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# Capsaicin mimics mechanical load-induced intracellular signaling events

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

Naoki Ito,[1,2] Urs T. Ruegg,[3] Akira Kudo,[2] Yuko Miyagoe-Suzuki[1] and Shin'ichi Takeda[1,*]

[1]Department of Molecular Therapy; National Institute of Neuroscience; National Center of Neurology and Psychiatry; Kodaira, Japan; [2]Department of Biological Information; Tokyo Institute of Technology; Yokohama, Japan; [3]Laboratory of Pharmacology; Geneva-Lausanne School of Pharmaceutical Sciences; University of Geneva; Geneva, Switzerland

**Mechanical 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 GSK3β. 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.**

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

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*Correspondence to: Shin'ichi Takeda; Email: takeda@ncnp.go.jp

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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.[1] Insulin-like growth factor-1 (IGF-1) activates mTOR through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is important for muscle growth and/or muscle hypertrophy.[2] 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,[1,3-5] 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 ($[Ca^{2+}]_I$),[6] 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.

### 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

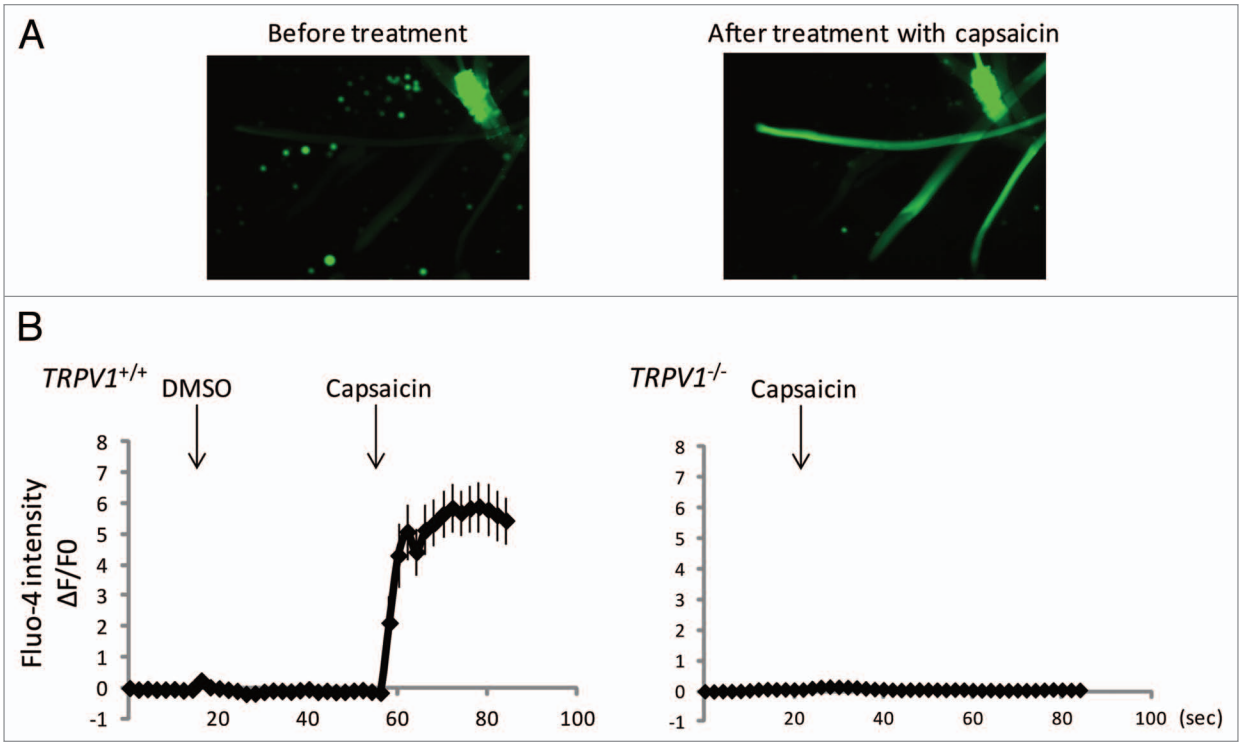


**Figure 1.** Capsaicin induces an increase of intracellular calcium levels in a TRPV1-dependent manner. (**A**) $Ca^{2+}$-imaging using Fluo-4 fluorescence of isolated wild-type single muscle fibers from extensor digitorum longus before or after treatment with capsaicin. (**B**) Quantitative analysis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

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

We previously showed that mTOR was activated by the TRPV1-induced increases in $[Ca^{2+}]_i$.[6] Because $[Ca^{2+}]_i$ regulates divergent intracellular signaling pathways,[7-9] we further analyzed the capsaicin-induced intracellular signaling events. Similar to previous observations,[6] intramuscular injection of capsaicin induced phosphorylation of p70S6K at Thr389, an mTOR-regulated phosphorylation site that is necessary for p70S6K activation[10] 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, AMPKα, or GSK3β (**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 $[Ca^{2+}]_i$.

Unlike the IGF-1-induced activation of the PI3K/Akt/mTOR pathway,[2] 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.[11] However, because transgenic mice that overexpressed a dominant-negative IGF-1 receptor showed normal overload-induced hypertrophy,[4] and relatively early activation of mTOR induced by overload occurred independently of PI3K/Akt signaling,[3] 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,[6] our study suggests the activation of TRPV1-mediated increase in $[Ca^{2+}]_i$ to be an important step in the induction of hypertrophy. Activation of the MAPK pathway upregulates several transcription factors.[12] Furthermore, previous studies indicate that mTOR is regulated by Erk1/2-induced regulation of TSC1/2.[3,9] 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 $[Ca^{2+}]_i$ 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

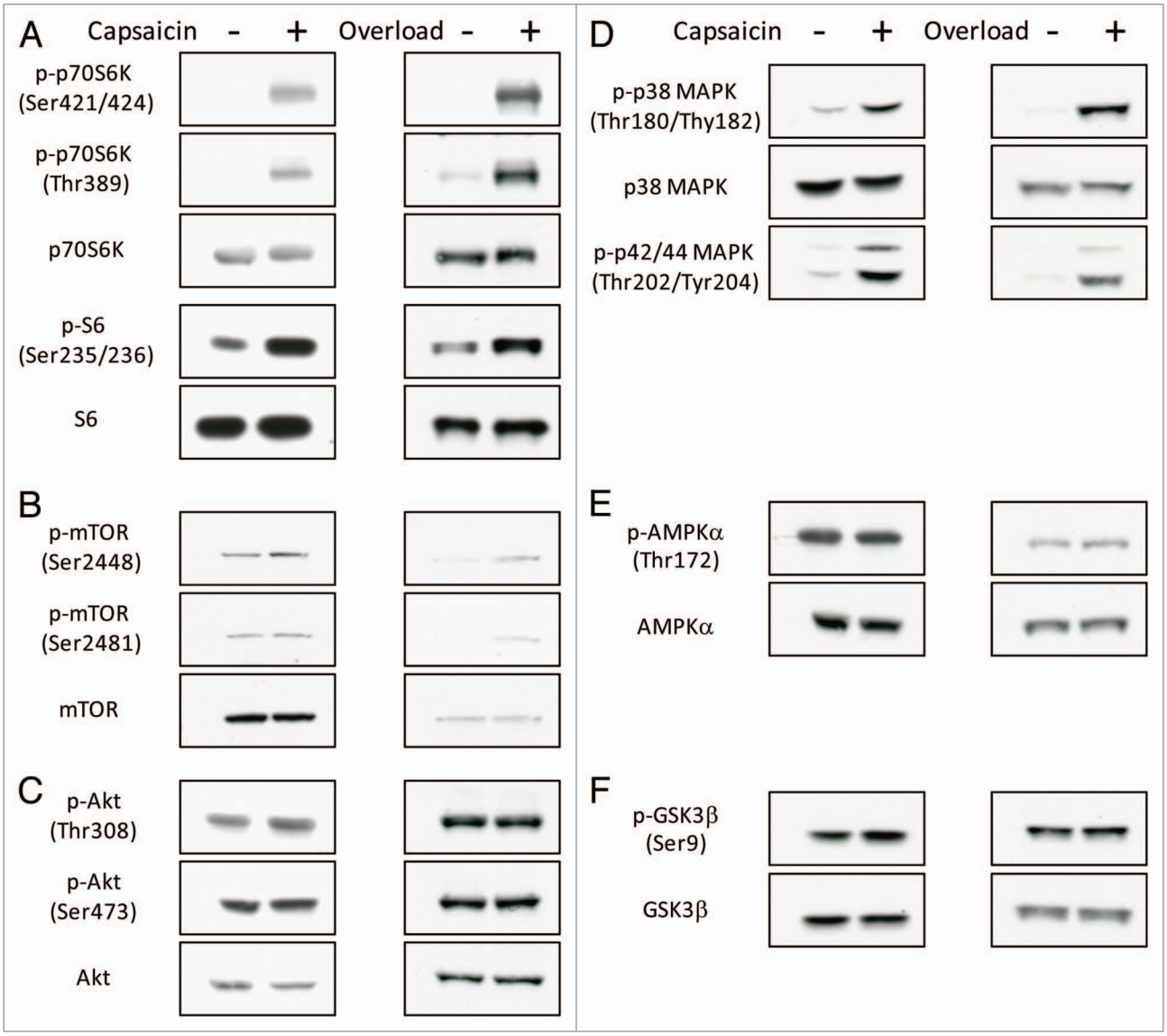


**Figure 2.** Mechanical overload and administration of capsaicin induce phosphorylation of p70S6K, S6, mTOR, Erk1/2 and p38 MAPK, but not Akt, AMPKα or GSK3β. Western blot analysis showing the effects of mechanical overload or administration of capsaicin on phosphorylation of p70S6K and S6 (**A**), mTOR (**B**), Akt (**C**), Erk1/2 and p38 MAPK (**D**), AMPKα (**E**) and GSK3β (**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 μl of a 10 μM 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 $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose (pH 7.0). $[Ca^{2+}]_i$ were monitored with Fluo-4 as previously described.[6] Briefly, single fibers were incubated with 4 μM 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 μM) were recorded every 2 sec. Data were calculated as normalized fluorescence $\Delta F/F_0$: $\Delta F/F_0 = (Fmax - F_0)/F_0$, where Fmax 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 $Na_4P_2O_7$, 100 mM glycerophosphate, 25 mM NaF, 5 mM $Na_2VO_4$ 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), AMPKα (2603), phospho-AMPKα (Thr172) (2535), phospho-ERK1/2 (Thr202/Tyr204) (9101), p38 MAPK (9212), phospho-p38 MAPK (Thr180/Tyr182) (9211), phospho-GSK-3β (Ser9) (9336) were purchased from Cell Signaling Technology. Antibody against GSK-3β (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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### References

1. Philp A, Hamilton DL, Baar K. Signals mediating skeletal muscle remodeling by resistance exercise: PI3-kinase independent activation of mTORC1. J Appl Physiol 2011; 110:561-8; PMID:21071597; http://dx.doi.org/10.1152/japplphysiol.00941.2010
2. Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, et al. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Nat Cell Biol 2001; 3:1009-13; PMID:11715022; http://dx.doi.org/10.1038/ncb1101-1009
3. Miyazaki M, McCarthy JJ, Fedele MJ, Esser KA. Early activation of mTORC1 signalling in response to mechanical overload is independent of phosphoinositide-3-kinase/Akt signalling. J Physiol 2011; 589:1831-46; PMID:21300751; http://dx.doi.org/10.1113/jphysiol.2011.205658
4. Spangenburg EE, Le Roith D, Ward CW, Bodine SC. A functional insulin-like growth factor receptor is not necessary for load-induced skeletal muscle hypertrophy. J Physiol 2008; 586:283-91; PMID:17974583; http://dx.doi.org/10.1113/jphysiol.2007.141507
5. Hornberger TA, Stuppard R, Conley KE, Fedele MJ, Fiorotto ML, Chin ER, et al. Mechanical stimuli regulate rapamycin-sensitive signalling by a phosphoinositide-3-kinase-, protein kinase B- and growth factor-independent mechanism. Biochem J 2004; 380:795-804; PMID:15030312; http://dx.doi.org/10.1042/BJ20040274
6. Ito N, Ruegg UT, Kudo A, Miyagoe-Suzuki Y, Takeda S. Activation of calcium signaling through Trpv1 by nNOS and peroxynitrite as a key trigger of skeletal muscle hypertrophy. Nat Med 2013; 19:101-6; PMID:23202294; http://dx.doi.org/10.1038/nm.3019
7. Gulati P, Gaspers LD, Dann SG, Joaquin M, Nobukuni T, Natt F, et al. Amino acids activate mTOR complex 1 via $Ca^{2+}$/CaM signaling to hVps34. Cell Metab 2008; 7:456-65; PMID:18460336; http://dx.doi.org/10.1016/j.cmet.2008.03.002
8. Tamás P, Hawley SA, Clarke RG, Mustard KJ, Green K, Hardie DG, et al. Regulation of the energy sensor AMP-activated protein kinase by antigen receptor and $Ca^{2+}$ in T lymphocytes. J Exp Med 2006; 203:1665-70; PMID:16818670; http://dx.doi.org/10.1084/jem.20052469
9. Wauson EM, Zaganjor E, Lee AY, Guerra ML, Ghosh AB, Bookout AL, et al. The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Mol Cell 2012; 47:851-62; PMID:22959271; http://dx.doi.org/10.1016/j.molcel.2012.08.001
10. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol 2009; 10:307-18; PMID:19339977; http://dx.doi.org/10.1038/nrm2672
11. Adams GR, Haddad F. The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy. J Appl Physiol 1996; 81:2509-16; PMID:9018499
12. Hazzalin CA, Mahadevan LC. MAPK-regulated transcription: a continuously variable gene switch? Nat Rev Mol Cell Biol 2002; 3:30-40; PMID:11823796; http://dx.doi.org/10.1038/nrm715