J Pharmacol Sci **122**, 278 – 288 (2013)

Journal of Pharmacological Sciences

Full Paper

Effects of Chronic Akt/mTOR Inhibition by Rapamycin on Mechanical Overload–Induced Hypertrophy and Myosin Heavy Chain Transition in Masseter Muscle

Daisuke Umeki[1,2], Yoshiki Ohnuki[1], Yasumasa Mototani[1], Kouichi Shiozawa[1], Takayuki Fujita[3], Yoshiki Nakamura[2], Yasutake Saeki[1], and Satoshi Okumura[1,3,*]

[1]*Department of Physiology,* [2]*Department of Orthodontics, Tsurumi University School of Dental Medicine, Yokohama 230-8501, Japan*
[3]*Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan*

Received August 29, 2012; Accepted June 4, 2013

Abstract. To examine the effects of the Akt/mammalian target of rapamycin (mTOR) pathway on masseter muscle hypertrophy and myosin heavy chain (MHC) transition in response to mechanical overload, we analyzed the effects of bite-opening (BO) on the hypertrophy and MHC composition of masseter muscle of BO-rats treated or not treated with rapamycin (RAPA), a selective mTOR inhibitor. The masseter muscle weight in BO-rats was significantly greater than that in controls, and this increase was attenuated by RAPA treatment. Expression of slow-twitch MHC isoforms was significantly increased in BO-rats with/without RAPA treatment, compared with controls, but the magnitude of the increase was much smaller in RAPA-treated BO-rats. Phosphorylation of p44/42 MAPK (ERK1/2), which preserves fast-twitch MHC isoforms in skeletal muscle, was significantly decreased in BO-rats, but the decrease was abrogated by RAPA treatment. Calcineurin signaling is known to be important for masseter muscle hypertrophy and fast-to-slow MHC isoform transition, but expression of known calcineurin activity modulators was unaffected by RAPA treatment. Taken together, these results indicate that the Akt/mTOR pathway is involved in both development of masseter muscle hypertrophy and fast-to-slow MHC isoform transition in response to mechanical overload with inhibition of the ERK1/2 pathway and operates independently of the calcineurin pathway.

Keywords: masseter muscle, Akt/mammalian target of rapamycin (mTOR) pathway, hypertrophy, myosin heavy chain, mechanical stress

Introduction

Mechanical overload induced by an increase in occlusal vertical dimension, produced by the use of interocclusal appliances (bite-opening: BO), is widely used to relieve masseter muscle spasm (1 – 3). Although BO treatment is recognized to be effective, its pathophysiological and molecular basis remains poorly understood.

Adult skeletal muscle is composed of muscle fibers that differ in their speed of contraction and predominant energy metabolism. Muscle fibers can be classified as type I (slow-twitch) and type II (fast-twitch) on the basis of their predominant myosin heavy chain (MHC) isoforms. Generally, MHC-I and MHC-IIa fibers utilize oxidative phosphorylation as their energy source, whereas MHC-IIx/d and MHC-IIb fibers harness anaerobic metabolism to generate ATP (4). The relative frequency of each fiber type defines the overall functional capabilities of a muscle. In response to mechanical overload or neuromuscular activity, however, muscles modify their functional characteristics by changing the fiber subtype (5). With minor exceptions in certain pathological conditions, muscle fibers switch to their "nearest neighbor type" following an obligatory pathway: I↔IIa↔IId/x↔IIb (5).

Calcineurin signaling is known to be important for the

*Corresponding author. okumura-s@tsurumi-u.ac.jp
Published online in J-STAGE on July 30, 2013
doi: 10.1254/jphs.12195FP

development of hypertrophy and for fast-to-slow MHC isoform transition after physiological stimulation of skeletal muscle (6). We have previously demonstrated that mechanical load on the masseter muscle also induces hypertrophy and fast-to-slow MHC isoform transition in BO-rats, and we showed that the calcineurin pathway plays an important role in this process by pharmacological inhibition of calcineurin with cyclosporin A (7, 8).

The Akt pathway also plays a role in the regulation of skeletal muscle hypertrophy and atrophy. Skeletal muscle hypertrophy is mediated through the activation of Akt and its downstream target mTOR (mammalian target of rapamycin), that is, the Akt/mTOR pathway, while skeletal muscle atrophy is mediated through the activation of Akt and its downstream target forkhead box subfamily O (Foxo), that is, the Akt/Foxo pathway (9). However, the role of the Akt/mTOR pathway in the development of masseter muscle hypertrophy and its influence on MHC isoform transition in response to mechanical overload have not been examined.

The p44/42 mitogen-activated protein kinase (MAPK) pathway, also known as the extracellular signaling regulated kinase 1/2 (ERK1/2) pathway, regulates cellular proliferation and differentiation and therefore may influence MHC isoform transition in masseter muscle, but its role remains poorly understood (10). Phosphorylation of ERK1/2 has been reported to play an important role in expression of slow-twitch fibers in skeletal muscle, while it has been reported to be necessary to preserve the expression of fast-twitch fibers with a concomitant repression of slow-twitch fibers in skeletal muscle (10 – 12). The ERK1/2 pathway is also important for regulating skeletal muscle mass, as well as the Akt/mTOR pathway and the calcineurin pathway, but the relationships among these pathways remains poorly understood (10).

We thus examined the role of the Akt/mTOR pathway in the development of hypertrophy and in the fast-to-slow MHC isoform transition in masseter muscle of BO-rats treated or not treated with rapamycin (RAPA), a selective mTOR inhibitor. The BO-rats have a gap of about 3 mm between the edges of the maxillary and mandibular incisors, produced by cementing a suitable appliance onto the incisors, and this causes mechanical overload in the masseter muscle similar to that seen in the case of splint therapy in humans (13), although the possibility cannot be ruled out that there might be some adverse changes specific to the rat model. We also examined the phosphorylation of ERK1/2 and expression of known calcineurin modulators, such as modulatory calcineurin-interacting protein 1 (MCIP1), and downstream molecules of the Akt/Foxo pathway, such as atrogin-1 and muscle Ring Finger-1 (MuRF1) (14 – 16), in order to see whether or not Akt/mTOR inhibition by RAPA also affects the ERK1/2 pathway and the calcineurin pathway.

Materials and Methods

Experimental animals

Animals were treated in accordance with institutional guidelines, and the experimental protocol was approved by the Animal Care and Use Committee of Tsurumi University. Twenty-eight Wistar rats, aged 8 weeks and weighing 243.9 ± 10.2 g, were divided into four groups: normal control group (Control), BO without RAPA treatment group (BO), RAPA treatment group (RAPA), and BO with RAPA treatment group (BO + RAPA). As in our earlier study, the bite was opened by cementing a suitable appliance onto the mandibular incisors under anesthesia with pentobarbital sodium (30 $mg \cdot kg^{-1} \cdot body\ weight^{-1}$; Abbott Laboratories, Chicago, IL, USA); further pentobarbital sodium was administered (10 – 20 $mg \cdot kg^{-1} \cdot body\ weight^{-1}$) if necessary (8, 17). The BO space between the edges of the maxillary and mandibular incisors was about 3 mm. BO-rats with a 3-mm space do not show a decrease of weight because, although they find it difficult to take the usual pellet food, they can take powered food. Also, the 3-mm increment of occlusal vertical dimension in BO-rats stretches the masseter muscle longitudinally by approximately 12%, which is within the physiological range (less than approximately 40% increase over its original length) during jaw-opening in rats (13, 18). The RAPA and BO + RAPA groups were treated with RAPA (LKT Laboratories, Inc., St. Paul, MN, USA) intraperitoneally (1.2 mg/kg dissolved in 10% ethanol and 2% carboxymethylcellulose) once daily for 14 days (19 – 21). Control and BO groups were injected intraperitoneally once daily for 14 days with vehicle containing 10% ethanol and 2% carboxymethylcellulose. The animals in each group were fed a powder diet ad libitum because BO-rats had difficulty in taking the usual pelleted diet and caloric restriction has been reported to affect the Akt/mTOR pathway in skeletal muscle (22).

After the completion of each treatment, all animals were killed by exsanguination under anesthesia with a fatal overdose of pentobarbital sodium (50 $mg \cdot kg^{-1} \cdot body\ weight^{-1}$). They were not fasted at the time of sacrifice.

The left and right masseter muscles were each excised and weighed; muscle mass was used as an index of muscle growth (23, 24). The central portion of the left superficial masseter muscle was divided into two pieces, which were rapidly frozen in liquid nitrogen and stored at −85°C for real-time quantitative PCR and SDS-PAGE analysis. The central portion of the right superficial

masseter muscle was fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4°C, and the fixed specimens were washed with PBS and then stored in 40% (w/v) sucrose in PBS at 4°C for immunohistochemical staining.

Histology

The specimens were embedded in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN, USA) and frozen in ethanol at −100°C. Cross sections (10-μm-thick) were cut from the middle portion of the specimens with a cryostat at −20°C and immunohistochemical staining was performed with monoclonal antibodies against skeletal fast (MY-32; Sigma, St. Louis, MO, USA) and slow (NOQ7.5.4D, Sigma) myosin. The immunoreaction was visualized with a Vectastain Universal Elite ABC kit (PK-6200; Vector Laboratories, Burlingame, CA, USA) and AEC Substrate Kit (SK-4200, Vector Laboratories), and observed under a light microscope (Nikon, Tokyo). The cross-sectional size of muscle fibers was evaluated by measuring the minimal diameter of muscle fibers to correct for obliquely cut muscle fibers (25). In total, 293 to 506 fibers obtained from 5 or 6 rats in each group were measured on micrographs of the immunostained sections taken with a digital camera (AxioCam; Carl Zeiss Japan, Tokyo) connected to the light microscope.

Real-time quantitative PCR

Total RNA was isolated from individual samples taken from superficial masseter in each group according to the manufacturer's instructions (FastRNATM Kit-GREEN; BIO 101, Vista, CA, USA). Real-time quantitative PCR was performed with oligonucleotide primer sets [neonatal MHC (MHC-Neo), MHC-I, MHC-IIa, MHC-IId/x, MHC-IIb, S16, peroxisome-proliferator-activated receptor-γ coactivator-1α (PGC-1α), MCIP1, atrogin-1, and MuRF1] based on published sequences (8).

Silver staining

MHC isoform composition in masseter muscle was analyzed with SDS-PAGE in masseter muscle, followed by silver staining of the bands of each MHC isoform (Silver Staining Kit; GE Healthcare, Uppsala, Sweden) and densitometric scanning as performed previously (13).

Western blotting

Western blotting was conducted with commercially available antibodies. The primary antibodies used in this study were purchased from Cell Signaling Technology (Boston, MA, USA) (26 – 29).

Statistical analyses

Data were expressed as means ± S.E.M. The statistical significance of differences in masseter muscle mass, MHC mRNA levels, MHC protein levels, and fiber diameter among the four groups was determined by one-way ANOVA followed by Tukey's test. The criterion of significance was taken as $P < 0.05$.

Results

Inhibition of the Akt/mTOR pathway with RAPA inhibited hypertrophy of masseter muscle in BO-rats

Body weights in the RAPA alone (263 ± 3.7 g) and BO + RAPA (223 ± 5.4 g) groups were significantly smaller than that in normal controls (Control: 303 ± 6.9, $P < 0.05$ vs. Control, n = 6 – 9), whereas BO-rats showed no significant difference from the controls (BO: 274 ± 8.0 g, P = not significant (NS) vs. Control, n = 5 – 9) (Fig. 1A).

BO-mediated mechanical overload resulted in masseter muscle hypertrophy (increase in mass from 1002 ± 21 to 1095 ± 24 mg, $P < 0.01$ vs. Control, n = 5 – 9), in agreement with previous findings (13). We then examined the effect of RAPA on the masseter muscle in BO-rats. RAPA alone slightly but significantly decreased the weight of masseter muscle (879 ± 24 mg, $P < 0.01$ vs. Control, n = 6). More importantly, RAPA also inhibited the development of masseter muscle hypertrophy in BO-rats (BO + RAPA: 927 ± 13 mg, $P < 0.01$ vs. BO, n = 6) (Fig. 1B).

We also performed immunohistochemical staining of masseter muscle with anti-monoclonal fast-myosin antibody to evaluate the fiber diameter. The fibers of masseter muscle were not stained with anti-slow myosin antibody (data not shown), as previously confirmed by SDS-PAGE analysis (13). The fiber diameter of the BO-rats was significantly increased, compared to that of the normal controls (Control vs. BO: 31.1 ± 0.4 vs. 35.1 ± 0.8 μm, $P < 0.01$, 293 – 329 fibers from each of 5 or 6 rats), but no abnormal organization of masseter muscle, such as fibrosis or muscle rupture, was observed (Fig. 1: C and D). RAPA significantly decreased the fiber diameter, compared with the control group (22.9 ± 1.9 μm, $P < 0.01$ vs. Control, 329 – 343 fibers from each of 5 or 6 rats). In accordance with the change of muscle weight, the fiber diameter of RAPA-treated BO-rats was significantly smaller than that of BO-rats not treated with RAPA (BO vs. BO + RAPA: 35.1 ± 0.8 vs. 29.8 ± 1.1 μm, $P < 0.01$ vs. BO, 293-506 fibers from each of 6 rats). These data indicate that RAPA inhibited the development of masseter muscle hypertrophy in BO-rats.

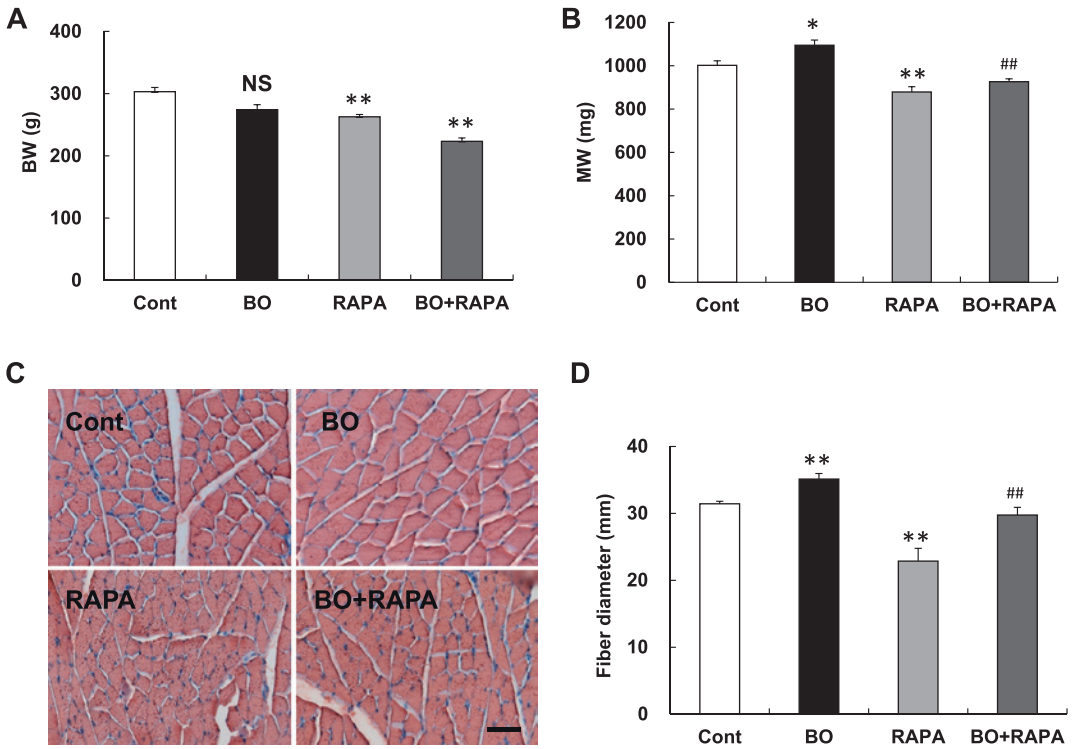

Fig. 1. RAPA inhibited BO-induced hypertrophy of masseter muscle. A) Body weights in the RAPA alone (263 ± 3.7 g) and BO + RAPA (223 ± 5.4 g) groups were significantly smaller than that in normal controls (Control: 303 ± 6.9 g, $^{**}P < 0.05$ vs. Control, n = 6 – 9), while BO-rat showed no such difference (BO: 274 ± 8.0 g, P = NS vs. Control, n = 5 – 9). B) The weight of masseter muscle in BO-rats was significantly greater than that in normal controls (Control vs. BO: 1002 ± 21 vs. 1095 ± 24 mg, $^{*}P < 0.05$ vs. Control, n = 5 – 9). RAPA alone significantly decreased the weight of masseter muscle at baseline (RAPA: 879 ± 24 mg, $^{**}P < 0.01$ vs. Control, n = 6) and also inhibited BO-induced masseter muscle hypertrophy (927 ± 13 mg, $^{\#\#}P < 0.01$ vs. BO, n = 6). C) Representative images of immunohistochemical staining of masseter muscle with anti-monoclonal fast-myosin antibody. Scale bar: 100 μm. D) Fiber diameter in BO-rats was significantly greater than that of the controls (Control vs. BO: 31.1 ± 0.4 vs. 35.1 ± 0.8 μm, $^{**}P < 0.01$ vs. Control, 293 – 329 fibers from each of 5 or 6 rats). RAPA significantly decreased the fiber diameter at baseline (RAPA: 22.9 ± 1.9 μm, $^{**}P < 0.01$ vs. Control, 329 – 343 fibers from each of 5 or 6 rats) and also inhibited the BO-mediated increase of the fiber diameter (BO + RAPA: 29.8 ± 1.1 μm, $^{\#\#}P < 0.01$ vs. BO, 293 – 506 fibers from each of 6 rats).

Inhibition of the Akt/mTOR pathway with RAPA attenuated fast-to-slow MHC isoform transition in masseter muscle of BO-rats

We recently showed that BO-mediated mechanical overload–induced hypertrophy of masseter muscle is accompanied with fast-to-slow MHC isoform transition (7, 13). We therefore examined the effect of RAPA on the composition of MHC mRNA isoforms in masseter muscle of BO-rats by real-time quantitative PCR. The mRNA expression levels of MHC-Neo (Fig. 2A), MHC-I (Fig. 2B), and MHC-IIa (Fig. 2C) were significantly increased in masseter muscle of BO-rats with or without RAPA treatment, compared with those of the control group ($P < 0.01$ vs. Control, n = 4 – 6), but the magnitudes of the increases in the BO + RAPA group were significantly smaller than those in the BO group ($P < 0.05$ or 0.01 vs. BO, n = 4 – 6).

The mRNA expression of MHC-IId/x was significantly increased by 1.6-fold in masseter muscle of BO-rats without RAPA treatment ($P < 0.01$, n = 6), compared with the control group, but that increase was abolished by RAPA (P = NS vs. Control, n = 6) (Fig. 2D).

The mRNA expression of MHC-IIb was significantly decreased by approximately 60% in masseter muscle of BO-rats without RAPA treatment versus the control group. However, RAPA treatment abrogated this decrease of MHC-IIb expression in masseter muscle of BO-rats (P = NS vs. Control, n = 6) (Fig. 2E).

We also analyzed the MHC composition at the protein level using SDS-PAGE, followed by silver staining and densitometric scanning of each MHC isoform (Fig. 3). Protein expressions of MHC-IIa was significantly increased in masseter muscle of BO-rats with or without RAPA treatment, compared with the control group ($P < 0.05$ or

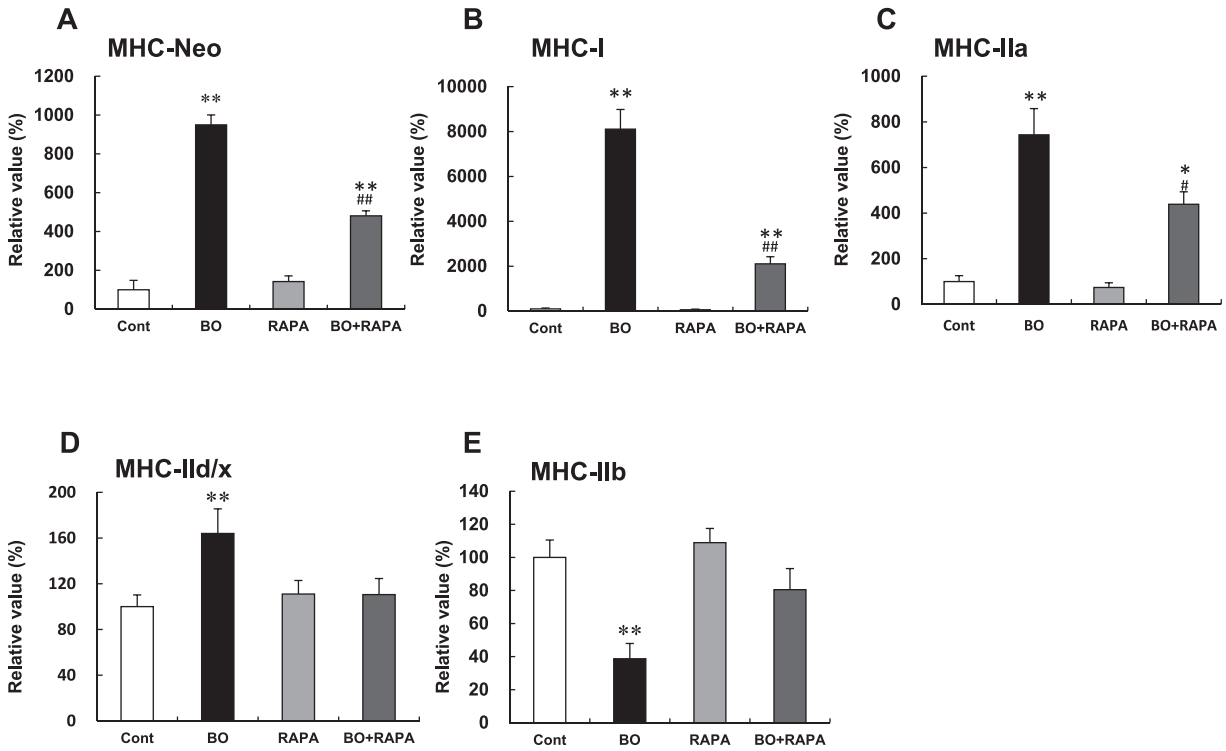

Fig. 2. RAPA attenuated fast-to-slow MHC isoform transition in masseter muscle of BO-rats at the mRNA level. A – C) mRNA expression levels of MHC-Neo (A), MHC-I (B), and MHC-IIa (C) were significantly increased in masseter muscle of BO-rats treated (BO + RAPA) or not treated (BO) with RAPA, compared with the normal controls (*$P < 0.05$ and **$P < 0.01$ vs. Control, n = 4 – 6). The expression increase in the BO + RAPA group was significantly smaller than that in the BO group ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. BO, n = 4 – 6). D) mRNA expression of MHC-IId/x was significantly increased in masseter muscle of the BO group (**$P < 0.01$, n = 6) versus the control group, but this increase was abolished in the BO + RAPA group (P = NS vs. Control, n = 6). E) mRNA expressions of MHC-IIb was significantly decreased in masseter muscle of the BO group versus the control group (**$P < 0.01$, n = 5), but the decrease was not significant in the BO + RAPA group (P = NS vs. Control, n = 6).

0.01 vs. Control, n = 3 – 5), but the magnitude of the increase in the BO + RAPA group was significantly smaller than that in the BO-rats without RAPA treatment ($P < 0.05$ vs. BO, n = 4 – 5) (Fig. 3A). Protein expression of MHC-IId/x was significantly increased by 1.2-fold in masseter muscle of BO-rats without RAPA treatment, compared with the control group ($P < 0.01$, n = 4 – 6), but that increase was abolished by RAPA (P = NS vs. Control, n = 5) (Fig. 3B). Protein expression of MHC-IIb was significantly decreased by approximately 60% in masseter muscle of BO-rats without RAPA treatment versus the control group, but RAPA treatment abrogated this decrease (P = NS vs. Control, n = 4 – 5) (Fig. 3C). Expression of MHC-Neo and MHC-I was barely detectable (Fig. 3D), in agreement with previous findings (13).

These results indicated that inhibition of the Akt/mTOR pathway with RAPA attenuated the fast-to-slow MHC isoform transition in masseter muscle of BO-rats at both the mRNA and protein levels.

Effects of RAPA on phosphorylation of Akt/mTOR downstream targets and ERK1/2 in BO-rats

In order to investigate the molecular signaling associated with the phenotypes induced in masseter muscle by mechanical overload and RAPA, we performed western blot analysis to examine the phosphorylation of Akt/mTOR downstream targets using total homogenate prepared from masseter muscle dissected at 3 – 7 days after the BO operation because phosphorylation of the Akt/mTOR downstream targets was easily detectable, rather than that at 14 days after the BO operation.

Two well-documented targets of Akt/mTOR are 70-kDa ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (30). Phosphorylations of S6K1 on threonine 389 and 4E-BP1 on threonine 37/46 were significantly increased by 2.6-fold ($P < 0.06$, n = 5 – 6) and 1.7-fold, respectively, in masseter muscle of BO-rats, compared with the control group ($P < 0.05$, n = 3 – 5), but these increases

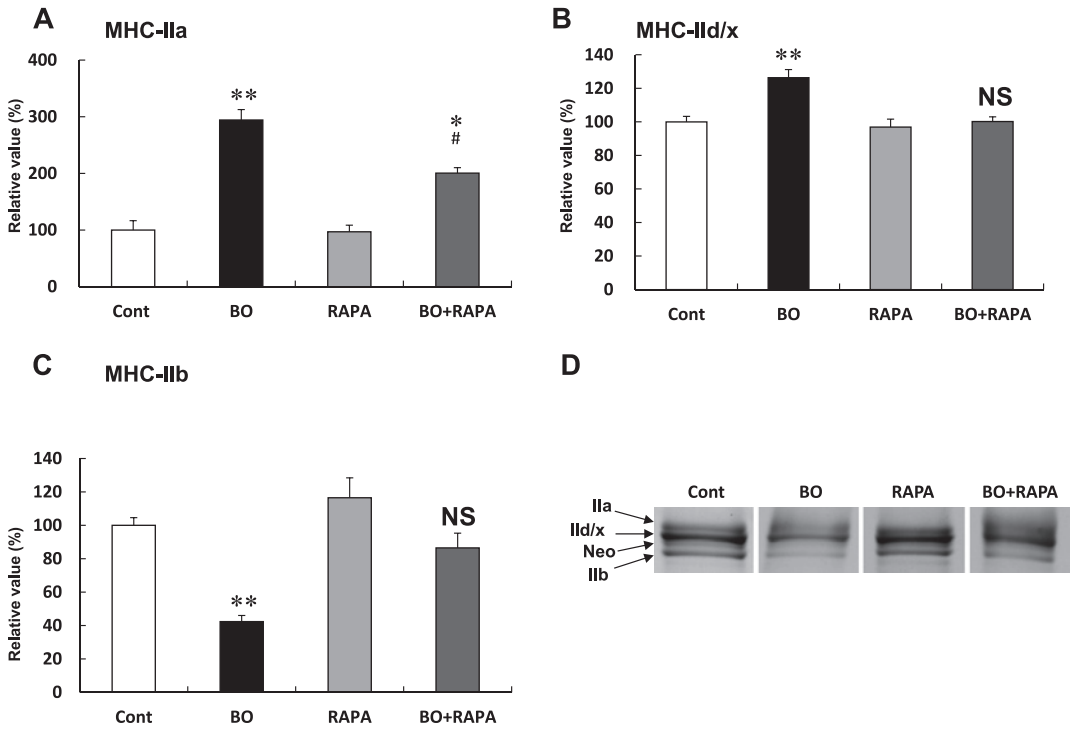

Fig. 3. RAPA attenuated fast-to-slow MHC isoform transition in masseter muscle of BO-rats at the protein level. A) Protein expression levels of MHC-IIa were significantly increased in masseter muscle of BO-rats treated (BO + RAPA) or not treated (BO) with RAPA, compared with the normal controls (*$P < 0.05$ and **$P < 0.01$ vs. Control, n = 3 – 5). The expression increase in the BO + RAPA group was significantly smaller than that in the BO group ($^{\#}P < 0.05$ vs. BO, n = 4 – 5). B) Protein expression of MHC-IId/x was significantly increased in masseter muscle of the BO-rats, compared with the control group (**$P < 0.01$, n = 4 – 6), but this increase was abolished in the BO + RAPA group (P = NS vs. Control, n = 5). C) Protein expressions of MHC-IIb was significantly decreased in masseter muscle of the BO group versus the control group (**$P < 0.01$, n = 5), but the decrease was not significant in the BO + RAPA group (P = NS vs. Control, n = 6). D) Representative silver staining of MHC isoform in the masseter muscle. Expression of MHC-I and MHC-Neo was barely detectable.

were significantly attenuated by RAPA ($P < 0.01$) (Fig. 4A, 4B, and 4D).

We also examined the phosphorylation of ERK1/2 on threonine 202 / tyrosine 204 because this has been reported to be important for regulating skeletal muscle mass and MHC isoform transition (10 – 12). The phosphorylation of ERK1/2 was significantly decreased in BO-rats, compared with the control group ($P < 0.01$, n = 5), but that decrease was abrogated by RAPA (Fig. 4: C and D).

Inhibition of the Akt/mTOR pathway with RAPA attenuated upregulation of PGC-1α in masseter muscle of BO-rats

We next examined the expression of PGC-1α, a transcriptional co-activator that is expressed preferentially in MHC-I, in masseter muscle of BO-rats treated or not treated with RAPA (31, 32). Expression of PGC-1α in the BO group was significantly increased by approximately two-fold compared to that in the control group ($P < 0.01$ vs. Control, n = 4 – 7) (Fig. 5A), while the increase in the BO + RAPA group was approximately 1.4-fold, which was significantly smaller than that in the BO group ($P < 0.05$ vs. BO, n = 6). These results indicate that inhibition of the Akt/mTOR pathway by RAPA attenuated the upregulation of PGC-1α mRNA expression in masseter muscle of BO-rats.

Inhibition of the Akt/mTOR pathway with RAPA did not alter upregulation of MCIP1 mRNA in masseter muscle of BO-rats

In order to examine the effects of RAPA treatment on the calcineurin pathway, we examined the expression of MCIP1 mRNA, which is regulated by calcineurin activity, in masseter muscle of the BO-rats treated or not treated with RAPA (14).

MCIP1 mRNA expression was significantly increased by 1.4-fold in masseter muscle of BO-rats, compared with the control group ($P < 0.05$ vs. Control) (Fig. 5B) and this increase was unaffected by RAPA treatment

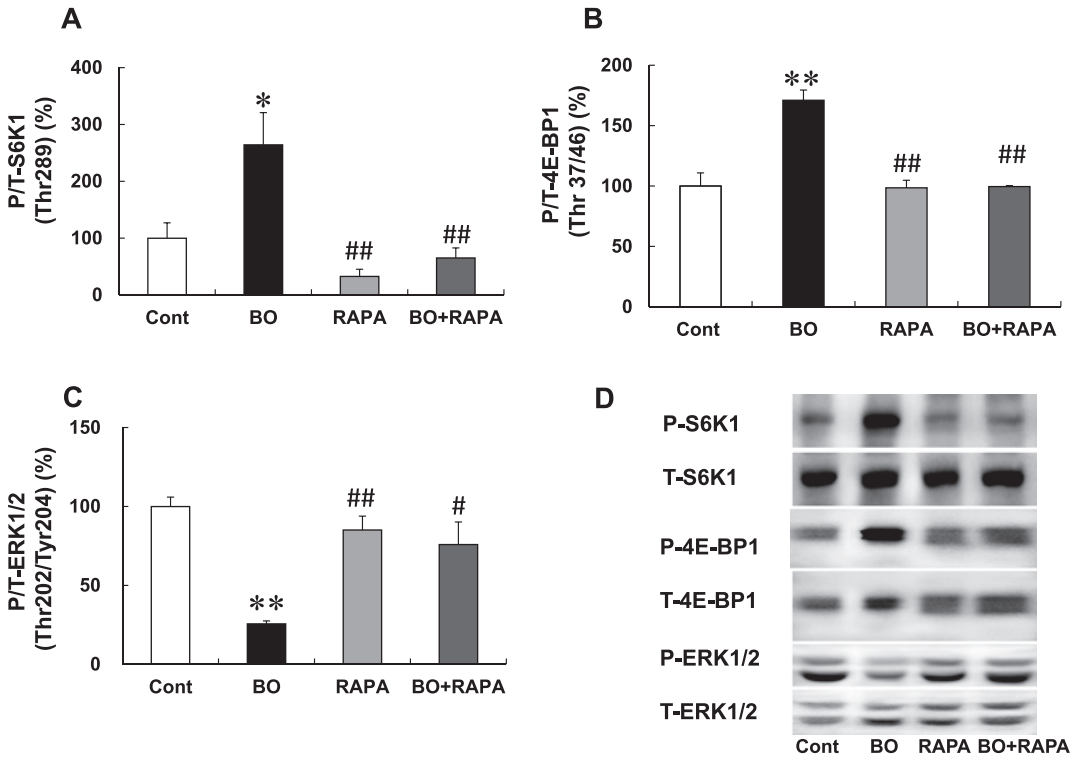

Fig. 4. Effects of Akt/mTOR inhibition with RAPA on phosphorylation of S6K1, 4E-BP1, and ERK1/2 in BO-rats. A) S6K1 phosphorylated on threonine 359 was significantly increased in BO-rats (Control vs. BO: 100% ± 27% vs. 264% ± 57%, $*P < 0.05$ vs. Control, n = 5 – 6), but that increase was abolished by RAPA in BO-rats (65% ± 18%, $^{\#\#}P < 0.01$ vs. BO, n = 5 – 6). The ratio of phosphorylated/total protein expression of S6K1 in the control group was taken as 100% in each determination. T-S6K1, total S6K1; P-S6K1 (Thr389), S6K1 phosphorylated on threonine 389. B) 4E-BP1 phosphorylated on threonine 37/46 was significantly increased in BO-rats (Control vs. BO: 100% ± 11% vs. 171% ± 9%, $**P < 0.01$, n = 3 – 5), but that increase was abolished by the treatment of RAPA in BO-rats (100% ± 1%, $^{\#\#}P < 0.01$ vs. BO, n = 3). The ratio of phosphorylated/total protein expression of 4E-BP1 in the control group was taken as 100% in each determination. T-4E-BP1, total 4E-BP1; P-4E-BP1 (Thr37/46), 4E-BP1 phosphorylated on threonine 37/46. C) ERK1/2 phosphorylated on threonine 202 / tyrosine 204 was significantly decreased in BO-rats (Control vs. BO: 100% ± 6% vs. 26% ± 2%, $**P < 0.01$, n = 5), but the decrease was abolished by the treatment of RAPA (76% ± 1%, $^{\#}P < 0.05$ vs. BO, n = 4 – 5). The ratio of phosphorylated/total protein expression of ERK1/2 in the control group was taken as 100% in each determination. T-ERK, total ERK; P-ERK (Thr202/Tyr204), phosphorylated ERK on threonine 202 / tyrosine 204. D) Representative immunoblotting results for phosphorylated forms and total proteins: S6K1 on threonine 359 (upper), 4E-BP1 on threonine 37/46 (middle), and ERK1/2 on threonine 202 / tyrosine 204 (lower).

(P = NS vs. BO, n = 7). These results indicated that inhibition of the Akt/mTOR pathway with RAPA did not influence the calcineurin pathway via modulation of MCIP1 upregulation in masseter muscle of BO-rats.

Inhibition of the Akt/mTOR pathway with RAPA did not affect mRNA expression of atrogin-1 and MuRF1

In order to examine the effects of RAPA treatment on the Akt/Foxo pathway, we examined the expression of atrogin-1 and MuRF1, which are downstream molecules of the Akt/Foxo pathway, in masseter muscle of the four groups. However, we found no difference among them (P = NS vs. Control, n = 7) (Fig. 5: C and D). These data indicated that RAPA has no effect on atrogin-1 and MuRF1 expression.

Discussion

In this study, we examined the influence of the Akt/mTOR pathway on the hypertrophy and fast-to-slow MHC isoform transition in masseter muscle of BO-rats by means of pharmacological inhibition with RAPA. Our results indicate that the Akt/mTOR pathway plays a significant role not only in the development of hypertrophy, but also in the fast-to-slow MHC isoform transition in this model, in addition to the calcineurin pathway, which we had previously demonstrated to be important in BO-rats by means of pharmacological inhibition with cyclosporine A (8).

However, the two pathways might be independent because the magnitude of the MCIP1 mRNA increase,

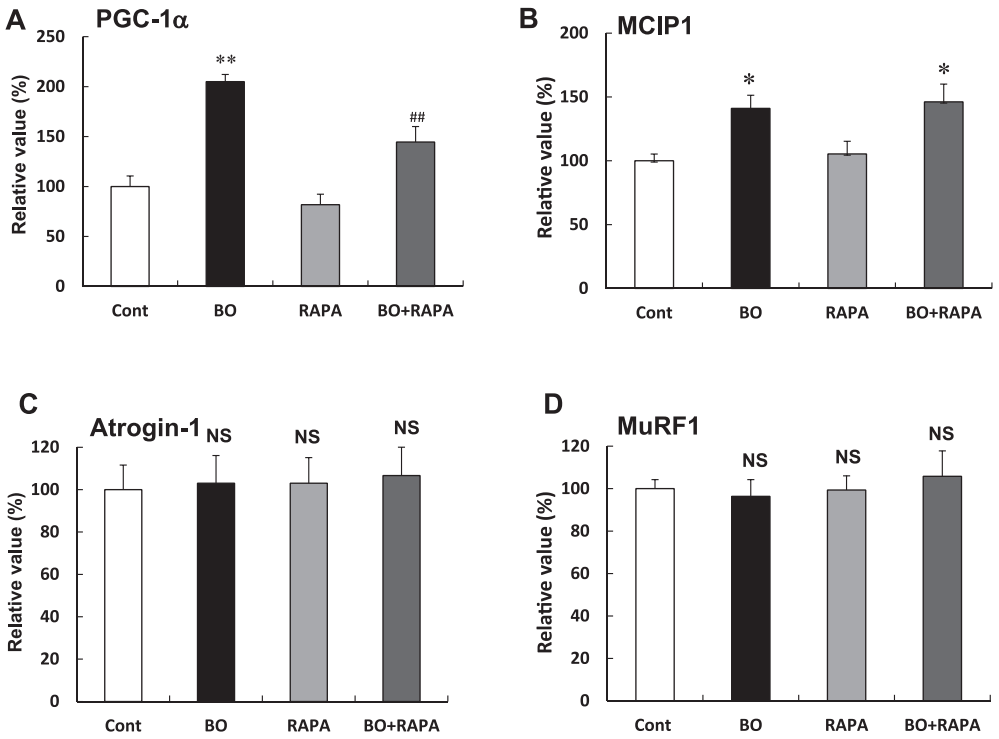

Fig. 5. Effects of Akt/mTOR inhibition with RAPA on expression of PGC-1α, MCIP1, Atrogin-1, and MuRF1 in BO-rats. A) Expression of PGC-1α in masseter muscle of the BO group was significantly greater by approximately 2-fold than that of the control group (**$P < 0.01$, n = 4), but the increase in the BO + RAPA group was smaller (1.4-fold) and was significantly attenuated compared with the BO group ($^{\#}P < 0.05$ vs. BO, n = 6). B) Expression of MCIP1 was significantly greater in masseter muscle of the BO group and the BO + RAPA group, compared with the control group (*$P < 0.05$ vs. Control, n = 7), and the magnitude of increase was similar in the two groups. C – D) Expression levels of atrogin-1 and MuRF1 were similar among the four groups (P = NS vs. Control, n = 7).

which is regulated via the calcineurin pathway, was similar in BO and BO + RAPA groups. Importantly, the calcineurin pathway was demonstrated to be involved in the transition from MHC-IIa to MHC-I without affecting the transitions among MHC-IIb, MHC-IId/x, and MHC-IIa (8), whereas the Akt/mTOR pathway has been shown to influence the transitions among all MHC isoforms, indicating that the Akt/mTOR pathway regulates masseter muscle hypertrophy and fast-to-slow MHC isoform transition, independently of the calcineurin pathway.

We also examined the activation (phosphorylation) of the ERK1/2 pathway because this has been reported to be necessary to preserve the fast-twitch fiber phenotype, with concomitant repression of the slow-twitch fiber phenotype in mouse C2C12 myoblasts in vitro and in skeletal muscle (11, 12). The ERK1/2 pathway and Akt/mTOR pathway are both known to be important for regulating skeletal muscle mass, but the relationship between the two pathways remains poorly understood (33 – 35). We thus examined the phosphorylation of ERK1/2 on threonine 202 / tyrosine 204 and found that it was significantly decreased in BO-rats, while the decrease was abrogated by the inhibition of the Akt/mTOR pathway with RAPA. These results indicate that the ERK1/2 pathway may contribute to the fast-to-slow MHC-I transition in BO-rats at least in part through the Akt/mTOR pathway.

It has been shown that mechanical overload–mediated hypertrophy induces reexpression of the neonatal MHC isoform in heart (36). Interestingly, we observed reexpression of MHC-Neo together with downregulation of MHC-IIb in masseter muscle of BO-rats, that is, the opposite change to that seen during postnatal development, and this phenomenon was attenuated by RAPA. The physiological significance of the induction of MHC-Neo in masseter muscle is presently unknown, but our current findings indicate that the MHC isoform transition from the MHC-Neo to adult type MHC isoforms (I, IIa, IId/x, IIb) at birth may be associated with activation of the Akt/mTOR pathway.

Slow-twitch MHC isoform contains a high level of mitochondria and oxidative metabolism is its chief energy source. In contrast, fast-twitch MHC isoforms such as MHC-IIb generate energy mainly through glycolysis.

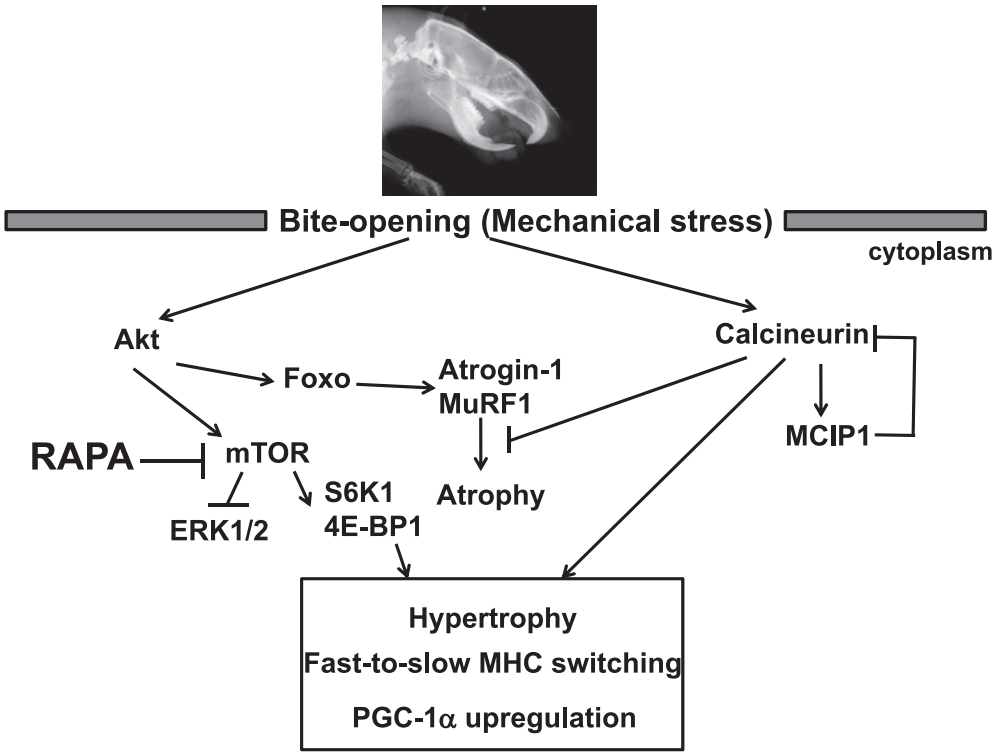

Fig. 6. Schematic illustration of the Akt/mTOR pathway, ERK1/2 pathway, and calcineurin pathway in masseter muscle of BO-rats. The Akt/mTOR pathway plays an important role in the development of hypertrophy and in the fast-to-slow MHC isoform transition, in addition to the role of the ERK1/2 pathway and calcineurin pathway, based on the present results and our previous studies.

The proportion of MHC-I was increased in masseter muscle of BO-rats. PGC-1α is a dominant regulator of mitochondrial content and increases mitochondrial biogenesis through upregulation of mitochondrial enzymes such as cytochrome c oxidase I (31, 32). We thus examined the mRNA expression of PGC-1α and found that it was significantly increased in both the BO and BO + RAPA groups, but the magnitude of the increase was much greater in the BO group. This is consistent with the decreased upregulation of MHC-I mRNA in the BO + RAPA group, compared with the BO group.

MCIP1, which is highly expressed in skeletal muscle, is induced by calcineurin and it inhibits calcineurin activity, thereby establishing a negative feedback loop. Consequently, altered upregulation of MCIP1 in response to mechanical overload may be related to a decrease of calcineurin activity (16). We therefore examined the mRNA expression of MCIP1 and found that it was similarly increased in masseter muscle of BO-rats treated or not treated with RAPA, suggesting that the calcineurin pathway was not affected by inhibition of the Akt/mTOR pathway through modulation of MCIP1 upregulation in BO-rats.

Foxo and mTOR are major downstream targets of Akt and activation of the Akt/Foxo pathway leads to inhibition of calcineurin activity (16). We therefore examined the involvement of the Akt/Foxo pathway by measuring mRNA expression of downstream molecules of Foxo, that is, E3 ubiquitin ligase atrogin-1 and MuRF1, which are involved in ubiquitin-mediated proteolysis of calcineurin in cardiac muscle (15, 16, 26, 27). There was no difference among the four groups, suggesting that the calcineurin pathway would not have been affected through modulation of the Akt/Foxo pathway in our experimental model.

Taken together, our experimental results indicate that the Akt/mTOR pathway plays an important role both in the development of hypertrophy and in fast-to-slow MHC isoform transition, independently of the calcineurin pathway (Fig. 6). Therefore, pharmacological activation of the Akt/mTOR pathway may provide a means to improve rhythmic movement and contractility by increasing the fast twitch fibers in masseter muscle for the treatment of masticatory dysfunction due to masseter muscle wasting and weakness.

Acknowledgments

This study was supported by funds from the Japanese Ministry of Education, Culture, Sports, Science and Technology; Takeda Science Foundation; Yokohama Foundation for Advancement of Medical Science; Yokohama City University (Grant for 2006 – 2007 Strategic Research Project: No. K19027); Mitsubishi Pharma Research Foundation; Yokohama Academic Foundation [Research for Promoting Technological Seeds A (discovery type) 2010]; Yokohama City University (Commercialization Promotion Program for Biotechnology-related Studies and Grants for Research and Development Project II No. 8 and 14); and the Research Foundation for Community Medicine.

References

1 Clark GT, Beemsterboer PL, Rugh JD. Nocturnal masseter muscle activity and the symptoms of masticatory dysfunction. J Oral Rehabil. 1981;8:279–286.
2 Manns A, Miralles R, Santander H, Valdivia J. Influence of the vertical dimension in the treatment of myofascial pain-dysfunction syndrome. J Prosthet Dent. 1983;50:700–709.
3 Boero RP. The physiology of splint therapy: a literature review. Angle Orthod. 1989;59:165–180.
4 Schiaffino S, Reggiani C. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiol Rev. 1996;76:371–423.
5 Pette D, Staron RS. Transitions of muscle fiber phenotypic profiles. Histochem Cell Biol. 2001;115:359–372.
6 Mallinson J, Meissner J, Chang KC. Chapter 2. Calcineurin signaling and the slow oxidative skeletal muscle fiber type. Int Rev Cell Mol Biol. 2009;277:67–101.
7 Ohnuki Y, Saeki Y, Yamane A, Yanagisawa K. Quantitative changes in the mRNA for contractile proteins and metabolic enzymes in masseter muscle of bite-opened rats. Arch Oral Biol. 2000;45:1025–1032.
8 Arai C, Ohnuki Y, Umeki D, Saeki Y. Effects of bite-opening and cyclosporin A on the mRNA levels of myosin heavy chain and the muscle mass in rat masseter. Jpn J Physiol. 2005;55:173–179.
9 Leger B, Cartoni R, Praz M, Lamon S, Deriaz O, Crettenand A, et al. Akt signalling through GSK-3β, mTOR and FOXO1 is involved in human skeletal muscle hypertrophy and atrophy. J Physiol. 2006;576:923–933.
10 Schiaffino S, Reggiani C. Fiber types in mammalian skeletal muscles. Physiol Rev. 2011;91:1447–1531.
11 Shi H, Zeng C, Ricome A, Hannon KM, Grant AL, Gerrard DE. Extracellular signal-regulated kinase pathway is differentially involved in β-agonist-induced hypertrophy in slow and fast muscles. Am J Physiol Cell Physiol. 2007;292:C1681–C1689.
12 Shi H, Scheffler JM, Pleitner JM, Zeng C, Park S, Hannon KM, et al. Modulation of skeletal muscle fiber type by mitogen-activated protein kinase signaling. FASEB J. 2008;22:2990–3000.
13 Ohnuki Y, Kawai N, Tanaka E, Langenbach GE, Tanne K, Saeki Y. Effects of increased occlusal vertical dimension on daily activity and myosin heavy chain composition in rat jaw muscle. Arch Oral Biol. 2009;54:783–789.
14 Yang J, Rothermel B, Vega RB, Frey N, McKinsey TA, Olson EN, et al. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res. 2000;87:E61–E68.
15 Willis MS, Patterson C. Into the heart: the emerging role of the ubiquitin-proteasome system. J Mol Cell Cardiol. 2006;41:567–579.
16 Ni YG, Berenji K, Wang N, Oh M, Sachan N, Dey A, et al. Foxo transcription factors blunt cardiac hypertrophy by inhibiting calcineurin signaling. Circulation. 2006;114:1159–1168.
17 Yang Y, Duan JZ, Gui DM, Yang HW, Gao DW. Effect of aminoguanidine on caspase-3 expression in rat retina after ischemia-reperfusion injury. Int J Ophthalmol. 2011;4:259–261.
18 Nordstrom SH, Bishop M, Yemm R. The effect of jaw opening on the sarcomere length of the masseter and temporal muscles of the rat. Arch Oral Biol. 1974;19:151–155.
19 Pallafacchina G, Calabria E, Serrano AL, Kalhovde JM, Schiaffino S. A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proc Natl Acad Sci U S A. 2002;99:9213–9218.
20 Houde VP, Brule S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, et al. Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. Diabetes. 2010;59:1338–1348.
21 Brewster AL, Lugo JN, Patil VV, Lee WL, Qian Y, Vanegas F, et al. Rapamycin reverses status epilepticus-induced memory deficits and dendritic damage. PLoS One. 2013;8:e57808.
22 Wang P, Zhang RY, Song J, Guan YF, Xu TY, Du H, et al. Loss of AMP-activated protein kinase-α2 impairs the insulin-sensitizing effect of calorie restriction in skeletal muscle. Diabetes. 2012;61:1051–1061.
23 Dunn SE, Chin ER, Michel RN. Matching of calcineurin activity to upstream effectors is critical for skeletal muscle fiber growth. J Cell Biol. 2000;151:663–672.
24 Hinkle RT, Donnelly E, Cody DB, Sheldon RJ, Isfort RJ. Activation of the vasoactive intestinal peptide 2 receptor modulates normal and atrophying skeletal muscle mass and force. J Appl Physiol. 2005;98:655–662.
25 Kiliaridis S, Engstrom C, Thilander B. Histochemical analysis of masticatory muscle in the growing rat after prolonged alteration in the consistency of the diet. Arch Oral Biol. 1988;33:187–193.
26 Bai Y, Tsunematsu T, Jiao Q, Ohnuki Y, Mototani Y, Shiozawa K, et al. Pharmacological stimulation of type 5 adenylyl cyclase stabilizes heart rate under both microgravity and hypergravity induced by parabolic flight. J Pharmacol Sci. 2012;119:381–389.
27 Okumura S, Suzuki S, Ishikawa Y. New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: Effects of targeted disruption of the type 5 adenylyl cyclase gene. J Pharmacol Sci. 2009;109:354–359.
28 Okumura S, Takagi G, Kawabe J, Yang G, Lee MC, Hong C, et al. Disruption of type 5 adenylyl cyclase gene preserves cardiac function against pressure overload. Proc Natl Acad Sci U S A. 2003;100:9986–9990.
29 Okumura S, Kawabe J, Yatani A, Takagi G, Lee MC, Hong C, et al. Type 5 adenylyl cyclase disruption alters not only sympathetic but also parasympathetic and calcium-mediated cardiac regulation. Circ Res. 2003;93:364–371.
30 Kline WO, Panaro FJ, Yang H, Bodine SC. Rapamycin inhibits the growth and muscle-sparing effects of clenbuterol. J Appl

Physiol. 2007;102:740–747.
31 Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, et al. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. 1999;98:115–124.
32 Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, et al. Transcriptional co-activator PGC-1α drives the formation of slow-twitch muscle fibres. Nature. 2002;418:797–801.
33 Dupont E, Cieniewski-Bernard C, Bastide B, Stevens L. Electrostimulation during hindlimb unloading modulates PI3K-AKT downstream targets without preventing soleus atrophy and restores slow phenotype through ERK. Am J Physiol Integr Comp Physiol. 2011;300:R408–R417.
34 Semsarian C, Wu MJ, Ju YK, Marciniec T, Yeoh T, Allen DG, et al. Skeletal muscle hypertrophy is mediated by a Ca^{2+}-dependent calcineurin signalling pathway. Nature. 1999;400:576–581.
35 Dunn SE, Burns JL, Michel RN. Calcineurin is required for skeletal muscle hypertrophy. J Biol Chem. 1999;274:21908–21912.
36 Izumo S, Nadal-Ginard B, Mahdavi V. Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. Proc Natl Acad Sci U S A. 1988;85:339–343.