Maslinic Acid Enhances mTOR Signaling Activity After Acute Resistance Exercise in Mouse Skeletal Muscle

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ABSTRACT

Background: Maslinic acid (MA) is an olive-derived extract with the structure of pentacyclic triterpenes, which potently anti-inflammatory effects. It has been reported that the combination of MA and resistance exercise increases skeletal muscle mass, but there are many unknowns regarding its detailed molecular mechanism. The present study aimed to clarify the effect of MA supplementation on muscle hypertrophic response to acute muscle contraction-induced resistance exercise using animal model.

Methods: Seven-week-old ICR (Institute of Cancer Research) male mice fed a diet containing 0.27% MA during an acclimatization of 1 week. After an overnight fast, the right gastrocnemius muscle was subjected to acute resistance exercise using percutaneous electrical stimulation-induced muscle contractions, while the left gastrocnemius muscle was saved as control. The muscle was excised at 1, 3, and 6 hours after exercise and protein synthesis-related signaling expressions were examined.

Results: MA was demonstrated to significantly activate the downstream targets of mammalian/mechanistic target of rapamycin (mTOR), phosphorylated ribosomal protein S6 (pS6) at Ser240/244 site particularly, while Akt/glycogen synthase kinase-3β (GSK-3β) pathway and mitogen-activated protein kinase (MAPK) signaling were unaffected.

Conclusion: Our results suggested that acute resistance exercise-induced muscle protein synthesis-promoting effect of MA is supported by the activation of downstream signaling of mTOR.

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Revised: June 29, 2022
Accepted: July 3, 2022

Introduction

Intake of nutritional supplement is one of the most common methods used for improving sports performance and health. Maslinic acid (MA) which is a rare ingredient extracted from Italian olive fruit and is a pentacyclic triterpene has been attracting attention as a supplement with various health benefits (1-4). MA has reduced the expression levels of inflammatory response associated genes, lipopolysaccharide-induced production of tumor...
necrosis factor-α, and reduced inflammation in the edema and arthritis model (2). Studies have indicated that MA has a powerful anti-inflammatory effect.

Moreover, a combination of MA and resistance exercise has improved skeletal muscle mass and physical function among elderly women (3). In another study, a combination of MA and whole-body vibration training suppressed knee muscle strength and knee joint inflammation in elderly women with knee osteoarthritis (4). Ursolic acid, which is also a member of the pentacyclic triterpene, increases skeletal muscle mass, grip strength and maximum running time in mice (5). Mammalian/mechanistic target of rapamycin (mTOR) signaling pathway is one of the molecular mechanisms that regulates muscle protein synthesis and hypertrophy and is potently activated by resistance exercise (6). Activation of P70 S6 kinase (P70S6K), ribosomal protein S6 (rpS6) and eukaryotic initiation factor 4E-bnding protein 1 (4E-BPI), downstream signals of mTOR, and enhances translational efficiency in ribosomes (7). Ursolic acid has augmented P70S6K and rpS6 phosphorylation after acute resistance exercise in rat skeletal muscle (8, 9). These reports suggest that the combination of resistance exercise and supplements containing pentacyclic triterpenes promote hypertrophy through the augmentation of mTOR signaling. Although ursolic acid induces activation of mTOR signaling, it is also known to elicit inflammatory response and is regarded as a double-edged sword (10). Conversely, MA has anti-inflammatory effects, making it easy to use as a supplement.

Several previous studies have supported that the combination of nutritional supplements and resistance exercise can enhance muscle hypertrophy (11-13). Dietary intake of casein protein or branched-chain amino acids has been reported to promote muscle hypertrophy in functionally overloaded mice (11). In our previous studies, MA has been previously reported to enhance the hypertrophic effect of functional overload through mTOR signaling activation in mouse skeletal muscle and that MA induces skeletal muscle hypertrophy in humans (12, 13). However, the effects of MA on mechanism concerning muscle protein synthesis and hypertrophy other than mTOR signaling, including mitogen-activated protein kinase (MAPK) signaling pathway, are unclear. Furthermore, the effects of MA intake on muscle protein synthesis signaling activated during acute resistance exercise have not been clarified. Therefore, this study aimed to determine the effects of MA intake on the muscle hypertrophic response that occurs during acute resistance exercise. MA has been hypothesized to promote the activation of mTOR and MAPK signaling during acute resistance exercise.

**Materials and Methods**

All experimental procedures performed in this study have been approved by the Institutional Animal Experiment Committee of the University of Tsukuba (19-380). Seven-week-old male Institute of Cancer Research mice (Tokyo Laboratory Animals Science Co, Tokyo, Japan) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and were housed at temperature- (22 °C±2 °C) and humidity (55%±5%)-controlled holding facilities under a 12-/12-h light/dark cycle and with ad libitum access to food and water. During acclimatization for 1 week, the mice were divided into two groups: normal diet (MF standard feed; Oriental Yeast Co., Ltd, Tokyo, Japan) and normal diet containing MA. Each sample size was five to six millimeter. MA was donated by Nippn Corp. (Tokyo, Japan) and was mixed to a concentration of 0.27% in the normal diet. As normal diet, laboratory animal diets MF (Oriental corp., Japan) containing 8.5% moisture, 22.9% protein, 3.9% fat, 5.6% crude ash, and 3.7% fiber were used in this study. MA content was determined based on previous reports (12, 13). Subsequently, the right gastrocnemius muscle was isometrically exercised after overnight fasting. The left gastrocnemius muscle was saved as sham-operated control. Under anesthesia, the mice were euthanized by cervical dislocation at 1, 3 and 6h after completion of resistance exercise, and the gastrocnemius muscle was excised, weighed, quickly frozen in liquid nitrogen, and stored at −80°C until analysis.

The resistance exercise protocol was carried out as previously described (14, 15). Briefly, under anesthesia with isoflurane (2.0-3.0% isoflurane in air) inhalation, the right lower limbs of each mouse were shaved and cleaned using alcohol wipes. The mice were positioned with their foot on a footplate (with an ankle joint angle of 90°C) in the prone position. The triceps and calf muscle were stimulated percutaneously with electrodes connected to an electric stimulator and isolator (Ag/AgCl, Vitrode V; Nihon Kohden, Tokyo, Japan). The right gastrocnemius muscle was isometrically exercised (stimulation for 3 s, 10 contractions, with intervals of 7 s between contractions; total of five sets with 3-min intervals between sets). The voltage (30 V) and stimulation frequency (100 Hz) have been adjusted to produce maximal isometric tension. This exercise protocol is known to activate the anabolic signaling and induces significant muscle hypertrophy, simulating long-term training (16).
Isolated gastrocnemius muscles were immediately frozen in liquid nitrogen, and total muscle protein was extracted in lysis buffer containing 50 mM of HEPEs (pH: 7.6), 150 mM NaCl, 10 mM EDTA, 10 mM Na₃PO₄, 10 mM NaF, 2 mM Na₂VO₃, 1% (v/v) NP-40, 1% (v/v) Na-deoxycholate, 0.2% (w/v) sodium dodecyl sulphate (SDS), and 1% (v/v) of a complete protease inhibitor cocktail. Protein concentrations were measured using a Protein Assay Bicinchoninate Kit (Nacalai Tesque Inc, Kyoto, Japan). Prior to SDS-polyacrylamide gel electrophoresis (PAGE), an aliquot of the extracted protein solution was mixed with an equal volume of sample loading buffer containing 1% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 125 mM Tris-HCl (pH: 6.8), 10% (w/v) sucrose, and 0.01% (w/v) bromophenol blue. Five micrograms of protein were separated in an SDS-PAGE gel and were electrically transferred from to an Immuno-Blot PVDF membrane (Bio-Rad laboratories, Hercules, CA, USA). The blot was blocked using Blocking One (Nacalai Tesque Inc.) for 1 h at room temperature and was incubated with primary antibodies overnight at 4°C in Tris-buffered saline (TBS) with 0.1% Tween-20. After overnight incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10000 dilution) for 60 min at room temperature. Signals were detected using the ImmunoStar Zeta or LD (FUJIFILM Wako Pure Chemical Co, Osaka, Japan), quantified by C-Digit (LI-COR Biosciences, Lincoln, Nebraska, USA), and expressed as arbitrary units. Coomassie Brilliant Blue staining was used to verify consistent loading.

The following primary antibodies were used for western blotting: 1:3000 dilution for anti-protein kinase B (Akt) (9272; Cell Signaling Technology, Danvers, MA, USA), 1:3000 dilution for anti-p-Akt (#4060S; Cell Signaling Technology, Danvers, MA, USA), 1:3000 dilution for anti-extracellular-related kinase 1/2 (ERK1/2) (#9102; Cell Signaling Technology, Danvers, MA, USA), 1:3000 dilution for anti-p-ERK1/2 (Thr204/Tyr202, #9101; Cell Signaling Technology, Danvers, MA, USA), 1:3000 dilution for anti-glycogen synthase kinase-3β (GSK-3β) (#9315; Cell Signaling Technology, Danvers, MA, USA), 1:3000 dilution for anti-mTOR (Ser2448) at any of the time points (Figure 1B and C). Similarly, no significant main effect of acute resistance exercise or MA had a significant effect on the expression levels of phosphorylated Akt at both Thr308 and Ser473 phosphorylation sites (Figure 1B and C). Additionally, MA significantly increased the expression levels of phosphorylated P70S6K (Thr389) at 6 hours after acute resistance exercise (main effect of RE, p<0.05 respectively, Figure 3B), while no significant main effect of acute resistance exercise or MA was observed on the expression levels of phosphorylated GSK-3β (Ser9, Figure 1D).

Next, the effects of acute resistance exercise or MA on the changes in the expression levels phosphorylated mTOR (Ser2448) have been examined. Neither acute resistance exercise nor MA had a significant effect on the expression levels of phosphorylated mTOR (Ser2448) at any of the time points (Figure 2B). The expression levels of phosphorylated P70S6K (Thr389 and Thr421/Ser424), rpS6 (Ser240/244 and Ser235/236) and 4E-BP1 (Thr37/46) as downstream signals of mTOR have also been measured. A significant main effect of acute resistance exercise was found on the expression levels of phosphorylated P70S6K (Thr389) at 1 and 6 hours after acute resistance exercise (main effect of RE, p<0.05 respectively, Figure 3B), while no significant main effect of MA was observed.

Additionally, MA significantly increased the
expression levels of phosphorylated P70S6K (Thr389) compared to the normal diet-fed group (main effect of MA, $p<0.05$, Figure 3B). However, acute resistance exercise or MA did not significantly affect the expression levels of phosphorylated P70S6K (Thr421/Ser424) at any of the time points (Figure 3C). We observed that acute resistance exercise tended to increase the phosphorylation of rpS6 (Ser240/244) at 1 and 3 hours after exercise compared with the control legs (main effect of RE, $p=0.064$; $p=0.074$ respectively, Figure 3D).

Acute resistance exercise was also found to significantly increase rpS6 (Ser240/244) phosphorylation at 6 h after exercise (main effect of RE, $p<0.05$, Figure 3D). Furthermore, MA significantly increased rpS6 (Ser240/244) phosphorylation at 1 and 3 h after acute resistance exercise compared with the normal diet-fed group (main effect of MA, $p<0.05$ respectively, Figure 3D). Ser235/236 phosphorylation, the other phosphorylation site of rpS6, was measured and found to be significantly increased by acute

Figure 1: Effect of MA on Akt/GSK-3β signaling after acute resistance exercise. (A) Representative western blot images. (B) Phosphorylated Akt at Thr308 site. (C) Phosphorylated Akt at Ser473 site. (D) Phosphorylated GSK-3β at Ser9 site. Values are mean±SE; n=6 samples each.

Figure 2: Effect of MA on mTOR phosphorylation after acute resistance exercise. (A) Representative western blot images. (B) Phosphorylated mTOR at Ser2448 site. Values are mean±SE; n=6 samples each.
resistance exercise at 1 h after exercise compared with the control legs (main effect of RE, \( p<0.05 \), Figure 3E). Furthermore, MA significantly increased rpS6 (Ser235/236) phosphorylation at 3 h after exercise compared with the normal diet-fed group (main effect of MA, \( p<0.05 \), Figure 3E). Moreover, a main effect of acute resistance exercise on 4E-BP1 (Thr37/46) phosphorylation at any of the time points was found, but a tendency for MA to increase 4E-BP1 (Thr37/46) phosphorylation at 1 hour after exercise compared with the normal diet-fed group was observed (main effect of MA, \( p=0.074 \), Figure 3F). Several previous studies have reported that resistance exercise caused by muscle contraction promotes phosphorylation of P38 mitogen-activated protein kinase (P38MAPK) and extracellular-related kinase 1/2 (ERK1/2), which are members of the MAPK family (19-21). Since activation of MAPK signaling has been suggested to induce activation of the mTOR complex 1 (22, 23), the effects of acute resistance exercise and MA on the phosphorylation of MAPK signaling have been evaluated. No significant effect of acute resistance exercise or MA on P38MAPK phosphorylation at any of the time points was found (Figure 4B). Similarly, the expression level of phosphorylated ERK1/2 was not significantly affected by acute resistance exercise or MA (Figure 4C).

**Discussion**

This study has examined the effects of MA supplementation on mTOR signaling and MAPK signaling activity associated with acute muscle contraction-induced resistance exercise. The main findings of this study were as follows: (i) Akt/GSK-3β and MAPK signaling activities in the...
Muscle subjected to acute resistance exercise were not affected by MA; (ii) the downstream signaling activity of mTOR, rather than mTOR itself. Akt is an upstream protein of mTOR and is phosphorylated upon activation of the IGF-1/phosphatidylinositol-3 kinase (PI3K) pathway (24). It has been reported that functional overload did not attenuate activation of mTOR signaling in Akt1-deficient mice, therefore, the role of Akt in the muscle hypertrophic response is controversial (25).

In this study, MA did not affect the Akt phosphorylation, suggesting that MA is not involved in the IGF-1/PI3K/Akt pathway-associated muscle hypertrophy. GSK-3β, a downstream target of Akt, is a negative regulator of muscle hypertrophy and promotes protein translation by phosphorylation (17, 18). GSK-3β phosphorylation has been reported to increase in an experiment employing an exercise protocol with increased muscle mass (26, 27), but did not change with acute resistance exercise (28). However, since a previous study of MA supplementation and the results of this study showed that MA did not affect Akt phosphorylation (12, 13), it is possible that MA does not affect the increase in the GSK-3β phosphorylation associated with muscle hypertrophy.

Our results showed that the phosphorylation level of mTOR did not change significantly at any time point after acute resistance exercise. Several studies have reported that acute resistance exercise or functional overload did not affect mTOR Ser2448 level (29, 30). In this study, MA did not affect the phosphorylation level of mTOR. MA increases the phosphorylation of mTOR during chronic functional overload (12), suggesting that MA may enhance the responsiveness of mTOR to prolonged stimulation of skeletal muscle.

MA was shown to increase the downstream signaling of mTOR, P70S6K Thr389 and rpS6 (Ser240/244, Ser235/236) at 1 and 3 h after acute resistance exercise. Furthermore, rpS6 Ser240/244 was also increased by acute resistance exercise, indicating that MA enhances rpS6 Ser240/244 by acute resistance exercise. In this study, MA alone increased the phosphorylation of P70S6K (Thr389) and rpS6 (Ser240/244). Although ursolic acid has the pentacyclic triterpene structure similar to that of MA, it did not affect the phosphorylation of P70S6K (Thr389) and rpS6 (Ser240/244) alone (8), suggesting that the results of this study are unique to MA. MA may induce activation of the P70S6K (Thr389)/rpS6 (Ser240/244) pathway and promote muscle protein synthesis after acute resistance exercise. Several studies have reported that two phosphorylation sites (Ser240/244 and Ser235/236) in rpS6 exhibited identical reactivity to acute resistance exercise or transient functional overload (14, 27, 30, 31).

Similarly, in this study, acute resistance exercise significantly increased the phosphorylation of rpS6 (Ser235/236) as well as rpS6 (Ser240/244). Furthermore, this study showed that MA alone increased the phosphorylation of rpS6 (Ser235/236),
which is the first report as few as we know. Canonically, mTOR/P70S6K/rpS6 pathway and mTOR/4E-BP1/eIF4E pathways are thought to synergistically contribute to protein synthesis (32). However, the results of this study showed that MA did not affect the phosphorylation levels of mTOR and 4E-BP1. Thus, at least for the protein synthesis response to acute resistance exercise, MA contributes to the activation of P70S6K (Thr389)/rpS6 (Ser240/244 or Ser235/236) pathway. Since our previous study reported that MA increased mTOR phosphorylation level during functional overload for 4, 7 and 14 days (12), it is possible that MA contributes the mTOR/4E-BP1/eIF4E pathway during chronic resistance exercise with muscle hypertrophy.

MAPK signaling molecules are upstream substrates of mTOR, and ERK1/2 and P38MAPK phosphorylation have been reported to be increased in a resistance exercise model of rat skeletal muscle (33). However, in this study, acute resistance exercise or MA did not affect the phosphorylation levels of ERK1/2 and P38MAPK. A previous study has reported that ERK1/2 MAPK activity is increased by the addition of insulin (34). In a previous study that showed increased phosphorylation levels of ERK1/2 and P38MAPK after acute resistance exercise, the phosphorylation level of Akt was also increased at the same time (33). Additionally, in a previous study examining the effect of ursolic acid on mTOR signaling, the phosphorylation level of ERK1/2 was increased under conditions where ursolic acid augmented the phosphorylation level of Akt (8, 9). Therefore, ERK1/2 and P38MAPK activation following acute resistance exercise may require activation of insulin or IGF-1/P3K/Akt pathway.

In this study, acute resistance exercise performed after 1 week of continuous MA intake was employed to evaluate mTOR signaling responses. Hence, the effect of a single bout of MA intake on the protein synthesis response after resistance exercise is unclear. Detailed studies are required on the amount and concentration of a single intake of MA, as well as time-dependent changes in blood MA concentrations. Also, our previous study suggests that muscle hypertrophy following 14 days of functional overload is facilitated via mTOR signaling activation (12). Long-term resistance exercise and MA ingestion should be performed in parallel to evaluate the effect of muscle hypertrophy. In this study, the effects of MA were investigated in a fasting state. The mTOR signaling and protein synthesis responses following resistance exercise are sensitive to amino acid and carbohydrate intake status (35). Thus, we need to examine the additive effects of MA and other anabolic response-promoting nutrients, and these data may lead to the development of nutritional strategies to enhance exercise effect.

**Conclusion**

MA was shown to promote the phosphorylation of rpS6, a downstream signal of mTOR, in skeletal muscle subjected to acute resistance exercise using electrical stimulation. This finding suggested that MA, the olive derived pentacyclic triterpene, is a functional nutrient that stimulates exercise-induced protein anabolic responses and promotes protein translation on the ribosome via mTOR-dependent signaling activation. In addition to the strong anti-inflammatory and antioxidant effects of MA, which are highly beneficial for human health, the results of this study are expected to lead to the future widespread use of MA in sports and health as functional supplement to promote muscle hypertrophy. As a future prospect, a new nutritional intake strategy can be proposed for athletes whose performance is directly related to muscle hypertrophy and suppression of age-related muscle atrophy.

**Acknowledgement**

This work was supported by the Japan Society for the Promotion of Science (20J15222) to TS.

**Conflict of Interest**

None declared.

**References**


Maslinic acids in mTOR signaling


