Maslinic Acid Promotes Hypertrophy Induced by Functional Overload in Mouse Skeletal Muscle

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Summary Nutritional supplements are sometimes important for athletes to improve their sports performance and maintain their condition. Maslinic acid (MA) is a type of compound with a pentacyclic triterpene structure extracted from olives, and has a strong anti-inflammatory effect and improves metabolic function. This study aimed to investigate the effects of MA on muscle hypertrophy by functional overload using an animal model. Mice plantaris muscles were overloaded by synergist ablation surgery with/without MA and they were sampled at 4, 7, and 14 d after the operation. We demonstrated that MA significantly increased plantaris’ cross-sectional area and activated the mechanistic target of rapamycin (mTOR) signaling compared with the non-supplemented group (main effect of MA, p<0.05). In addition, MA also significantly reduced catabolic proteins compared with the non-supplemented group. MA supplementation increased muscle fiber size and promoted muscle hypertrophy via mTOR signaling. Our results indicate that MA supplementation may be useful for promoting hypertrophy of skeletal muscle.

Key Words maslinic acid, protein synthesis, hypertrophy, functional overload, mTOR signaling

Maslinic acid (MA) is a pentacyclic triterpene found in olives, and has been attracting attention as a supplement with a various beneficial functions for health (1–4). A previous study has reported that MA suppressed inflammation and necrosis of synovial tissue at the knee joint in arthritis-induced mice, and suppressed gene expression of inflammation-related factors (Tnf-α, Il-1, Ifn-γ) (1). Furthermore, in a human study, MA also suppressed high-sensitive-CRP (C-reactive protein) in blood and the visual analogue scale degree of pain in an elderly with knee joint pain (2). These results indicate that MA has a strong anti-inflammatory effect.

Also, MA enhances skeletal muscle mass and motor function in the elderly when combined with resistance exercise (3). Furthermore, a study combining MA intake with whole-body vibration training in elderly women with arthralgia reported that it improved knee muscle strength in addition to inhibiting knee joint inflammation (4). Previous studies using rainbow trout have reported that trout phosphate promotes protein synthesis and muscle mass gain (5, 6), but this study did not clarify the detailed molecular mechanism. Ursolic acid, which is also a member of the pentacyclic triterpenes, increases skeletal muscle mass and improves grip strength and exercise performance in mice (7, 8). Resistance training mainly changes the morphology of skeletal muscle, improves muscle strength, and promotes hypertrophy (9–11), by activating the mechanistic target of rapamycin (mTOR) signaling pathway, which induces the p70 S6 kinase (p70S6K) and S6 ribosomal protein (S6) (12). Ursolic acid can also enhance exercise-induced protein synthesis (8). Some report suggest that the combination of exercise and supplements containing pentacyclic triterpenes can improve hypertrophy and motor performance.

Previous studies indicate that a combination of some kinds of supplements and exercise promote muscle hypertrophy. A combination of MA and resistance exercise is also reported to improve muscle strength in elderly (3). However, the mechanism by which MA exerts these effects is unclear, and detailed analyses are needed. Therefore, we thought that a combination of taking MA as a supplement with pentacyclic triterpenes intake and exercise would enhance muscle hypertrophy. This study aims to investigate whether MA intake promote muscle hypertrophy in overloaded mice. We hypothesized that MA would promote muscle hypertrophy via mTOR signaling in overloaded mice.

Materials and Methods

Animals and synergist ablation surgery. All experi-
Mental procedures performed in this study were approved by the Institutional Animal Experiment Committee of the University of Tsukuba (20-407). Male ICR mice aged 7 to 8 wk (Tokyo Laboratory Animals Science Co., Tokyo, Japan) were used in this study. Mice were kept at a temperature of 22 ± 2˚C and humidity of 55 ± 5% in controlled facilities under a 12/12-h light/dark cycle with ad libitum access to food and water. The experimental protocol is shown in Fig. 1. We performed bilateral synergist ablation surgeries, as previously described under anesthesia with 2.0% isoflurane air inhalation (13, 14). This in vivo model induces hypertrophy of the plantaris muscle by mechanical overload (OL) through the surgical removal of synergist muscles (gastrocnemius and soleus). All mice were divided into the OL and OL+MA groups as follows: the OL group was fed normal foods (Oriental Yeast Co., Ltd., Tokyo, Japan) and the OL+MA group was fed normal food containing 0.27% MA (Nippun Corp., Tokyo, Japan). On days 4, 7 and 14 after surgery, the mice were anesthetized and the plantaris muscle was excised, weighed, quickly frozen in liquid nitrogen, and stored at −80˚C until needed for analyses.

**Cross-sectional area (CSA) quantification.** The plantaris muscle was covered with optimal cutting temperature (OCT) compound (Sakumra Finetek, Tokyo, Japan), and then quickly frozen in liquid nitrogen-cooled isopentane and stored at −20˚C until sectioning. The frozen muscle was sectioned at a thickness of 10 μm, air dried, and stored at −20˚C. Images were captured with an Olympus DP-74 microscope (Tokyo, Japan).

**Western blotting.** Excised plantaris muscles were immediately frozen in liquid nitrogen and total muscle protein was extracted by lysis buffer containing 50 mM HEPES (pH: 7.6), 150 mM NaCl, 10 mM EDTA, 10 mM Na2P2O7, 10 mM NaF, 2 mM Na3VO4, 1% (vol/vol) NP-40, 1% (vol/vol) Na-deoxycholate, 0.2% (wt/vol) SDS, and 1% (vol/vol) complete protease inhibitor cocktail. Protein concentrations were measured using a Protein Assay Bicinchoninate Kit (Nacalai Tesque Inc., Kyoto, Japan). Before SDS-PAGE, an aliquot of the extracted protein solution was mixed with an equal volume of sample loading buffer containing 1% (vol/vol) 2-mercaptoethanol, 4% (wt/vol) SDS, 125 mM of Tris-HCl (pH: 6.8), 10% (wt/vol) sucrose, and 0.01% (wt/vol) bromophenol blue. The mixture was then heated at 97˚C for 3 min. Ten micrograms of each protein sample was separated on an SDS-polyacrylamide gel and electrically transferred to an ImmunoBlot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blot was blocked by Blocking One (Nacalai Tesque Inc.) for 1 h at room temperature and incubated with primary anti-
bodies overnight at 4°C in TBS containing 0.1% Tween-20. After overnight incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. Signals were detected using the Immunostar Zeta or LD (Wako Chemicals, Osaka, Japan), quantified with C-Digit (LI-COR Biosciences, Lincoln, NE, USA), and expressed as arbitrary units.

Primary antibodies for western blotting. The following primary antibodies were used for western blotting: protein kinase B (Akt) (9272; Cell Signaling Technology, Danvers, MA, USA), p-Akt (#4060S; Cell Signaling Technology), mTOR (#2983; Cell Signaling Technology), p-mTOR (#2971; Cell Signaling Technology), p70 S6 kinase (p70S6K) (#9202; Cell Signaling Technology), p-p70S6K (#9205; Cell Signaling Technology), S6 ribosomal protein S6 (S6) (#2217; Cell Signaling Technology), p-S6 (#4858S; Cell Signaling Technology), Muscle Atrophy F-box (MAFbx) (sc-33782, Santa cruz), Muscle RING-Finger Protein (MuRF1) (sc-32920, Santa cruz), and ubiquitin (sc-166553, Santa cruz).

Statistical analyses. Data are shown as means±standard error. Two-way analysis of variance were conducted for all measurements. When a significant p-value was obtained, statistical significance was calculated according to Tukey’s method. The GraphPad Prism 7 software (GraphPad, Inc., San Diego, CA, USA) was used for all statistical calculations, and the significance level was set to p<0.05 for all cases.

RESULTS

There was no difference in food consumption between the OL and OL+MA groups after 14 d (Fig. 2A). Also, the body weight and plantaris wet weight are almost the same between the OL and OL+MA groups at each sampling point (main effect of time, p<0.05, Fig. 2B, C, D). These data showed that MA intake does not affect food consumption, body weight, and skeletal muscle mass.

To examine the effect of MA on muscle hypertrophy induced by functional overload, we measured the CSA of plantaris muscle between OL and OL+MA groups at 4, 7, and 14 d. Hematoxylin and eosin stain revealed a difference in the muscle fibers’ gross morphology between the OL and OL+MA at each time point (Fig. 3A). As for CSA, those of the OL+MA group were much larger than those of the OL group on 4, 7, and 14 d (main effect of MA, p<0.05; main effect of time, p<0.05; interaction, p<0.05, Fig. 3B).

Next, we examined the effect of MA on muscle hypertrophy induced by functional overload, we measured Akt/mTOR signaling pathway by using western blot. We evaluated the phosphorylation levels of Akt, mTOR, p70S6K and S6, known as related to muscle hypertrophy. The phosphorylation levels of Akt was not significant in all group (Fig. 4A). The phosphorylation levels of mTOR, p70S6K and S6 were significantly increased in the OL+MA group compared with those in the OL group at each time point (main effect of MA, p<0.05, Fig. 4B, C, D). These data demonstrated that MA intake promotes the phosphorylation levels of mTOR and down regulate proteins in plantaris by functional overload.

We also examined whether MA affects muscle protein synthesis as well as degradation. We measured ubiquitin-proteasome system. Muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1) were known to key regulators of skeletal muscle atrophy.
The expression levels of MAFbx was not significant in all groups (Fig. 5A), but MuRF1 was significantly decreased in the OL+MA group compared with those in the OL group at 4, 7 and 14 d (main effect of MA, \( p<0.05 \), Fig. 5B). In addition, the expression levels of total ubiquitinated proteins were also significantly decreased in the OL+MA groups compared with those in OL group regardless of the sampling points (main effect of MA, \( p<0.05 \), Fig. 5C). These results indicate that MA has not only muscle protein synthesis but also inhibitory effect on degradation.

**DISCUSSION**

This study investigated whether MA could promote muscle hypertrophy associated with functional overload by synergist ablation surgery. In this study, there was no difference in the weight of muscle tissue but there was a difference between groups in CSA. A previous study reported that elderly mice fed royal jelly had an increase in muscle fiber CSA even though muscle weight was unchanged (16, 17). Muscle weight during pathological conditions such as obesity, type 2 diabetes, or age-related sarcopenia can be affected by the infiltration of adipose and/or connective tissue (18). Overload stimulation by synergist ablation not only causes muscle hypertrophy, but also breaks muscle fibers, resulting in significant changes in the extracellular environment such as connective tissue. It may be interesting to explore the effect of MA on the infiltration of muscle by connective tissue.

The mTOR signaling proteins, p70S6K and S6, play an important role in muscle hypertrophy (19). The phosphorylation levels of these signaling molecules were significantly increased in the OL+MA group compared with those in the OL group. It has been shown that the phosphorylation of p70S6K correlates with the...
magnitude of muscle hypertrophy after resistance exercise (20). Furthermore, p70S6K and S6, which are downstream of mTOR signaling, are dependent on exercise volume (21). These reports suggest that activation of mTOR signaling is necessary for muscle hypertrophy. In the present study, we found that MA intake increased the phosphorylation of p70S6K and S6 and promoted muscle hypertrophy induced by functional overload. Previous study in humans have reported that a combination of daily both 20 g of the MA jelly (containing 60 mg of MA) intake and resistance training for 12 wk improves muscle mass and motor function in the elderly (3). Our present data provide a detailed mechanism for the combination of exercise and MA intake for skeletal muscle hypertrophy, and these results provide data that demonstrate the effectiveness of MA as a supplement for promoting muscle hypertrophy.

In a previous study, ursolic acid, which has the same pentacyclic triterpene structure as MA, increased the expression of the molecules in Akt/mTOR signaling pathway effectors associated with transient resistance exercise. In this report, ursolic acid intake enhanced Akt/mTOR signaling due to increased insulin-like growth factor in the blood, an upstream factor of mTOR signaling (22). However, MA did not activate Akt in the present study, but activated mTOR and its downstream signals, mTOR is known to a nutrient sensor protein that acts when amino acids and glucose are ingested (23). The present study suggests that MA may act directly on mTOR, rather through the IGF-1/Akt cascade, to increase muscle protein synthesis and promote muscle hypertrophy. Our data showed that MA increased the phosphorylation of mTOR, p70S6K and S6 in OL+MA group. Interestingly, MA did not change the expression of Akt, but had a direct effect on mTOR signaling. These results suggest that MA may have stimulated those proteins by a pathway other than Akt, such as through mitogen activated protein kinase (MAPK). Although ursolic acid promotes muscle hypertrophy, it is also known to enhance inflammatory response, so it has been regarded as a double-edged sword supplement (24). In contrast, MA has an anti-inflammatory effect and can be an easy supplement for athletes to use.

Our results indicate that the phosphorylation of mTOR, p70S6K and S6 remained elevated for 14 d. In a previous study, functional overload increased phosphorylation of p70S6K and S6 and promoted muscle hypertrophy for 14 d (14). Furthermore, in a study examining the time course of compensatory overload, the overload also increased phosphorylation of p70S6K at each time point (13). Therefore, MA intake in combination with continuous overload muscle contraction may enhance the effect of exercise. We thus conclude that MA intake promotes muscle hypertrophy associated with functional overload.

We also examined muscle proteolytic signals. The results showed that MA decreased the expression of MuRF1 and ubiquitinated proteins. Previous studies have shown that MuRF1 is upregulated by inflammation-induced muscle atrophy (25). In this study, we used synergist ablation, which is a method of muscle hypertrophy accompanied by excessive inflammation (26), and found that ingestion of MA, which has an anti-inflammatory effect, may have suppressed the inflammation-induced increase in MuRF1. Therefore, total ubiquitinated proteins may have been reduced as well as MuRF1. These results indicate that MA not only promotes muscle hypertrophy but also suppresses muscle atrophy associated with functional overload. However, this study was conducted in a muscle hypertrophy model, and future studies using muscle atrophy models such as denervation and hindlimb suspension are necessary to examine the effects of MA on muscle atrophy.

Many athletes take supplements to maintain their condition and to improve their athletic performance (27). We demonstrated that MA could be a good supplement for skeletal muscle hypertrophy. Previous studies in humans and animals have shown that MA can inhibit inflammatory responses, with strong anti-tumor, anti-bacterial, and antioxidant effects (1, 28, 29). Overall, MA has been reported to have many beneficial effects in vivo and human study (1, 2, 4, 30), and considering that it also has muscle hypertrophy effects, as revealed in this study, it has the potential to be an ideal supplement especially for power athletes. In future studies, based on the results of this study, it is necessary to examine athletes the effects of MA for athletes in more detail. As a limitation of the study, this is a initial study that demonstrated the positive effects of MA on muscle hypertrophy. We believe that future studies will provide more robust data on MA and muscle hypertrophy by examining acute molecular signaling responses, changes in skeletal muscle mass and muscle protein synthesis rates in athletes. Furthermore, combining MA with different exercise modalities, such as running and swimming, would present the usefulness of exercise supplementation.

Authorship

TS and TT conceived and designed the project; TS, KK and KU performed the experiments; TS analyzed the data; TS wrote the manuscript and revisions, and were checked by YY and TT.

Disclosure of state of COI

The authors declare no conflict of interest.

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