

# Capsaicin mimics mechanical load-induced intracellular signaling events

## Involvement of TRPV1-mediated calcium signaling in induction of skeletal muscle hypertrophy

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**M**echanical load-induced intracellular signaling events are important for subsequent skeletal muscle hypertrophy. We previously showed that load-induced activation of the cation channel TRPV1 caused an increase in intracellular calcium concentrations ( $[Ca^{2+}]_i$ ) and that this activated mammalian target of rapamycin (mTOR) and promoted muscle hypertrophy. However, the link between mechanical load-induced intracellular signaling events, and the TRPV1-mediated increases in  $[Ca^{2+}]_i$  are not fully understood. Here we show that administration of the TRPV1 agonist, capsaicin, induces phosphorylation of mTOR, p70S6K, S6, Erk1/2 and p38 MAPK, but not Akt, AMPK or GSK3 $\beta$ . Furthermore, the TRPV1-induced phosphorylation patterns resembled those induced by mechanical load. Our results continue to highlight the importance of TRPV1-mediated calcium signaling in load-induced intracellular signaling pathways.

mTOR through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is important for muscle growth and/or muscle hypertrophy.<sup>2</sup> However, recent studies have revealed that mechanical load-induced activation of mTOR was not mediated by PI3K/Akt, especially at early stages of muscle hypertrophy,<sup>1,3-5</sup> suggesting the presence of another route that converts mechanical load into the activation of mTOR. We previously showed that mechanical load-induced activation of mTOR and subsequent muscle hypertrophy required a TRPV1-mediated increase in intracellular calcium concentrations ( $[Ca^{2+}]_i$ ),<sup>6</sup> implicating calcium signaling as a crucial step for mechanical load-induced activation of mTOR. However, it is still unclear to what extent the TRPV1-mediated increase in  $[Ca^{2+}]_i$  is involved in intracellular signaling after mechanical load. Here, we show additional data that further highlight the significance of the calcium-induced intracellular signaling in response to mechanical load in skeletal muscle.

**Keywords:** skeletal muscle, muscle hypertrophy, calcium signaling, TRPV1, capsaicin, mTOR

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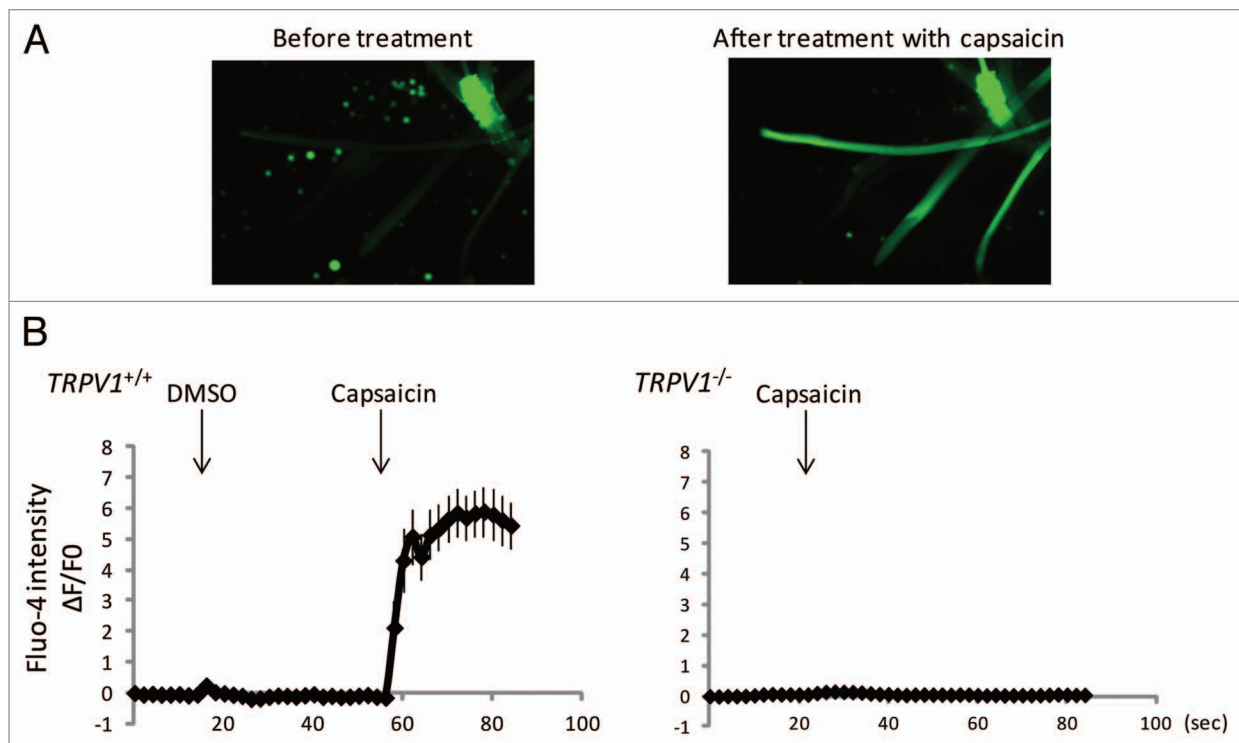
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### Introduction

Skeletal muscle mass is regulated by a balance of protein synthesis and degradation. Increased muscle activity by exercise or weight training induces activation of mammalian target of rapamycin (mTOR), which promotes protein synthesis and subsequent muscle hypertrophy.<sup>1</sup> Insulin-like growth factor-1 (IGF-1) activates

### Results and Discussion

To investigate the effect of TRPV1-induced calcium signaling on intracellular signaling events, we treated muscle cells with the TRPV1 agonist, capsaicin. We isolated single muscle fibers from the extensor digitorum longus muscle of wild-type or *TRPV1*-null mice and analyzed



**Figure 1.** Capsaicin induces an increase of intracellular calcium levels in a TRPV1-dependent manner. **(A)**  $\text{Ca}^{2+}$ -imaging using Fluo-4 fluorescence of isolated wild-type single muscle fibers from extensor digitorum longus before or after treatment with capsaicin. **(B)** Quantitative analysis of Fluo-4 signal intensity. Approximately 10 fibers were analyzed.

the effect of capsaicin on  $[\text{Ca}^{2+}]_i$  using Fluo-4.  $[\text{Ca}^{2+}]_i$  were increased by capsaicin in wild-type but not in *TRPV1*-null muscle fibers, indicating that the capsaicin-induced increases in  $[\text{Ca}^{2+}]_i$  were mediated by TRPV1 (Fig. 1A and B).

We previously showed that mTOR was activated by the TRPV1-induced increases in  $[\text{Ca}^{2+}]_i$ .<sup>6</sup> Because  $[\text{Ca}^{2+}]_i$  regulates divergent intracellular signaling pathways,<sup>7-9</sup> we further analyzed the capsaicin-induced intracellular signaling events. Similar to previous observations,<sup>6</sup> intramuscular injection of capsaicin induced phosphorylation of p70S6K at Thr389, an mTOR-regulated phosphorylation site that is necessary for p70S6K activation<sup>10</sup> in addition to phosphorylation of the downstream target S6 (Fig. 2A). Capsaicin also induced phosphorylation of p70S6K at Thr421/Ser424 and mTOR at Ser2448 and Ser2481. In addition to p70S6K, capsaicin induced phosphorylation of Erk1/2 and p38 MAPK, but not Akt, AMPK $\alpha$ , or GSK3 $\beta$  (Fig. 2B–F). Interestingly, similar changes in phosphorylation were observed within 3 min of mechanical overload, an experimental model of hypertrophy,

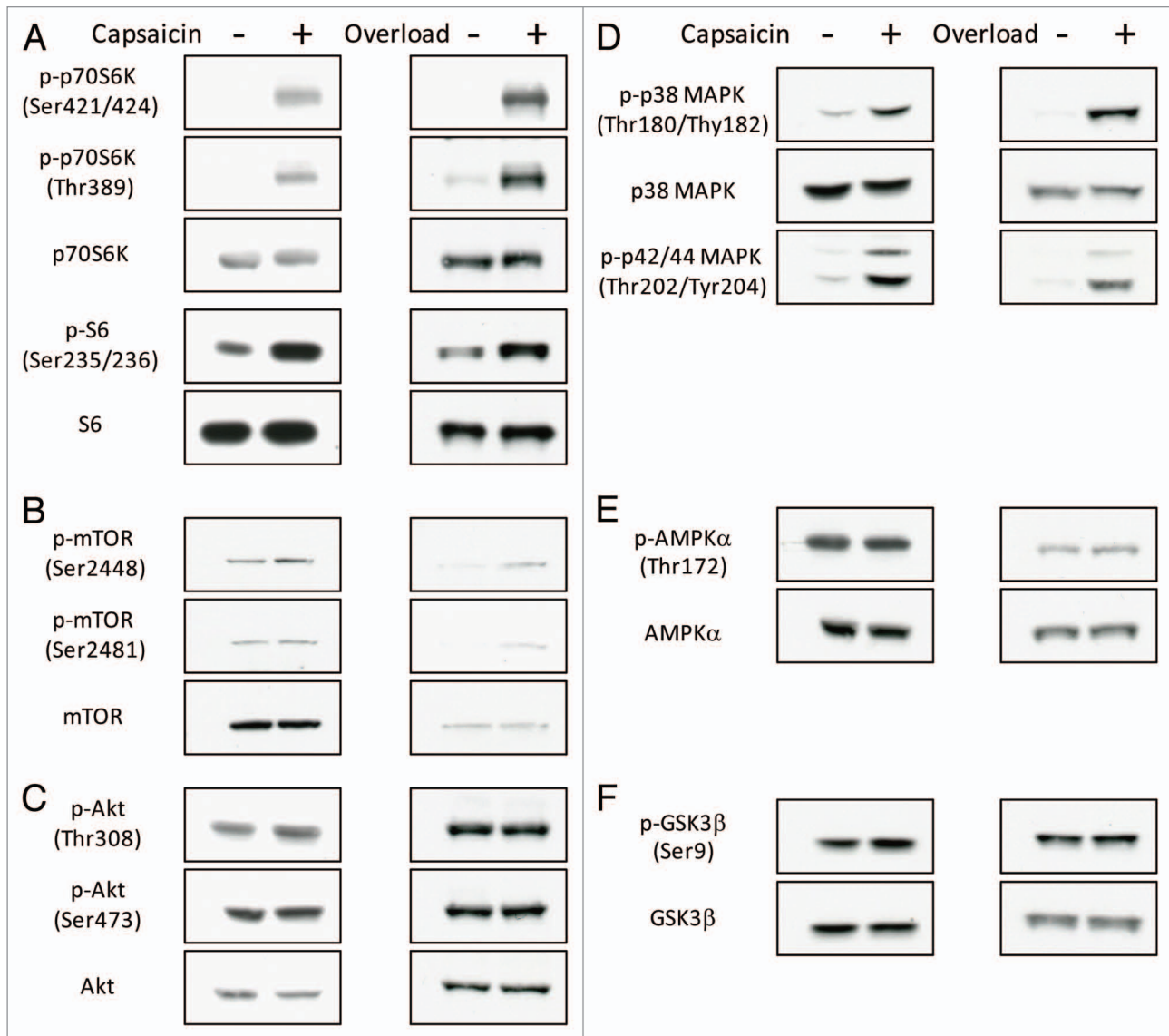
which relies on compensatory adaptation of the plantaris muscle following ablation of the tendons of the functionally synergistic muscles (Fig. 2). This suggests that load-induced intracellular signaling is regulated by TRPV1-mediated increase in  $[\text{Ca}^{2+}]_i$ .

Unlike the IGF-1-induced activation of the PI3K/Akt/mTOR pathway,<sup>2</sup> the load- or the TRPV1-induced activation of mTOR occurred independently of the phosphorylation of Akt (Fig. 2C). IGF-1 is responsible for the induction of muscle hypertrophy.<sup>11</sup> However, because transgenic mice that overexpressed a dominant-negative IGF-1 receptor showed normal overload-induced hypertrophy,<sup>4</sup> and relatively early activation of mTOR induced by overload occurred independently of PI3K/Akt signaling,<sup>3</sup> at least one other signaling pathway is likely to be present. Because capsaicin mimicked overload-induced activation of intracellular signaling events (Fig. 2), and we previously showed that treatment with capsaicin was sufficient to induce hypertrophy,<sup>6</sup> our study suggests the activation of TRPV1-mediated increase in  $[\text{Ca}^{2+}]_i$  to be an

important step in the induction of hypertrophy. Activation of the MAPK pathway upregulates several transcription factors.<sup>12</sup> Furthermore, previous studies indicate that mTOR is regulated by Erk1/2-induced regulation of TSC1/2.<sup>3,9</sup> A loss-of-function approach for Erk1/2 will be required to investigate any molecular link between a potential load-induced activation of the Erk1/2 pathway and the mTOR pathway. The elucidation of the molecular mechanisms by which mechanical load activates mTOR, Erk1/2 and p38 MAPK will lead to a better understanding of the mechanisms underlying load-induced muscle hypertrophy and might contribute to the development of therapeutics to counteract muscle atrophy. Furthermore, elucidation of the mechanisms involved in co-activation of these molecules by mechanical load and TRPV1-induced regulation of  $[\text{Ca}^{2+}]_i$  will lead to a better understanding of load-induced muscle hypertrophy.

## Materials and Methods

**Animals.** Twelve- to forty-week-old male *TRPV1*-null mice and C57BL/6



**Figure 2.** Mechanical overload and administration of capsaicin induce phosphorylation of p70S6K, S6, mTOR, Erk1/2 and p38 MAPK, but not Akt, AMPK $\alpha$  or GSK3 $\beta$ . Western blot analysis showing the effects of mechanical overload or administration of capsaicin on phosphorylation of p70S6K and S6 (A), mTOR (B), Akt (C), Erk1/2 and p38 MAPK (D), AMPK $\alpha$  (E) and GSK3 $\beta$  (F) (n = 3–4).

mice were purchased from Charles River Laboratories and Nihon CREA, respectively. These mice were housed at the institutional animal facility. A 150  $\mu$ l of a 10  $\mu$ M solution of capsaicin (Sigma-Aldrich) was injected intramuscularly 30 min before isolation of muscles.

**Isolation of single fibers and intracellular calcium level measurement.** Extensor digitorum longus muscles from wild-type and *TRPV1*-null mice were isolated and dissociated by digestion using type 1 collagenase in a buffer containing 140 mM NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM

HEPES, 10 mM glucose (pH 7.0). [Ca<sup>2+</sup>]<sub>i</sub> were monitored with Fluo-4 as previously described.<sup>6</sup> Briefly, single fibers were incubated with 4  $\mu$ M Fluo-4 AM (Dojindo) for 30 min at room temperature to allow homogenous intracellular distribution of the dye. After raising the temperature to 37°C, the fibers were placed onto the stage of an inverted microscope (Olympus), and fluorescence intensity changes by vehicle (DMSO) or capsaicin (10  $\mu$ M) were recorded every 2 sec. Data were calculated as normalized fluorescence  $\Delta F/F_0$ :  $\Delta F/F_0 = (F_{max} - F_0)/F_0$ , where  $F_{max}$  is the maximum fluorescence

and  $F_0$  is the fluorescence before exposure to a reagent.

**Western blot analysis.** Total muscle protein was extracted by sample buffer containing 0.1% Triton X-100, 50 mM HEPES (pH 7.4), 4 mM EGTA, 10 mM EDTA, 15 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 100 mM glycerophosphate, 25 mM NaF, 5 mM Na<sub>2</sub>VO<sub>4</sub> and a complete protease inhibitor cocktail (Roche). The protein concentration was determined using Coomassie brilliant blue G-250 (Bio-Rad). Just before SDS-PAGE, an aliquot of the extracted protein solution was mixed with an equal volume of sample

loading buffer containing 30% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, 62.5 mM TRIS-HCl (pH 6.8) and 0.05% bromophenol blue, and the mixture was heated at 60°C for 10 min. Thirty micrograms of protein was separated on an SDS-polyacrylamide gel and electrically transferred to a polyvinylidene difluoride membrane (Millipore). The blot was incubated with primary antibodies. The signals were detected using the ECLTM Western Blotting Detection system (GE Healthcare). Antibodies against Akt (9272), phospho-Akt (Ser473) (9271), phospho-Akt (Thr308) (9275), p70S6K (9202), phospho-p70S6K (Thr389) (9205), phospho-p70S6K (Thr421/Ser424) (9204), S6 (2217), phospho-S6 (Ser235/236) (4858), mTOR (2972), phospho-mTOR (Ser2448) (2971), phospho-mTOR (Ser2481) (2974), AMPK $\alpha$  (2603), phospho-AMPK $\alpha$  (Thr172) (2535), phospho-ERK1/2 (Thr202/Tyr204) (9101), p38 MAPK (9212), phospho-p38 MAPK (Thr180/Tyr182) (9211), phospho-GSK-3 $\beta$  (Ser9) (9336) were purchased from Cell Signaling Technology. Antibody against GSK-3 $\beta$  (sc-9166) was purchased from Santa Cruz.

**Synergist ablation surgery.** Mice were anesthetized with diethyl ether. Functional overload of the plantaris muscle was induced by bilateral surgical ablation of the tendons of the gastrocnemius and

soleus muscles. Briefly, a midline incision was made in the skin on the hind limbs. The distal tendons of both the gastrocnemius and soleus were transected. The incision was closed with a 7–0 silk suture (Matsuda Ika Kogyo). Plantaris muscles were isolated immediately 3 min after mice recovered from the anesthetic and started walking. For the sham-operated group, similar incisions were made in the skin, but the tendons were not transected.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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