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ORIGINAL ARTICLE

Repetitive restriction of muscle blood flow enhances mTOR signaling pathways in a rat model

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Abstract Skeletal muscle is a plastic organ that adapts its mass to various stresses by affecting pathways that regulate protein synthesis and degradation. This study investigated the effects of repetitive restriction of muscle blood flow (RRMBF) on microvascular oxygen pressure ($PmvO_2$), mammalian target of rapamycin (mTOR) signaling pathways, and transcripts associated with proteolysis in rat skeletal muscle. Eleven-week-old male Wistar rats under anesthesia underwent six RRMBF consisting of an external compressive force of 100 mmHg for 5 min applied to the proximal portion of the right thigh, each followed by 3 min rest. During RRMBF, $PmvO_2$ was measured by phosphorescence quenching techniques. The total RNA and protein of the tibialis anterior muscle were obtained from control rats, and rats treated with RRMBF 0–6 h after the stimuli. The protein expression and phosphorylation of various signaling proteins were determined by western blotting. The mRNA expression level was measured by real-time RT-PCR analysis. The total muscle weight increased in rats 0 h after RRMBF, but not in rats 1–6 h. During RRMBF, $PmvO_2$ significantly decreased (36.1 ± 5.7 to 5.9 ± 1.7 torr), and recovered at rest period. RRMBF significantly increased phosphorylation of p70 S6-kinase (p70S6k), a downstream target of mTOR, and ribosomal protein S6 1 h after the stimuli. The protein level of REDD1 and phosphorylation of AMPK and MAPKs did not change. The mRNA expression levels of FOXO3a, MuRF-1, and myostatin were not significantly altered. These results suggested that RRMBF significantly decreased $PmvO_2$, and enhanced mTOR signaling pathways in skeletal muscle using a rat model, which may play a role in diminishing muscle atrophy under various conditions in human studies.

Keywords Blood flow restriction · Mammalian target of rapamycin (mTOR) · Microvascular pO_2 · Muscle atrophy · Myostatin · p70 S6-kinase · Rat skeletal muscle · Hypoxia

Abbreviations

RMBF	Restriction of muscle blood flow
RRMBF	Repetitive restriction of muscle blood flow
$PmvO_2$	Microvascular oxygen pressure
HIF-1α	Hypoxia-inducible factor-1α
mTOR	Mammalian target of rapamycin
p70S6k	p70 S6-kinase
ERK1/2	Extracellular signal-regulated kinase 1/2
FOXO3a	Forkhead box O3A
MuRF-1	Muscle ring finger-1
VEGF	Vascular endothelial growth factor

Introduction

It has been well known that muscle size is mainly determined by the balance between muscle protein synthesis and

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degradation [1, 2]. Muscle hypertrophy occurs when the protein synthesis exceeds the protein degradation, while muscle atrophy occurs when the protein degradation overcomes the protein synthesis. After muscle disuse, for example during long-term bed rest, spaceflight, and simulated models of no-bearing activity, skeletal muscle atrophy develops due to the altered protein metabolism, leading to decreased muscle contractile protein content. To prevent it and increase muscle protein synthesis, resistance exercise, an established potent stimulus for enhancing muscle protein synthesis and subsequent muscle hypertrophy, is usually used. The increased muscle protein synthesis is associated with enhanced phosphorylation events on numerous kinases to regulate mRNA translation, including mammalian target of rapamycin (mTOR1)/p70 S6-kinase (p70S6k) signaling pathways [3, 4].

The conventional strength training pursued at intensity lower than 60–70 % 1RM usually does not increase muscle size, compared with high-intensity exercise. However, high-intensity loading entails a risk of injury to the moving organs or circulatory system in elderly individuals and patients with various diseases. On the other hand, several human studies have shown that low-intensity (20–30 % 1RM) resistance exercise under restriction of muscle blood flow (RMBF), also known as KAATSU training [5, 6], is a new strength training method which can stimulate numerous muscle fibers and produce increased muscle size and strength even with low-intensity loading [6, 7]. Fujita et al. [8] reported that the protein synthesis increased in healthy young adults 3 h after a brief session of RMBF knee extension exercise. Additionally, they showed that phosphorylation of p70 S6-kinase, a key regulator of the mTOR pathway, was enhanced after RMBF exercise, compared with control exercise. Moreover, reduced expression of the proteolysis-related genes such as forkhead box O3A (FOXO3a), atrogin-1 and muscle ring finger-1 (MuRF-1) as well as myostatin, a negative regulator of muscle mass, has been reported after acute RMBF exercise [9, 10]. These mechanisms may play a role on hypertrophic effects of RMBF exercise, even by using low-intensity resistance exercise. On the other hand, skeletal muscle is a plastic organ that adapts its mass to various stresses by affecting pathways that regulate protein and cellular turnover. Takarada et al. [11] reported that repetitive restriction of muscle blood flow (RRMBF) even without exercise effectively diminished postoperative disuse atrophy of knee extensors in patients who underwent reconstructive surgery of the anterior cruciate ligament. They used RRMBF stimulus, each consisting of five repetitions of RMBF (mean maximal pressure, 238 mmHg) for 5 min, and release of pressure for 3 min. Kubota et al. [12] also reported that the application of RRMBF to the lower extremity prevented disuse muscular weakness induced by immobilization without weight bearing. They used RRMBF at an external compressive force of 200 mmHg for 5 min, followed by 3 min rest, a regimen repeated five times in a single session. Thus, RRMBF appears to be a novel stimulus of skeletal muscle to induce a net positive protein balance and prevent atrophy. However, the underlying mechanisms of RRMBF have not been investigated.

Therefore, we investigated the effects of RRMBF on mTOR signaling pathways involved in initiating skeletal muscle translation, and transcripts associated with proteolysis as well as microvascular pO_2 ($PmvO_2$) in skeletal muscle using a rat model. Here we documented for the first time that RRMBF significantly decreased $PmvO_2$, and enhanced mTOR signaling pathways in skeletal muscle using a rat model.

Materials and methods

Animals

Male Wistar rats ($n = 34$, age 11 weeks) were used in our study (Japan SLC, Inc., Shizuoka Lab. Animal Center). All rats were housed in a temperature-controlled room at 22 ± 2 °C with a light–dark cycle of 12 h, and were maintained on rat feed and water ad libitum as previously described [13]. All procedures for microvascular PO_2 ($PmvO_2$) measurement and blood flow restriction experiments were performed under isoflurane anesthesia (50 mg/kg body wt). All experiments were conducted under the guidelines established by the Physiological Society of Japan and were approved by University of Electro-Communications Institutional Animal Care and Use Committee.

Repetitive restriction of muscle blood flow (RRMBF)

Under isoflurane anesthesia, rats were secured in a supine position, and the hip and knee joint of the right leg were bent in 90° flexion. The right leg was fixed mechanically and positioned on a firm and stable pedal as shown in Fig. 1a. Blood flow restriction to the right lower extremity was induced by compressing the proximal end of the right thigh with a Durable Digit Cuff (latex cuff; overall cuff size 16 mm width, 90 mm length; D.E. Hokanson, Inc.). The RRMBF stimulus, which consisted of an external compressive force of 100 mmHg for 5 min, followed by 3 min of rest, was repeated six times during a 48-min interval (Fig. 1b) as described in the previous clinical studies [11, 12].

Experimental protocol

Microvascular PO_2 ($PmvO_2$) measurement

Phosphorescence quenching as described previously [14] was utilized to determine $PmvO_2$ during a blood flow

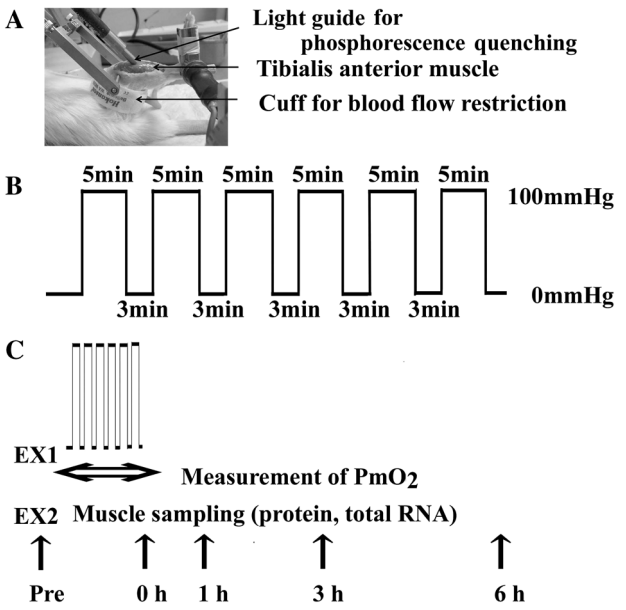

Fig. 1 Experimental protocols. **a** Blood flow restriction (BFR) using a durable digit cuff. Under isoflurane anesthesia, rats were kept at supine position and the right leg was fixed on a firm and stable pedal at 2–3 cm above the chest level with both hip and ankle joint of 90° (0° at full extension). **b** Protocols of repetitive restriction of muscle blood flow (RRMBF). The RRMBF stimuli, consisting of external compressive force of 100 mmHg for 5 min followed by 3 min of rest, were repeated six times for 48 min. **c** Experimental protocol for microvascular PO_2 ($PmvO_2$) measurement during RRMBF (*Experiment 1* EX1) and muscle tissue isolation (*Experiment 2* EX2). Phosphorescence quenching method was utilized for the determination of Pmv_{O2} during RRMBF protocol within the medial region of the tibialis anterior muscle. In EX2, rats in each group ($n = 6$) were sacrificed in control (pre), immediately after (0 h), 1, 3, and 6 h after RRMBF, and the total tibialis anterior muscles were dissected from both right leg (BFR side) and left leg (control side)

restriction protocol applied to the medial region of the tibialis anterior muscle. Rats were anesthetized with isofurane anesthesia. To monitor arterial blood pressure and heart rate, the left carotid artery was cannulated (PE50). This catheter was also used to infuse a phosphorescent probe [palladium meso-tetra (4-carboxyphenyl) porphyrin dendrimer (R2)] at 15 mg/kg body wt. A lateral incision of the skin and overlying fascia was made to expose the tibialis anterior muscle of the right leg (Fig. 1a). The muscle surface was superfused with Krebs-Henseleit solution, equilibrated with 5 % CO_2–95 % N_2 at 38 °C, and adjusted to pH 7.4. The phosphor R2 was applied via the arterial cannula 15 min before the beginning of experiments, which were performed in a darkened room to prevent contamination by ambient light. The total duration of the experiments did not exceed 1 h.

$PmvO_2$ was determined at 1-s intervals before and during RRMBF, and for 5 min during recovery [Fig. 1c, Experiment (EX) 1]. A PMOD 2000 frequency domain phosphorometer (Oxygen Enterprises, Philadelphia, PA) was used to determine $PmvO_2$. The common end of the bifurcated light guide was placed approximately 2–3 mm above the medial region of the tibialis anterior muscle. The theoretical basis for phosphorescence quenching has been reported previously [15, 16]. Briefly, the Stern–Volmer relationship describes the quantitative O_2 dependence of the phosphorescent probe. R2 is a nontoxic dendrimer that binds to albumin at 38 °C and pH 7.4, with a quenching constant of 409 $mmHg^{-1}$ s^{-1} and a 601 μs lifetime of decay of in the absence of O_2, under the physiological conditions extant herein [17]. The net negative charge of R2 also facilitates restriction of the compound to the vascular space. Thus, $PmvO_2$ reflects the pO_2 within capillary blood, which constitutes the principal intramuscular vascular space [15, 18].

Isolation of muscle tissue

Male Wistar rats ($n = 30$) underwent RRMBF under isoflurane anesthesia (50 mg/kg body wt) in 100 % O_2. Rats in each group ($n = 6$) were sacrificed immediately (~approximately 5 min after RRMBF) (0 h), 1 h, 3 h, and 6 h after RRMBF, and the entire tibialis anterior muscle was collected from both the right leg (blood flow restriction side) and left leg (control side). The harvested tissue was immediately flash frozen in liquid nitrogen and stored at −80 °C until use in experiments [Fig. 1c, Experiment (EX) 2].

RNA extraction and real-time quantitative reverse transcriptase/polymerase chain reaction (RT-PCR)

All cellular RNA was extracted from each tibialis anterior muscle using an RNeasy mini kit (Qiagen, Cambridge, MA). For RT-PCR, complementary DNA (cDNA) was synthesized from 1 μg of total RNA using a reverse transcriptase with random primers (Toyobo, Osaka). Real-time quantitative RT-PCR was performed using real-time Taq-Man technology and a sequence detector (ABI PRISM®7000, Applied Biosystems, Foster City, CA). Gene-specific primers and Taq-Man probes were used to analyze transcript quantity. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript quantity of mRNA. The probes used in this study were purchased as part of the Assay-on-Demand service from Applied Biosystems (Foster City, CA): Assay ID Rn01441087_m1 for forkhead box O3A (FOXO3a), Rn00590197_m1 for muscle ring finger-1 (MuRF-1), Rn00569683_m1 for myostatin, Rn00577560_m1 for hypoxia-inducible factor-1α (HIF-1α), and 4310893E for 18S rRNA endogenous control.

Western blotting analysis

Proteins were separated on 10 % polyacrylamide gels for 60 min at 200 V and then transferred to Amersham Hypond-P membranes (GE Healthcare UK Ltd., Buckinghamshire, England) for 60 min at 72 mA using the semi-dry method. After the transfer, the membrane was blocked with 2–3 % skim milk in PBS (0.01 M phosphate buffer, 0.138 M NaCl, 0.027 M KCl, pH 7.4) with 0.1 % Tween 30 (PBS-T) at room temperature for 1 h. The membrane was then exposed to antibodies at the appropriate dilutions in blocking buffer overnight at 4 °C. The probed membrane was then washed three times in PBS-T for 15 min each time and was subsequently incubated with peroxidase-linked anti-rabbit IgG (Santa Cruz Biotechnology, Inc., CA; diluted 1:5000 with blocking buffer) for 1 h at room temperature. After three additional washes, bound antibodies were detected with a Chemi-Lumi One Super kit (Nacalai Tesque, Kyoto, Japan) and analyzed with an LAS-3000 mini-image analyzer (Fuji-Film, Tokyo, Japan).

Phosphorylation of Akt (Protein kinase B, PKB) on Ser473 was assessed using anti-phospho-Akt antibody (#4060, Cell Signaling Technology, Beverly, MA, USA) and anti-total Akt antibody (#4691, Cell Signaling Technology). Phosphorylation of mTOR on Ser2448 was measured using anti-phospho-mTOR antibody (#2971, Cell Signaling Technology) and anti-total mTOR antibody (#2972, Cell Signaling Technology). Phosphorylation of p70 S6 kinase (p70S6 k) on Thr389 was determined using anti-phospho-p70 S6 kinase antibody (#9234, Cell Signaling Technology) and anti-total p70 S6 kinase antibody (#2708, Cell Signaling Technology). Ribosomal protein S6 phosphorylation (rpS6) on Ser240/244 was evaluated using anti-phospho-S6 antibody (#5364, Cell Signaling Technology) and anti-total S6 antibody (#2317, Cell Signaling Technology). Antibody dilutions were 1:1000 in each case. Phosphorylation of eEF2 on Thr56 was determined using anti-phospho-eEF2 antibody (#2331, Cell Signaling Technology) and anti-total eEF2 antibody (#2332, Cell Signaling Technology). 4E-BP1 phosphorylation on Thr37/46 was assessed using anti-phospho-4E-BP1 antibody at a dilution of 1:4000 (#2855, Cell Signaling Technology) and anti-total 4E-BP1 antibody (#9644, Cell Signaling Technology). AMPK phosphorylation on Thr172 was assessed using anti-phospho-AMPKα antibody (#2535, Cell Signaling Technology) and anti-total AMPKα antibody (#2532, Cell Signaling Technology). Phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) at Thr202/Tyr204, p38 mitogen-activated protein kinase (p38MAPK) at Thr180/Tyr182, and c-Jun N-terminal kinase 1 (JNK1) at Thr183/Tyr185 was measured using anti-phospho-ERK1/2 antibody (#4370, Cell Signaling Technology) and anti-total ERK1/2 antibody (#4695, Cell Signaling Technology), anti-phospho-p38MAPK antibody (#4511, Cell Signaling Technology) and anti-total p38MAPK antibody (#2307, Cell Signaling Technology), and anti-phospho-JNK1/2 antibody (# 9255, Cell Signaling Technology) and anti-total JNK1/2 antibody (#3708, Cell Signaling Technology), respectively. No change in total protein content was observed for any of the variables during these experiments.

Anti-REDD1 (regulated in development and DNA damage responses) rabbit polyclonal antibodies (NOVUS, NBP1-95,188) were obtained from Novus Biological. Anti-HIF-1α antibody and anti-vascular endothelial growth factor (VEGF) rabbit polyclonal antibody were purchased from Abcam. Blots were loaded according to protein concentration (approx. 100–150 μg).

Data analysis

All values are expressed as mean ± SEM. Differences between groups were compared by one-way ANOVA and Bonferroni's post hoc test. The level of significance was set at $P < 0.05$.

Results

$PmvO_2$ response during RRMBF

Figures 2a, b show dynamic $PmvO_2$ profiles in response to RRMBF. The typical data obtained from a rat are illustrated in Fig. 2a. During the application of RRMBF, dynamic $PmvO_2$ decreased rapidly and reached a level of approximately 5–8 torr. After release of the cuff pressure, dynamic P $PmvO_2$ returned to a control level. During RRMBF, similar dynamic $PmvO_2$ profiles were observed. Figure 2b shows mean $PmvO_2$ profiles during RRMBF obtained from four different rats. Baseline values of $PmvO_2$ were 36.1 ± 5.7 torr ($n = 4$). The minimum $PmvO_2$ level during RRMBF changed in the range of 4.8–9.0 torr ($n = 4$). The mean value was 5.9 ± 1.7 torr ($n = 4$), and gradually recovered during a rest period.

Animal data

Figures 2c, d show the effects of RRMBF on total weight of the tibialis anterior muscle and total muscle weight/body weight. Total weight of the tibialis anterior muscle (Fig. 2c) and total muscle weight/body weight (Fig. 2d) increased significantly in rats sacrificed immediately after RRMBF, compared with control rats. It is therefore likely that the application of RRMBF using 100 mmHg in the present model induced venous pooling of blood in the lower leg and swelled the tibialis anterior muscle, a known phenomenon of KAATSU training [5, 19]. The increase in

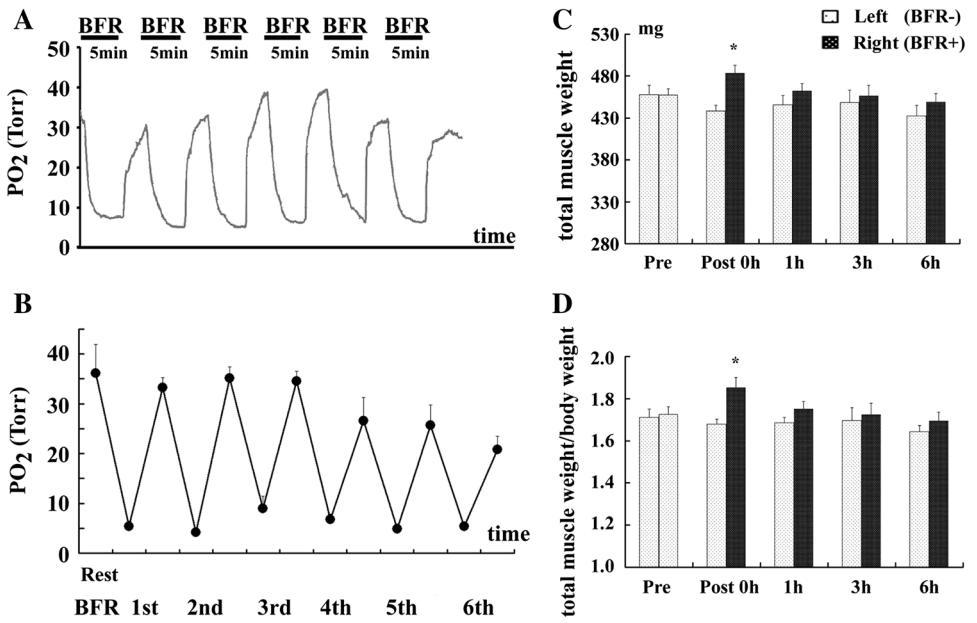

Fig. 2 **a**, **b** Effects of RRMBF on microvascular pO_2 ($PmvO_2$) measurement. **a** Typical data recording of the dynamic Pmv_{O2} profiles in response to RRMBF. Note that during the application of RRMBF, the dynamic $PmvO_2$ rapidly decreased, and reached to a level of approximately 4.8–9.0 torr. After the release of BFR, the dynamic $PmvO_2$ returned almost to a control level. **b** The mean $PmvO_2$ profiles during RRMBF obtained from four different rats. The values of $PmvO_2$ at rest were 36.1 ± 5.7 torr ($n = 4$), and significantly decreased to 5.9 ± 1.7 torr significantly ($n = 4$) during RRMBF. **c**, **d** Effects of RRMBF on total muscle weight (**c**) and total muscle weight/body weight of the tibialis anterior muscle (**d**). The total muscle weight and total muscle weight/body weight of the tibialis anterior muscle were measured in control rats, and rats immediately (0 h), 1, 3, and 6 h after RRMBF. Each data were obtained from both sides of the muscles ($n = 6$). Note that the total muscle weight and total muscle weight/body weight of the tibialis anterior muscle increased significantly in rats sacrificed immediately after RRMBF. *$P < 0.05$ vs. control (pre)

total weight of the tibialis anterior muscle and total muscle weight/body weight had returned to control levels at 1 h after RRMBF.

Effects of RRMBF on mTOR signaling pathways

Figure 3 shows the effects of RRMBF on phosphorylation of $p70^{S6k}$, a downstream target of mTOR and a factor in synthesis regulation of some ribosomal proteins. As shown in Fig. 3a, p70S6 k phosphorylation at Thr389, a phosphorylation site associated with maximal activation of the kinase [20], in the right tibialis anterior muscle (BFR leg) was increased significantly 1 h after RRMBF, compared with control rats ($p < 0.05$). However, $p70^{S6k}$ phosphorylation in the left tibialis anterior muscle (non-BFR leg) was not changed significantly at any time point in response to RRMBF (Fig. 3b). The enhanced phosphorylation of $p70^{S6k}$ had returned to control levels 3 h after the stimuli (Fig. 3c). S6 phosphorylation at Ser240/244, a downstream target of $p70^{S6k}$, was also increased significantly 1 h after RRMBF ($P < 0.05$, Fig. 4a), which returned to the control level 3 h after the stimuli.

Figures 4b, c illustrate the effects of RRMBF on phosphorylation of 4E-BP1 and eEF2. Phosphorylation of 4E-BP1 at Thr37/46 (Fig. 4b) and phosphorylation of eEF2 at Thr56 (Fig. 4c) did not change significantly at any time point in response to RRMBF, compared with control rats.

As shown in Fig. 5a, Akt phosphorylation at Ser473, a marker for Akt activation (Alessi et al. 1996), did not change significantly at any time point in response to RRMBF. Phosphorylation of mTOR on Ser2448, a site directly phosphorylated by Akt [21], tended to increase at 1 h after RRMBF ($P = 0.06$, Fig. 5b), but not statistically significantly.

Effects of RRMBF on AMPK phosphorylation and REDD1

AMPK phosphorylation at Thr172 did not change significantly at any time point in response to RRMBF (Fig. 5c). There was no difference in REDD1 total protein content in controls and after RRMBR (Fig. 5d).

Effects of RRMBF on myostatin, MuRF-1, and FOXO3a mRNA expression

We also investigated the effects of RRMBF on MuRF-1 and FOXO3a mRNA expression, factors associated with

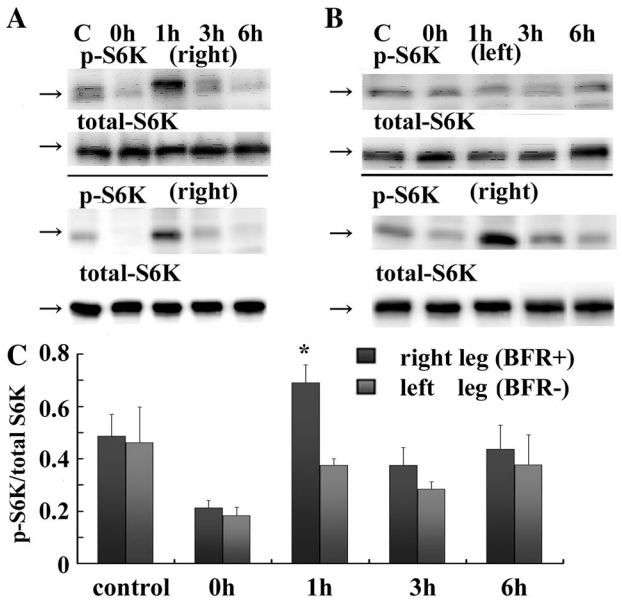

Fig. 3 Phosphorylation of p70 S6-kinase (p70S6k) on Thr389 in rat tibialis anterior skeletal muscle before (control, **c**), immediately after (0 h), 1, 3 and 6 h after RRMBF. In **a**, **b** the representative immunoblots (phospho- p70S6k and total p70S6k) are shown above each group. The data in **a** were obtained from the right tibialis skeletal muscle (BFR side). The data in **b** were obtained from the right (BFR side) and left tibialis anterior muscle (non-BFR side). In this and following figures, proteins with a phosphorylation site are shown as a phospho to total ratio. Proteins without a regulatory phosphorylation site are indicated as total protein content. The ratio of p- p70S6k/total p70S6k is shown in **c**. The data for each time were obtained from six different rats. Note that RRMBF enhanced the phosphorylation of p70S6k in right tibialis anterior muscle (BFR leg), but not left muscle (non-BFR leg) 1 h after the stimuli. *$P < 0.05$ vs. control (pre)

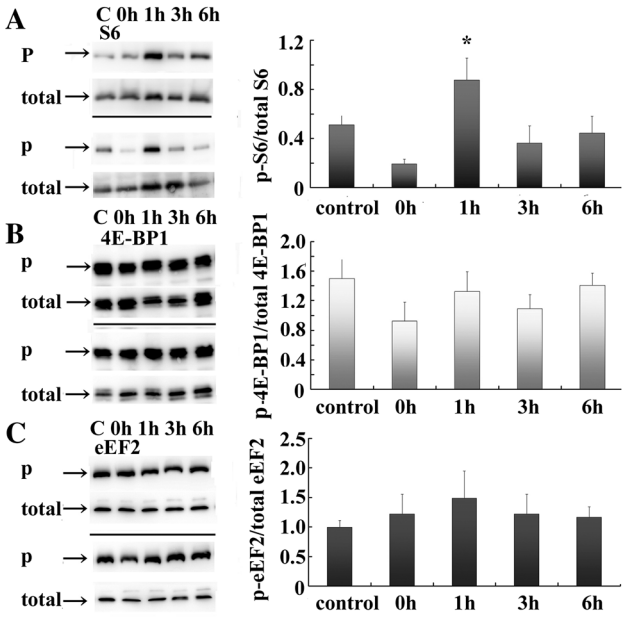

Fig. 4 Phosphorylation of S6 at Ser240/244 (**a**), 4E-BP1 at Thr37/46 (**b**), eEF2 at Thr56 (**c**) in rat tibialis anterior skeletal muscle before (control), immediately after (0 h), 1, 3, and 6 h after RRMBF. Two different representative immunoblots (phosphorylation and total) at times indicated are shown. The data were obtained from the right tibialis anterior muscle (BFR side) of six different rats. The phospho to total ratio is shown on the right. *$P < 0.05$ vs. control (pre)

the regulation of muscle protein breakdown. As shown in Fig. 6, there were no significant differences in MuRF-1 (Fig. 6a, $p > 0.05$) and FOXO3a mRNA (Fig. 6b, $P > 0.05$) levels in controls and after RRMBF. Figure 6c shows the effects of RRMBF on myostatin mRNA level. There were no difference in myostatin mRNA in controls and after RRMBF.

Effects of RRMBF on muscle HIF-1α mRNA, HIF-1α and VEGF protein

To determine whether hypoxia induced by RRMBF stimulates hypoxia-related genes and proteins, we investigated the effects of RRMBF on HIF-1α protein and gene expression. As shown in Fig. 6d, HIF-1α mRNA expression was not significantly changed after RRMBF ($P > 0.05$, Fig. 6d). HIF-1α protein expression was increased 1 h after RRMBF ($P < 0.05$), compared with controls (Figs. 6e, f), while it was not significantly changed at 0 h (~ approximately 5 min) after RRMBF. VEGF protein expression was not altered at any time point in response to RRMBF (Fig. 6g).

Effects of RRMBF on MAP kinase phosphorylation

Figure 7 shows the effects of RRMBF on phosphorylation of ERK1/2, p38MAPK, and JNK1/2. As shown in Fig. 7, phosphorylation of ERK1/2 (Fig. 7a) at Thr202/Tyr204, p38 MAPK (Fig. 7b) at Thr180/Tyr182, and JNK1/2 (Fig. 7c) at Thr183/Tyr185 did not change significantly at any time point in response to RRMBF.

Discussion

The major findings of our study are as follows. (1) During RRMBF, $PmvO_2$ decreased from 36.1 ± 5.7 to 5.9 ± 1.7 torr, and recovered gradually during a rest period. (2) RRMBF significantly increased p70S6k phosphorylation, a downstream target of mTOR, and ribosomal protein S6 phosphorylation at 1 h after stimulation. (3) Protein levels of REDD1, AMPK and MAPKs phosphorylation did not change significantly. (4) The mRNA expression level of FOXO3a, MuRF-1 and myostatin was not significantly altered during RRMBF. These results showed for the first time that RRMBF significantly decreased $PmvO_2$, and enhanced mTOR signaling pathways in skeletal muscle using a rat model, which may play a role in diminishing muscle atrophy under various conditions in human studies.

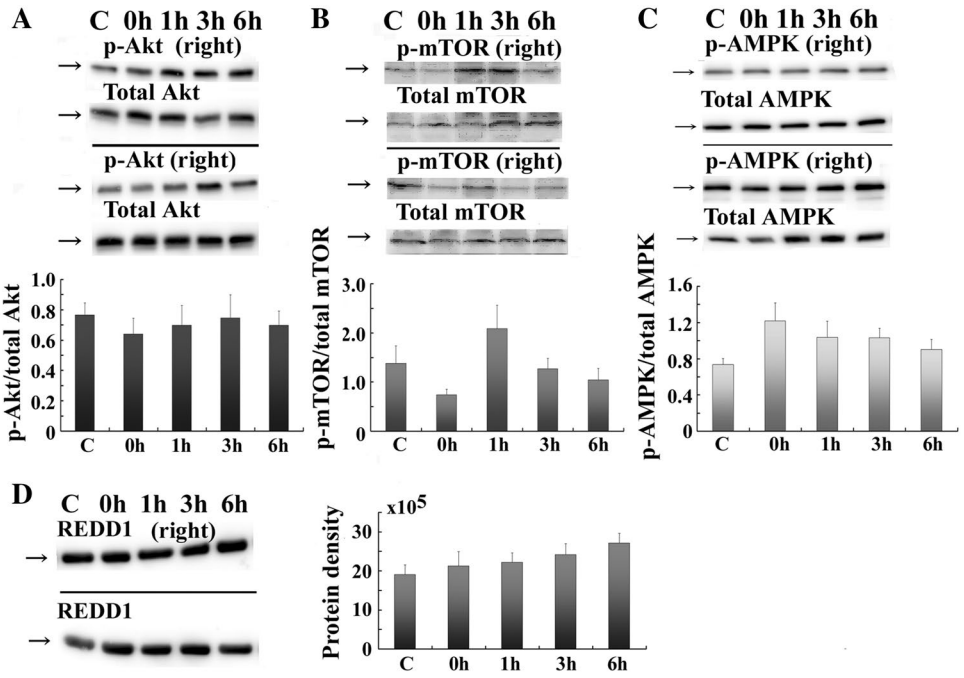

Fig. 5 Phosphorylation of Akt at Ser473 (**a**), mTOR at Ser2448 (**b**), AMPK (Thr172) (**c**) and REDD1 protein (**d**) expression in rat tibialis anterior skeletal muscle before (control), immediately after (0 h), 1, 3, and 6 h after RRMBF. Two different representative immunoblots (phosphorylation and total) are shown above each group in (**a**–**c**). The data were obtained from right tibialis anterior muscle (BFR side) at times indicated. The phospho to total ratio is shown in *lower part*. In **d**, two different representative immunoblots (REDD1) obtained from right muscle are shown. The protein density of REDD1 is shown in D (*right part*). Each datum was obtained from six different rats

Our study showed the first model of RMBF using rats. In this model, we applied an RRMBF stimulus without exercise comprising an external compressive force of 100 mmHg, which is lower than systolic blood pressure of rats to avoid hemostasis. With this restriction, the total weight of tibialis anterior muscle and total muscle weight/body weight ratio increased significantly in rats sacrificed immediately after RRMBF, suggesting that the application of RRMBF at 100 mmHg pressure does not completely block blood flow, and induces venous pooling of blood in the lower legs and swelling of the tibialis anterior muscle. It is a characteristic phenomenon of KAATSU training [5, 19, 22]. During RRMBF, $PmvO_2$ decreased significantly from 36.1 ± 5.7 to 5.9 ± 1.7 torr, and gradually recovered during a rest period. NMR techniques for myoglobin saturation have shown that resting intracellular PO_2 in human skeletal muscle is estimated to approximately 34 mmHg, and drops to approximately 23 mmHg, when subjects breathe ambient 10 % O_2 [23]. However, during near-maximal exercise, intracellular PO_2 drops to values as low as 2–5 mmHg [24]. In addition, mean tissue PO_2 in lower limb muscle made ischemic by complete obstruction of blood flow drops to less than 1.9 mmHg [25]. Thus, the level of $PmvO_2$ reached in our study is higher than that induced by complete obstruction of blood flow, and that reached during the near-maximal exercise as described previously [24, 25].

Oxygen is an essential regulator of cellular metabolism. Under hypoxic conditions, cells rapidly activate a variety of adaptive responses that limit energy expenditure by inhibiting energy-intensive processes including protein translation [26, 27]. One related mechanism involves inhibition of mTOR activity, an anabolic pathway for skeletal muscle, observed following exposure to hypoxia (1 % O_2) [28]. HIF-1α activity is known to increase under continuous hypoxia due to decreased rates of oxygen-dependent proline hydroxylation, ubiquitination, and proteasomal degradation of the HIF-1α subunit. And, hypoxic regulation of mTOR activity occurs through a pathway involving the REDD1 gene [29], where REDD1 expression is highly induced in response to hypoxia via HIF-1α [29, 30]. In our study employing RRMBF protocols, expression level of REDD1 protein did not change significantly. In general, the increased HIF-1α protein elicited by hypoxia quickly returns to control levels immediately after the re-oxygenation [31]. In the present study, expression level of HIF-1α protein did not significantly change immediately after RRMBF, while it increased 1 h after RRMBF. Hypoxia is also known to increase the expression of VEGF via HIF-1α [32], and VEGF protein expression did not significantly differ at any time point in response to RRMBF. Thus, it is likely that RRMBF used in our protocol did not activate HIF-1α activity enough to inhibit mTOR pathways and enhance VEGF expression. Alternatively, AMPK is an energy-sensing serine/threonine kinase activated by metabolic stressor that depletes ATP and increases AMP during exercise, hypoxia and glucose deprivation. Once activated, AMPK inhibits ATP consuming anabolic processes such as protein translation largely through inhibition of mTOR signaling [33]. However, AMPK phosphorylation also did not change significantly at any time point in response to RRMBF.

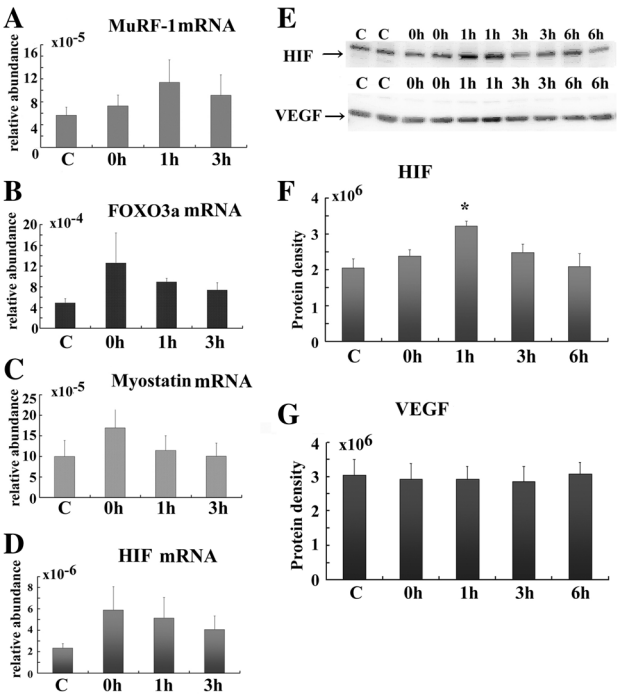

Fig. 6 **a**–**c** Skeletal muscle MuRF-1 (**a**), FOXO3a (**b**), and myostatin (**c**) mRNA in responses to RRMBF. The levels of MuRF-1 (**a**), FOXO3a (**b**) and myostatin (**c**) mRNAs were measured by the quantitative real-time RT-PCR, and normalized to those of the 18S ribosomal RNA levels. The relative abundance of mRNA is shown before (control), immediately after (0 h), 1, and 3 h after RRMBF. $^*P < 0.05$ vs. control (pre). **d**–**f** Skeletal muscle HIF-1α mRNA (**d**), HIF-1α protein (**e**, **f**) and VEGF protein (**g**) in responses to RRMBF. The levels of HIF-1α mRNA are normalized to those of the 18S ribosomal RNA levels, and the relative abundance of mRNA is shown before (control), immediately after (0 h), 1, and 3 h after RRMBF. $^*P < 0.05$ vs. control (pre). In **e**, two different representative immunoblots obtained from right muscle are shown above each group. The protein density of HIF-1α and VEGF is illustrated in **f** and **g**, respectively. The data were obtained from six different rats. $^*P < 0.05$ vs. control (pre)

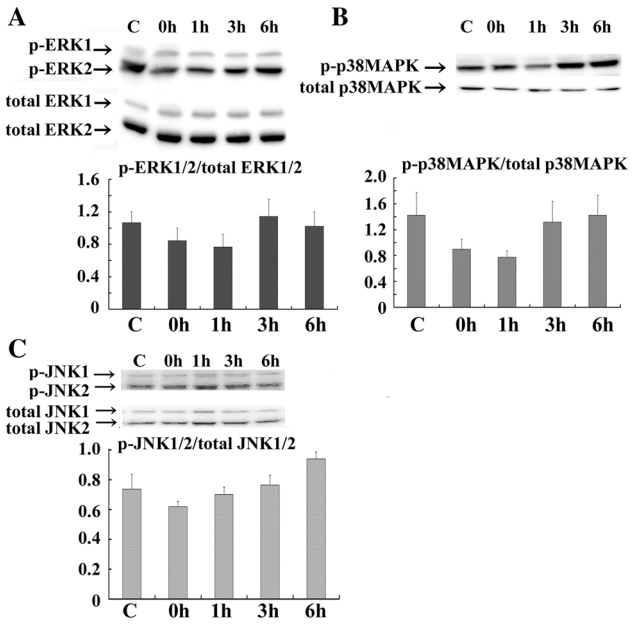

Fig. 7 Phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2, **a**), p38 MAP kinase (p38MAPK, **b**) and c-Jun N-terminal kinase 1/2 (JNK1/2, **c**) before (control), immediately after (0 h), 1, 3, and 6 h after RRMBF. A representative immunoblot (phosphorylation and total) at time indicated is shown. The data were obtained from right tibialis anterior muscle ($n = 6$, BFR side) at times indicated. The phospho to total ratio is shown in *lower part*

Several studies have shown that activation of mTOR and p70S6 k phosphorylation in the recovery phase after high-intensity resistance exercise is associated with increased protein synthesis [3, 34–36]. And, phosphorylation of p70S6k leads to S6 protein phosphorylation, which is thought to promote translation of the mRNAs that encodes ribosomal proteins and other translation factors. S6 protein activation leads to increased cellular capacity for protein synthesis. Recently, low-intensity resistance exercise under RMBF in healthy subjects has been reported to increase muscle strength and size even with low-intensity loads [6, 7]. Sessions of RMBF exercise have also been reported to increase protein synthesis through post-translation regulation in the Akt/mTOR pathway [8, 37]. They showed that the RMBF exercise increased p70S6k phosphorylation. Previous human studies have also reported that RRMBF even without a combination of exercise effectively diminishes post-operation disuse atrophy of knee extensors [11], and muscle weakness induced by immobilization without weight-bearing [12], but the underlying mechanisms have not been investigated. Here, we showed for the first time that RRMBF had significantly increased p70S6k phosphorylation, a downstream target of mTOR, and ribosomal protein S6 phosphorylation in a rat model. The degree of p70S6k phosphorylation in the first few hours after a session of high-intensity resistance exercise has been reported to be correlated with percentage change in muscle mass after several weeks of exercise in both rodents and humans [35, 38]. S6 phosphorylation is also regarded as a stimulation of protein synthesis [35, 36, 38]. Thus, RRMBF appears to increase protein synthesis due to enhanced p70S6k and S6 phosphorylation, possibly resulting in diminishing muscle atrophy as described previously in human studies [11, 12].

Several mechanisms for the effects of RRMBF on p70S6k and S6 phosphorylation in rat skeletal muscle may be proposed. Resistance exercise is an established and potent stimulus for enhancing muscle protein synthesis and subsequent muscle hypertrophy. The activation of mTOR signaling pathways is a primary mechanism by which resistance exercise stimulates translation initiation, elongation, and the rate of muscle protein synthesis [39]. Akt is an

upstream positive regulator of mTOR. However, RRMBF did not increase phosphorylation of Akt. Phosphorylation of mTOR on Ser2448 tended to increase at 1 h after RMBF, but not statistically significant. Thus, our study was unable to determine the involvement of Akt on increased p70S6k and S6 phosphorylation after RRMBF. However, we showed for the first time that RRMBF enhanced p70S6k phosphorylation, a downstream target of mTOR, in a rat model. RRMBF did not enhance the phosphorylation in control legs, suggesting that the local factors induced by RRMBF, but not systemic factors, are involved in enhanced p70S6k phosphorylation.

MAPK cascades are potential physiological mechanisms involved in the exercise-induced regulation of gene expression and protein synthesis in skeletal muscle [40]. The MAPK family members are separated into distinct parallel pathways, including ERK1/2, stress-activated protein kinase cascades (SAPK1/JNK) and SAPK2/p38 [41]. Downstream cytosolic targets for ERK1/2 include p90 ribosomal S6 kinase [42], and ERK1/2 is capable of phosphorylating S6 on its Ser235/236 regulatory site [43]. The kinases JNK and p38 MAPK are known as stress-activated protein kinases because of their activation by environmental stressors including osmotic shock and heat shock [44, 45]. RMBF induces an acute fluid shift from the vascular space into the muscles, and then muscle volume changes [5, 19, 22], which may be involved in the BFR exercise–induced anabolic response of skeletal muscle as proposed recently [22]. In our study, the total weight of the tibialis anterior muscle increased significantly in rats immediately after RRMBF, suggesting that application of RRMBF in the present model induced venous pooling of blood into the lower legs, and swelling the muscle. However, RRMBF did not increase phosphorylation of MAPK including ERK1/2. Thus, it is most likely that RRMBF enhances mTOR signaling pathways in rat skeletal muscle independently of MAPK signaling pathways. The formation of reactive oxygen species (ROS) in skeletal muscle has been also described in response to muscle stimulation, heat stress, and ischemia–reperfusion injury [46]. ROS have been proposed to play physiological roles on cell signaling in skeletal muscle [47]. The activity of ROS within muscle may increase in a hypoxic environment [48]. Also, because ischemia and reperfusion of skeletal muscle are associated with an increase of ROS release [49], ROS could be produced when muscle is kept hypoxic and subsequently exposed to reperfusion. Therefore, both lowered and elevated muscle oxygenation levels during RRMBF may enhance ROS production in skeletal muscle. ROS has been reported to activate the downstream target of phosphoinositide 3-kinase (PI3K)-Akt signaling [50]. However, RRMBF in the present study did not increase phosphorylation of Akt. Therefore, the basic mechanisms underlying the enhancement of mTOR signaling pathways following RRMBF remain to be clarified.

Proteolytic events involve the ubiquitination and subsequent degradation of proteins induced by E3 ligases [51, 52]. Exercise-responsive genes involved in the muscle cell ubiquitin/proteolysis pathway include MuRF-1 and atrogin-1 (muscle atrophy F-box), both of which share a common transcription factor, FOXO3a. Atrogin-1 and MuRF-1 negatively regulate muscle mass. Recently, reduced expression of the proteolysis-related genes FOXO3a, atrogin-1, MuRF-1, and myostatin has been reported after acute BFR exercise [9, 10]. Suppression of myostatin has been found to induce muscle hypertrophy in rodents [53]. Also, immobilization increases the expression of myostatin in mice, while re-loading muscle decreases myostatin expression [54]. From these observations, it is likely that BFR combined with exercise inhibits skeletal muscle protein degradation and promotes myogenesis via ubiquitin/proteolysis pathways, resulting in increasing muscle mass. However, Drummond et al. [55] showed that mRNA expression level for myostatin and MuRF-1 3 h after performing BFR knee extension at 20 % 1RM was not different from that after control 20 % 1RM knee extension. In our study using animal model, RRMBF without exercise did not significantly change the mRNA expression level of FOXO3a, MuRF-1, and myostatin. Thus, the muscle cell ubiquitin/proteolysis pathways do not appear to be involved in the RRMBF-induced decrease of muscle atrophy as reported previously in human studies [11, 12].

Kaatsu training is an exercise training under the conditions with RMBF [5, 6]. The training is distinctive for producing increases in muscle size and strength through short-duration, and low load-intensity training. This training has been reported to enhance protein synthesis through post-translation regulation in the Akt/mTOR pathway [8, 37]. But, several papers have shown that RRMBF even without exercises appears to be a novel stimulus of skeletal muscle to induce a net positive protein balance and prevent atrophy. In the present paper, we showed the first evidence that RRMBF without exercises enhanced mTOR signaling pathways in skeletal muscle using a rat model. The hypertrophic effects of KAATSU training cannot be obtained by the continuous application of RMBF alone in clinical studies [56], suggesting that hypoxia only appears to be not enough to activate the mTOR pathway. In contrast, RRMBF appears to be a novel stimulus of skeletal muscle to induce a net positive protein balance and prevent atrophy. However, the further studies are needed to investigate the potencies of the hypertrophic effects of the RMBF exercises and RRMBF. In addition, we applied an RRMBF stimulus comprising an external compressive force of 100 mmHg, and used five repetitions for 5 min, and release of pressure for 3 min as described in the previous clinical

studies [11, 12]. Therefore, the further detailed studies using different protocols of RRMBF including numbers and duration of RRMBF are needed to clarify the effects of RRMBF of the mTOR signaling pathways.

Exercise such as aerobic exercise is a well-established method for improving quality of life, and decreasing cardiovascular risk [57, 58]. However, it is difficult to apply to bedridden or postoperative patients. On the other hand, RRMBF can be used in such patients, and may prevent muscle atrophy in such patients as shown in the previous papers [11, 12]. But, further clinical studies are needed to clarify this possibility.

Conclusions

RRMBF significantly decreased $PmvO_2$, and enhanced mTOR signaling pathways in skeletal muscles using a rat model, which may play a role in preventing muscle atrophy in skeletal muscle under various conditions in human studies.

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Compliance with ethical standards

Conflict of interest There is no conflict of interests to disclose.

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