Ursolic acid stimulates mTORC1 signaling after resistance exercise in rat skeletal muscle

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—A recent study identified ursolic acid (UA) as a potent stimulator of muscle anabolism. The mammalian target of rapamycin complex 1 (mTORC1) is recognized as a key regulator of translation initiation and has been shown to be important in muscle protein synthesis and muscle hypertrophy (4, 8, 9). Recent studies have indicated that resistance exercise activates mTORC1 in contracted skeletal muscle through a mechanism independent of PI3K/Akt signaling (15, 26), although the latter pathway is a well-known upstream signaling pathway involved in the activation of mTORC1 (7, 20, 25). Although little is known about the mechanisms of UA-induced muscle anabolism, a previous study reported that the mRNA levels of IGF-I and Akt phosphorylation are upregulated in the skeletal muscle of mice after daily UA consumption over a long period (13); this implies that UA may acutely activate mTORC1 through IGF-I/Akt signaling. Because of these findings, we hypothesized that UA may additively increase Akt-independent mTORC1 activation induced by resistance exercise through Akt activation.

Therefore, the purpose of the present study was to investigate the effect of UA on mTORC1 activation induced by resistance exercise. To this end, we measured the degree of phosphorylation of p70S6K at Thr389 increased 1 h after resistance exercise but attenuated to the controls levels 6 h after the completion of exercise. On the other hand, prolonged phosphorylation of p70S6K was maintained even 6 h after exercise. These results indicate that UA is able to sustain resistance exercise-induced mTORC1 activity.

MATERIALS AND METHODS

Animals and Experimental Protocol
Twenty male Sprague-Dawley rats, aged 10 wk (330–360 g), were obtained from CLEA Japan (Tokyo, Japan). All animals were housed for 1 wk in an environment maintained at 22–24°C with a 12:12-h light-dark cycle and were allowed food (CE2; CLEA Japan) and water ad libitum. Subsequently, the rats were exercised after a 12-h overnight fast, and UA (250 mg/kg in corn oil) or placebo (PLA; corn oil only) was injected intraperitoneally immediately after exercise. UA was dissolved in corn oil at a concentration of 25 mg/mL. The rats were killed 1 or 6 h after the completion of exercise, and the target tissues were removed immediately (Fig. 1). After their masses were measured, the tissue samples were rapidly frozen in liquid N2 and stored at −80°C until use. The study protocol was approved by the Ethics Committee for Animal Experiments at Ritsumeikan University.

Iso metric Exercise
Under isoflurane anesthesia, hair was shaved off the right lower leg of each rat, and the shaved leg was cleaned with alcohol wipes. The rats were then positioned with their right foot on the footplate (the ankle joint angle was positioned at 90°) in the prone posture. The triceps surae muscle was stimulated percutaneously with electrodes (Vitrode V, Ag/AgCl; Nihon Kohden, Tokyo, Japan), which were cut to measure 10 × 5 mm, and connected to an electric stimulator and an

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Measurement of Serum and Muscle IGF-I Concentrations

IGF-I levels in the serum and skeletal muscle extracts were determined using the mouse/rat IGF-I quantikine ELISA kit (MG100, R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

A two-way analysis of variance (ANOVA) was used to evaluate changes in phosphorylation of signaling proteins with the condition (PLA, PLA + exercise, UA, and UA + exercise) and time (1 h and 6 h) as factors. Post hoc analyses were performed using r-tests with the Benjamini and Hochberg false discovery rate correction for multiple comparisons when a significant main effect and/or interaction was observed. All values are expressed as means ± SE. Significance was accepted at P < 0.05.

RESULTS

Serum and Muscle IGF-I Concentrations

UA alone did not change the muscle IGF-I concentrations, but resistance exercise increased muscle IGF-I concentrations 1 h and 6 h after resistance exercise (Table 1). Furthermore, UA tended (P = 0.051) to further increase the exercise-induced muscle IGF-I concentrations at 6 h after resistance exercise. Serum IGF-I concentrations were not changed with resistance exercise alone, UA alone, or with UA in combination with the resistance exercise (Table 1).

Intracellular Signaling

Representative blots of signaling proteins are shown in Fig. 2. Akt. UA injection increased the Akt phosphorylation atThr308 both 1 h and 6 h after the injection, whereas resistance exercise alone did not significantly change the phosphorylation of Akt at Thr308 (Fig. 3). Akt phosphorylation at Ser473 was not affected significantly by UA alone, resistance exercise alone, or the combination of both.

PRAS40. UA alone did not increase PRAS40 phosphorylation at any time point (Fig. 4). In contrast, resistance exercise increased PRAS40 phosphorylation 1 h after resistance exercise. At 6 h after resistance exercise, the increased PRAS40 phosphorylation was maintained when UA was administered immediately after resistance exercise, while the degree of PRAS40 phosphorylation decreased to that in the controls when exercise alone was performed.

p70S6K. In the case of resistance exercise alone, the phosphorylation of p70S6K increased at 1 h after resistance exercise (Fig. 5A); at 6 h after resistance exercise, the level remained high but lower than that at 1 h after resistance exercise. On the other hand, the increased p70S6K phosphorylation was maintained at 6 h after resistance exercise when UA was adminis-

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Table 1. Serum and muscle IGF-I concentrations

<table>
<thead>
<tr>
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<th>1 h</th>
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<th>6 h</th>
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<tbody>
<tr>
<td>UA</td>
<td>- +</td>
<td>-</td>
<td>- +</td>
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<tr>
<td>EX</td>
<td>- -</td>
<td>+</td>
<td>+ +</td>
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<tr>
<td>Serum, ng/ml</td>
<td>1.294 ± 4</td>
<td>NA</td>
<td>1.261 ± 28</td>
<td>1.189 ± 32</td>
</tr>
<tr>
<td>Muscle, ng/g</td>
<td>170 ± 9</td>
<td>142 ± 17</td>
<td>448 ± 79*</td>
<td>448 ± 75*</td>
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<tr>
<td></td>
<td>138 ± 13</td>
<td>168 ± 18</td>
<td>378 ± 56*</td>
<td>546 ± 47*#</td>
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Values are means ± SE; n = 5 per group. UA, ursolic acid; EX, exercise; NA, not analyzed. *P < 0.05 vs. control (without UA or EX); #P = 0.051 vs. corresponding EX only group.
tered immediately after resistance exercise. UA alone did not increase the phosphorylation of p70S6K at any time point.

rpS6. Increase in the phosphorylation of rpS6 was observed 1 and 6 h after resistance exercise, but not with UA injection (Fig. 5B). There was no additional or synergistic effect of resistance exercise and UA on rpS6 phosphorylation.

DISCUSSION

In this study, we first investigated the acute effects of UA alone and in combination with resistance exercise on the phosphorylation of mTORC1 signaling proteins in in vivo skeletal muscle and observed that UA sustained mTORC1 activity after resistance exercise.

A previous in vivo study using mice reported that chronic daily UA consumption increased Akt phosphorylation at Ser473 and mRNA expression of IGF-I (13). Therefore, it was concluded that UA-induced muscle anabolism is mediated by the activation of Akt through the IGF-I/PI3K signaling pathway. However, it is currently unclear whether UA can significantly increase IGF-I protein level. Furthermore, the direct link be-

Fig. 3. Phosphorylated Akt Thr308 (A) and Ser473 (B) relative to total protein during the protocol (n = 5 per group). Values are means ± SE. *P < 0.05 vs. control muscle (no exercise and only treated with corn oil).
Although we observed an acute increase in Akt phosphorylation at Thr\textsuperscript{308} by UA alone, these did not contribute to an increase in the phosphorylation of downstream signaling proteins. That is, UA alone did not increase the phosphorylation of p70S6K or rpS6. In contrast, previous in vitro studies observed that acute UA injection increased the phosphorylation of those proteins with the concomitant increase in Akt phosphorylation at Ser\textsuperscript{473} (5, 13). It is known that Thr\textsuperscript{308} phosphorylation is necessary for Akt activation and that Ser\textsuperscript{473} phosphorylation is required for the complete activation of Akt (19). Therefore, increase in the Akt phosphorylation at Ser\textsuperscript{473} may be required to activate mTORC1 and phosphorylate downstream proteins in vivo. The discrepancies between the findings of this and previous studies may be attributable to the differences in the experimental design (i.e., in vivo vs. in vitro studies). Nonetheless, because previous in vivo studies observed that chronic UA injection led to an increase in the phosphorylation of Akt at Ser\textsuperscript{473} (12, 13), we can infer that accumulative UA stimulation may be required to phosphorylate the Ser\textsuperscript{473} site of Akt in vivo. Further studies are required to investigate the effect of chronic UA injection on mTORC1 in vivo.

High-intensity muscle contraction is known to increase the phosphorylation of the mTORC1 downstream targets p70S6K and rpS6 for more than 18 h after muscle contraction (15, 17). In contrast, our preliminary test using the present muscle activation protocol and the findings of previous studies together indicate that, although high-intensity muscle contraction leads to Akt phosphorylation at Ser\textsuperscript{473} transiently (its peak activation state is often observed ~30 min after muscle contraction and return to the baseline level within 1 h of muscle contraction), the phosphorylation of Akt at Thr\textsuperscript{308} is not altered with acute muscle contraction (15, 17, 18). This suggests that muscle contraction-induced mTORC1 activation is independent of the activation of the PI3K/Akt signaling pathway. In accord with the findings of previous studies, our findings indicated that resistance exercise alone increased the phosphorylation of p70S6K at Thr\textsuperscript{389} and rpS6 at Ser\textsuperscript{240/244}, but Akt phosphorylation at either residue remained unchanged at 1 h and 6 h after resistance exercise.

PRAS40 is a known component of mTORC1 and acts as a negative regulator of its activity (22, 27, 29). The phosphorylation of PRAS40 weakens its interaction with mTORC1 and results in mTORC1 activation (23, 27). In the present study,
Akt phosphorylation did not change with resistance exercise but PRAS40 Thr246, a downstream target of Akt, was phosphorylated 1 h after resistance exercise. Although insulin has consistently shown the ability to stimulate Akt and PRAS40 concomitantly (22), the discrepancy between Akt and PRAS40 phosphorylation after resistance exercise has also been reported previously (26). Taken together, PI3K/Akt-independent PRAS40 activation (dissociation from mTORC1) may have contributed to mTORC1 activation induced by resistance exercise.

Interestingly, in the present study, the phosphorylation of p70S6K at Thr389 at 6 h after resistance exercise was maintained at the same level as that 1 h after resistance exercise only when UA was injected immediately after the exercise, whereas UA alone did not alter its phosphorylation levels. A similar trend was observed for muscle IGF-I. Although it remains unclear whether IGF-I per se or IGF-I/PI3k/Akt signaling pathway is involved in the muscle hypertrophy induced by mechanical load, the overexpression of IGF-I has been shown to stimulate mTORC1 and induce muscle hypertrophy without increasing Akt phosphorylation (2, 21). Furthermore, a previous in vitro study reported that IGF-I is necessary to stimulate p70S6K phosphorylation by UA, thereby indicating that high muscle IGF-I concentration contributed to the sustained p70S6K phosphorylation. However, the upstream mechanisms are relatively unclear, and further studies are needed to elucidate this. Nonetheless, because the phosphorylation of p70S6K reflects the mTORC1 activity and its associated muscle protein synthesis and subsequent muscle hypertrophy (1, 8, 11, 24), the combination of mTORC1 activity and its associated muscle protein synthesis and mTORC1 signaling may have contributed to subsequent muscle hypertrophy induced by resistance exercise.

In conclusion, we suggest that UA has anabolic effects against lengthening contraction-induced muscle injuries. Future study should be conducted to determine whether resistance exercise and UA have additive or synergistic effects on muscle mass and strength. In conclusion, we suggest that UA has the potential to serve as an anabolic agent for facilitating acute muscle anabolism induced by resistance exercise.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: R.O., K.S., and K.H. performed experiments; R.O. analyzed data; R.O., K.S., and K.H. interpreted results of experiments; R.O. prepared figures; K.S., K.H., K.N., and S.F. edited and revised manuscript.

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