-BP1, when active, is bound to eIF-4E, inhibiting protein synthesis (57). When phosphorylated by upstream signaling proteins, 4E-BP1 becomes inactive and disassociates from the eIF-4E complex, allowing eIF-4E to bind and form a complex with eIF-4G and thus promote protein synthesis via post-transcriptional mechanisms (24).

Experimental protocols that elicit changes in the activation levels of the hyper-phosphorylated form of 4E-BP1 are common, as much work has been performed to elucidate the signaling mechanisms involved in the mTOR pathway (2, 9, 16, 21-23, 51, 57). Limesand *et al.* observed a significant increase in total 4E-BP1 expression with fetal sheep exposed to hypoglycemia (39) and Thomson *et al.* showed LKB1 knock-out mice to also express higher levels of total 4E-BP1 (52). This confirms our finding that under a chronic stress, such as missing an integral component of the mechanical signal propagation system, total signaling proteins can increase, possibly to become more sensitive and create normal down stream effects despite the interruption of the mechanical signaling pathway. In theory, changes in 4E-BP1 levels would indicate changes in protein synthesis such as was observed in Thomson's study where LKB1 knock-out mice had lower body weight, however, Limesand's group observed no differences in protein synthesis with exposure to hypoglycemic conditions. Limesand's findings agree with our findings that despite higher 4E-BP1 levels in KO, overall body size did not vary compared to WT, suggesting similar muscle protein synthesis rates.

## Conclusion

In conclusion, we have demonstrated that in the absence of desmin, irregularities appear in content and activation of signaling proteins involved in the mTOR pathway. We suggest that an up-regulation of mTOR and 4E-BP1 compensate for a potential decrease in sensitivity to mechanical stress due to the absence of desmin. This up-regulation of total signaling protein content allows downstream signals such as p70S6k to maintain normal responses resulting in similar protein synthesis rates between WT and KO animals. Due to the alterations in signaling content and patterns of activation when desmin is gone, we conclude desmin indirectly influences the mTOR hypertrophy pathway.

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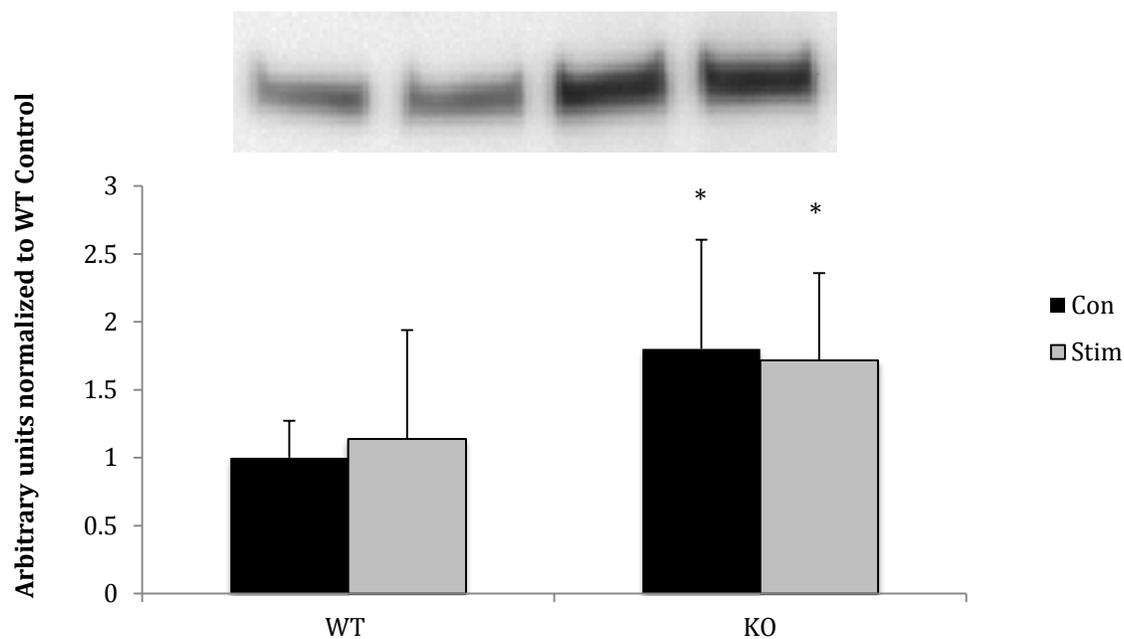
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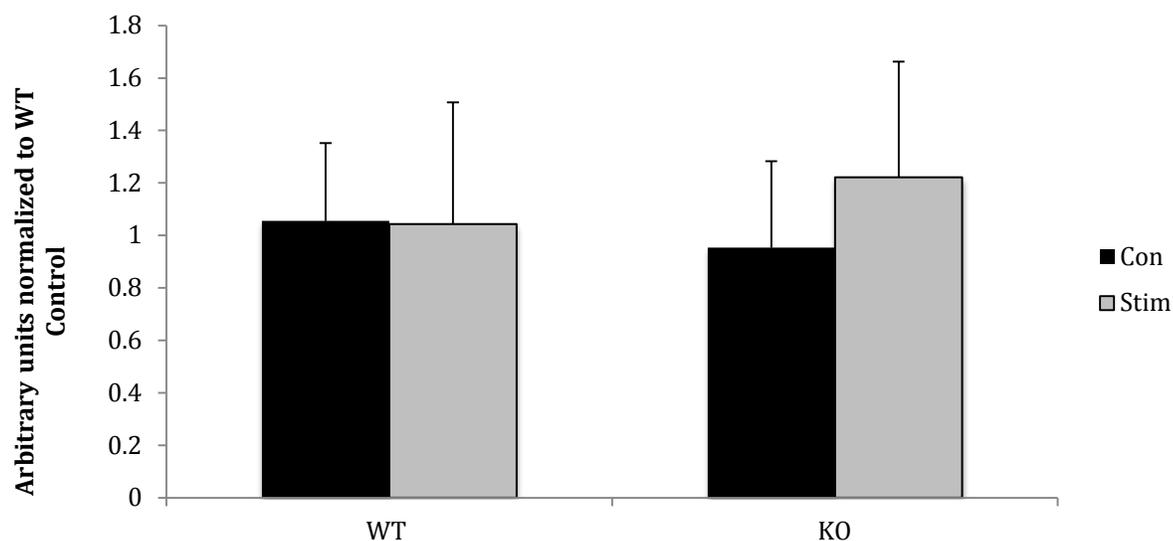
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## Figures



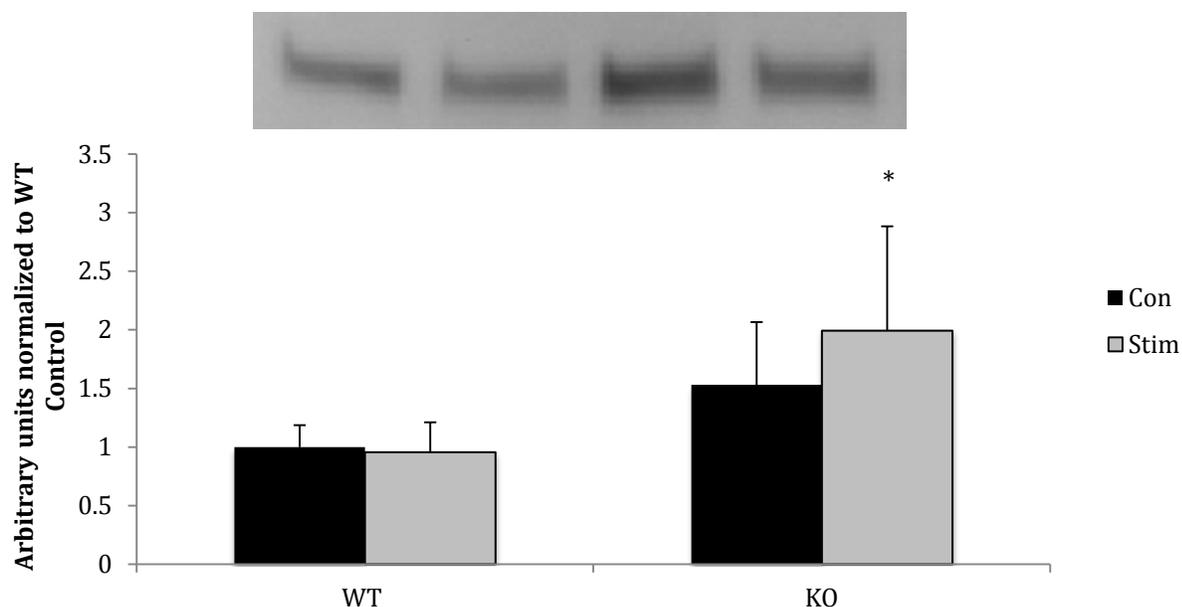
**Figure 1. Total mTOR content.**

Control values were  $80.0 \pm 26.4\%$  higher in KO than WT ( $p < 0.05$ ) and stimulated values were  $50.0 \pm 20.8\%$  higher in KO than WT ( $p < 0.05$ ) animals.



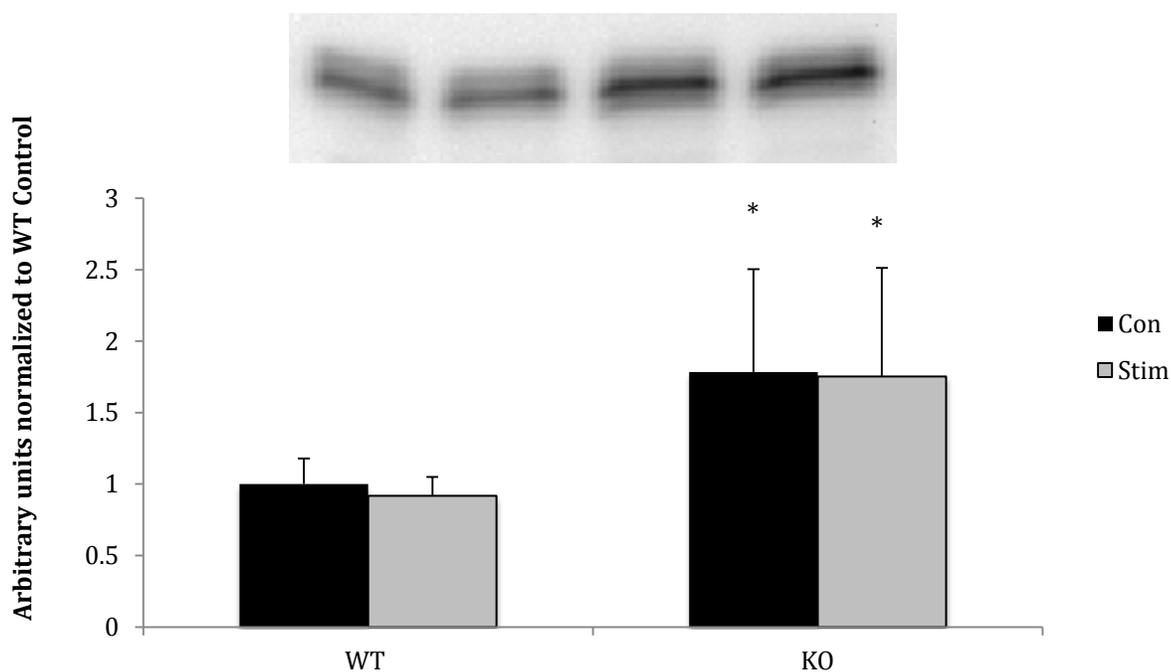
**Figure 2. Phosphorylated mTOR normalized to total mTOR content.**

No statistically significant changes were detected in phosphorylation levels of mTOR in either WT or KO groups with electrical stimulation of the lower limb.



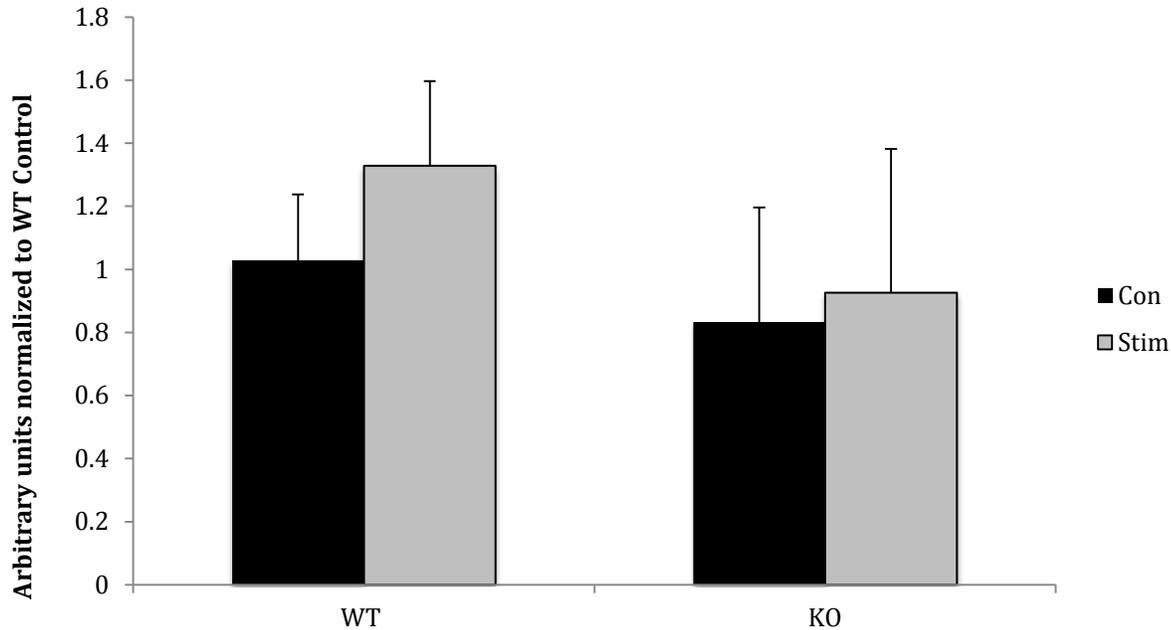
**Figure 3. Phosphorylated levels of mTOR.**

Phosphorylated mTOR was higher in the stimulated limbs of KO animals than the stimulated limbs of WT animals by  $2.08 \pm 0.57$  fold ( $p < 0.001$ ).



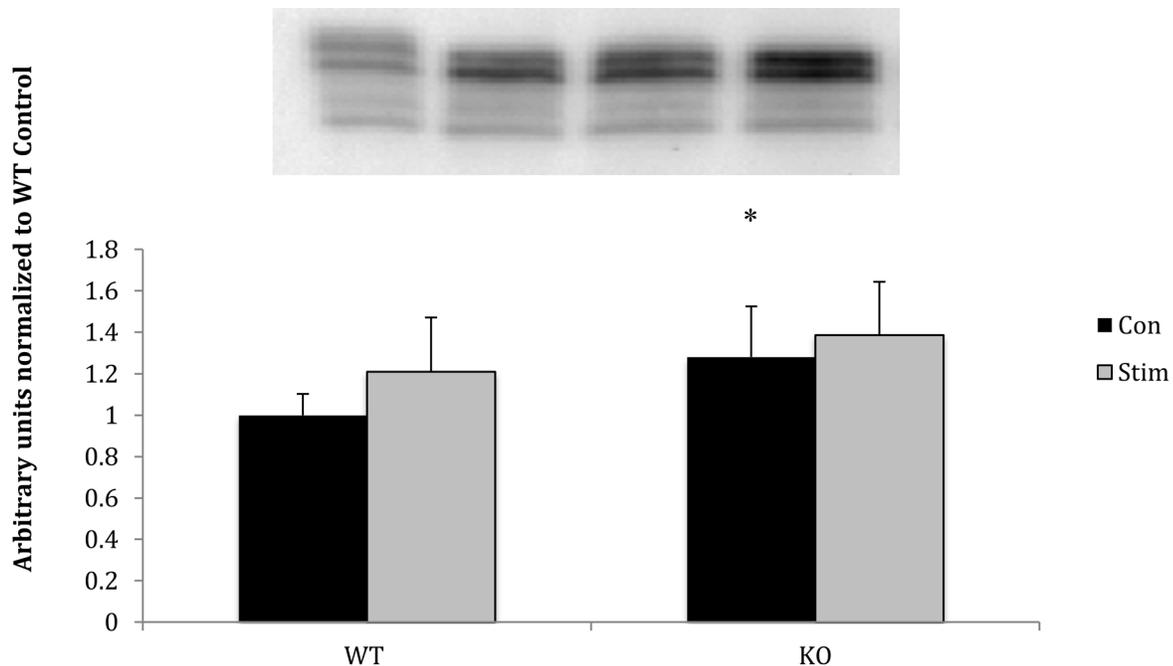
**Figure 4. Total 4E-BP1 content.**

Total 4E-BP1 in the control limbs was  $78.5 \pm 12.0\%$  higher ( $p < 0.05$ ) in KO than WT animals, while the KO stimulated limbs had  $90.0 \pm 23.2\%$  more ( $p < 0.01$ ) 4E-BP1 than the WT stimulated limbs.



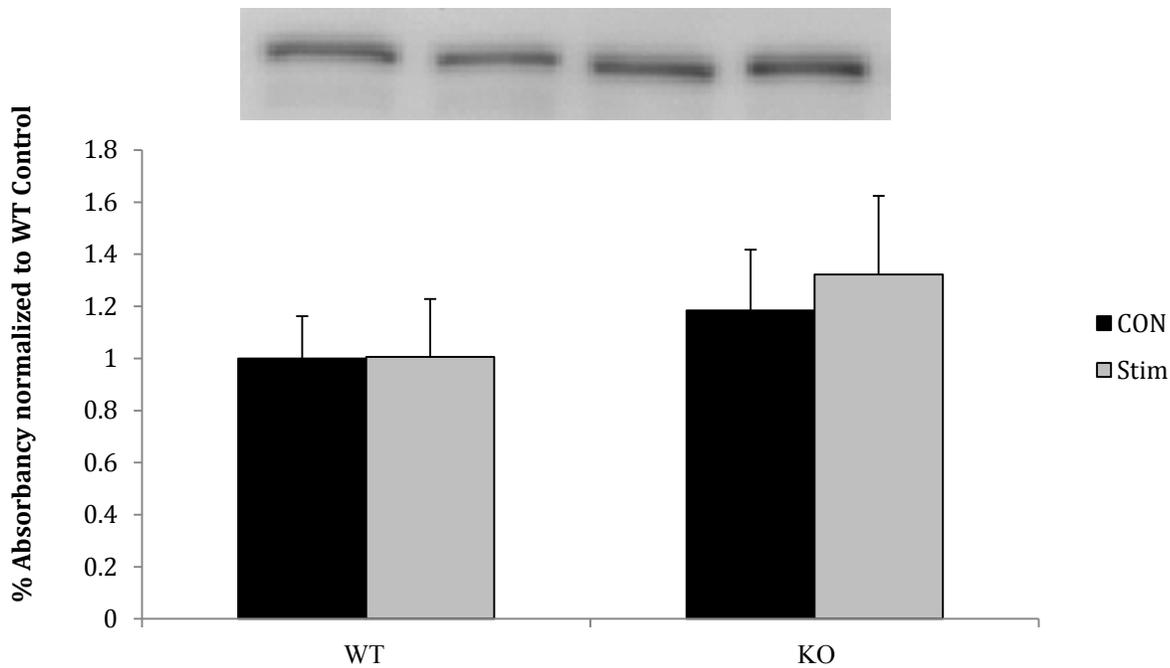
**Figure 5. Phosphorylated 4E-BP1 normalized to total 4E-BP1 content.**

Phosphorylated levels of 4E-BP1 trended toward an increase of  $29.9 \pm 12.0\%$  ( $p=0.06$ ) in WT animals with electrical stimulation.



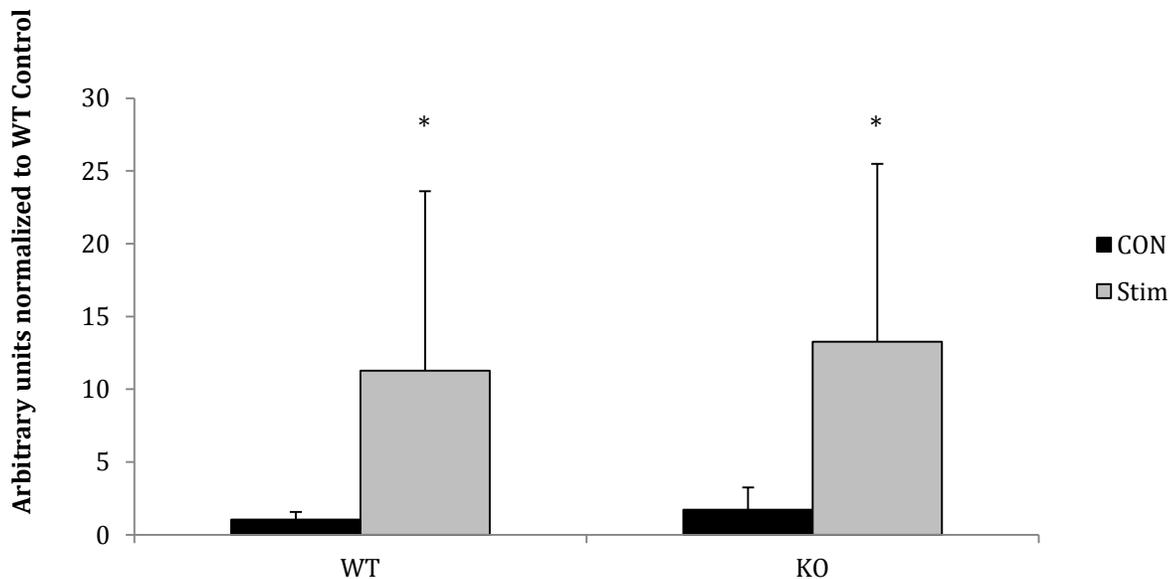
**Figure 6. Phosphorylated levels of 4E-BP1.**

Phosphorylated 4E-BP1 was  $28.0 \pm 24.7\%$  greater ( $p<0.05$ ) in the KO control limbs than WT control limbs.



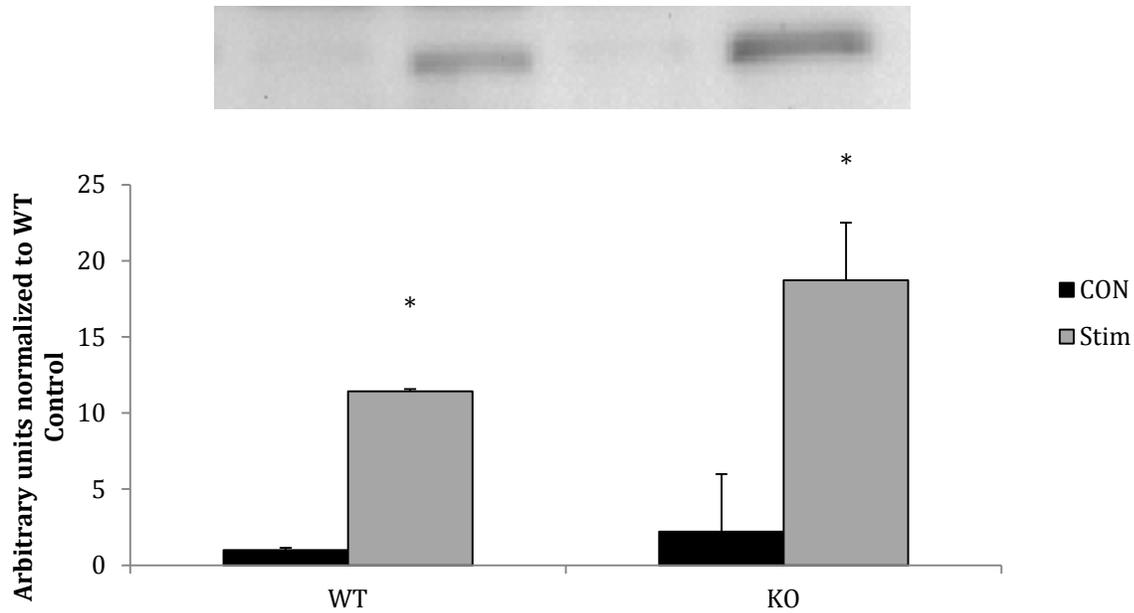
**Figure 7. Total p70S6k content.**

No difference was observed in the total p70S6k content between group control limbs.



**Figure 8. Phosphorylated p70S6k normalized to total p70S6k content.**

The electrically stimulated limbs of both WT and KO groups experienced significant increases in phosphorylated p70S6k levels; WT animals increased  $10.2 \pm 4.3$  fold ( $p < 0.05$ ) while the KO animals increased  $11.6 \pm 11.7$  fold ( $p < 0.01$ ). No significant difference was detected between the increases observed in the WT and KO groups.



**Figure 9. Phosphorylated levels of p70S6k.**

Phosphorylated p70S6k was significantly higher in the stimulated limbs of both WT ( $p < 0.01$ ) and KO groups ( $p < 0.05$ ) compared to control groups by  $12.3 \pm 10.1$  fold and  $12.8 \pm 14.0$  fold respectively.