Elucidation of Mechanisms Underlying the Preventive Effects of Olive Fruit-Derived Maslinic Acid on Muscle Atrophy

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Abstract

Skeletal muscle accounts for approximately 40% of body weight and 50% of protein and is the most abundant tissue in the human body. Muscle atrophy can be caused by inactivity, sarcopenia, and various neuromuscular diseases. It can be atrophy when synthesis is inferior to degradation. Sarcopenia is mechanistically implicated in multiple pathophysiological processes, including inflammation, oxidative stress, and subsequent activation or inhibition of signaling pathways, including the mTOR signaling pathway and autophagy-lysosome, and ubiquitin-proteasome system. In the elderly, low-grade inflammation is considered to be one of the main determinants of health status in the elderly and is suspected to be a favorable environment for the development of sarcopenia. The marked increase in the elderly population in Japan, where the aging rate is estimated to reach approximately 40% by 2040, is concerning. The loss of muscle mass and strength must be mitigated among the elderly related muscle wasting is closely related to life expectancy and quality of life (QOL). Among the elderly, sarcopenia is a major health challenge that increases an individual's risk of disability, falling, fall-related injuries, being hospitalized, dependency, and death. Sarcopenia must be treated at early stages as muscle mass decreases by approximately 1% each year after 40 years of age. To lead an independent life, the functions required for engaging social life must be improved and maintained among the elderly. Further studies are needed to prevent sarcopenia and improve a healthy lifestyle.

The Mediterranean diet is reported to promote various health benefits, including the prevention of inflammation-associated diseases. Olives and their oils are considered an important part of the Mediterranean diet. The extract residues of olive oil contain high amounts of pentacyclic triterpenes, which include maslinic acid (MA; 2α,3β-

dihydroxyolean-12-en-28-oic acid). MA exerts potent anti-inflammatory effects by inhibiting the activity of inflammatory cytokines. Additionally, MA enhances synovial tissue formation and repair of cartilage damage caused by arthritis through the NF-κB canonical pathway of signaling. Activated NF-κB pathways as well as inflammatory cytokines promote muscle atrophy. NF-κB stimulates proteolysis by upregulating the expression levels of MuRF1 and Atrogin-1, which are skeletal muscle-specific ubiquitin ligases and function downstream of the FOXO family transcription elements. A recent clinical trial demonstrated that MA supplementation enhanced knee muscle strength and alleviated inflammation of the knee joint in elderly women suffering from osteoarthritis of the knee who underwent a whole-body resistance vibration training. In another clinical trial, the combination of MA intake and resistance training demonstrated effective increases in skeletal muscle mass in the elderly. But the concentration-dependent benefits of dietary MA and the comprehensive underlying molecular mechanisms of MA on the prevention of muscle atrophy have not been elucidated. Therefore, this study aimed to examine the therapeutic effects of olive-derived MA on muscle atrophy. The findings of this study may aid in developing strategies to prevent sarcopenia.

The differential blood MA absorption resulting from varying amounts of MA intake in humans was evaluated to determine the effects of MA intake concentrations on skeletal muscles and QOL in healthy elderly individuals. Five healthy adult men (37.0 ± 10.7 years) were administered test diets containing 30, 60, or 120 mg of MA, and the plasma MA content was analyzed. The test diets concentration-dependently increased the blood MA level. Next, a randomized, double-blind, three-dose parallel-group trial was conducted with 69 healthy adult men and women (66.6 ± 8.5 years) who were prescribed a placebo or 30 or 60 mg MA continuously for 12 weeks and advised physical exercise.

The MA (60 mg) group had higher trunk muscle mass and vitality scores on the Short Form-8, a QOL assessment index, than the placebo group. Additionally, the grip strength in the MA (30 mg)-administered and MA (60 mg)-administered groups was higher than in the placebo group. These results indicated that the combination of MA intake and physical exercise improved muscle strength and that MA intake dose-dependently improved muscle mass and QOL.

Next, animal experiments were performed to investigate the effects of MA on skeletal muscle atrophy and strength of degenerated muscles and elucidate the underlying molecular mechanisms. Mice were fed on a control diet or a diet containing 0.27% MA. At week 1 post-intervention, muscle atrophy was induced by severing the sciatic nerve in both legs. Mice were observed on day 14 post-denervation. MA inhibited the decrease in gastrocnemius muscle mass and skeletal muscle strength caused by denervation. The results of quantitative real-time PCR and enzyme-linked immunosorbent assay revealed that MA upregulated *Igf1* levels and downregulated *Atrogin-1* and *Murf*1 levels. Microarray analysis of gastrocnemius muscle revealed a number of possible mechanisms for muscle maintenance. By Gene set enrichment analysis (GSEA), several biological processes were found to be enriched in MA-treated mice, including TNFα signaling via NF-κB, TGF-β signaling, myogenesis, and PI3K/ACT/mTOR signaling.

Microarray results suggest that MA suppresses inflammation due to denervation. Therefore, *in vitro* studies were performed to evaluate the influence of MA on inflammation-induced muscle atrophy. Lipopolysaccharide (LPS) and dexamethasone (DEX) induce muscle atrophy by upregulating the muscle protein degradation pathway. The expression levels of *Atrogin-1*, *Murf1*, and *Tnfα* in LPS-treated C2C12 myotubes were significantly higher than in the untreated control. Treatment with MA significantly

inhibited the LPS-induced upregulation of *Atrogin-1*, *Murf1*, and *Tnfα* levels. Next, the effect of MA on DEX-induced denervation in mice was examined. Treatment with DEX significantly decreased the diameter of C2C12 myotubes. However, treatment with MA effectively mitigated the DEX-induced downregulation of myotube diameter. These results suggest that MA suppresses DEX-induced protein proteolysis and myotubular atrophy.

In conclusion, this study revealed that MA is a potential novel therapeutic and protective dietary ingredient for skeletal muscle atrophy and strength for muscular atrophy and strength through anti-inflammatory response.

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List of Abbreviations and Acronyms

ANOVA: analysis of variance

Atrogin-1: muscle atrophy F-box protein 32

AUC: area under the curve

BDNF: brain-derived neurotrophic factor

BIA: bioelectrical impedance analysis

BMI: body mass index

BP: bodily pain

C_{max}: maximum plasma concentration

CRP: C-reactive protein

DEX: dexamethasone

DXA: dual-energy X-ray absorptiometry

ELISA: Enzyme-linked immnosorbent assay

EWGSOP: European Working Group on Sarcopenia in Older People

FOXO: forkhead box O

GH: general health

GR: Glucocorticoid receptor

GSEA: gene set enrichment analysis

IGF1: insulin-like growth factor 1

IL6: interleukin 6

LC-MS/MS: liquid chromatography-mass spectrometry

LPS: lipopolysaccharides

MA: maslinic acid

MCS: mental component summary

MH: mental health

mTOR: mechanistic target of rapamycin

MuRF1: muscle RING finger 1

NF-κB: nuclear factor-kappa B

OA: oleanolic acid

PCS: physical component summary

PF: physical functioning

RE: role emotional

RP: role physical

QOL: quality of life

SF-8: Short-Form 8 Healthy Survey

SF: social functioning

TGF-β: transforming growth factor beta

TLR4: toll-like receptor-4

T_{max}: time point at maximum plasma concentration

TNFα: tumor necrosis factor alpha

VT: vitality

CHAPTER 1: General Introduction

Skeletal muscle accounts for approximately 40% of body weight and 50% of protein and is the most abundant tissue in the human body. Muscle atrophy can be caused by sarcopenia, inactivity, and various neuromuscular diseases, and it can be atrophy when synthesis is inferior to degradation. Sarcopenia is mechanistically implicated in multiple pathophysiological processes, including inflammation, oxidative stress, mitochondrial dysfunction, and subsequent activation or inhibition of signaling pathways, including the mTOR signaling pathway and autophagy-lysosome, and ubiquitin-proteasome system. In the elderly, low-grade inflammation is considered to be one of the main determinants of health status (Franceschi et al. 2018) and is suspected to be a favorable environment for the development of sarcopenia (Argiles et al. 2015). The marked increase in the elderly population in Japan, where the aging rate is projected to reach approximately 40% by 2040, is concerning. Age-related inflammation is associated with decreased motor function and muscle mass, as well as with muscle dysfunction (Walston 2012, Wang and Bai 2012). The loss of muscle mass and strength must be mitigated among the elderly related muscle wasting is closely related to life expectancy and quality of life (QOL) (Evans 1995). In Japan, patients suffering from locomotive disorders require special assistance or nursing care (Fig. 1-1). Sarcopenia is a state in which skeletal muscle mass gradually decreases, resulting in a loss of muscle mass and strength, which decreases the overall muscle quality (Cruz-Jentoft et al. 2010). Among the elderly, sarcopenia is a major health challenge that increases an individual's risk of disability, falling, fall-related injuries, being hospitalized, dependency, and death (Senior et al. 2015). Sarcopenia must be treated at early stages as muscle mass

decreases by approximately 1% each year after 40 years of age (Janssen et al. 2000). The etiological factors for sarcopenia include decreased physical activity, age-related anorexia, weight loss, low vitamin D, and increased inflammatory cytokines (Morley, Anker, and von Haehling 2014). The pathogenesis of sarcopenia involves several processes, including denervation, mitochondrial dysfunction, hormonal and inflammatory changes, and enhanced risk of falls. In 2018, the European Working Group on Sarcopenia in Older People (EWGSOP) identified probable sarcopenia due to low muscle strength as a strong predictor of sarcopenia (Cruz-Jentoft et al. 2019, Cruz-Jentoft et al. 2010). The molecular mechanisms underlying skeletal muscle changes have not been elucidated. Current evidence suggests that a complex network regulates sarcopenia. The upregulation of the inflammatory status in the elderly, especially increased levels of IL6, TNFα, and C-reactive protein (CRP), is considered to be the markers of predictors of mortality or frailty (Michaud et al. 2013). Alleviating inflammation in the systemic and muscular systems may contribute to improving muscle strength and physical function by promoting muscle hypertrophy and inhibiting muscle atrophy.

Studies on the effectiveness of pharmacotherapy in sarcopenia are limited. Hence, resistance training and nutritional supplements are the major conventional treatments for sarcopenia. Exercise is an essential component of the treatment plan for sarcopenia because it increases muscle mass, decreases body fat, and enhances the immune and cardiovascular system functions, muscle strength, and endurance. Resistance training, which is vital to minimize skeletal muscle loss, promotes muscle growth and improves muscle strength by promoting muscle protein synthesis and

reducing muscle protein breakdown (Johnston, De Lisio, and Parise 2008). Appropriate exercise and diet are essential to improve muscle function. However, maintaining an adequate exercise routine is challenging for the elderly. The effects of food on the regulation of muscle function have yet to be fully understood. To improve muscle function through diet, the biological mechanisms linking food components and muscle function must be elucidated.

The world faces several food-related social issues, such as food shortages due to global population growth. Food resources are limited. Health problems related to diet and lifestyle, such as lifestyle-related diseases and malnutrition, are increasing worldwide, which is a growing concern. The Mediterranean diet provides various health benefits, including the prevention of inflammation-associated diseases. Olives and their oils are considered important foods in the Mediterranean diet (Keys 1995, Rosillo et al. 2016, Willett et al. 1995). Dietary patterns, such as the Mediterranean diet intake, and physical activity are associated with sarcopenia. The Mediterranean diet affects some metabolic changes, such as obesity, especially abdominal obesity, diabetes, and inflammatory factors (Abete et al. 2019). Diet and physical activity are essential mediators regulating systemic inflammation that play a direct role in the sarcopenic process. The combination of healthy dietary patterns and physical activity is a promising strategy for reducing age-related sarcopenia. The demand for food is expected to increase with the growing global population. Thus, reducing food loss and waste is a critical issue. The production of olive oil is escalating owing to its organic properties and significant health benefits. The olive fruit is subjected to several processes, such as grinding, mixing, and centrifugation during the isolation and

extraction of olive oil (Fig. 1-2). Furthermore, the yield of olive oil is 15%–20%, while the remaining raw material (olive pomace) constitutes 80%–85% (Fig. 1-3) (Di Giovacchino et al. 2017). Many residual components are produced during the extraction process. The yield of olive pomace is approximately 200,000 tons per year although this resource is not effectively utilized (Pagnanelli, Viggi, and Toro 2010). The components of olive pomace include maslinic acid (MA; 2α,3β-dihydroxyolean-12-en-28-oic acid) and oleanolic acid (OA) (a triterpenoid), hydroxytyrosol, and various phenolic compounds (Fig. 1-4). Olive oil extraction residues contain high amounts of pentacyclic triterpenes, including MA. MA exerts potent anti-inflammatory effects and inhibits the activity of inflammatory cytokines. Additionally, MA downregulates the expression levels of inflammatory response-related genes, mitigates the lipopolysaccharide (LPS)-induced production of TNFα, and suppresses inflammation in edema and arthritis models (Fukumitsu, Villareal, Fujitsuka, et al. 2016). Furthermore, MA enhances synovial tissue formation and repair of cartilage damage caused by arthritis thorough the NF-κB canonical pathway of signaling (Shimazu et al. 2019). Muscle atrophy is caused by the activation of the NF-κB pathway and inflammatory cytokines. NF-κB stimulates protein degradation by promoting the expression levels of Atrogin-1 and MuRF1 , two skeletal muscle-specific ubiquitin ligases that act downstream of the FOXO family of transcription factors. Chronic inflammation (indicated based on high-sensitivity CRP (hs-CRP) levels) might be involved in the pathogenesis of sarcopenia. Muscle strength loss was related to higher levels of hs-CRP, a sensitive marker for detecting low-grade chronic inflammation (Shokri-Mashhadi et al. 2021). A previous clinical trial in the elderly reported that the

decrease in serum hs-CRP levels of the MA intake group was greater than that of the placebo group after the intervention. However, the serum hs-CRP levels were not significantly different between the groups at week 12 of treatment (Fukumitsu, Villareal, Aida, et al. 2016). A recent clinical study revealed that 50 mg of MA supplementation enhanced knee muscle strength and reduced knee joint inflammation in elderly women suffering from osteoarthritis of knee who participated in a whole-body vibration resistance training (Yoon et al. 2018). In another clinical trial, the combination of 60 mg MA intake and resistance training demonstrated effective increases skeletal muscle mass in the elderly (Nagai et al. 2019). Some clinical evidence supports the beneficial effects of natural products, including phytoproducts such as curcumin, resveratrol, catechins, and ginseng, on sarcopenia (Bagherniya et al. 2022). These compounds are reported to exert various beneficial effects on inflammatory stress. However, the clinical benefits of plant-derived natural products are inconclusive due to limited clinical trials in humans. Our group identified MA as an active ingredient in olive pomace and developed an industrial method to extract and purify MA. The effective use of industrial by-products is important as it contributes to a recycling-based society.

The mechanism of action of MA on muscle proteins has been partly elucidated (Bagchi, Sreejayan, and Sen 2018, Fernandez-Navarro et al. 2008, Murata et al. 2021, Shirai et al. 2021). MA induces protein synthesis and increases white muscle weight in rainbow trouts fed on MA-containing diets. This can be attributed to the activity of MA as a growth factor, which involves the binding of MA to specific receptors in the cytoplasm involved in protein synthesis to promote the expression of protein synthesis-related genes(Bagchi, Sreejayan, and Sen 2018, Fernandez-Navarro et al. 2008).

Recent studies have suggested MA supplementation exerts therapeutic effects on skeletal muscle hypertrophy through the activation of mTOR signaling pathway, which performs a central role in the regulation of protein synthesis (Murata et al. 2021, Shirai et al. 2021). However, the concentration-dependent beneficial effects of dietary MA and the mechanisms of preventive effects of MA on muscle atrophy have not been elucidated. Therefore, this study aimed to evaluate the beneficial effects of olive-derived MA on muscle atrophy.

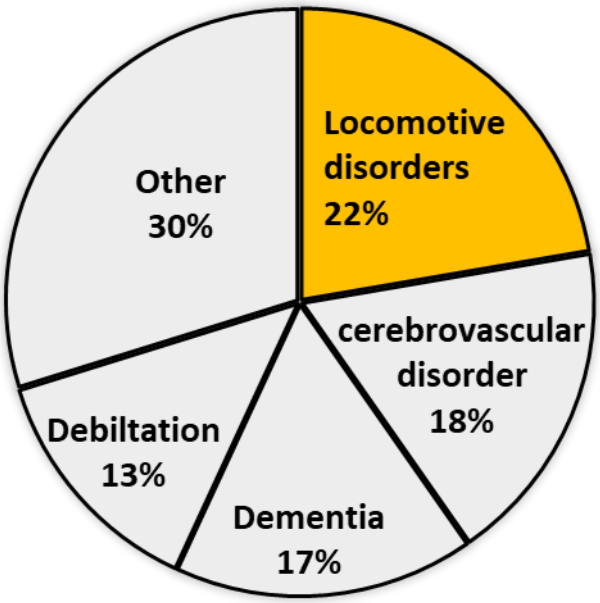


Figure 1-1. Locomotive disorders are serious health issues.

Locomotive disorders are associated with sarcopenia, osteoarthritis, and osteoporosis.

Source: Japanese Ministry of Health, Labor and Welfare, Comprehensive survey of living conditions 2016.

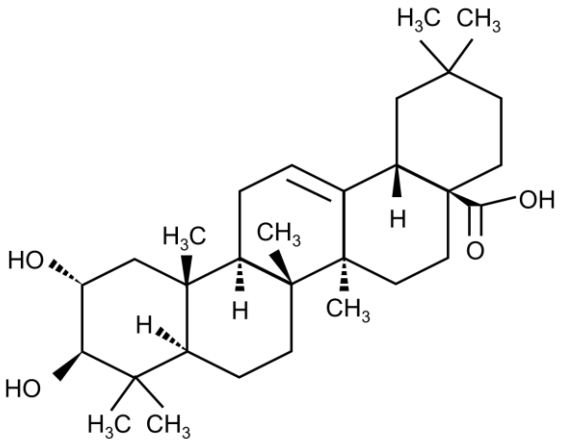


Figure 1-2. Chemical structure of maslinic acid.

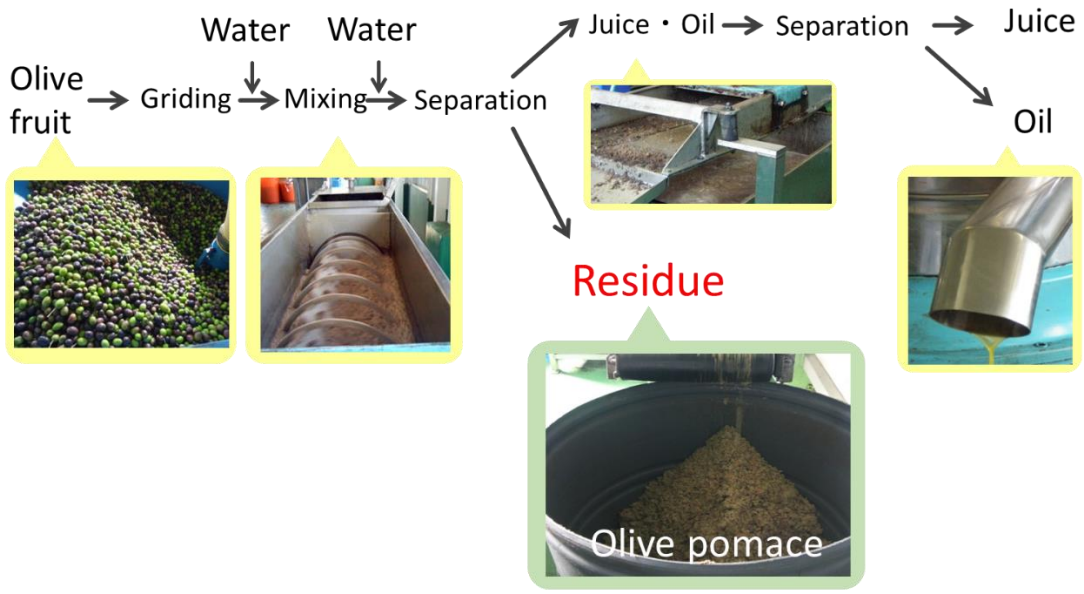


Figure 1-3. Chemical components in fraction of olive oil production process.

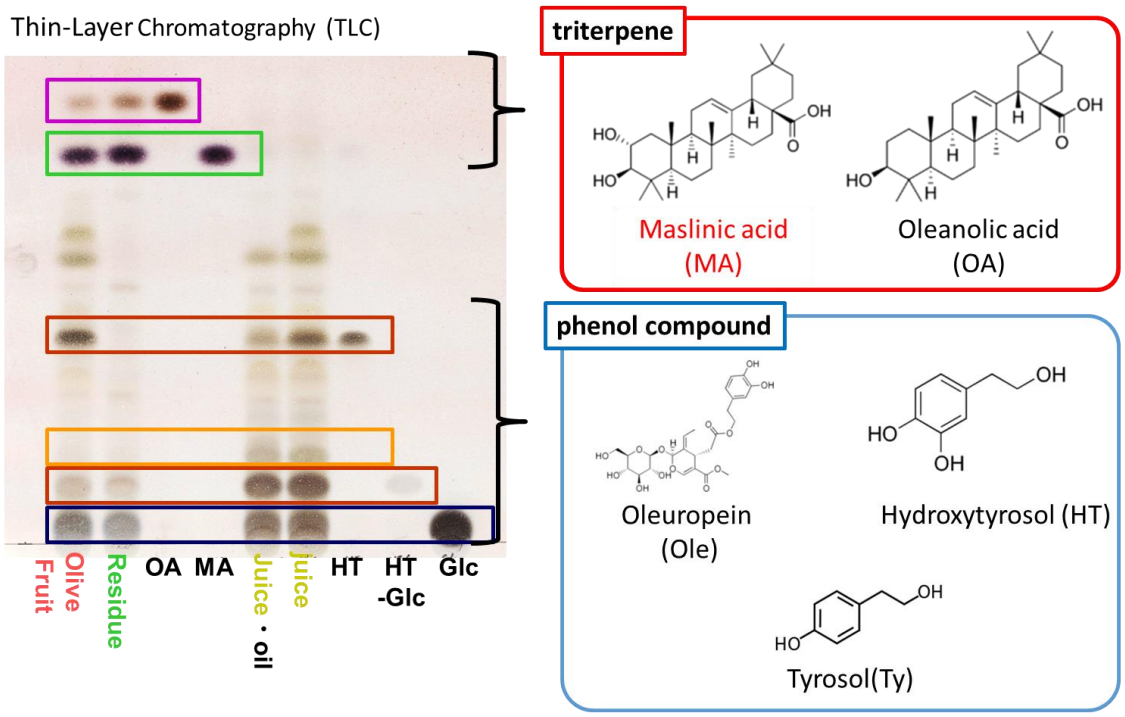


Figure 1-4. Major functional components in olive fruit.

Images showing the analysis of components in olive fruit using thin-layer chromatography. These components include maslinic acid and oleanolic acid (a triterpene), hydroxytyrosol (HT), and several phenolic compounds.

CHAPTER 2: Pharmacokinetics of maslinic acid and effects of daily maslinic acid intake in combination with physical exercise on muscle strength and mass in the healthy Japanese population

2.1. Introduction

The aging rate in Japan is expected to reach approximately 40% by 2040, which is a major concern. Additionally, the proportion of elderly people has been increasing worldwide in recent years. Generally, age-related inflammation is associated with a loss of mobility and muscle mass, resulting in impaired muscular performance (Walston 2012, Wang and Bai 2012). Muscle mass decreases by approximately 1% each year after the age of 40 years (Janssen et al. 2000). Thus, strategies need to be developed to mitigate muscle mass decline among the elderly and middle-aged adults. Sarcopenia is a condition characterized by the gradual loss of skeletal muscle mass, strength, and quality (Cruz-Jentoft et al. 2010).

Several studies have demonstrated that regular physical activity, primarily resistance training, delays the onset of sarcopenia (Peterson and Gordon 2011, Pillard et al. 2011). However, the elderly cannot train at high intensity for prolonged durations due to physical and environmental constraints. Therefore, there is a need to develop efficient methods to maintain and improve muscle mass and strength in the elderly. Nutritional supplementation in combination with low-intensity to moderate-intensity exercise has been demonstrated to promote physical strength in the elderly. Additionally, regular low-intensity exercise in combination with amino acid and polyphenol supplementation improves muscle mass and strength in the elderly (Kim et al. 2012, Kinoshita et al. 2021).

The Mediterranean diet provides various health benefits, including the

prevention of inflammation-associated diseases; olives and their oils are essential foods in the Mediterranean diet (Keys 1995, Rosillo et al. 2016, Willett et al. 1995). Olive oil extraction residue is a rich source of various pentacyclic triterpenes, including maslinic acid (MA). Previous studies have demonstrated that 30 mg MA intake relieves knee pain and improves the quality of life (QOL) in the elderly (Fukumitsu et al. 2017). One study reported that the combination of 50 mg MA intake and resistance training improves knee muscle strength and alleviates knee joint inflammation in elderly women with knee osteoarthritis (Yoon et al. 2018). Meanwhile, another clinical trial demonstrated that the combination of resistance exercise and 60 mg MA supplementation effectively improved skeletal muscle mass in the elderly (Nagai et al. 2019). Therefore, MA intake alone or in combination with resistance exercise can be expected to exert therapeutic effects. However, appropriate and effective minimum intake levels have yet to be determined.

This study hypothesized that differential blood MA absorption resulting from different MA intakes would be associated with increased muscle mass and strength. To verify this hypothesis, the effects of different MA intake levels on MA blood absorption, muscle mass, and strength were examined to determine effective MA intake levels. Blood MA concentrations and pharmacokinetic parameters were measured in healthy men who orally ingested MA. A randomized, double-blind, placebo-controlled trial was conducted to determine the effects of different doses of MA intake (0, 30, and 60 mg/day) on muscle mass and strength in healthy adults undergoing daily resistance training.

2.2. Materials and methods

2.2.1. Test diets

2.2.1.1 Experiment 1

MA-containing jelly was used to evaluate MA absorption in the blood after the administration of a single dose. This study used jelly containing 30, 60, or 120 mg MA derived from olive fruit extract (NIPPN CORPORATION, Tokyo, Japan).

2.2.1.2 Experiment 2

The participants were administered three capsules containing olive fruit extract. Each capsule comprised 0, 10, or 20 mg MA to confirm the efficacy of MA intake via supplements. The placebo capsules contained highly branched cyclic dextrin instead of olive extract. However, all test diets were visually identical.

2.2.2. Study design

2.2.2.1 Experiment 1

This experiment was conducted as a single-blind crossover study. The objective of this experiment was to confirm the blood absorption of MA from the test diet and determine the MA intake in the efficacy study. The participants were instructed to fast overnight and consume jelly containing 30, 60, and 120 mg MA. The participants ingested standardized meals at 3 and 8 h post-test diet consumption. A 1-week washout period was allowed before ingestion of the MA jelly to minimize carryover effects. The blood samples were collected before test diet ingestion and at 0.5, 1, 2, 3, 4, 8, 12, and 24 h post-test diet ingestion using an indwelling venous needle (10 mL). Next, the blood

samples were centrifuged at 1,200 *g* and 4°C for 15 min. The plasma was collected and immediately reserved at −80°C. Plasma MA content was measured using liquid chromatography-mass spectrometry (LC-MS/MS).

2.2.2.2 Experiment 2

The experiment was conducted as a randomized, double-blind, placebo-controlled trial with the following three groups: placebo group, 30 mg MA-treated group (MA30 group), and 60 mg-treated MA group (MA60 group). The objective of this experiment was to evaluate the effects of different amounts of MA intake on physical function and QOL. The participants in each group consumed 3 capsules each day containing 0, 10, or 20 mg of MA simultaneously after breakfast for 12 weeks. The study participants were instructed not to make any lifestyle changes during the study period.

The participants received low-intensity to moderate-intensity resistance training that could be performed at home. The training comprised the following eight components: (1) lower body squats, (2) thigh exercises, (3) back muscle exercises, (4) back and buttock exercises, (5) upper body abdominal exercises, (6) lower body abdominal exercises, (7) arm exercises, and (8) calf muscle training. An experienced sports scientist supervised the series of training sessions. For the upper body training, resistance training with rubber tubing was used. The participants were instructed to perform each exercise three times per week during the study period. Additionally, the participants were instructed to walk 1,000 to 2,000 steps daily using a walking pole. To record the number of steps walked each day, participants were asked to wear a pedometer (Calori Scan HJA-401F, Omron Corporation, Kyoto, Japan). During the intervention period, the participants were

instructed to record daily whether or not they consumed the test diets, changes in their physical condition, and the type of exercise they performed.

2.2.3. Participants

2.2.3.1 Experiment 1

Five volunteers were recruited by SOUKEN Co., Ltd. (Tokyo, Japan). The participants offered written informed consent to participate in the clinical trial. The inclusion criteria were as follows: Japanese men (1) aged 20–59 years at the time of providing consent, (2) can provide multiple blood samples, (3) living a regular lifestyle (no binge drinking or disordered lifestyle), and (4) not suffering from any disease. The exclusion criteria are as follows: (1) allergic to olive, (2) participation in other clinical trials, and (3) history of severe hepatic, renal, or heart disease. Experiments were approved by the Shiba Palace Clinic Ethical Review Committee (Approval No. 20181108) and conducted following the Declaration of Helsinki for ethical considerations.

2.2.3.2 Experiment 2

Based on the results of a previous study (Nagai et al. 2019), 75 participants were needed to evaluate the effects of MA intake in the three groups. The inclusion criteria were as follows: (1) age 40 years or older at the time of providing consent, (2) capable of exercising regularly, and (3) capable of consenting to participation after fully understanding the explanations. Meanwhile, the exclusion criteria are as follows: (1) allergic to olive, (2) pregnant or breastfeeding, (3) undergoing orthopedic rehabilitation for treatment of knee or back pain, (4) participation in another clinical trial within 3

months before providing consent, or (5) deemed by the principal investigator to be inappropriate for participation for other reasons.

The participants were recruited from members of the health promotion organization “Iyo-city exercise class” in Ehime Prefecture. Sixty-nine participants (10 men and 59 women, aged 43–83 years) participated in the baseline study. Next, the participants were then randomly assigned to the placebo, MA30, and MA60 groups (n = 23 each) using gender and age as allocation factors.

The study was approved by the Sapporo Yurinokai Hospital Clinical Trials Committee (Approval No. 20190220) and conducted following the ethical guidelines for medical and health research involving human subjects. It was registered in the UMIN Clinical Trial Registry under the ID UMIN000035980. The participants offered written informed consent for the study.

2.2.4. Materials and method of evaluation

2.2.4.1 Chemicals and reagents

Experiment 1: MA used in the analysis was obtained from Funakoshi Co., Ltd. (Tokyo, Japan). LC/MS grade acetonitrile, formic acid, and 1 mol/L ammonium formate solution were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2.4.2 Measurement of MA concentration in blood samples

Experiment 1: Plasma MA concentrations were measured using a Shimadzu Prominence UFLC System (Shimadzu Corporation, Kyoto, Japan). MA was extracted from the plasma using acetonitrile. The extract was filtered through a 0.2-μm membrane

filter to prepare the analysis sample. Chromatography was performed using L-column2 ODS (150 mm × 2.1 mm, 5 μm; GL Science Inc., Tokyo, Japan) under the following conditions: column temperature, 40°C; mobile phase A, 0.1% formic acid + 10 mM ammonium formate; mobile phase B, acetonitrile; linear gradient, 80% A to 100% A for 12 min; flow rate, 0.2 mL/min; injection volume, 5 μL. Mass spectrometry analysis was performed using the ion trap mass spectrometer 4500QTRAP (ABSciex Co, Ltd., Tokyo, Japan) equipped with an electron spray ionization (ESI) ion source. The MS conditions were as follows: mode, negative ion mode (ESI−); reaction type, multiple reaction monitoring; m/z, 471.3/393.1 (MA); drying and nebulizing gas, nitrogen.

2.2.4.3 Pharmacokinetic analysis

Experiment 1: A non-compartmental pharmacokinetic analysis was conducted. Maximum plasma concentration (C_{max}) and time to reach maximum plasma concentration (T_{max}) were determined directly from the plasma concentration-time data. The elimination half-life ($t_{1/2}$) at the log-linear endpoint was calculated using linear regression analysis from the elimination rate constant. The area under the curve (AUC) of the plasma concentration-time curve up to 24 h after ingestion was calculated using the linear trapezoidal method.

2.2.4.4 Body composition and physical function

Experiment 2: Body composition and physical functions were assessed by the management staff before and after the intervention. Body composition parameters (body weight, body mass index (BMI), body fat%, and muscle mass) were determined using a

bioelectrical impedance analyzer (MC-780A; Tanita, Tokyo, Japan). Grip strength was measured using a digital dynamometer (TKK5401; Takei Scientific Instruments Co., Ltd, Niigata, Japan). Leg strength was measured with an isometric knee extension force dynamometer (TKK5715; Takei Scientific Instruments Co., Ltd, Niigata, Japan). The participant was instructed to walk straight for 12 m to measure the 10 m walking speed. Walking speed was measured as the time taken to walk 10 m, excluding 1 m from the start point and 1 m before the goal point.

2.2.4.5 Subjective questionnaire

Experiment 2: To assess the subjective health status of participants, the participants were instructed to complete the Short-Form 8 Healthy Survey (SF-8) questionnaire before and after the intervention. SF-8 is a widely used questionnaire to assess the subjective physical and mental QOL (Fukuhara and Suzukamo 2004, Tokuda et al. 2009). In particular, SF-8 measures physical functioning (PF), role physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH), and provides a mental component summary (MCS) scores and physical component summary (PCS). For each item, 50 points were the national standard score. High scores are directly proportional to QOL.

2.2.4.6 Safety

Information was collected on all adverse events, clinical symptoms, syndromes, and illnesses that occurred or worsened relative to the start of the study. Adverse events

were determined based on life diary records. Events that appeared to be causally related to the study diet were considered adverse events.

2.2.5. Statistical analysis

Results are presented as mean ± standard deviation. In Experiment 1, pharmacokinetic parameters were analyzed using multiple comparison tests with Bonferroni's method. Dose dependence was analyzed using linear regression analysis. In Experiment 2, baseline characteristics were analyzed using one-way analysis of variance and the Chi-square test. The efficacy of MA intake was analyzed using the paired *t*-test for within-group comparisons before and after the intervention and the Williams test for between-group comparisons. SPSS Statistics ver25 for Windows (IBM Inc., Tokyo, Japan) and SAS ver9.4 (SAS Institute Inc., Cary, NC, USA) were used for statistical analysis. Differences were considered significant at $p < 0.05$.

2.3. Results

2.3.1. Participant characteristics in Experiment 1

Five participants completed the study without reporting any adverse events. The mean age and weight of the participants were 37.0 ± 10.7 years (26–54 years) and 76.2 ± 9.8 kg (62.0–85.0 kg), respectively.

2.3.2. Pharmacokinetic parameters in Experiment 1

Figure 2-1A shows the plasma concentration-time curves for single oral doses of 30, 60, or 120 mg MA. The pharmacokinetic parameters are summarized in Table 2-1.

The MA concentration peaked at approximately 3 h post-administration. All doses were below the detection limit at 24 h post-administration. The C_{max} values at doses of 30, 60, and 120 mg were 146.4 ± 21.9, 280.0 ± 48.3, and 477.8 ± 86.4 ng/mL, respectively, which were significantly higher than the administered dose, whereas the AUC values were 9,811.5 ± 3,492.3, and 21,042.8 ± 3374.3, and 34,841.3 ± 9,508.2 ng·h /mL, respectively. There was no dose dependency to the time at T_{max} and $t_{1/2}$. The AUC and C_{max} values after administration were almost proportional to MA intake, confirming a dose-dose correlation (Fig. 2-1B, D). No dose correlation was observed for the AUC (AUC/dose) or C_{max} (C_{max}/dose) corrected for dose (Fig. 2-1C, E). Generally, the AUC/dose ratio should be constant over the dose range in linear pharmacokinetics. The low value suggests that MA exhibits nonlinear pharmacokinetics at high doses.

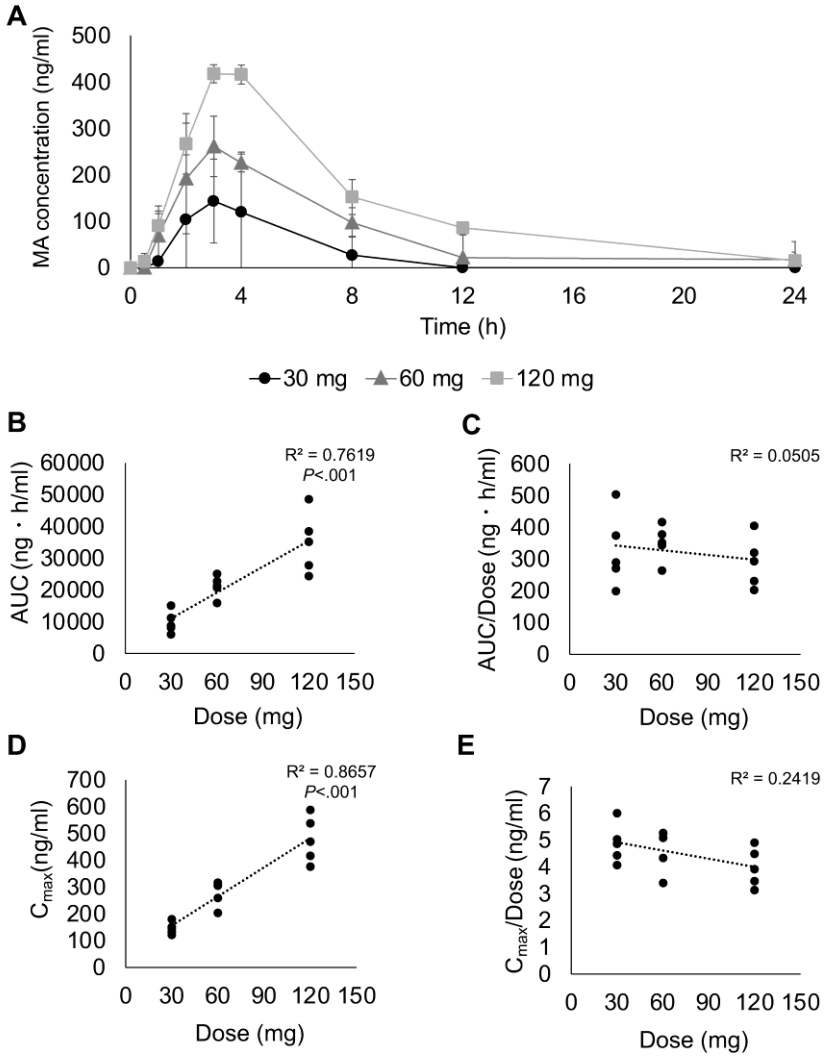


Figure 2-1. Plasma concentrations of MA.

Plasma concentration of MA after oral administration of test diet containing MA (A). Values are expressed as mean ± standard deviation (n = 5). The correlation between MA bioavailability and MA intake was determined based on the area under the curve of test diets for individual participants. The slopes of (B), (C), (D), and (E) indicate dose proportionality or linear pharmacokinetics. MA, maslinic acid; AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration.

Table 2-1 Effect of oral MA administration on the pharmacokinetic parameters in healthy participants

MA	AUC_{0-24} (ng·h/mL)		C_{max} (ng/mL)		T_{max} (h)	$t_{1/2}$ (h)
30 mg	9811.5 ± 3492.3	a	146.4 ± 21.9	a	3.0 ± 0.7	3.3 ± 1.2
60 mg	21042.8 ± 3374.3	b	280.0 ± 48.3	b	3.0 ± 0.7	4.5 ± 2.2
120 mg	34841.3 ± 9508.2	c	477.8 ± 86.4	c	3.4 ± 0.5	3.3 ± 0.3

Values are expressed as mean ± standard deviation. Different superscripts (a, b, c) simultaneously indicate significant differences ($p < 0.05$) between the groups (multiple *t*-tests with Bonferroni correction following analysis of variance). MA, maslinic acid; AUC, area under the plasma concentration-time curve; C_{max}, maximum plasma concentration; T_{max}, time at maximum plasma concentration; $T_{1/2}$, the elimination half-life.

2.3.3. Participant characteristics and compliance in Experiment 2

Based on the ease of MA intake, the benefits of MA intake can be achieved at lower doses. MA pharmacokinetic analysis revealed that a dose of 120 mg was considered excessive overall. Thus, the MA intake dose in this study was fixed at 30 and 60 mg. Maximum absorption occurred at approximately 3 h post-MA intake. The blood MA level was below the detection limit at approximately 12 h. Therefore, in Experiment 2, the intake timing was set in the morning instead of the evening to ensure that MA circulated in the blood during the activity period. Fig. 2-2 shows the flowchart of participant monitoring. Sixty-nine participants fulfilled the inclusion criteria and were randomly allocated to the three groups. As shown in Fig. 2-2, 1, 1, and 2 participants from the placebo, MA30, and MA60 groups, respectively, dropped out during the intervention period. Thus, the number of participants in the placebo, MA30, and MA60 groups was 22, 22, and 21, respectively.

Table 2-2 shows the baseline characteristics of the participants. All tested indicators were not significantly different between the three groups. Adherence to intake was high in the placebo (98.3% ± 3.8%), MA30 (99.2% ± 3.7%), and MA60 groups (97.1% ± 2.3%). The average number of exercise days per week was approximately 3 days in all groups and was not significantly different between the groups.

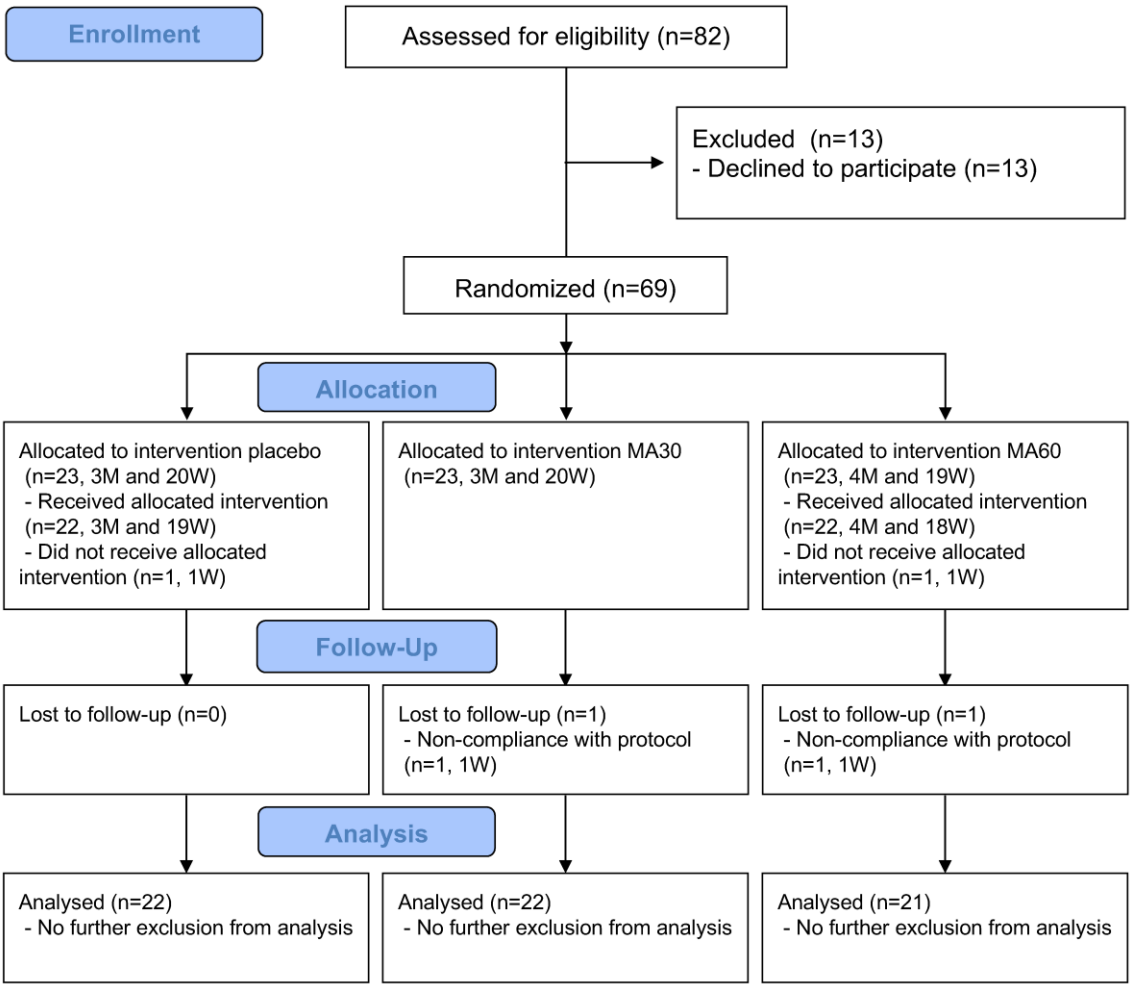


Figure 2-2. CONSORT flow diagram of participant recruitment and dropouts before and during the study.

CONSORT, Consolidated Standards of Reporting Trials; MA30, 30 mg maslinic acid-treated group; MA60, 60 mg maslinic acid-treated group; W, women; M, men

Table 2-2 Baseline characteristics of participants

Characteristics	Placebo (n = 22)	MA30 (n = 22)	MA60 (n = 21)	*p**
Number of mem	3	3	4	
Age (years)	68.7 ± 9.7	69.5 ± 8.1	67.7 ± 8.5	0.8
Body composition				
Body mass (kg)	55.0 ± 11.2	55.4 ± 10.8	54.4 ± 10.0	0.95
Body mass index (kg/m^2)	22.8 ± 3.1	23.1 ± 3.4	22.1 ± 3.3	0.62
Fat mass (%)	29.2 ± 6.0	30.1 ± 7.3	28.4 ± 7.7	0.74
Muscle mass (kg)	36.7 ± 7.9	36.2 ± 6.0	36.5 ± 6.4	0.97
Physical performance				
Grip strength (dominant side, kg)	27.9 ± 9.0	25.9 ± 6.5	25.7 ± 6.3	0.54
Leg strength (dominant side, kg)	35.9 ± 12.2	36.7 ± 12.7	35.9 ± 10.5	0.97
10-m gait speed (m/s)	2.5 ± 0.5	2.5 ± 0.4	2.8 ± 0.4	0.054
SF-8 (points)				
PCS	49.9 ± 6.5	48.7 ± 5.8	48.8 ± 7.1	0.81
MCS	51.2 ± 6.1	50.9 ± 5.9	48.2 ± 7.3	0.26

Values are expressed as mean ± standard deviation. MA, maslinic acid; SF-8, Short-Form 8 Healthy Survey; PCS, physical component summary; MCS, mental component summary. *One-way analysis of variance for continuous variables among groups; Chi-square test for categorical variables.

2.3.4. Body composition and physical performance in Experiment 2

Table 2-3 shows the body composition and parameters of participants at baseline and week 12. The participants exhibited significantly decreased body weight, BMI ($p <$ 0.01 for the placebo, MA30, and MA60 groups), and percent body fat ($p < 0.01$ for the placebo, MA30, and MA60 groups) after the intervention. The effect of MA intake was not significantly different from that of placebo intake. The whole-body muscle mass was not significantly different between the groups. The rates of increase in muscle mass after intervention relative to before intervention were 0.4%, 0.9%, and 0% in the MA30, MA60, and placebo groups, respectively. The trunk muscle mass in the MA60 group was significantly higher than in the placebo group ($p < 0.05$). Furthermore, the effect of MA intake on limb muscle mass was not significantly different from that of placebo intake.

The grip strength (dominant arm) after intervention relative to before intervention in the MA intake groups was significantly higher than in the placebo group. Overall improvement in leg muscle strength and 10-m gait speed was observed post-intervention relative to pre-intervention. Additionally, leg muscle strength and 10-m gait speed in the MA intake groups were not significantly different from those in the placebo group.

2.3.5. QOL score in Experiment 2

The QOL scores for each domain in the placebo group exhibited an overall decreasing tendency, whereas those in the MA groups exhibited an increasing tendency (Table 2-4). The VT score in the MA60 group was significantly higher than in the placebo group ($p < 0.05$). Furthermore, the PCS score did not significantly change after MA intake.

However, the MCS scores post-treatment in the MA30 ($p < 0.05$) and MA60 ($p < 0.1$) groups were higher than those at baseline.

2.3.6. Adverse events

MA supplementation caused no serious side effects.

Table 2-3 Changes in the body composition and physical performance parameters relative to the baseline

	Placebo (n = 22)		MA30 (n = 22)		$P^{\#}$	MA60 (n = 21)		$P^{\#}$
	Baseline	Week 12	Baseline	Week 12		Baseline	Week 12	
Body composition								
Body mass (kg)	55.0 ± 11.2	54.1 ± 11.4**	55.4 ± 10.8	54.8 ± 10.6*	0.77	54.4 ± 10.0	53.5 ± 9.8**	0.59
Body mass index (kg/m^2)	22.8 ± 3.1	22.4 ± 3.2**	23.1 ± 3.4	22.9 ± 3.4*	0.82	22.1 ± 3.3	21.8 ± 3.1**	0.62
Fat mass (%)	29.2 ± 6.0	28.1 ± 6.3**	30.1 ± 7.3	29.1 ± 7.5**	0.57	28.4 ± 7.7	26.8 ± 7.8**	0.13
Muscle mass (kg)	36.7 ± 7.9	36.7 ± 8.1	36.2 ± 6.0	36.4 ± 6.1	0.27	36.5 ± 6.4	36.9 ± 7.0**	0.11
Segmental muscle mass								
Right arm (kg)	1.78 ± 0.59	1.77 ± 0.59	1.74 ± 0.36	1.75 ± 0.38	0.15	1.75 ± 0.40	1.74 ± 0.41	0.36
Left arm (kg)	1.66 ± 0.51	1.67 ± 0.53	1.66 ± 0.35	1.68 ± 0.39*	0.50	1.66 ± 0.37	1.66 ± 0.40	0.70
Trunk (kg)	21.06 ± 3.72	20.60 ± 3.69*	20.84 ± 2.96	20.58 ± 3.02**	0.20	20.93 ± 3.00	21.16 ± 3.75	0.01
Right leg (kg)	6.12 ± 1.68	6.33 ± 1.75**	6.01 ± 1.25	6.19 ± 1.30*	0.69	6.12 ± 1.44	6.16 ± 1.37	0.96
Left leg (kg)	6.05 ± 1.66	6.29 ± 1.74**	5.95 ± 1.28	6.15 ± 1.29**	0.68	6.07 ± 1.40	6.13 ± 1.37	0.95
Physical performance								
Grip strength (dominant side, kg)	27.9 ± 9.0	27.8 ± 8.5	25.9 ± 6.5	27.1 ± 6.0**	0.02	25.7 ± 6.3	26.7 ± 5.2†	0.04
Leg strength (dominant side, kg)	35.9 ± 12.2	41.5 ± 14.2**	36.7 ± 12.7	39.3 ± 10.7	0.91	35.9 ± 10.5	41.8 ± 14.7**	0.53
10-m gait speed (m/s)	2.5 ± 0.5	2.8 ± 0.4**	2.5 ± 0.4	2.8 ± 0.4**	0.89	2.8 ± 0.4	3.0 ± 0.5**	0.98

Values are expressed as mean ± standard deviation, †$p < 0.1$, *$p < 0.05$, **$p < 0.01$ compared with baseline (within-group; paired *t*-test).

#*p* value, compared with the placebo group (Williams test). MA, maslinic acid.

Table 2-4 Changes in the physical and mental quality of life parameters relative to the baseline

	Placebo (n = 22)		MA30 (n = 22)		$P^{\#}$	MA60 (n = 21)		$P^{\#}$
	Baseline	12 weeks	Baseline	12 weeks		Baseline	12 weeks	
SF-8 (points)								
PF	51.1 ± 4.4	48.0 ± 6.9*	50.0 ± 4.8	48.8 ± 7.0	0.21	48.4 ± 6.9	48.6 ± 6.8	0.12
RP	51.7 ± 4.9	48.9 ± 7.1	49.8 ± 5.7	50.1 ± 5.7	0.13	49.4 ± 6.8	48.7 ± 8.2	0.22
BP	50.6 ± 6.8	51.4 ± 7.3	50.7 ± 8.5	51.4 ± 8.6	0.51	52.4 ± 7.3	51.5 ± 7.5	0.69
GH	51.3 ± 4.1	51.2 ± 7.9	50.4 ± 5.9	51.8 ± 7.6	0.24	49.0 ± 5.9	50.1 ± 6.0	0.28
VT	51.3 ± 4.1	50.1 ± 7.0	52.2 ± 4.7	52.2 ± 6.0	0.11	49.3 ± 5.2	50.8 ± 4.9	0.04
SF	51.9 ± 6.4	51.1 ± 7.5	51.0 ± 6.6	53.1 ± 5.4	0.16	48.9 ± 8.6	51.3 ± 7.2	0.15
RE	51.1 ± 4.1	50.1 ± 6.1	50.2 ± 7.2	50.9 ± 4.8	0.17	49.1 ± 6.3	49.9 ± 5.9	0.22
MH	51.8 ± 5.5	51.6 ± 7.8	51.6 ± 6.0	53.6 ± 4.1†	0.17	48.9 ± 8.3	52.0 ± 6.9	0.13
PCS	49.9 ± 6.5	47.8 ± 7.8	48.7 ± 5.8	48.2 ± 8.4	0.28	48.8 ± 7.1	47.7 ± 7.2	0.38
MCS	51.2 ± 6.1	51.0 ± 8.3	50.9 ± 5.9	53.0 ± 4.7*	0.15	48.2 ± 7.3	51.2 ± 7.3†	0.09

Values are expressed as mean ± standard deviation, †$p < 0.1$, *$p < 0.05$, vs. baseline (within-group; paired *t*-test). #*p* value, vs. placebo (Williams test). MA, maslinic acid; SF-8, Short-Form 8 Healthy Survey; PF, physical functioning; RP, role physical; BP, bodily pain; GH, general health; VT, vitality; SF, social functioning; RE, role emotional; MH, mental health; PCS, physical component summary; MCS, mental component summary

2.4. Discussion

This study revealed a significant dose-response relationship between blood MA concentrations and intake quantity. The combination of 30 mg MA intake, which is lower than the previously reported dose, and daily exercise improved grip strength. To the best of our knowledge, the present study is the first to examine the effects of different amounts of MA intake on muscle mass and strength and estimate the minimum effective MA dose to increase muscle strength in the healthy elderly population. These results confirm our hypothesis.

However, this study did not investigate the effects of daily MA intake in the regular diet. MA is abundant in various natural products, including vegetables and fruits, used in the Mediterranean diet. For example, olives are a typical natural source of MA. However, 15–25 olives are estimated to be required to obtain 30–60 mg MA (Romero et al., 2010). Additionally, based on the dietary patterns of the Japanese population, MA intake from a regular diet is likely to be low. Therefore, pentacyclic triterpenes, including MA and its derivatives, have attracted attention as nutraceuticals (Sheng and Sun 2011). However, the efficacy of MA as a nutraceutical cannot be established without human bioavailability studies. The bioavailability of MA is higher than that of OA, a pentacyclic triterpene (de la Torre et al. 2020). Single-dose crossover studies have demonstrated that T_{max} is achieved at approximately 3 h post-test meal administration. A significant dose-response relationship between AUC and intake was observed when an MA intake of 30–120 mg was maintained (Fig. 2-1A, B; Table 2-1).

The efficacy evaluation test did not confirm the effect of MA intake on whole-body muscle mass. However, the combination of 60 mg MA intake and exercise significantly increased trunk muscle mass (Table 2-3). Previous studies have reported a

significant relationship between dynamic trunk balance and the risk of falls (Takahashi et al. 2020). Hence, the consumption of 60 mg MA and exercising daily may help prevent falls. Although the consumption of 30 mg MA did not significantly increase the muscle mass in this study, the grip strength increased significantly with MA intake (Table 2-3). These were the main findings of this study. Additionally, MA intake in combination with exercise dose-dependently increased muscle mass. However, the grip strength improved even at lower quantities of MA intake. Grip strength decreases with age, and elderly individuals with low grip strength are at high risk for needing long-term care (Marsh et al. 2011). Hence, the combination of exercise and 30 mg MA intake may decrease the need for caregiving among the elderly.

The mechanism of action of MA on muscle proteins has been partly elucidated (Bagchi, Sreejayan, and Sen 2018, Fernandez-Navarro et al. 2008, Murata et al. 2021, Shirai et al. 2021). Diets containing MA are reported to increase white muscle weight in rainbow trout. This effect can be attributed to the function of MA as a growth factor, which involves the binding of MA to specific receptors in the cytoplasm involved in protein synthesis, inducing the expression of protein synthesis-related genes (Bagchi, Sreejayan, and Sen 2018, Fernandez-Navarro et al. 2008). Recent studies have suggested MA supplementation exerts therapeutic effects on skeletal muscle hypertrophy through the activation of the mTOR signaling pathway, which plays a major role in the regulation of protein synthesis (Murata et al. 2021, Shirai et al. 2021).

MA intake did not significantly alter arm muscle mass but significantly increased grip strength (Tables 2-3). MA injection enhances nitric oxide production and exerts vasodilatory effects in rats (Hussain Shaik et al. 2012). Human muscle strength is enhanced by exercise and caffeine ingestion, which exerts vasodilatory effects (Goldstein

et al. 2010). Hence, the combination of MA intake and exercise can improve NO production, blood flow, and muscle strength. Additionally, MA administration increases brain-derived neurotrophic factor (BDNF) levels in the mouse brain (Bae et al. 2020). BDNF administration improves skeletal muscle mitochondrial function and exercise performance in mice (Matsumoto et al. 2018). MA induces protein synthesis, NO production, and BDNF production. A small dose (30 mg) of MA may improve muscle strength by promoting blood flow, while a high dose (60 mg) may induce protein synthesis. Grip strength is less responsive to training than lower leg muscle strength (Tieland et al. 2015). The correlation between muscle strength and age-related mortality can vary depending on the body part (e.g. upper or lower body). MA improved grip strength, a particularly unresponsive indicator (Garcia-Hermoso et al. 2018).

The overall leg muscle strength tended to increase post-intervention relative to pre-intervention although it was not significantly different between the treatment groups. This can be attributed to the increased effectiveness of training and the overall increase in muscle mass of both legs. Future studies must examine the appropriate type and intensity of exercise to determine the effectiveness of MA. One diagnostic criterion for sarcopenia in the Asian population is a walking speed of 0.8 m/s or less in the elderly (Panita, Praew, and Chatlert 2015). Here, healthy adults were examined. In each treatment group, the mean walking speed post-intervention was significantly higher than that pre-intervention (2.5–2.8 m/s) (Table 2-2). The overall increase in walking speed can be attributed to daily walking and lower body training. Similar to the results of leg strength analysis, the effect of an exercise intervention on gait function was superior to the effect of MA intake.

Previously, we had reported that 30 mg MA intake improves the QOL in the

elderly (Fukumitsu et al. 2017). However, according to SF-8, only VT scores in the MA60 group were significantly higher than those in the placebo group (Table 2-4). Differences in participant attributes can explain this finding. The previous study included elderly participants with mild joint discomfort and SF-8 scores well below the national standard (50 points). However, this study included healthy participants with baseline SF-8 scores close to the national standard.

This study has several limitations. First, the improvement in motor function with MA intake was limited to grip strength, indicating the lack of overall effect. Future studies should determine the optimal MA intake for improving physical performance, including confirmation of the appropriate type, intensity, and exercise frequency. Additionally, body composition was determined by bioelectrical impedance analysis (BIA) rather than dual-energy X-ray absorptiometry (DXA), which is widely used in various research fields as the most reliable body composition measurement method. Furthermore, the portable scale MC-780A (Tanita Corporation, Japan) equipped with BIA technology was used to measure body composition (Verney et al. 2015), the accuracy of which has been validated with DXA. This study did not confirm the findings with histological analyses, such as skeletal muscle biopsy, to elucidate the mechanism of action of MA in the muscle. Additionally, the biomarkers, such as TNF-α, related to anti-inflammatory marker or IGF1 were not assessed in the efficacy evaluation test. These objective measures will enable the monitoring of increased muscle strength even when muscle mass remains unchanged. The findings of this study must be verified using an alternate study design to generalize the effects of MA intake on individuals with difficulties in exercising, such as those undergoing medical therapy or rehabilitation.

The highlight of this study was measuring the blood MA concentrations to

confirm MA pharmacokinetics. The significant increase in grip strength even at low doses (30 mg) of MA intake is a novel finding and demonstrates the versatility of MA.

In conclusion, this study demonstrated that daily exercise in combination with an intake of 30 mg MA, a dose lower than that used in previous studies, can improve grip strength. Consistent with the study hypothesis, a positive correlation between MA dose-dependent physical performance improvement and blood MA absorption was observed. These results suggest that the combination of MA intake (30 mg/day) and exercise can prevent sarcopenia by maintaining a good QOL in healthy adults.

CHAPTER 3: Effect of maslinic acid on skeletal muscle mass and strength in denervation-treated mice

3.1 Introduction

Skeletal muscle accounts for approximately 40% of body weight and 50% of protein and is the most abundant tissue in the human body. It is a critical tissue for maintaining an ideal QOL and achieving enhanced athletic performance (Tsekoura et al. 2017). The high plasticity of skeletal muscles enables their adaptation to changing environmental and physiological conditions (Stewart and Rittweger 2006). Muscle atrophy can be induced by sarcopenia, inactivity, and various neuromuscular diseases. Sarcopenia is a condition characterized by the gradual loss of skeletal muscle mass with age, resulting in a decline in muscle mass, strength, and overall muscle quality (Evans 1995). In the elderly, sarcopenia is a major health problem and increases the risk of disability, falling, falls-related injuries, hospitalization, dependency, and death (Landi et al. 2012).

Muscle atrophy is the loss of muscle mass due to the imbalance between protein synthesis and degradation (Leger et al. 2006). Skeletal muscle component proteins are constantly being synthesized and degraded. IGF1 enhances protein synthesis in skeletal muscles and prevents muscle hypertrophy by regulating PI3K/AKT/mTOR signaling. Additionally, IGF1 prevents skeletal muscle atrophy by suppressing the phosphorylation of forkhead box O (FOXO), which is a downstream mediator of AKT. Thus, two distinct AKT signaling pathways initiated by IGF1 maintain the dynamic balance between muscle protein degradation and synthesis (Glass 2005, Ruegg and Glass 2011).

The cellular mechanisms mediating muscle atrophy are complex. Conditions of low mechanical stress that cause skeletal muscle atrophy include bed-ridden, immobile,

and disuse states. Low mechanical stress causes skeletal muscle atrophy by primarily activating the ubiquitin-proteasome pathway (Jagoe and Goldberg 2001). Activation of the NF-κB pathway and inflammatory cytokines also induces muscle atrophy. NF-κB induces protein degradation by promoting the expression levels of MuRF1 and Atrogin-1, which are muscle-specific E3 ubiquitin ligases (Li et al. 1998). *Atrogin-1* and *Murf1* are upregulated in atrophied muscles and accelerate the ubiquitin-proteasome pathway in disused atrophied muscles. Mice in which *Atrogin-1* and *Murf1* are knocked out are resistant to denervation-induced muscle atrophy (Bodine et al. 2001).

Denervation is implicated in the development of skeletal muscle atrophy. Skeletal muscle atrophy caused by denervation is associated with serious health consequences. Although denervation-induced muscle atrophy is widespread and severe, the underlying molecular pathogenesis has not been completely elucidated, hindering the development of pharmacological therapies. Several interrelated pathways, including the ubiquitin-proteasome system, autophagy-lysosome system, protein synthesis pathway, and myofiber regeneration pathway, are involved in the development of skeletal muscle atrophy resulting from peripheral nerve injury. Denervation is widely used as a model for muscle atrophy.

MA exerts potent anti-inflammatory effects and inhibits the activity of inflammatory cytokines. Additionally, MA enhances the formation of synovial membrane tissue and the repair of cartilage damage caused by arthritis via the NF-κB signaling pathway (Fukumitsu, Villareal, Fujitsuka, et al. 2016, Shimazu et al. 2019). A recent clinical study demonstrated that 50 mg MA supplementation increased knee muscle strength and alleviated knee joint inflammation in elderly women suffering from osteoarthritis of knee who participated in a whole-body vibration resistance training

(Yoon et al. 2018). Furthermore, another clinical trial demonstrated that the combination of 60 mg MA supplementation and resistance exercise effectively improved skeletal muscle mass in the elderly (Nagai et al. 2019). Recent studies have suggested MA supplementation is a novel therapeutic strategy for skeletal muscle hypertrophy and that MA exerts its effect through activation of mTOR signaling pathway, which plays a major role in the regulation of protein synthesis (Murata et al. 2021, Shirai et al. 2021). However, the overall molecular mechanisms through which MA has a protective effect on muscle atrophy have not been elucidated. The objective of this chapter was to assess the effects of MA on the loss of muscle mass and strength due to denervation in a mouse model.

3.2 Materials and methods

3.2.1. Preparation of the MA purified fraction in olive pomace

The MA fraction was extracted from olive pomace for 3 h using 90% (*v/v*) ethanol at 85 °C. The aqueous ethanol extract was vaporized and resolved in chloroform. The dissolved extract was held in chloroform on a silica column (Chromatorex FL100D; Fuji Silysia Chemical Ltd., Aichi, Japan). Subsequently, it was eluted with a solution of chloroform and methanol (49:1, v/v) to obtain the purified fraction with 97.9% MA purity. No peaks of other triterpenes, such as oleanolic acid and ursolic acid were detected in the purified fraction. The MA contents were analyzed using a Shimadzu Nexera UHPLC system (Shimadzu Co., Kyoto, Japan) under the following conditions: column, Infinitylab poroshell 120 column (4.6 × 100 mm, 2.7 μm; Agilent Technologies Japan Ltd., Tokyo, Japan); temperature, 30 °C; mobile phase, acetonitrile/methanol/water/phosphoric acid (500:400:100:0.5, *v/v/v/v*); flow rate, 1 mL/min. MA was measured at 210 nm using a UV detector (Shimadzu). The standard MA reagent was obtained from Funakoshi Co., Ltd.

(Tokyo, Japan).

For the *in vivo* experiment, a diet containing 0.27% MA was prepared. MA intake concentrations were decided based on a previous report (Kunkel et al. 2011).

3.2.2. Animal experimental procedures

The Ethical Committee of NIPPN Corporation approved all animal experimental procedures (permission number: 2020-6). Male Slc: 7-week-old ICR mice (n = 30) were purchased from Japan SLC Inc. (Shizuoka, Japan) and allowed to acclimatize under the following conventional conditions: room temperature, 24°C ± 1°C; humidity, 50 ± 10%; circadian cycle, 12-h light-dark cycle. Mice were fed on an AIN-93G diet (Funabashi Farm Ltd., Chiba, Japan) and allowed free access to food and water. After one week of acclimatization, the mice were randomly divided into a control group fed a standard diet and an MA group fed a diet containing 0.27% MA (n = 15; 0, 7, 14 days, n = 3, 6, 6, respectively). One week later, the sciatic nerves of both legs were transected and a 5 mm section was excised under anesthesia. During injury-induced muscle atrophy, mice were continued to be fed on control or MA diet until the endpoint. Overall grip strength was measured using a grip strength meter (GPM-100; Melquest, Toyama, Japan). Mice were put on a metal mesh to tightly grip the metal mesh using their whole four limbs. Then, the tail of each mouse was then pulled to the side of the measurer with the same strength. The average of the three central scores from the five tests conducted was calculated. The mice were anesthetized on days 0, 7, and 14 post-surgery. Muscle tissue from the gastrocnemius, plantaris, and soleus muscles were collected, weighed, rapidly flash-frozen in liquid nitrogen, and reserved at −80 °C until analysis.

3.2.3. Total RNA extraction from gastrocnemius muscle

Total RNA was isolated from gastrocnemius muscle using Isogen reagent (Nippon Gene Co., Ltd., Tokyo, Japan). Purification of RNA samples was performed using Qiagen's RNeasy Mini kit (Qiagen K.K., Tokyo, Japan). RNA quality and quantity were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Tokyo, Japan).

3.2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA (1.0 μg) was reverse-transcribed into complementary DNA (cDNA) using the PrimeScript RT Reagent kit (RR037A, TaKaRa Bio Inc., Shiga, Japan). cDNA was amplified by SYBR Premix EX Taq (RR041A, TaKaRa Bio) on a Thermal Cycler Dice TP950 (TaKaRa Bio). The thermal cycling conditions were as follows: 95 °C, 30 s, followed by 40 cycles of 95 °C, 5 s, 60 °C, 30 s. The expression levels of target genes were normalized to those of *Tbp*. The following primers were used for qRT-PCR analysis: *Igf1*, 5′-TGCTCTTCAGTTCGTGTG-3′ (forward) and 5′-ACATCTCCAGTCTCCTCAG-3′ (reverse); *Atrogin-1*, 5′-ACTTCTCGACTGCCATCCTG-3′ (forward) and TTCTTTTGGGCGATGCCACT (reverse); *Murf1*, 5′-GGGCCATTGACTTTGGGACA-3′ (forward) and 5′-TGGTGTTCTTCTTTACCCTCTGTG-3′ (reverse).

3.2.5. Protein extraction from gastrocnemius muscles

Total proteins were extracted from tissue samples using radio immunoprecipitation assay buffer containing a protease inhibitor cocktail (Sigma, Tokyo, Japan). Centrifugation of the homogenate was performed at 10,000 *g* and 4 °C for 10 min.

The concentration of protein in the supernatant was determined using the protein assay rapid kit (FUJIFILM, Tokyo, Japan).

3.2.6. Enzyme-linked immunosorbent assay (ELISA)

The IGF1, Atrogin-1, and MuRF1 levels in the gastrocnemius muscle were quantified using the mouse/rat IGF-I Quantikine (R&D system, Minneapolis, MN, USA), mouse F-box only protein 32 (FBXO32) (CUSABIO, Wuhan, China), and mouse MuRF1 (muscle-specific RING-finger protein 1) ELISA kits, respectively, following the manufacturer's instructions.

3.2.7. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). Data were compared using analysis of variance and Welch's *t*-test. SPSS Statistics ver. 25 for Windows (IBM Inc., Tokyo, Japan) was used for statistical analyses. Differences were considered significant at $p < 0.05$.

3.3. Results

3.3.1. Effects of MA on denervation-induced skeletal muscle atrophy

At week 1 post-MA diet ingestion, denervation induced muscle atrophy. The average daily intake of food (Fig. 3-1A) and body weight (BW) (Fig 3-1B) were not significantly different between the two groups. The grip strength was not significantly difference between the groups at the start of feeding. In contrast, grip strength after denervation among the MA group was significantly higher than that in the control group on days 7 (3.68 ± 0.17 and 5.29 ± 0.57 mg/g bodyweight in the control and MA groups,

respectively, $p < 0.05$) and 14 (3.82 ± 0.18 and 4.54 ± 0.23 mg/g bodyweight in the control and MA groups, respectively, $p < 0.05$) (Fig. 3-1C). Fourteen days post-denervation, the MA group gastrocnemius wet weight (2.29 ± 0.11 mg/g bodyweight) was significantly higher than in the control group (1.95 ± 0.09 mg/g bodyweight) on day 14 post-denervation (Fig. 3-1D, $p < 0.05$). MA intake mitigated the denervation-induced decrease in plantaris muscle weight on day 7 post-denervation (Fig. 3-1E, 0.36 ± 0.02 and 0.43 ± 0.01 mg/g bodyweight in the control and MA groups, respectively, $p < 0.05$). There was no significant difference in the soleus muscle wet weight on days 7 and 14 compared to the control group (Fig. 3-1F). These results demonstrate that MA intake attenuated the denervation-induced decrease in skeletal muscle and improved skeletal muscle strength.

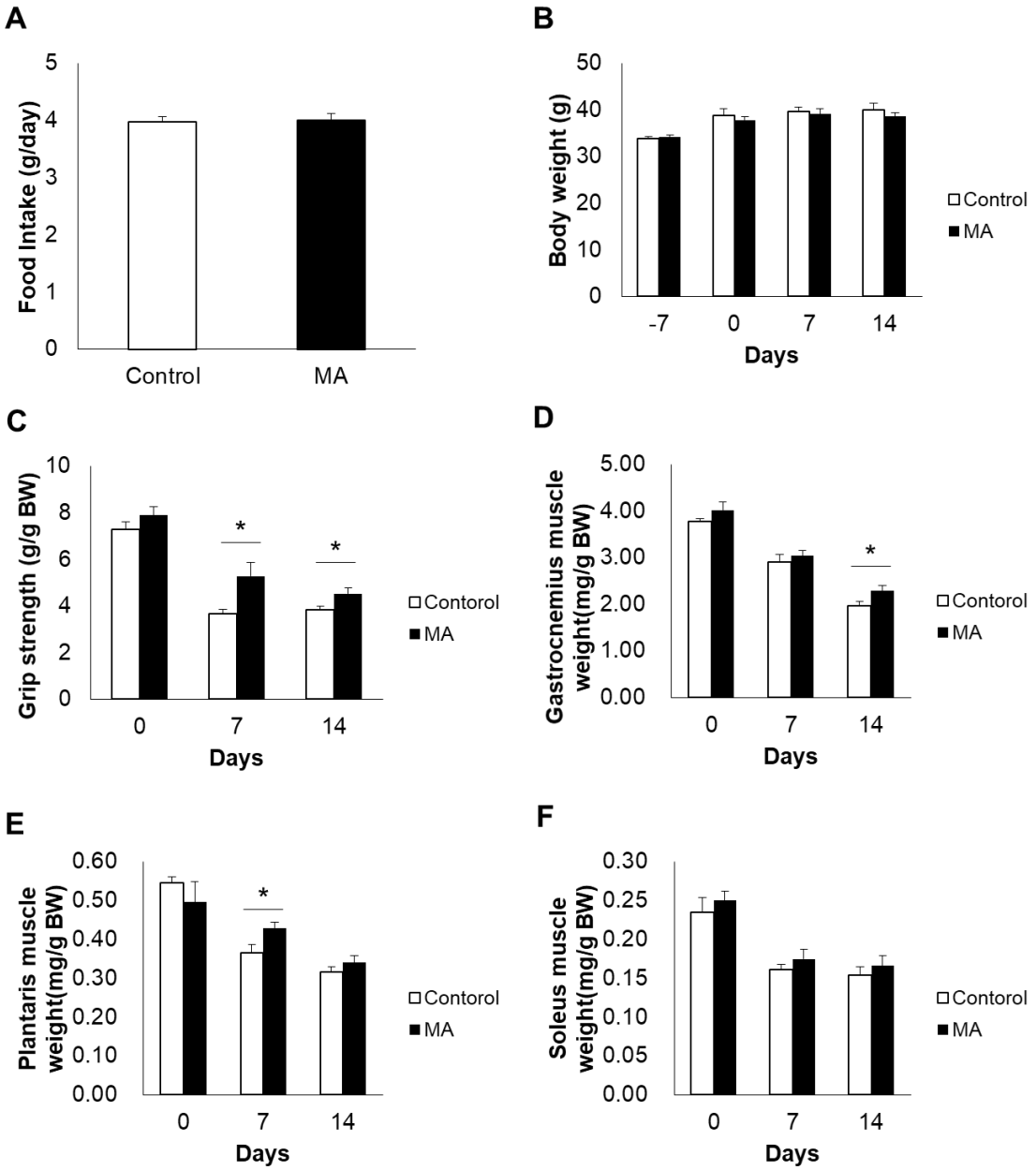


Figure 3-1. MA mitigated denervation-induced skeletal muscle atrophy.

(A) Food intake, (B) body weight (BW), (C) grip strength, (D) gastrocnemius muscle weight (E) p lantaris muscle weight, and (F) soleus muscle weight. Values are represented as mean ± SEM (n = 3–15), *$p < 0.05$ vs. control.

3.3.2. Effect of MA on atrophy-related gene and protein expression

To determine the mechanism underlying the preventive effects of MA on the loss of muscle strength and mass, the expression levels of atrophy-related genes were quantified. MA upregulated *Igf1* expression on day 14 post-denervation ($p < 0.05$) (Fig. 3-2A). MuRF1 and Atrogin-1 are specific E3 ligases involved in protein degradation. Denervation markedly increased the mRNA expression levels of *Murf1 and Atrogin-1*. However, MA suppressed the denervation-induced upregulation of *Murf1* ($p < 0.05$ on day 7 post-denervation) and *Atrogin-1* ($p < 0.05$ on days 7 and 14 post-denervation). The Igf1 levels in the MA group were significantly higher than in the control group ($p < 0.05$ on day 14 post-denervation). Meanwhile, MA suppressed the denervation-induced upregulation of catabolic factors, such as MuRF1 ($p < 0.05$ on day 14 post-denervation) and Atrogin-1 ($p < 0.01$ on day 14 post-denervation). Thus, MA suppressed the denervation-induced upregulation of mRNA and protein levels of MuRF1 and Atrogin-1, which are involved in muscle protein degradation, and the downregulation of Igf1 associated with muscle protein synthesis.

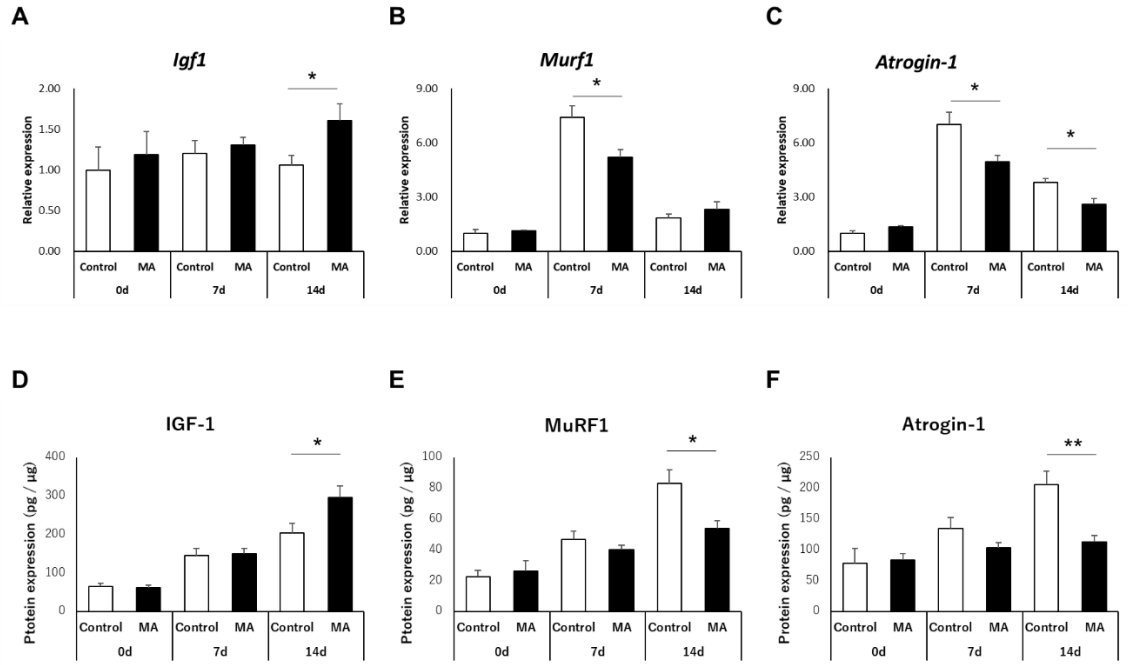


Figure 3-2. Changes in muscle protein degradation-related factors.

qRT-PCR of relative mRNA expression levels of *Igf1* (A), *Murf1* (B), and *Atrogin-1* (C). Enzyme-linked immunosorbent assay analysis of protein expression levels of Igf1 (D), MuRF1 (E), and Atrogin-1 (F). Data are expressed as mean ± SEM (n = 3–6), *$p < 0.05$, **$p < 0.01$ vs control.

3.4. Discussion

The results of this study revealed that daily intake of MA-supplemented diet mitigated decline in skeletal muscle mass and muscle strength in mice caused by denervation and had an effect on muscle atrophy.

The sciatic nerve transection rodent model is a widely employed and well-studied tool to induce denervation (Sarukhanov et al. 2014). In this study, the dietary supplementation of MA successfully attenuated muscle strength and weight decline on day 14 post-sciatic nerve denervation (Fig. 3-1C-E). Previous studies have reported that MA supplementation in combination with resistance exercise maintained skeletal muscle mass in the elderly (Nagai et al. 2019), which is consistent with *in vivo* findings of this study. Different muscle types exhibit varying properties. Muscle fibers are classified as fast and slow muscle fibers. Plataris and gastrocnemius muscles comprise fast muscle fibers that provide rapid strength, whereas the soleus muscles comprise slow muscle fibers that provide stamina. MA intake may attenuate denervation-induced grip strength loss by maintaining fast muscle mass.

Protein metabolism serves an essential role in the regulation of skeletal muscle mass. The amount of skeletal muscle mass is upregulated when protein synthesis outweighs protein breakdown. Conversely, skeletal muscle mass is downregulated when protein degradation exceeds protein synthesis. A recent study suggested MA supplementation exerts therapeutic effects on skeletal muscle hypertrophy by activating mTOR signaling pathway, which takes an essential role in regulating protein synthesis (Murata et al. 2021, Shirai et al. 2021). Moreover, ursolic acid, another member of pentacyclic triterpenoids, is reported to enhance muscle hypertrophy (Kunkel et al. 2011).

qRT-PCR and ELISA analyses revealed that MA significantly upregulated the expression of IGF1, a critical anabolic growth factor involved in modulating muscle hypertrophy by enhancing PI3K/AKT signaling, in gastrocnemius muscle on day 14 after denervation (Timmer, Hoogaars, and Jaspers 2018) (Fig. 3-2A, D). Moreover, IGF1 is reported to suppress protein degradation and the mRNA expression levels of *Murf1* and *Atrogin-1* (Frost et al. 2009, Sacheck et al. 2004). In this study, MA downregulated MuRF1 and Atrogin-1 mRNA and protein levels in denervation-treated and might consequently suppress muscle atrophy (Fig. 3-2B, C, D, E).

The gastrocnemius muscle mass was not significantly different between groups on day 7. However, the grip strength of the MA group mice was significantly greater than in the control group on day 7 after denervation treatment, (Fig. 3-1C, D). The capillary network is altered in disuse-induced atrophic muscle (Fujino et al. 2005). Additionally, the combination of caffeine intake and training, which exerts vasodilatory effects, can improve muscle strength without increasing muscle weight (Goldstein et al. 2010). MA intake has been reported to exert a vasodilatory effect and enhance nitric oxide production in rats (Hussain Shaik et al. 2012). Although the effects of MA on vascular function have not been evaluated in this study, dietary MA supplementation may improve blood flow and enhance muscle strength irrespective of muscle mass weight (Fig. 3-1C, D).

Previous studies have demonstrated that the aging process of neuromuscular or motor function is similar in humans and mice (Barreto, Huang, and Giffard 2010, Graber et al. 2013, Parks et al. 2012). Sarcopenia is studied in mice because owing to their short lifespan and relative ease of genetic manipulation (Yuan, Peters, and Paigen 2011, Yuan et al. 2009). Muscle atrophy is of the following three types: diffuse deconditioning like denervation, natural aging, or microgravity, chronic diseases, and immobilization (Cohen,

Nathan, and Goldberg 2015, Dutt et al. 2015, Pokorski 2016).

In summary, this chapter demonstrated that dietary intake of MA suppresses injury-induced muscle atrophy in denervation-treated mice. This is the initial study to report that MA may regulate muscle protein degradation and synthesis by suppressing atrophy-related genes, such as MuRF1 and Atrogin-1 and boosting IGF1 production. However, the detailed mechanisms of the beneficial effects of MA are unknown. The regulatory pathways of the factors whose expression varies upon treatment with MA must be investigated in the future.

CHAPTER 4: Analysis of the mechanism of muscle atrophy prevention by maslinic acid

4.1. Introduction

The cellular mechanisms involved in muscle atrophy are complex. Denervation promotes inflammation, glucocorticoid effect, myostatin activation, and PI3K/AKT inhibition (Duan, Gao, and Zhu 2021). Lipopolysaccharide (LPS)-activated TLR4 induces C2C12 myotube atrophy by upregulating the expression of the ubiquitin ligase Atrogin-1 (Doyle et al. 2011). Maslinic acid (MA) exerts a preventive effect on arthritis by promoting tissue formation and suppressing inflammation within glucocorticoid receptors through the inactivation of TLR signaling and downregulation of leukotriene in the synovium (Shimazu et al. 2019). The health benefits of dietary flavonoids, which are ubiquitously found in plant foods, have piqued the interest of the scientific community (Terao 2009). The two primary flavones apigenin and luteolin exert potent inhibitory effects on Atrogin-1 expression (Shiota et al. 2015). Additionally, apigenin and luteolin suppress LPS-induced Atrogin-1 expression in C2C12 myotubes by inhibiting the JNK signaling pathway. Activation of the NF-κB pathway and inflammatory cytokines mediate muscle atrophy. Subsequently, NF-κB promotes the proteolysis of the muscle-specific E3 ubiquitin ligases MuRF1 and Atrogin-1 by upregulating their expression. MA exerts anti-inflammatory effects through the inactivation of NF-κB in RAW 264.7 cells by suppressing the phosphorylation of IκB-α (Fukumitsu, Villareal, Fujitsuka, et al. 2016). Based on these findings, this study hypothesized that MA can prevent or mitigate inflammatory-induced muscle atrophy by inhibiting the expression of MuRF1 and Atrogin-1.

In skeletal muscles, glucocorticoid decreases the rate of muscle protein synthesis

by increasing the rate of protein breakdown (Lofberg et al. 2002, Schakman et al. 2013). These effects are expected to contribute to atrophy. Glucocorticoids bind to glucocorticoid receptors, which are reported to act by interfering with the insulin/IGF1 signaling pathway or stimulating the transcription of genes, including transcriptional factors, especially FOXO, involved in muscle atrophy. Dexamethasone (DEX) is widely used to promote glucocorticoid-induced muscle atrophy. DEX-induced muscle atrophy is related with upregulated ubiquitin-proteasome pathway-related factors, such as MuRF1 and Atrogin-1 (Son et al. 2017, Wang et al. 2017). Resveratrol is a natural polyphenol resent in grape skins and red wine (Burns et al. 2002). Previous studies have reported that resveratrol significantly suppresses the DEX-induced upregulation of MuRF1 in C2C12 myotubes *in vitro*. However, NF-κB knockdown mitigated the resveratrol-induced suppression of DEX-induced MuRF1 upregulation, suggesting that the beneficial effects of resveratrol are modulated by NF-κB (Sun et al. 2017).

As shown in chapter 3, the intake of MA significantly attenuated the denervation-induced skeletal muscle mass loss and muscle weakness. Nevertheless, the overall molecular mechanism of the protective effect of MA on muscle atrophy has yet to be elucidated. This chapter aims to reveal the molecular mechanism of preventive effects of MA on muscle atrophy using whole-genome microarray analysis in mice and DEX-stimulated mouse C2C12 myotubes. The inhibitory effects of MA on the expression of LPS-induced atrophic factors were investigated to examine the mechanism underlying the prevention of inflammation-induced muscle atrophy.

4.2. Materials and methods

4.2.1. DNA Microarray

Whole-genome microarray analysis was performed using Affymetrix's GeneAtlas® System, following the manufacturer's instructions (Affymetrix Inc., Santa Clara, CA, USA). Total RNA (100 ng) was isolated from the gastrocnemius muscles of both MA-treated and control mice. The quantity and quality of RNA were measured by the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). GeneChip 3neChip (Affymetrix Inc., Santa Clara, CA, USA) was used to amplify poly(A) RNA in total RNA samples by reverse transcription priming and to prepare biotin-labeled cRNA samples (complementary RNAs) prepared from the poly(A) RNA in the total RNA sample by reverse transcription priming. Fragmented and labeled cRNA samples were then prepared for hybridization by GeneAtlas® hybridization, wash, and stain Kit for 3' IVT Array Strips (901531). All samples were loaded onto Affymetrix MG-430 PM Array Strips (901570). The samples were loaded onto hybridization array strips and hybridized on the GeneAtlas® Hybridization Station at 45°C for 16 h. Lastly, hybridization arrays were washed, stained, and scanned using the GeneAtlas® Fluidics Station and the GeneAtlas® Imaging Station.

Affymetrix Expression Console™ software was used for raw data processing. Robust Multi-array Analysis algorithms were employed for gene-level normalization and signal summarization (http://www.affymetrix.com). Transcriptome Analysis Console software version 4 (Thermo Fisher Scientific Inc.) was employed for subsequent differential expression analysis. Data were collected from the differentially expressed genes (DEGs), identified as genes with a fold change higher than 1.2 and *p*-values less than 0.05 (one-way analysis of variance (ANOVA); between subjects) in linear space.

The Hallmark gene set was determined using the Gene Set Enrichment Analysis (GSEA) web tool (https://software.broadinstitute.org/gsea/index.jsp)' Molecular Signature Database (MSigDB) (Liberzon 2014, Subramanian et al. 2005). Database for Annotation, Visualization and Integrated Discovery (DAVID) ver. 6.8, an online data mining tool, was used to perform gene ontology (GO) analysis to determine the significant biological processes in which the DEGs were enriched (Huang da, Sherman, and Lempicki 2009). Lastly, heat maps were generated using the online data visualization software Heatmapper (http://www.heatmapper.ca/expression/). The microarray data, including the CHP and CEL files, have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus database (accession number: GSE181031) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE181031).

4.2.2. Cells and cell culture

C2C12 myoblast cells (ATCC CRL 1772) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, Tokyo, Japan) containing 10% fetal bovine serum with 1% penicillin and streptomycin. When the cultures reached a confluency of 70%–80%, the culture medium was replaced with DMEM containing 2% horse serum. The medium was changed once every 2 days until the differentiation of C2C12 myotubes for 4–5 days. The cells were grown at 37°C in a moistened atmosphere with 5% CO_2 and 95% air.

4.2.3. Cell viability assay

Cells were harvested in 96-well culture plates at a density of 5×10^3 cells/mL and differentiated into myotubes. C2C12 myotubes were treated with MA (1, 5, 10, 25, or 50 μM). Cell viability was determined using the Cell WST-8 reagent (Dojindo Laboratories,

Kumamoto, Japan), with the manufacturer's instructions. In brief, each cell was placed in an incubator with CCK-8 solution for 1 hour, and the absorbance at 450 nm was measured.

4.2.4. LPS-induced muscle atrophy

On days 4–5, the myotubes were pretreated with dimethyl sulfoxide (FUJIFILM, Tokyo, Japan), MA (1, 5, or 10 μM), or Apigenin (positive control, FUJIFILM, Tokyo, Japan) for 1 h, and the cells were collected after 2 h of 0.1 μg/mL LPS stimulation. The samples were harvested to extract protein and mRNA. The protein and mRNA samples were reserved at −80°C for subsequent analysis.

4.2.5. Measurement of myotube diameter

Differentiated myotubes were co-cultured with dimethyl sulfoxide, MA (1, 5, or 10 μM), or resveratrol (positive control, FUJIFILM, Tokyo, Japan) in the absence or presence of 10 μM DEX(FUJIFILM, Tokyo, Japan) for 24h. C2C12 myotubes were visualized under a microscope (BZ X700, Keyence, Osaka, Japan), and images were captured. The diameters of myotubes were determined semi quantitatively in 20 random fields in each well of a 6-well plate.

4.2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from C2C12 myotubes using Isogen reagent (Nippon Gene Co., Ltd., Tokyo, Japan). Purification of RNA samples was performed using Qiagen's RNeasy Mini kit (Qiagen K.K., Tokyo, Japan). RNA quality and quantity were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Tokyo, Japan). Purified total RNA samples (1.0 μg) were reverse-transcribed into complementary

DNA (cDNA) by the PrimeScript RT reagent kit (RR037A, TaKaRa Bio Inc., Shiga, Japan). cDNA was amplified using SYBR Premix EX Taq (RR041A, TaKaRa Bio) with a Thermal Cycler Dice TP950 (TaKaRa Bio). Conditions for thermal cycling were as follows: 95 °C, 30 s, followed by 40 cycles of 95 °C, 5 s, 60 °C, 30 s. The expression levels of target genes were normalized to those of *Tbp*. The following primers were used for real-time PCR analysis: *Igf1*, 5′-TGCTCTTCAGTTCGTGTG (forward) and 5′-ACATCTCCAGTCTCCTCAG-3′ (reverse); *Atrogin-1*, 5′-ACTTCTCGACTGCCATCCTG-3′ (forward) and TTCTTTTGGGCGATGCCACT (reverse); *Murf1*, 5′-GGGCCATTGACTTTGGGACA-3′ (forward) and 5′-TGGTGTTCTTCTTTACCCTCTGTG-3′ (reverse); *Tnfa*, 5′-TTCCAGATTCTTCCCTGAGGT-3′ (forward) and 5′-TAAGCAAAAGAGGAGGCAACA-3′ (reverse).

4.2.7. Statistical analysis

The data are presented in terms of mean ± standard error of mean. Means between two groups were compared using Student's t-test, whereas those between multiple groups were compared using ANOVA, followed by Tukey's post-hoc test. SPSS Statistics ver. 25 for Windows (IBM Inc., Tokyo, Japan) or GraphPad Prism 8 (GraphPad, Inc., San Diego, CA, USA) was used for statistical analysis. Differences were statistically significant at $p < 0.05$.

4.3. Results

4.3.1. Effect of MA on gene expression in gastrocnemius muscle atrophy due to

denervation

Microarray analysis was performed to examine the effects of MA intake on the gene expression profile of gastrocnemius muscle on day 14 after denervation. In total, 45,078 probe sets were identified. In the MA group, 3172 unique DEGs were identified (fold change > ± 1.2 and *p*-value < 0.05 vs. control). The DEG significance and magnitude of fold change are shown in the volcano plot (Fig. 4-1A). Red and green dots indicate upregulated and downregulated DEGs, respectively. In total, 1491 DEGs were upregulated in the MA group compared to the control group, whereas 1681 DEGs were downregulated (Fig. 4-1B). The heat map displays rows of z-scores of DEGs between the MA and control groups on day 14 post-denervation and before denervation (Fig. 4-1C). MA modulated denervation-induced gene expression changes.

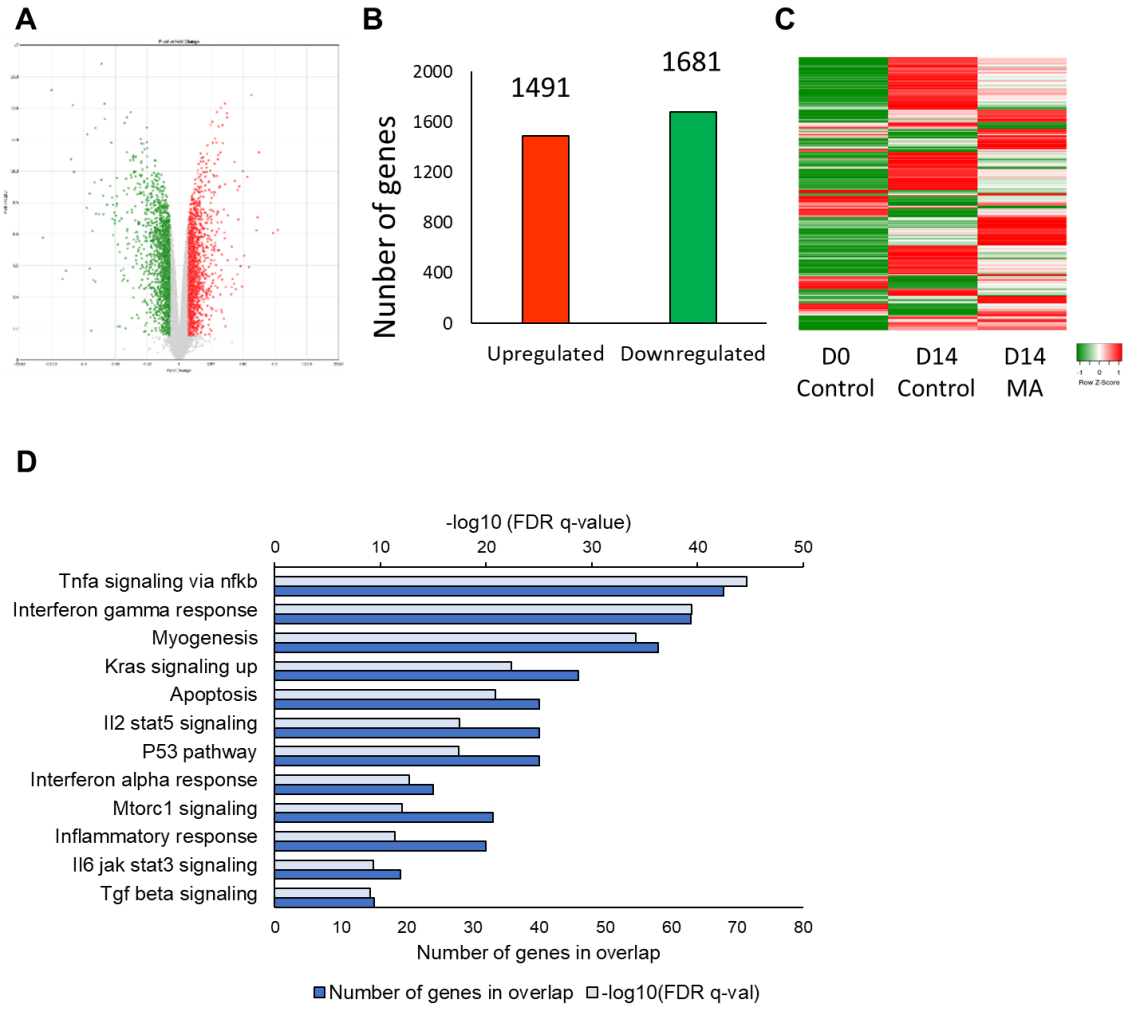
4.3.2. Effects of MA on biological functions in mice with denervation induced skeletal muscle atrophy

In Fig. 4-1D, we can see that the MA group was significantly enriched in the hallmark gene set compared with the control group. The term 'hallmark gene set' is defined as 'a set of genes that represent a summary of a specific, well-defined biological state or process and that exhibit consistent expression' (MSigDB of GSEA). Hallmark gene sets are preferred due to the relatively low noise and redundancy they are considered to have. The Hallmark gene sets, such as muscle differentiation-related mTORC1 signaling, myogenesis, and KRAS signaling were significantly enriched in the MA group relative to the control group. Meanwhile, muscle degradation-associated TNFα signaling via NF-κB, inflammatory response, IL6/JAK/STAT3, IL2/STAT5 signaling pathways, TGF-β signaling, immune responses such as interferon α response and interferon γ

response, as well as P53 pathway and apoptosis were significantly enriched in the MA group relative to the control group.

4.3.3. Effects of MA on biological processes and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway in denervated mice

GO analysis revealed that several biological processes associated to muscle organization, tissue formation, and repair were significantly upregulated in the MA group (from DAVID). The overlapped genes were strongly related with skeletal muscle tissue development (GO:0007519), sarcomere organization (GO:0045214), collagen fibril organization (GO:0030199), heart development (GO:0007507), regulation of release of sequestered calcium ion into cytosol by sarcoplasmic reticulum (GO:0010880), actin cytoskeleton organization (GO:0030036), cardiac muscle contraction (GO:0060048), cardiac muscle cell development (GO:0055013), microtubule-based process (GO:0007017), and wound healing (GO:0042060) (Fig. 4-1E). In contrast, biological processes involved in immune and inflammatory response were significantly downregulated in the MA group. The overlapped genes were strongly related with immune system process (GO:0002376), positive regulation of transcription from RNA polymerase II promoter (GO:0045944), inflammatory response (GO:0006954), positive regulation of transcription, DNA-templated (GO:0045893), response to bacterium (GO:0009617), negative regulation of transcription from RNA polymerase II promoter (GO:0000122), cellular response to interferon-gamma (GO:0071346), innate immune response (GO:0045087), positive regulation of gene expression (GO:0010628), negative regulation of cell proliferation (GO:0008285) (Fig. 4-1F).


A
B
C
2000
1600
1200
800
400
0
Number of genes
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1681
Upregulated
Downregulated
D0
Control
D14
Control
D14
MA
Row Z-Score
D
-log10 (FDR q-value)
0
10
20
30
40
50
Tnfa signaling via nfkb
Interferon gamma response
Myogenesis
Kras signaling up
Apoptosis
Il2 stat5 signaling
P53 pathway
Interferon alpha response
Mtorc1 signaling
Inflammatory response
Il6 jak stat3 signaling
Tgf beta signaling
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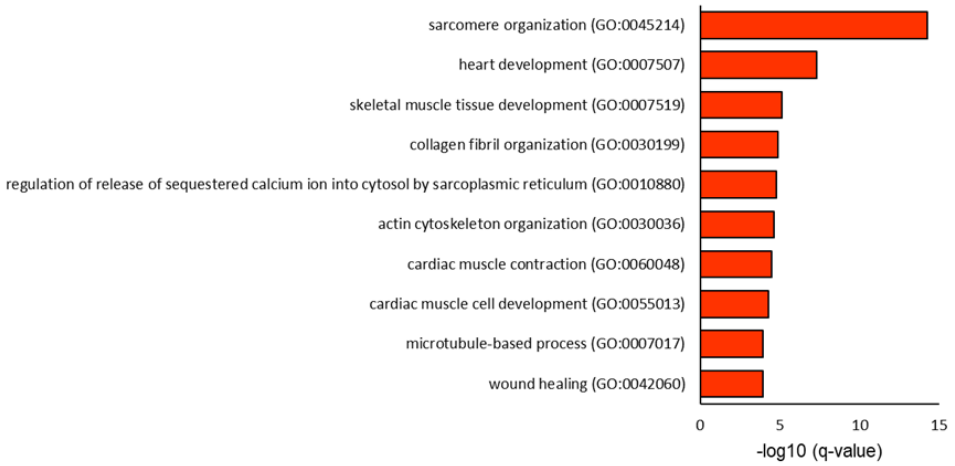
E



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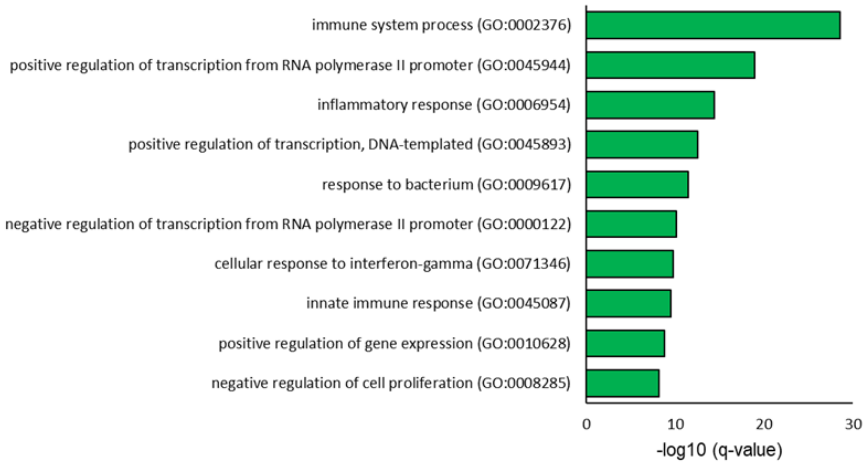

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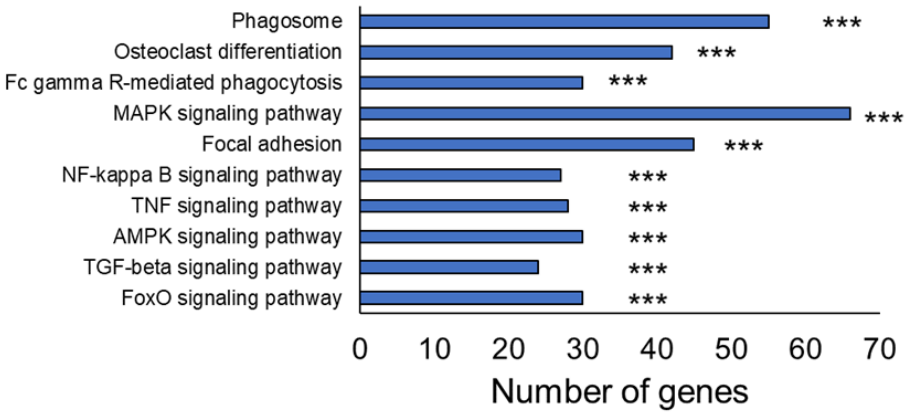

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Figure 4-1. Microarray analysis of gastrocnemius muscle gene expression profile on day 14 after denervation.

(A) The volcano plot represents the DEGs between MA and control groups, where the Y-axis represents -log10 p-value (analysis of variance) and the X-axis represents fold change. Upregulated DEGs are indicated in red, and downregulated DEGs are shown in green dots. (B) Column graph displays the number of DEGs. (C) Heat map displays the row z-score of DEGs. (D) Bar graph displays the Hallmark gene sets in which the DEGs are significantly enriched (MSigDB of GSEA). The criteria for significance were as follows: false discovery rate q-value < 0.05. (E) Bar graph displays the significantly upregulated biological processes in which the DEGs were enriched (from DAVID). (F) Bar graph displays the significantly downregulated biological processes in which the DEGs were enriched (from DAVID). (G) Bar graph displays significantly enriched selected KEGG pathways. DEGs, differentially expressed genes; MA, maslinic acid; GSEA, gene set enrichment analysis; DAVID, Database for Annotation, Visualization and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes.

4.3.4. Effect of MA on LPS-induced genes expression in mouse C2C12 skeletal myotubes

Microarray results suggest that MA suppresses inflammation due to denervation. Therefore, *in vitro* studies were performed to evaluate the beneficial influence of MA on inflammation-induced muscle atrophy. The effect of treatment with various concentrations of MA (0, 1, 5, 10, 25, or 50 μM) for 24 h on C2C12 myotube cell viability was evaluated. MA treatment at concentrations > 25 μM exerted cytotoxic effects on myotubular cells (Fig. 4-2A). Therefore, in the following experiments, the concentrations of MA were set at 1, 5, and 10 μM. LPS induces muscle atrophy by upregulating the muscle protein degradation pathway. The *Atrogin-1*, *Murf1*, and *Tnfa* levels in LPS-treated C2C12 myotubes were significantly higher than in the untreated control. Apigenin (positive control) suppressed the LPS-induced upregulation of *Atrogin-1*, *Murf1*, and *Tnfa* (Fig. 4-2B-D). Treatment with 5 and 10 μM MA significantly mitigated the LPS-stimulated upregulation of *Atrogin-1*, *Murf1*, and *Tnfa*. This indicates that MA suppresses the LPS-induced upregulation of inflammation and associated E3 ligase expression.

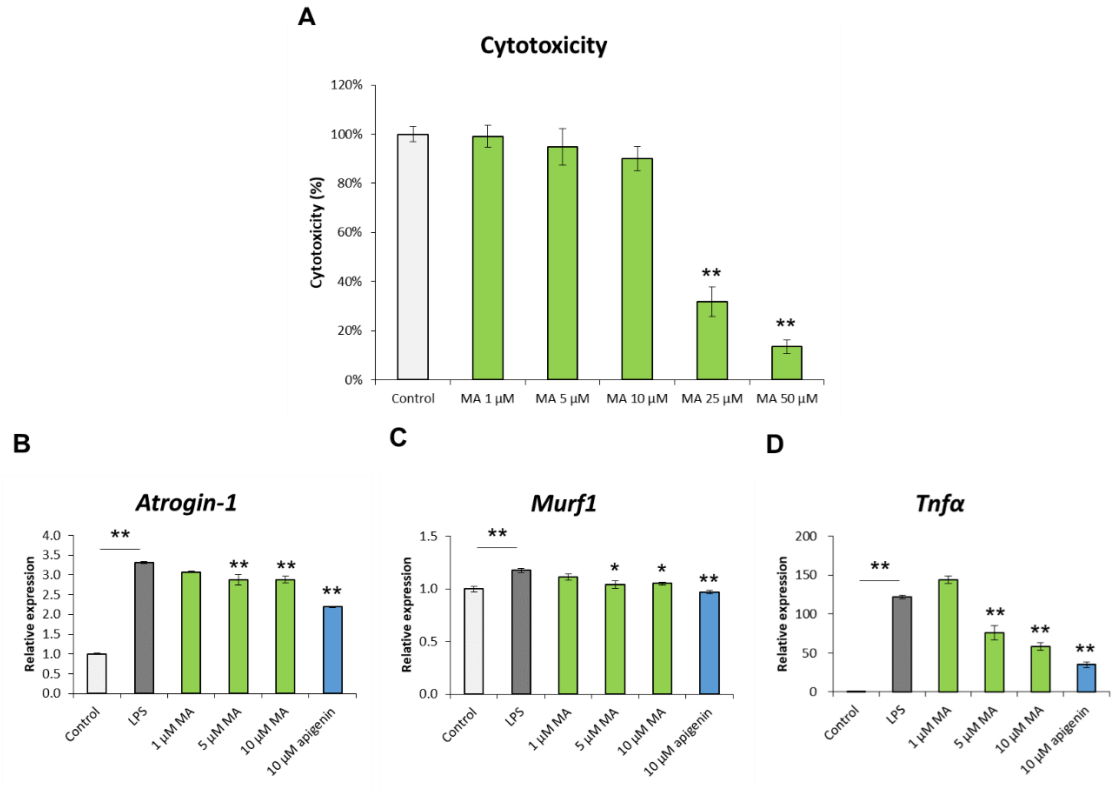

Figure 4-2. MA suppresses the LPS-induced upregulation of *Atrogin-1*, *Murf1,* and *Tnfa* in mouse C2C12 skeletal myotubes.

(A) Effect of MA on the viability of C2C12 myotube cells. (B, C, D) qRT-PCR of relative mRNA expression levels of, *Atrogin-1*, *Murf1*, and *Tnfa*. Data are expressed as mean ± SEM (n = 3–4), *$p < 0.05$, **$p < 0.01$ vs control. apigenin: positive control. MA, maslinic acid; LPS, lipopolysaccharide.

4.3.5. Effect of MA on DEX-stimulated C2C12 myotubes

To validate the effect of MA on the glucocorticoid effect of denervation in mice, the preventive effects of MA on myotube atrophy were examined in the DEX-induced C2C12 cells. Treatment with 10 μM of DEX for 24 h significantly decreased the myotube diameter. Meanwhile, treatment with resveratrol (positive control) alleviated the DEX-induced decreased myotube width (Fig. 4-3). Treatment with 5 or 10 μM of MA for 24 h effectively mitigated the DEX-induced decreased myotube diameter. The balance of protein turnover determines muscle mass that allows adaptation to different pathophysiological conditions (Bonaldo and Sandri 2013, Sartorelli and Fulco 2004). Muscle atrophy is characterized by the shrinkage of muscle tissues or organs caused by cellular atrophy, resulting from imbalances in myocytes, organelles, cytoplasm, and proteins that form the following two primary cellular proteolytic systems: the autophagy-lysosome system and the ubiquitin-proteasome system (Blaauw et al. 2009, McCarthy et al. 2011, Raffaello et al. 2010). These results indicate that MA reversed DEX-stimulated muscle atrophy and prevented myotubular cell contraction.

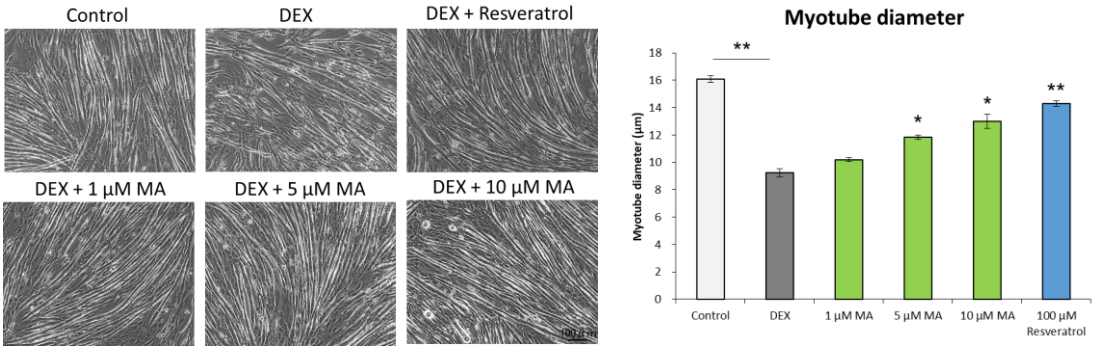

Figure 4-3. MA ameliorated DEX-induced C2C12 myotube atrophy.

Representative images of C2C12 myotubes are shown after treatment with 10 μM DEX and/or MA. Showing on the right is a comparison of myotube diameters between the groups. Data are represented as mean ± SEM (n = 3), *$p < 0.05$, **$p < 0.01$ vs control. resveratrol: positive control. MA, maslinic acid; DEX, dexamethasone.

4. 4. Discussion

Skeletal muscle atrophy due to denervation is accompanied by complex biochemical and physiological changes. The gene expression profiling of denervated muscle at different time points revealed the following four distinct transcriptional stages: oxidative stress, inflammation, atrophy, and atrophic fibrosis stages (Shen et al. 2019). To elucidate the molecular mechanisms underlying the preventive effects of MA on denervation-induced muscle atrophy, the gastrocnemius muscle was subjected to whole-genome microarray analysis on day 14 after denervation. Several possible mechanisms by which MA maintains muscle have been suggested. GSEA revealed that various biological processes, such as myogenesis, PI3K/AKT/mTOR signaling, TNFα signaling via NF-κB, and TGF-β signaling were significantly enriched in MA-intake mice when compared with control mice (Fig. 4-1D). Inflammation is considered to be a major factor in the physiological response to muscle atrophy. Consistent with the hypothesis of the study, MA suppressed denervation-induced inflammation as evidenced by the marked variation in the gene sets related to inflammatory responses, such as the Hallmark sets "TNFα signaling via NF-κB," "interferon gamma response," and "TGF-β signaling," as well as in GO terms, such as cellular response to TNFα and IFNγ (Fig. 4-1D, F). The overexpression of TGF-β, which can directly induce muscle fiber atrophy, results in muscle fiber atrophy and fibrosis and upregulates the expression of MuRF1 in skeletal muscles (Narola et al. 2013). Furthermore, TGF-β activity was observed to contribute to muscle weakness in muscle atrophy caused by cancer cachexia (Waning et al. 2015). A previous study reported that MA treatment downregulated TGF-β expression in a colorectal cancer mouse model (Sanchez-Tena et al. 2013). However, the effect of MA on TGF-β regulation in muscle atrophy models has not been previously reported. Thus, the

findings of this study suggest that MA exerts preventive and therapeutic effects on muscle atrophy by downregulating the TGF-β signaling pathway (Fig. 4-1D). *In vitro* studies were performed to demonstrate the beneficial influence of MA in inflammation-induced muscle atrophy. Treatment with MA significantly suppressed the LPS-induced downregulation of *Atrogin-1*, *Murf1*, and *Tnfa* (Fig. 4-2B-D).

Protein metabolism takes an essential role in the regulation of skeletal muscle mass. Skeletal muscle mass gains when protein synthesis is greater than protein breakdown. Conversely, when protein breakdown outweighs protein synthesis, skeletal muscle mass decreased. MA enhances protein synthesis and muscle mass increase in rainbow trout (Fernandez-Navarro et al. 2008). A recent study suggested MA exerts therapeutic effects on skeletal muscle hypertrophy by activating mTOR signaling pathway, which plays a major role in regulating protein synthesis (Murata et al. 2021, Shirai et al. 2021). Microarray data revealed that MA intake promotes myogenesis and regulates PI3K/AKT/mTOR signaling, contributing to muscle maintenance (Fig. 4-1D). Additionally, MA downregulated the expression of *Redd1* (Fig. 4-4). REDD1 negatively regulates muscle mass through the inhibition of the AKT/mTORC1 signaling pathway. The expression of REDD1 is significantly upregulated in response to glucocorticoid secretion, a component of the energy stress response (Britto et al. 2014). Further studies are needed to confirm whether MA promotes the degradation REDD1 and evaluate the effect of MA on REDD1 activity. Hennebry et al. demonstrated that IGF1 stimulates significant muscle hypertrophy in mice without myostatin (Hennebry et al. 2017), a secreted member of the TGF-β superfamily. Thus, MA-induced simultaneous *Igf1* upregulation and *Tgfb* downregulation in the denervation-treated muscle may have contributed to muscle maintenance. Furthermore, IGF1 inhibits protein degradation and

mRNA expression levels of *MuRF1* and *Atrogin-1* (Frost et al. 2009, Sacheck et al. 2004).

FOXO-activated kinases can enhance the nuclear translocation and activation of FOXO. JNK is a family member of oxidative stress-activated mitogen-activated protein kinases. Activated JNK can phosphorylate FOXO and antagonize the AKT signaling pathway to promote FOXO transcription and nuclear translocation (Wang, Chen, and Xing 2012). MA upregulated PGC-1α expression by modulating AMPK and CaMK2 activities and regulated antioxidant-related catalase and MnSOD expression (Fig. 4-5). Although calcium ions were not directly evaluated in this study, MA is reported to increase the concentration of calcium ions in adipose cells (Perez-Jimenez et al. 2016). PGC-1α inhibits the nuclear translocation of NF-κB and FOXO, which is consistent with MA-induced suppression of genes, such as *MuRF1* and *Atrogin-1* (Sandri et al. 2006).

MA directly affects muscle composition and consequently maintains the muscles. GO analysis shows that the MA group significantly upregulated several biological processes related to muscle organization, tissue formation, and repair. Consistent with this result, MA is reported to promote collagen synthesis (Tan, Sonam, and Shimizu 2017).

MA also modulated immune responses in the gastrocnemius muscle, which is an overlap of factors related to the inflammatory response. Furthermore, MA regulated the pathways involved in phagocytosis (Fig. 4-1E, G). Generally, mitophagy activity increases in disuse-induced muscle atrophy (Yamashita et al., 2021) but intake of MA changed the activity of phagosome in denervated mice.

Denervation promotes inflammation, glucocorticoid effect, myostatin activation, and PI3K/AKT inhibition (Duan, Gao, and Zhu 2021). In this chapter, the effects of MA on denervation-induced muscle atrophy and drug-treated C2C12 myotubes were

investigated. The effect of MA on the glucocorticoid effects of denervation treatment in mice was examined. MA reversed DEX-induced muscle atrophy and prevented myotubular cell shrinkage.

Further studies using different muscle atrophy mouse models or muscle atrophy cell models are required to elucidate and clarify the preventive effects of MA on muscle atrophy and sarcopenia. Additionally, the effect of MA on muscle recovery without pre-treatment after denervation must be examined to enable clinical applications.

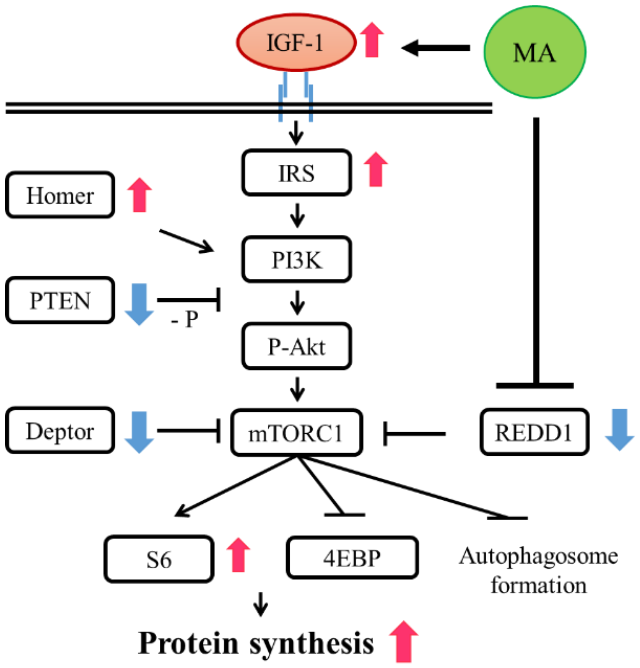

Figure 4-4. Schematic representation of the cascade of reactions involving the muscle protein synthesis-related genes in denervated mice fed with MA.

MA enhanced protein synthesis by upregulating Igf1 expression and regulating Redd1, which is involved in mTORC1 activation. MA, maslinic acid; Igf1, insulin-like growth factor 1; IRS, insulin receptor substrate; S6, ribosomal protein S6; 4EBP, eukaryotic translation initiation factor 4E-binding protein; Redd1, regulated DNA damage and development 1.

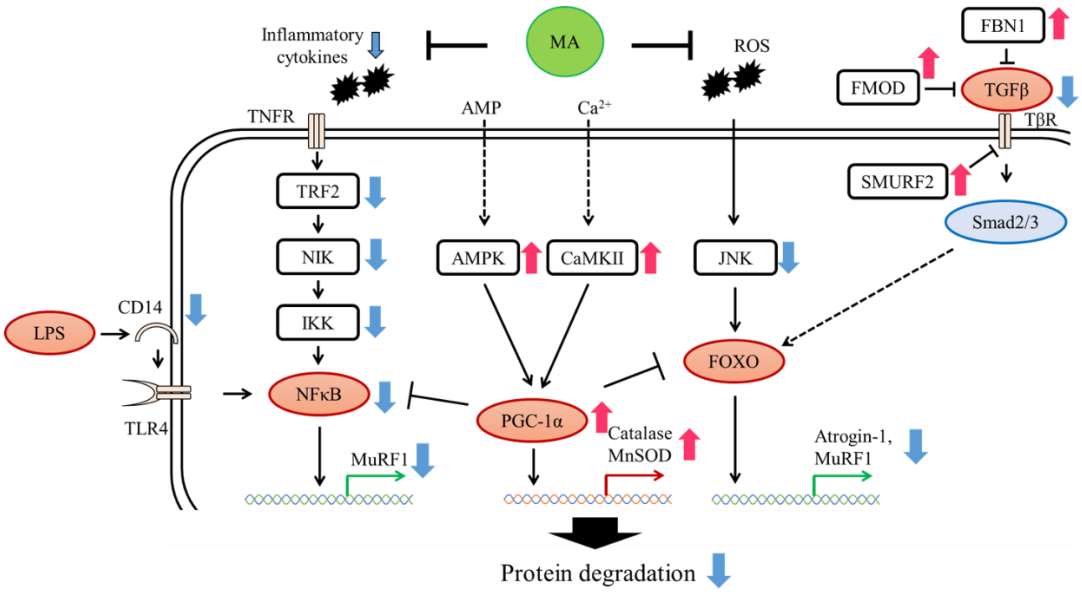

Figure 4-5. Schematic representation of the cascade of reactions involving the muscle protein degradation-related genes in denervated mice fed with MA.

MA exerted its effects on the anti-inflammation and anti-oxidation systems, and inhibited TGF-β expression, resulting in the modulation of protein degradation. Additionally, MA mitigates muscle atrophy and muscle strength. MA, maslinic acid; ROS, reactive oxygen species; LPS, lipopolysaccharide; TNFR, tumor necrosis factor receptor; TNF2, TNF receptor-associated factor 2; NF-κB, nuclear factor-kappa B; NIK, NF-κB-inducing kinase; IKK, I kappa B kinase; AMPK, AMP-activated protein kinase; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase kinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; JNK, c-Jun N-terminal Kinase; FoxO, Forkhead box O transcription factors; FMOD, fibromodulin; FBN1, fibrillin 1; TGF-β, transforming growth factor β; TβR, TGF-β receptor type; SMURF2, SMAD specific E3 ubiquitin protein ligase 2; MuRF1, Muscle Ring Finger 1.

CHAPTER 5: General Discussion

In Japan, locomotive disorders are the major reason for requiring special assistance or nursing care. Sarcopenia, which is characterized by decreased muscle mass and function, is related with an increased risk of disability, falling, fall-related injuries, hospitalization, and mortality. Various studies have focused on alleviating sarcopenia with food ingredients, preventing QOL decline, and extending healthy life expectancy. The Mediterranean diet provides various health benefits, including the prevention of inflammation-associated diseases. Olives and their oils are important foods in the Mediterranean diet (Keys 1995, Rosillo et al. 2016, Willett et al. 1995). MA exerts potent anti-inflammatory effects and inhibits the activity of inflammatory cytokines (Fukumitsu, Villareal, Fujitsuka, et al. 2016, Shimazu et al. 2019). In the elderly, the presence of low-grade inflammation is considered to be one of the main determinants of health status (Franceschi et al. 2018) and is suspected to be a favorable environment for the development of sarcopenia (Argiles et al. 2015). Therefore, this research aimed to investigate the beneficial effects of olive-derived MA on muscle atrophy.

In Chapter 2, the clinical trial aimed to understand the beneficial effect of MA on the motor system of the elderly. The hypothesis of the clinical trial was that differential MA absorption in the blood resulting from varying amounts of MA intake increases muscle mass and strength. To verify this hypothesis, the efficacy of MA was examined at appropriate and effective intake doses. This study was conducted as a single-blind crossover trial. A significant dose-response relationship between blood MA level and intake quantity was observed. Based on the results of MA pharmacokinetics, 120 mg MA was considered an excessive dose. Hence, the MA intake dose in this study was fixed at 30 and 60 mg. A randomized, double-blind, placebo-controlled trial was performed to

evaluate the beneficial effect of different amounts of daily MA intake (0, 30, and 60 mg/day) on muscle mass and strength of healthy adults undergoing resistance training. MA intake in combination with exercise improved grip strength and muscle mass. To the best of our knowledge, the present study is first to assess the effect of various amounts of MA intake on muscle mass and strength and estimate the minimum effective concentration of MA that can increase muscle strength in the healthy elderly population undergoing resistance training. These results confirmed the study hypothesis. Diet and physical activity are essential regulatory mediators of systemic inflammation that are directly involved in the sarcopenic process. Combining healthy dietary patterns and physical activity is a promising strategy to mitigate age-related sarcopenia.

The previous clinical trial was conducted with healthy participants. Hence, there was a need to evaluate the direct effect of MA on muscle atrophy. Several factors can lead to muscle atrophy. Sarcopenia is studied in mouse models, including denervation-based, aging-based, hindlimb unloading-based, and immobilization-based models. The denervation model is widely used in muscle atrophy research. Denervation promotes inflammation, glucocorticoid effect, myostatin activation, and PI3K/AKT inhibition. This activates the ubiquitin proteasome system and the autophagy-lysosome system, enhances protein degradation, suppresses new protein synthesis, and consequently promotes muscle atrophy. MA exerts potent anti-inflammatory effects and inhibits the activity of inflammatory cytokines. Therefore, Chapter 3 aimed to assess the effects of MA on the loss of muscle mass in the denervation mouse model. MA prevented the denervation-induced decrease in skeletal muscle strength and gastrocnemius muscle mass in mice. Additionally, MA suppressed the mRNA and protein expression levels of MuRF1 and Atrogin-1, which are involved in muscle protein degradation, and upregulated the mRNA

and protein expression levels of Igf1, which is involved in muscle protein synthesis, after denervation. IGF1 is a critical anabolic growth factor involved in regulating muscle hypertrophy by enhancing PI3K/AKT signaling (Timmer, Hoogaars, and Jaspers 2018). MA upregulated the expression of IGF1. MuRF1 and Atrogin-1, which are muscle-specific E3 ubiquitin ligases (Li et al. 1998), are associated with the maintenance of muscle mass. However, the detailed mechanisms of the beneficial effects of MA are unknown. Future studies must investigate the regulatory pathways of the factors whose expression varied upon treatment with MA.

Chapter 4 aimed to reveal the molecular mechanism underlying the preventive effects of MA on muscle atrophy using whole-genome microarray analysis in mice. Additionally, the inhibitory effects of MA on gene expression in LPS-induced and/or DEX-stimulated mouse C2C12 myotubes were investigated to elucidate the mechanisms underlying its preventive effects on inflammation-induced muscle atrophy. Microarray analysis revealed that biological processes, such as myogenesis, PI3K/AKT/mTOR signaling, TNFα signaling via NF-κB, and TGF-β signaling were significantly enriched in MA-intake mice when compared with control mice.

Several reports have demonstrated the effectiveness of MA on muscle synthesis (Murata et al. 2021, Shirai et al. 2021) although its effects on muscle atrophy and the underlying mechanisms of action have not been elucidated. This study examined the effects of MA supplementation on muscle mass and strength and elucidated a mechanism. However, this study did not perform histological analysis of skeletal muscle fibers and examine the protein phosphorylation level in skeletal muscle. A previous western blotting analysis revealed that MA exhibits anti-inflammatory effects by inactivation of NF-κB in RAW 264.7 cells through the inhibition of IκB-α phosphorylation (Fukumitsu, Villareal,

Fujitsuka, et al. 2016). In addition, Kunkel et al. have reported by immunoblot analysis that ursolic acid, a triterpene, promotes muscle conservation by boosting IGF1-mediated signaling cascades in serum-starved C2C12 myotubes (Kunkel et al. 2011). Consequently, further *in vivo* studies of MA processing effects on the protein levels of these markers are indicated.

MA prevented the denervation-induced decrease in skeletal muscle strength and gastrocnemius muscle mass. Global genomic analysis of the gastrocnemius muscles using microarray revealed multiple potential mechanisms for muscle maintenance. GSEA revealed that biological processes, such as TNFα signaling via NF-κB, TGF-β signaling, and PI3K/AKT/mTOR signaling were significantly enriched in MA-intake mice when compared with control mice.

Profiling of gene expression in denervated muscle at different time points identified the following four distinct transcriptional stages: oxidative stress, inflammation, atrophy, and atrophic fibrosis stages (Shen et al. 2019). After denervation, the skeletal muscle lost its contractile function, which results in decreased blood perfusion and induction of hypoxia in the target muscle. Subsequently, this leads to oxidative stress-related injury due to excessive production of reactive oxygen species (ROS). Overproduction of ROS causes muscle damage, increases inflammatory factors, and further activates inflammatory response pathways. Excessive activation of inflammation accelerates skeletal muscle atrophy and fibrosis. Inflammation plays an important role in the process of muscle atrophy caused by denervation. Celecoxib (known as Celebrex), a nonsteroidal anti-inflammatory drug, selectively inhibits PTGS2 and is prescribed as a treatment for osteoarthritis and rheumatoid arthritis. Celecoxib extensively ameliorates inflammation and oxidative stress and relieves denervation-induced muscle atrophy by

improving microcirculation (Zhang et al. 2022). MA effectively attenuates some major inflammatory mediators, such as prostaglandin E2, and inhibits the expression of IL6, PTGS2, and TNFα in a concentration-dependent manner (Chen et al. 2021). Moreover, MA attenuates ischemia/reperfusion-induced inflammation by downregulating NF-κB-mediated adhesion molecule expression (Ampofo et al. 2019). Based on the findings of this study, MA and Celecoxib may ameliorate muscle atrophy caused by denervation through reducing inflammation and improving microcirculation.

The results of *in vivo* and *in vitro* experiments in this study and previous studies (Murata et al. 2021, Shirai et al. 2021) suggest that MA regulates the synthesis and degradation of muscle proteins and maintains muscle mass and strength (Fig. 5-1). Moreover, the clinical trial examined the effects of different amounts of MA intake on muscle mass and strength and estimated the minimum effective concentration of MA that can increase muscle strength in healthy adults undergoing resistance training. This study demonstrated that daily MA intake can prevent sarcopenia by regulating the imbalance between protein breakdown and synthesis.

In conclusion, the results presented in this thesis reveal the potential of MA as a new preventive and therapeutic dietary ingredient for alleviating muscular atrophy and improving muscle strength. Sarcopenia increases the risk of disability, falling, falls-related injuries, hospitalization, and death. Hence, alleviating sarcopenia is essential to mitigate decreased quality of life and extend healthy life expectancy. Our group identified MA as the active component of olive pomace and developed an industrial-scale MA extraction and purification method. The effective use of industrial by-products is important as it contributes to a recycling-based society. Regular supplementation of

MA derived from by-products such as olive pomace might help protect from sarcopenia and extend healthy life span.

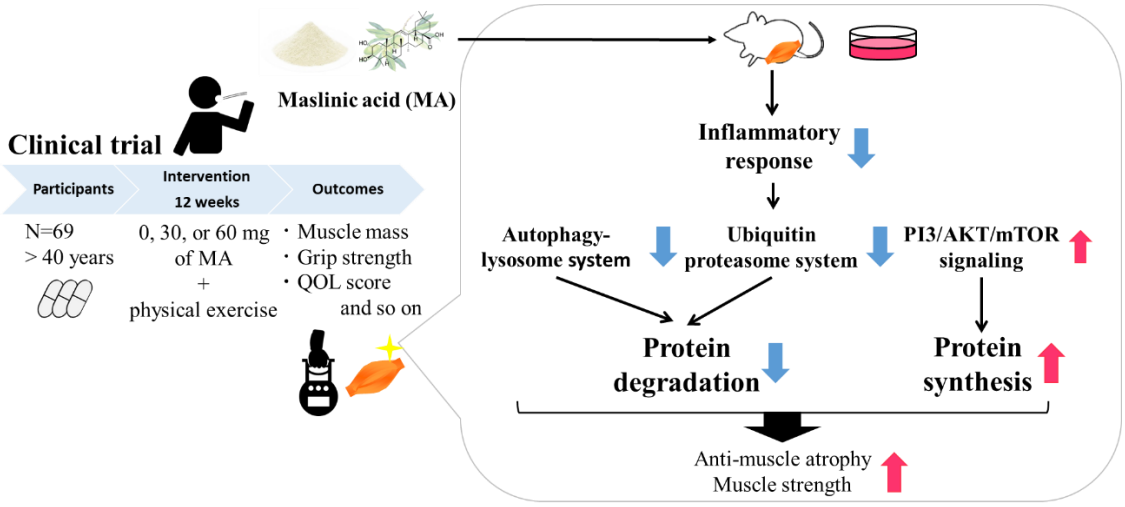

Figure 5-1. Schematic diagram of the mechanisms of MA in preventing muscle atrophy. Dietary MA can modulate the dynamic balance of muscle protein degradation and synthesis by inducing IGF1 expression. Upregulated IGF1 promotes enhanced protein synthesis via PI3K/AKT/mTOR signaling. Additionally, MA exerts its effects on the anti-inflammation system and consequently regulates protein degradation. Thus, MA alleviates muscle atrophy and decreased muscle strength. MA, maslinic acid; QOL, quality of life.

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List of Publication

International publications

Yamauchi Y, Ferdousi F, Fukumitsu S, Isoda H. Maslinic Acid Attenuates Denervation-Induced Loss of Skeletal Muscle Mass and Strength. Nutrients. 2021 Aug 25;13(9):2950. doi: 10.3390/nu13092950. PMID: 34578826; PMCID: PMC8468537