

**Differential activation of mTOR signaling by contractile activity in skeletal muscle.**

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**Abstract**

The cellular mechanisms by which contractile activity stimulates skeletal muscle hypertrophy are beginning to be elucidated and appear to include the activation of the PI3 kinase signaling substrate, mammalian target of rapamycin (mTOR). In the present study, we examined the time course and location of mTOR phosphorylation in response to an acute bout of contractile activity. Rat hindlimb muscle contractile activity was elicited by high frequency electrical stimulation (HFES) of the sciatic nerve. Plantaris (PLA), tibialis anterior (TA), and soleus (SOL) muscles from stimulated and control limbs were collected immediately after or 6 hr after stimulation. HFES resulted in mTOR phosphorylation immediately after ( $3.4 \pm 0.9$ -fold,  $p < 0.01$ ) contractile activity in PLA whereas the TA was unchanged compared to controls. mTOR phosphorylation remained elevated in the PLA ( $3.6 \pm 0.6$ -fold) and increased in the TA ( $4.6 \pm 0.9$ -fold,  $p < 0.05$ ) 6 hr following HFES. Interestingly, mTOR activation occurred predominantly in fibers expressing the type IIa but not type I MHC isoform. Further, HFES induced modest p70<sup>S6K</sup> phosphorylation immediately after exercise in PLA ( $0.4 \pm 0.1$ -fold,  $p < 0.05$ ) but not TA, and more markedly 6 hr after in both the PLA and TA ( $1.4 \pm 0.4$  vs.  $2.4 \pm 0.3$ -fold, respectively,  $p < 0.01$ ). Akt/PKB phosphorylation was similar to controls at both time points. These results suggest that mTOR signaling is increased after a single bout of muscle contractile activity. Despite reports that mTOR is activated downstream of Akt/PKB, in this study, HFES induced mTOR signaling independent of Akt/PKB phosphorylation. Fiber type-dependent mTOR phosphorylation may be a molecular basis by which some fiber types are more susceptible to contraction-induced hypertrophy.

## Introduction

The maintenance of skeletal muscle mass is regulated by a balance between protein synthesis and protein degradation. Contractile activity and mechanical overload in skeletal muscle stimulate protein synthesis, leading to enhanced fiber size, strength, and alterations in metabolic properties (4,21). Although the biochemical mechanisms responsible for these adaptations are not fully understood, recent evidence suggests that the phosphoinositol-3 (PI3) kinase signaling cascade plays an essential role.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates muscle cell growth and myogenesis (3,8). Control of cell growth by mTOR appears to be dependent on its ability to enhance translation initiation, whereas the mechanism by which it regulates myogenesis is unknown. mTOR is activated by a variety of stimuli, including insulin, growth factors, and amino acids. In response to insulin and growth factors, mTOR activation is believed to occur through sequential activation of PI3 kinase, phosphoinositide-dependent kinase-1 (PDK-1), and protein kinase B (Akt/PKB), the later of which phosphorylates Ser2448 of mTOR in vitro (10,16). In contrast, there is evidence that activation of mTOR by amino acids does not require Akt/PKB, suggesting that mTOR is a point of convergence for nutrient and growth factor signaling pathways (2,14,16).

When activated, mTOR influences translation initiation by at least two distinct mechanisms. The first involves 5' cap binding via phosphorylation of the eukaryotic initiation factor binding protein (eIF-4E BP1). Upon hyperphosphorylation, this protein releases from a complex with eIF4E and allows eIF4G to bind eIF4E, thereby increasing the affinity of eIF4E for the 5' cap (11). In addition, mTOR phosphorylates the

ribosomal protein S6 kinase (p70<sup>S6K</sup>) which, in turn, phosphorylates the S6 ribosomal protein and allows the upregulation of a subclass of mRNA's encoding the translational apparatus (11).

p70<sup>S6K</sup> appears to play an important role in regulating contraction induced muscle hypertrophy. A single bout of high frequency electrical stimulation (HFES) results in prolonged phosphorylation of p70<sup>S6K</sup> for up to 36 hours (1,9). The degree of p70<sup>S6K</sup> phosphorylation after a single bout of HFES is closely associated with the increase in muscle weight after 6 weeks of chronic stimulation (1). Therefore, the acute pattern of p70<sup>S6K</sup> activation is likely preserved during training and is essential for muscle growth. This is supported by evidence that if mTOR is pharmacologically blocked, loading fails to activate p70<sup>S6K</sup> and hypertrophy is prevented (3).

The role of Akt/PKB in regulating contraction-induced muscle hypertrophy is less clear. While Akt/PKB is an important regulator of muscle size and can prevent muscle atrophy *in vivo* (3,17), the downstream targets of Akt/PKB after muscle contractile activity are unknown. Akt/PKB phosphorylation does not follow a similar time course as p70<sup>S6K</sup> after a single bout of muscle contractions (15). Rather, Akt/PKB activation appears after 1 min and returns to baseline within 15-30 minutes after the onset of contraction (19). While the time course of mTOR phosphorylation is not known, this information may prove useful in identifying the putative signaling pathways involved in stimulating protein synthesis following muscle contractile activity.

The purpose of this study was to determine if mTOR is phosphorylated in response to a single bout of muscle contractile activity and to compare the pattern of activation to both upstream (Akt/PKB) and downstream (p70<sup>S6K</sup>) kinases. Based on

evidence that p70<sup>S6K</sup> is not activated in contracting soleus muscle (1,15), we were further interested in determining if contraction-induced mTOR phosphorylation is fiber type dependent. We hypothesized that mTOR phosphorylation would be increased primarily six hours after rather than immediately after muscle contractile activity and would be associated with p70<sup>S6K</sup> phosphorylation in both the plantaris (PLA) and tibialis anterior (TA). We further hypothesized that an early induction of Akt/PKB phosphorylation could be dissociated from that of mTOR.

### **Materials and Methods**

**Materials.** Primary antibodies (anti-phospho Akt (Ser308), anti-phospho p70<sup>S6K</sup> (Thr389), anti-phospho mTOR (Ser2448), anti-Akt, anti-mTOR) were from Cell Signaling Technologies (Beverly, MA) and anti-p70<sup>S6K</sup> was from Santa Cruz Biotech (Santa Cruz, CA). Type I and IIa myosin heavy chain (MHC) antibodies were grown from hybridoma cell lines A4.951 (ATCC, Manassas, VA) and SC-71 (DSMZ, Braunschweig, Germany). Anti-rabbit and anti-mouse HRP conjugated secondary antibodies were from Amersham Biosciences (Piscataway, NJ). Biotinylated secondary antibodies for immunofluorescence experiments were from Vector Laboratories (Burlingame, CA). All other chemicals were from Sigma (St. Louis, MO).

**Animals.** Protocols for animal use were approved by the Institutional Animal Care and Use Committee of Boston University. Six month male Fischer 344 x Brown Norway rats (383 ± 5 g) were purchased from the National Institute on Aging. Upon arrival, animals were acclimatized for 3 days prior to experimentation and were given normal laboratory

chow and water ad libitum. Animals were fasted overnight prior to the experimental protocol.

***Electrical Stimulation.*** The HFES model was chosen based on its efficacy in stimulating protein translation and muscle hypertrophy *in vivo* (1). This protocol results in concentric (shortening) contraction of the PLA and SOL and eccentric (lengthening) contraction of the TA. In rats, the fiber type composition of these muscle is as follows: PLA: type I, 7%; type IIa, 52%; type IIb, 41%; TA: type I, 3%; type IIa, 61%; type IIb, 36%; SOL: Type I, 83%; Type IIa, 17%; Type IIb, 0% (12). Animals (n=10) were anesthetized with an intraperitoneal injection 75-mg/kg ketamine plus 5-mg/kg xylazine and the sciatic nerves of both legs were exposed. Platinum wire electrodes were attached to the sciatic nerve of the experimental hindlimb immediately superior to the point of trifurcation . Animals were secured in a fashion to allow the experimental limb to freely move. Each contraction was elicited by stimulating the experimental nerve with a 3 sec train of pulses (frequency, 100 Hz; duration, 1 ms at 10-12 V) using a Grass S48 stimulator (Grass Telefactor, Quincy, Ma). Animals performed a total of 100 contractions grouped into 10 sets of 10 contractions. Each contraction was followed by 10 sec rest and a 60 sec rest interval separated each set, resulting in a protocol time of 30 min. Animals were sacrificed by a lethal dose of sodium pentobarbital either immediately after or 6 hours after HFES.

***Preparation of skeletal muscle tissue lysates.*** TA, PLA, and SOL muscles were rapidly dissected, trimmed of connective tissue, weighed, frozen in liquid nitrogen and stored at -

80°C. Muscle pieces for western blotting analyses were homogenized in 10 volumes of buffer containing (in mM) 50 Tris-HCL, 100 NaF, 10 EDTA, 50  $\beta$ -glycerophosphate, 1  $\text{Na}_3\text{VO}_4$ , 3 benzamide, 1 PMSF, and 10  $\mu\text{g}/\text{ml}$  each of aprotinin, leupeptin, pepstatin. Homogenates were centrifuged at 10,000 x g for 10 min at 4°C and aliquots were stored at -80°C. The protein concentration of the supernatant was determined by the Bradford assay with BSA as standard (BioRad, Hercules, CA). Muscle pieces for immunohistochemical analyses were embedded in Tissue-Tek mounting medium and frozen in isopentane cooled to the temperature of liquid nitrogen.

**Western Blotting.** Equal amounts of protein (20 or 40  $\mu\text{g}$ ) were resolved by SDS-PAGE using either 10% (p70<sup>S6K</sup>/Akt) or 7.5% (mTOR) gels. Proteins were transferred to PVDF membranes (BioRad, Hercules, CA) and equal protein loading was verified by Ponceau S staining. Membranes were blocked for 1 hr in TTBS containing 5% milk followed by incubation with the appropriate primary antibody (diluted 1:1000 in 2% BSA in TTBS) overnight at 4°C. After several washes in TTBS, membranes were incubated with either anti-rabbit or anti-mouse HRP conjugated secondary antibodies (1:10,000 in blocking buffer) for 1 hr at room temperature. Protein signals were detected with ECL Plus reagents (Amersham, Piscataway, NJ). Images were scanned and band intensities were quantified by densitometry (Bioquant Image Analysis, Nashville, TN).

**Double Immunofluorescence.** 10  $\mu\text{m}$  cross sections of TA muscles were cut at -20°C, placed on slides, and dried for 30 min at room temperature. Sections were fixed in acetone for 10 min at -20°C and blocked in PBS containing 5% normal goat serum

(Jackson ImmunoResearch, West Grove, PA) for 30 min. Endogenous biotin binding sites were blocked with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) according to the manufactures instructions. Slides were then incubated with anti-phospho mTOR antibody (1:100) overnight at 4°C. Slides were rinsed and then incubated for 30 min with biotinylated anti-rabbit IgG secondary antibody diluted 1:400 in TBS containing 2% normal goat serum. Slides were then incubated with Flourescein Avidin DCS (Vector Laboratories, Burlingame, CA) diluted 1:200 in 10 mM Hepes, pH 7.4, 150 mM NaCl for 20 min. After repeating the blocking steps, sections were incubated with either anti-MHC I or anti-MHC IIa antibodies for 1 hr at room temperature. The MHC signal was visualized as described for phospho-mTOR using a biotinylated anti-mouse IgG secondary antibody and Texas Red Avidin DCS (Vector Laboratories, Burlingame, CA). Control slides were performed with either the second or both primary antibodies omitted. Slides were coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined using a Nikon Eclipse E400 microscope (Melville, NY). Images were captured at 10x magnification using the Bioquant Nova Image analysis system (Bioquant Image Analysis, Nashville, TN). Overlay images were created in Adobe Photoshop.

***Statistical Analyses.*** The fold-change in kinase phosphorylation was calculated between experimental muscles and corresponding control muscles (fold-change = [experimental-control]/control). Data are presented as means  $\pm$  standard error of 5 observations per group. Differences between the experimental and control muscles were determined using a paired Students t-test. Differences were considered significant at  $P < 0.05$ .

## Results

***Contractile activity did not alter Akt/PKB phosphorylation.*** Immediately following and 6 hr following an acute bout of HFES, we failed to detect any change in Akt/PKB phosphorylation using western blotting strategy in either TA or PLA muscle (figure 1). Total Akt/PKB protein in response to HFES did not differ between experimental and control muscles at either timepoint.

***Contractile activity increases mTOR phosphorylation in type IIa muscle fibers.***

Compared to controls, mTOR phosphorylation was increased  $3.4 \pm 0.9$ -fold immediately following HFES in PLA ( $p < 0.01$ ) whereas there was no change in TA muscle (figure 2). By six hours of recovery, mTOR phosphorylation remained elevated in PLA ( $3.6 \pm 0.6$ -fold,  $p < 0.05$ ) and increased in the TA ( $4.6 \pm 0.9$ -fold,  $p < 0.05$ ). mTOR phosphorylation was not altered in SOL at either time point. Furthermore, immunofluorescence experiments in TA muscle showed that mTOR phosphorylation after HFES was localized to both the membrane and intracellular region of the muscle fiber (figure 3). Strikingly, 6 hr following stimulation, an increase in mTOR phosphorylation was observed in a subset of fiber types. Using antibodies for the MHC isoforms, mTOR phosphorylation was determined to be specific to fibers predominantly expressing the MHC IIa isoform, whereas the increase in phosphorylation in type I fibers was negligible.

***p70<sup>S6K</sup> activation with contractile activity.*** The increase in mTOR phosphorylation was associated with an increase in the phosphorylation state of p70<sup>S6K</sup> (figure 4).

Immediately after contractile activity, p70<sup>S6K</sup> phosphorylation was modestly increased

( $0.4 \pm 0.1$ -fold,  $p < 0.05$ ) in PLA but was not significantly different in TA. After 6 hours of recovery, p70<sup>S6K</sup> phosphorylation increased  $1.4 \pm 0.4$  -and  $2.4 \pm 0.3$ -fold in PLA and TA respectively ( $p < 0.01$ ). p70<sup>S6K</sup> phosphorylation was not altered in SOL at either time point. Total p70<sup>S6K</sup> and mTOR proteins did not change in response to HFES or differ between muscles examined.

## Discussion

The present study is the first to describe mTOR phosphorylation after a single bout of contractile activity in adult skeletal muscle. HFES resulted in mTOR phosphorylation that was fiber type dependent, and this response was delayed in the TA. p70<sup>S6K</sup> followed a similar pattern of activation as mTOR whereas Akt/PKB phosphorylation remained unchanged. The finding that contractile activity regulates mTOR signaling is in agreement with Reynolds et al., who reported that 2 weeks of synergist ablation increases mTOR phosphorylation approximately 2-fold in rat PLA (18). Furthermore, treatment with a specific inhibitor of mTOR during synergist ablation abolishes 95% of muscle fiber growth, suggesting that mTOR signaling is required for loading-induced hypertrophy (3). The specific mechanisms by which mTOR regulates loading-induced muscle growth is unclear but likely involves stimulation of translation initiation through its ability to phosphorylate p70<sup>S6K</sup> and eIF-4E BP1.

A further novel finding of this study is that contraction-mediated mTOR phosphorylation is localized primarily to type IIa muscle fibers. Since the HFES protocol recruits all motor units of the contracting muscle, this finding appears not to be the result of differential recruitment of specific motor units during HFES. Another possibility is

that muscle damage induced by the eccentric contractions performed by the TA activates mTOR in “injury-sensitive” type II fibers. However, this is unlikely because we observed a similar degree of total mTOR phosphorylation in the concentrically contracting PLA as we observed in the eccentrically lengthening TA. Furthermore, the injury hypothesis fails to explain the lack of mTOR phosphorylation in type IIb fibers (by process of elimination), which also incur damage during eccentric contractions (13). It is important to note that fiber type-dependent activation of mTOR may be unique to the acute nature of the stimulus or the stimulation patterns employed in the present study. For example, hypertrophy of both type I and type II fibers during chronic loading is blocked by treatment with the mTOR inhibitor rapamycin (3). However, fiber type-dependent activation of mTOR signaling in the present study is supported by the observation that mTOR and p70<sup>S6K</sup> phosphorylation is unchanged in SOL following contractile activity.

Both mTOR and p70<sup>S6K</sup> followed similar time courses in the TA and PLA after contractile activity, suggesting that mTOR may activate p70<sup>S6K</sup> following muscle contractile activity *in vivo*. The finding that p70<sup>S6K</sup> phosphorylation is increased after muscle contractile activity is in agreement with others (1,15). The degree of p70<sup>S6K</sup> phosphorylation is strongly correlated with the increase in wet muscle mass after chronic HFES, whereas rapamycin treatment inhibits p70<sup>S6K</sup> and muscle fiber hypertrophy (1,3). Together, these data suggest that mTOR dependent activation of p70<sup>S6K</sup> is required for contraction-induced muscle hypertrophy.

Contrary to our hypothesis, we failed to detect a change in Akt/PKB phosphorylation in response to contractile activity at either time point. Sherwood et al

(1999) did not show any change in Akt/PKB activity with 15 s to 60 min of HFES (20). However, previous studies have reported that Akt/PKB phosphorylation is induced briefly (1-30 min) by acute muscle contractile activity *in vivo* (15,19) and during compensatory hypertrophy (3). The discrepancy in the literature may reflect that different modes of contractile activity (acute vs. chronic), stimulation patterns, duration/number of contractions, and/or length of stimulation protocols can influence Akt/PKB activity. However, the observation that Akt/PKB phosphorylation is unchanged following 6 hours of recovery from a single bout HFES is consistent with previous reports (15).

Although Akt/PKB phosphorylates mTOR *in vitro* (10,16), its role in regulating mTOR activity after contractile activity *in vivo* remains unclear. Multiple Akt isoforms (Akt1/Akt2/Akt3) are present in skeletal muscle and appear to have distinct physiological roles (5-7). While Akt1 knockout mice display growth retardation and normal glucose homeostasis (5,7), Akt2 deficient mice have impaired insulin stimulated glucose uptake in skeletal muscle (6). Though all three isoforms are briefly activated by muscle contraction (19), the downstream signaling molecules by which Akt/PKB exerts its effects in contracting skeletal muscle are unknown. Overexpression of active Akt/PKB induces muscle growth that is inhibited by rapamycin (17), suggesting that Akt/PKB control of muscle growth is mediated through mTOR. However, the observation that increased mTOR and p70<sup>S6K</sup> phosphorylation are not associated with Akt/PKB phosphorylation following muscle contractile activity suggests that these events may occur independent of Akt/PKB kinase activity. In addition, the dissociation between Akt/PKB and mTOR phosphorylation may reflect that cellular localization of Akt/PKB,

rather than total kinase activity, may be critical for mTOR activation following muscle contractile activity.

In summary, we report that mTOR phosphorylation is elevated following muscle contractile activity and is associated with p70<sup>S6K</sup> but not Akt/PKB. Furthermore, mTOR phosphorylation in TA is localized to intracellular and membrane regions of type IIa fibers, suggesting that this subpopulation of fibers may be more responsive to hypertrophy. Presently, the stimulus responsible for increasing mTOR and p70<sup>S6K</sup> phosphorylation in contracting skeletal muscle is unknown.

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**Figure Legends:**

Figure 1. Akt/PKB phosphorylation following *in situ* muscle contractile activity. Rat TA (A, C) and PLA (B, D) muscles were contracted via sciatic nerve stimulation and collected either immediately after (0 hr) or 6 hrs after stimulation. Akt/PKB phosphorylation (Thr308) in stimulated muscle (Stim) and sham operated controls (Con) was determined by western blotting using an anti-phospho Akt/PKB antibody. There were no differences in Akt/PKB phosphorylation or total protein at either timepoint.

Figure 2. Increased mTOR phosphorylation following *in situ* muscle contractile activity. Rat TA (A, D), PLA (B, E), and SOL (C, F) muscles were assayed for mTOR phosphorylation using an anti-phospho mTOR (Ser2448) antibody. Data are means  $\pm$  S.E. of n=5 per group. \*, significantly different from control (p<0.05).

Figure 3. Panel A, cross section of TA muscle six hours after contractile activity showing mTOR phosphorylation that is localized to the cell membrane and intracellular region of a subset of muscle fibers. Panel B, muscle fibers expressing the MHC IIa isoform display the same pattern as A. Panel C, overlay. Panels D-F, control muscle shows minimal staining for phospho-mTOR that does not overlay with type IIa fibers (sections representative of 5 animals, 10x magnification). Stim, stimulated TA. Con, control TA.

Figure 4. Increased p70<sup>S6K</sup> phosphorylation following *in situ* muscle contractile activity. TA (A, D), PLA (B, E), and SOL (C, F) muscle lysates were probed using anti-phospho p70<sup>S6K</sup> (Thr389) and anti-p70<sup>S6K</sup> antibodies either immediately after (0 hr) or 6 hrs after stimulation. Data are means  $\pm$  S.E. of n=5 per group. \*, significantly different from control (p<0.05).

Figure 1.

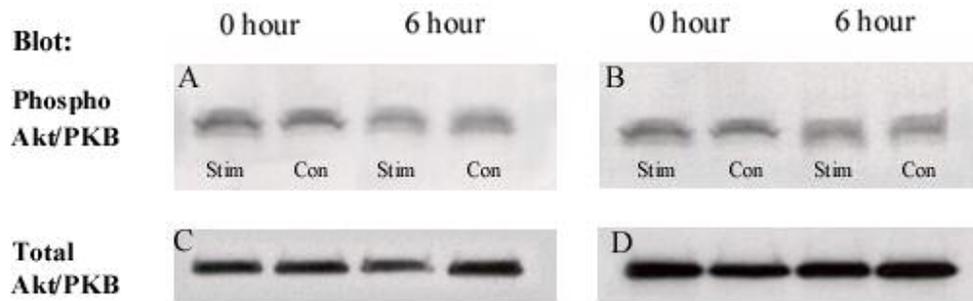


Figure 2.

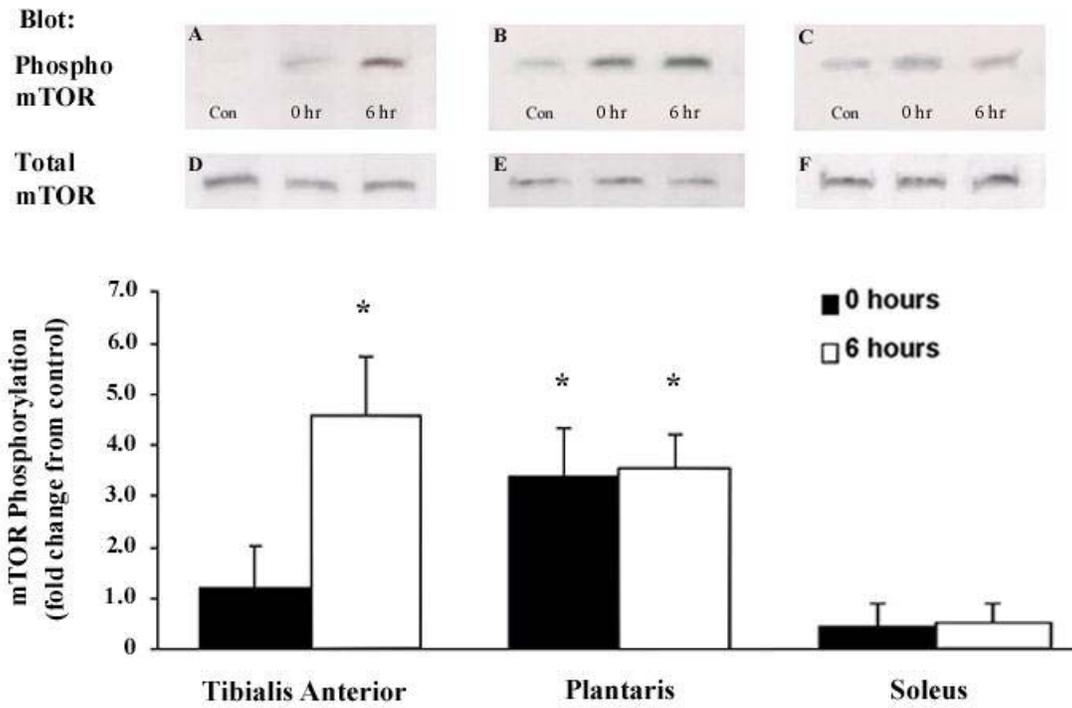


Figure 3.

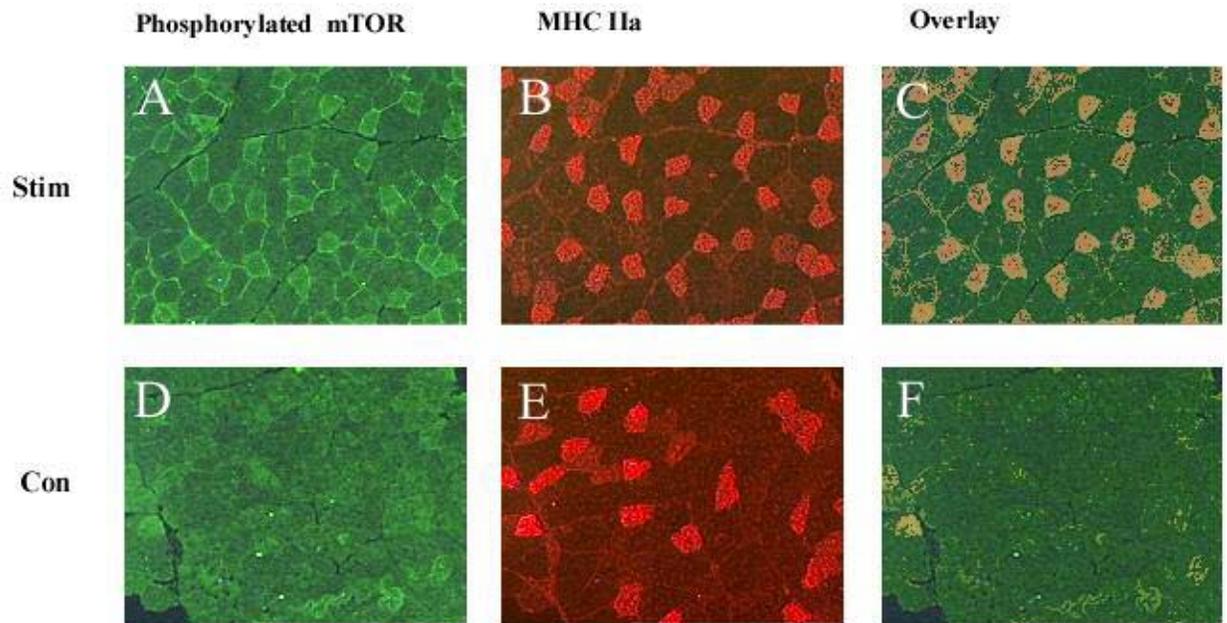


Figure 4.

