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Glycyrrhiza uralensis attenuates TNF-α-induced muscle atrophy in myoblast cells through the Nrf2 and MAFbx signaling cascades

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Abstract

Muscle atrophy is associated with chronic diseases, such as heart failure diabetes, and aging-related diseases. *Glycyrrhiza uralensis* (GU) extract is widely used in traditional medicine. However, no studies have evaluated the effects of GU on muscle atrophy. Thus, in this study, we assessed the effects of GU on prevention of muscle atrophy. GU reduced the levels of the TNF-α-induced muscle atrophy markers, muscle RING-finger protein-1(Murf-1) and muscle atrophy F-box (MAFbx), and upregulated myosin heavy chain expression (MyHC). It also reduced the phosphorylation of nuclear factor kappa B, and downregulated Smad3 proteins, which are involved in protein ubiquitination. When we examined whether GU exhibits antioxidant activities. GU suppressed TNF-α-induced muscle atrophy by increasing the translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), which regulates the expression of antioxidant factors such as heme oxygenase-1 (HO-1) as well as apoptosis-related factors, such as caspase-3/7. These results suggest that GU extract is potentially an important agent in the regulation of TNF-an induced muscle atrophy.

Keywords: *Glycyrrhiza uralensis*, Muscle atrophy, Anti-oxidants, Nrf2, C2C12 myoblast cells

Introduction

Sarcopenia is defined as the loss of muscle mass and strength during the natural course of aging. The term was first introduced by Rosenberg in 1989 [1, 2]. Although the condition is multifactorial, elevated oxidative stress and consequent increase in proinflammatory cytokines due to aging induces apoptosis of the muscle cells, thereby damaging them. Loss of muscle cells alters muscle mass and may cause physiological dysfunction, cancer, degenerative brain disorders, kidney diseases, and heart diseases [3–7]. The etiology of sarcopenia includes reduced muscle cell count and the breakdown of muscle proteins related to protein intake, hormones, and proinflammatory cytokines [8–11]. Muscle atrophy occurs as proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6, facilitate the degradation of myofibrillar proteins and reduce protein synthesis, which directly causes muscle wasting. In particular, TNF-α induces ubiquitin-dependent proteolysis by activating several intracellular factors and triggers apoptosis [12–16].

Treatments for sarcopenia include increased mitochondrial production, suppression of muscle proteolysis, and use of anti-inflammatory agents; however, since an effective therapeutic agent is lacking and existing drugs cause various adverse reactions (cytotoxicity), there is a need for measures to maintain muscle mass [17–19]. In recent years, protein supplements have been the chosen alternative for many. However, protein supplements lead to excessive protein intake and have been linked to an increased risk of adverse events. Moreover, individuals with kidney disease cannot consume high-protein diets and aging deteriorates kidney function as well. Therefore, there is an urgent calling for other alternatives that can

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increase protein intake to prevent sarcopenia. Recently, many studies have attempted to attenuate sarcopenia using various natural products [20].

Glycyrrhiza uralensis (GU) is a plant belonging to the Fabaceae family that is widely utilized in several herbal medicines and foods in Asia. The main components of GU include glycyrrhizin, flavonoids, liquiritigenin, isoliquiritigenin, and isoflavonoid licoricidin [21]. GU extracts have been investigated for their various bioactivities, such as antioxidant, immune-enhancing, antibacterial, and anti-viral effects [22–25]. In relation to muscles, studies have reported that the flavonoids of GU regulate smooth muscle function, increase muscle mass, and inhibit cardiomyocyte injuries [22, 26, 27]; however, research on the effects of GU extracts on inhibiting inflammation-induced muscle atrophy is lacking. This study aimed to evaluate the effects of GU extract on an in vitro model of muscle atrophy using myoblasts.

Methods

Materials

Recombinant TNF-α, formaldehyde, formic acid (FA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media: Dulbecco's Modified Eagle's Medium (DMEM), antibiotic–antimycotic solution, fetal bovine serum (FBS), horse serum, and other ingredients required to culture cells were supplied by Life Technologies (Grand Island, NY, USA). Protease and phosphatase inhibitor cocktail tablets were supplied by Thermo Scientific (Rockford, IL, USA). The nuclear/cytosol fraction kit was obtained from Biovision (Milpitas, CA, USA). DNA, RNA, and protein purification kits were purchased from MACHEREY–NAGEL (Neumann, DU, Germany). Primary and secondary antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA), Abcam (Cambridge, MA, USA), and Developmental Studies Hybridoma Bank (Iowa City, IA, USA). All kits required for real-time PCR were obtained from Bio-Rad (Hercules, CA, USA). High-performance liquid chromatography (HPLC)-grade water and acetonitrile (ACN) (JT Baker, Phillipsburg, NY, USA) were used as solvents for the liquid chromatography-mass spectroscopy (LC–MS) experiments. The lyophilized extracts (8 mg) were dissolved in 1 mL of 0.1% FA water, and the samples were sonicated for 2 h. After sonication, 1 mL of 0.1% FA water was added, and the solutions were filtered using a 0.45 μm polyvinyl difluoride syringe filter.

Preparation of GU

The roots of *Glycyrrhiza uralensis* (2 years old) were collected from Yeongwol-gun, Gangwon-do, Republic of Korea in November 2017. The sample was washed with distilled water and air-dried at 50 °C. It was ground and extracted twice with 70% ethanol at 50 °C for 3 h and then filtered. The supernatant was vacuum-evaporated and freeze-dried to prepare the root extract.

High-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS)

All HPLC-ESI-MS experiments for chemical identification in the samples were performed in positive ion mode using a Synapt G2-Si HDMS QTOF (Waters, Manchester, UK) equipped with an ACQUITY UPLC HSS C18 VanGuard pre-column (1.8 μm, 2.1 × 5 mm) and an ACQUITY UPLC HSS C18 column (1.8 μm, 2.1 × 100 mm). Two solvents, FA water (0.1%, solvent A) and FA ACN (0.1%, solvent B), were used at a flow rate of 0.2 mL/min. The gradient of the two solvents were set as follows: linear increase of B from 0 to 5% at 0–3.0 min; 5–75% at 3.0–23.5 min; 75% at 23.5–27.5 min; linear decrease from 75 to 0% at 27.5–28.5 min; 0% B at 28.5–32.0 min. The injection volume and column oven temperature were 10 μL and 40 °C, respectively. For intact ionization, the capillary voltage, sampling cone, and source offset were set to 3.0, 20.0, and 80.0 kV, respectively. Moreover, the source temperature and desolvation temperature were set to 150 and 400 °C, respectively. The cone gas flow, desolvation flow, and nebulizer gas flow were set at 10.0 L/h, 650.0 L/h, and 650 kPa bar, respectively.

Cell culture and treatment

C2C12 muscle cells were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM supplemented with 5% FBS and 1% antibiotic–antimycotic solution in a humidified incubator with 5% CO_2 at 37 °C. To initiate differentiation, cells were allowed to reach 80% confluence, and the medium was changed to DMEM supplemented with 2% horse serum and replenished every 2 days. Full differentiation characterized by myotube fusion and spontaneous twitching was observed after 5 days. GU was dissolved in DMSO for a final concentration of 10 mM of the stock solution and used for each dilution. Differentiated myotubes were treated for 1 h with 10 ng/mL TNF-α, different concentrations of GU, or both drugs combined.

Measurement of myotube diameter

Myotubes were photographed at 200 × magnification after 1 h of treatment with TNF-α and GU using an OLYMPUS CKX41 microscope (Olympus, Tokyo, Japan). At least 10 diameters per group were measured using SOLUTION Lite 9.1 software (Medline scientific, Oxon, UK).

Western blot analysis

C2C12 myotubes were seeded in 6-well plates. The cells were lysed with protein extraction solution (Elpis Biotech, Daejeon, Korea) and cell lysates were separated into nuclear and cytosolic fractions using a nuclear/cytosol fraction kit. Protein concentration was measured using a protein assay dye reagent with bovine serum albumin as the standard. The same amount of protein from each sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% skimmed milk and incubated overnight at 4 °C with the following primary antibodies: myosin heavy chains (MyHCs), myogenin, muscle atrophy F-box (MAFbx), β-actin, muscle RING-finger protein-1 (MuRF1), phospho-p65 (p-p65), p-65, p-p38, p-38, p-smad3, samd3, heme oxygenase (HO)-1, nuclear factor erythroid 2-related factor 2 (Nrf2), and lamin B. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Antibody detection was carried out using the ECL reagent (Thermo Scientific) and visualized using ChemiDoc (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of the bands were normalized to the β-actin or lamin B bands using Image Lab Software 4.0.1 (Bio-Rad).

Apoptosis analysis (Caspase-3/7)

C2C12 cells were plated onto 6-well plates at a density of 5×10^5 cells/well. They were treated with different concentrations (10, 50, and 100 μg/mL) of GU or 10 ng/mL of TNF-α for 12 or 24 h. Caspase-3/7 activity was quantified using the Muse™ Caspase-3/7 assay kit (Merck Millipore Burlington, MA, USA). Cells were collected using centrifugation (3000 rpm, 5 min) and washed with phosphate-buffered saline. Cells were resuspended in a 1 × Assay Buffer BA, mixed with the Muse™ Caspase-3/7 reagent, and incubated for 30 min at 37 °C in a 5% CO_2 atmosphere, in the dark. Then, the cells were incubated with Muse Caspase 7-AAD, a dead cell marker, for 5 min at room temperature. Cell suspensions were used to estimate the proportions of four cell populations, including percentages of live cells, apoptotic cells exhibiting caspase-3/7 activity, late apoptotic/dead cells, and necrotic cells according to the Muse cell analyzer (Merck KGaA, Darmstadt, Germany).

Statistics analysis

All statistical parameters were calculated using GraphPad Prism software (version 3.0; GraphPad Software Inc., San Diego, CA, USA). Values are expressed as the mean ± standard error of the mean (SEM). The results were analyzed using one-way analysis of variance followed by Tukey's post hoc test. Differences were considered statistically significant at $P < 0.05$, $P < 0.001$.

Results

HPLC–ESI–MS analysis of GU

Two abundant constituents of *G. uralensis*, glycyrrhizin and licoricidin, were identified using ultra-performance liquid chromatography electrospray ionization quadrupole time-of-flight tandem mass spectrometry in the positive ion mode. As shown in Fig. 1A glycyrrhizin and licoricidin ions were found at 15.62 and 22.11 min, respectively. The MS spectrum of glycyrrhizin (Fig. 1B) shows both protonated molecular ions (experimental m/z 823.4110; theoretical m/z 823.4111) and sodiated molecular ions (experimental and theoretical m/z 845.3930) [28]. The fragment peaks obtained from the two molecular ions were observed at m/z 647.37, 471.35, 453.34, 669.37, and 493.33, corresponding to $[M+H-C_6H_8O_6]^+$, $[M+H-C_{12}H_{18}O_{12}]^+$, $[M+H-C_{12}H_{17}O_{13}]^+$, $[M+Na-C_6H_8O_6]^+$, and $[M+Na-C_{12}H_{18}O_{12}]^+$, respectively (Fig. 1B) [29]. The MS spectrum of licoricidin showed protonated molecular ions at m/z 425.2320 (calculated m/z 425.2323), and the MS^2 spectra of the molecular ions (Fig. 1C) showed two fragment ions at m/z 369.14 and 301.07 [29]. The UPLC-ESI-MS analyses indicated that our extract originated from *G. uralensis*.

GU ameliorated the effect of TNF-α on myotube differentiation

After differentiating C2C12 cells for 5 days, the cells were treated with TNF-α alone or with GU for 24 h. The thickness of the muscle fibers was compared between the two treatment groups. The control group had a thickness of 16.824 ± 1.684 μm, and the TNF-α-induced muscle atrophy group showed a reduction to 9.023 ± 0.930 μm; when

(See figure on next page.)

Fig. 1 Extracted ion chromatograms of glycyrrhizin and licoricidin in the *Glycyrrhiza uralensis* extract. The base peak chromatogram of the glycyrrhiza uralensis extract (0.4 mg/mL) obtained by UPLC-ESI-qTOF in positive ion mode. Expected UPLC-MS peaks of glycyrrhizin and licoricidin were marked as the black circle (●) and the white circle (○), respectively (**A**). MS (top) and MS^2 (middle, bottom) spectra of glycyrrhizin ions. Singly protonated and sodiated glycyrrhizin ions were fragmented to identify glycyrrhizin in the extract (**B**). MS (top) and MS^2 (bottom) spectra of licoricidin in the extract. Singly protonated licoricidin ions were fragmented to identify licoricidin in the extract (**C**)

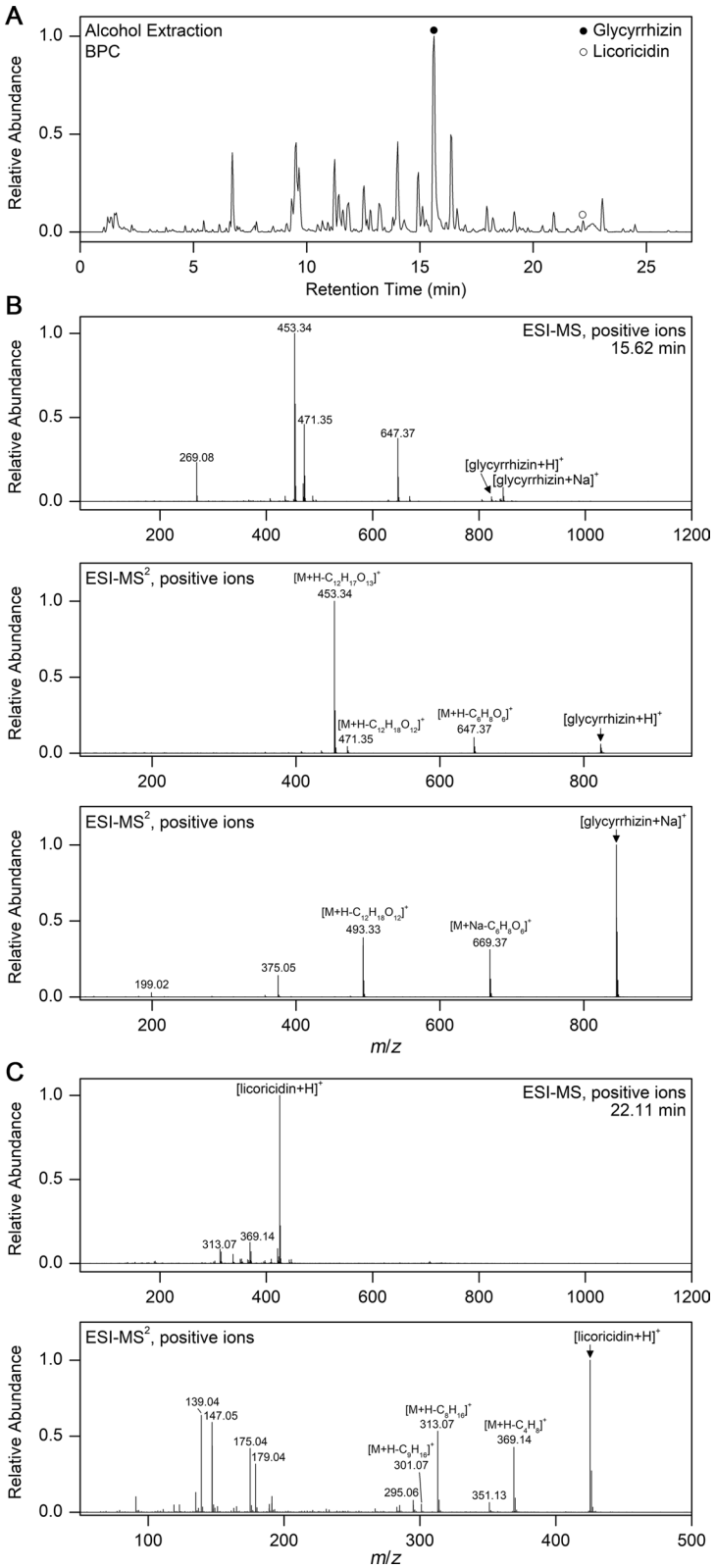


Fig. 1 (See legend on previous page.)

treated with GU, the reduction of myoblast thickness was attenuated in a dose-dependent manner. The thickness at GU concentrations of 10, 50, and 100 μg/mL were 10.441 ± 1.389, 12.622 ± 1.933, and 18.776 ± 3.728 μm, respectively. The thickness in the GU-treated groups was more than that of the control group, confirming the protective effects of GU against myotube injuries (Fig. 2A). MyHC protein expression was assessed using western blot analysis. The GU treatment group showed 1.7 times greater MyHC expression than the control, confirming that GU effectively increases muscle volume with increased myotubes and prevents muscle atrophy (Fig. 2B).

GU attenuated TNF-α induced muscle atrophy

The protein expression of the atrophy markers MuRF1 and MAFbx was assessed using western blot analysis. The TNF-α-treated group showed five times greater MuRF1 expression compared to the control group, while the TNF-α and GU-treated groups showed reduced MuRF1 expression (Fig. 3). The MuRF1 expression was markedly reduced to approximately 50% at a GU concentration of 100 μg/mL. MAFbx expression increased threefold in the TNF-α-treated group, and was downregulated in the TNF-α and GU co-treated group; however, the change was not significant. We also examined the activity of p-Smad, a transcriptional activator of MuRF1, and found that MAFbx-p-Smad activity increased with TNF-α treatment and reduced with GU treatment.

Myotubes were induced for 5 days and treated with TNF-α to establish a model of atrophy. C2C12 cells were treated in the presence or absence of TNF-α (10 ng/mL) and GU (10, 50, and 100 μg/mL) for 24 h. The expression levels of muscle atrophy F-box, muscle RING-finger protein-1, and Smad3 proteins were measured by western blot analysis. Data are presented as the means ± standard error of the means (SEMs) of three independent experiments.

Effect of GU on TNF-α-induced NF-κB signaling in C2C12 cells

Oxidative stress, characterized by elevated levels of reactive oxygen species (ROS), induced by TNF-α

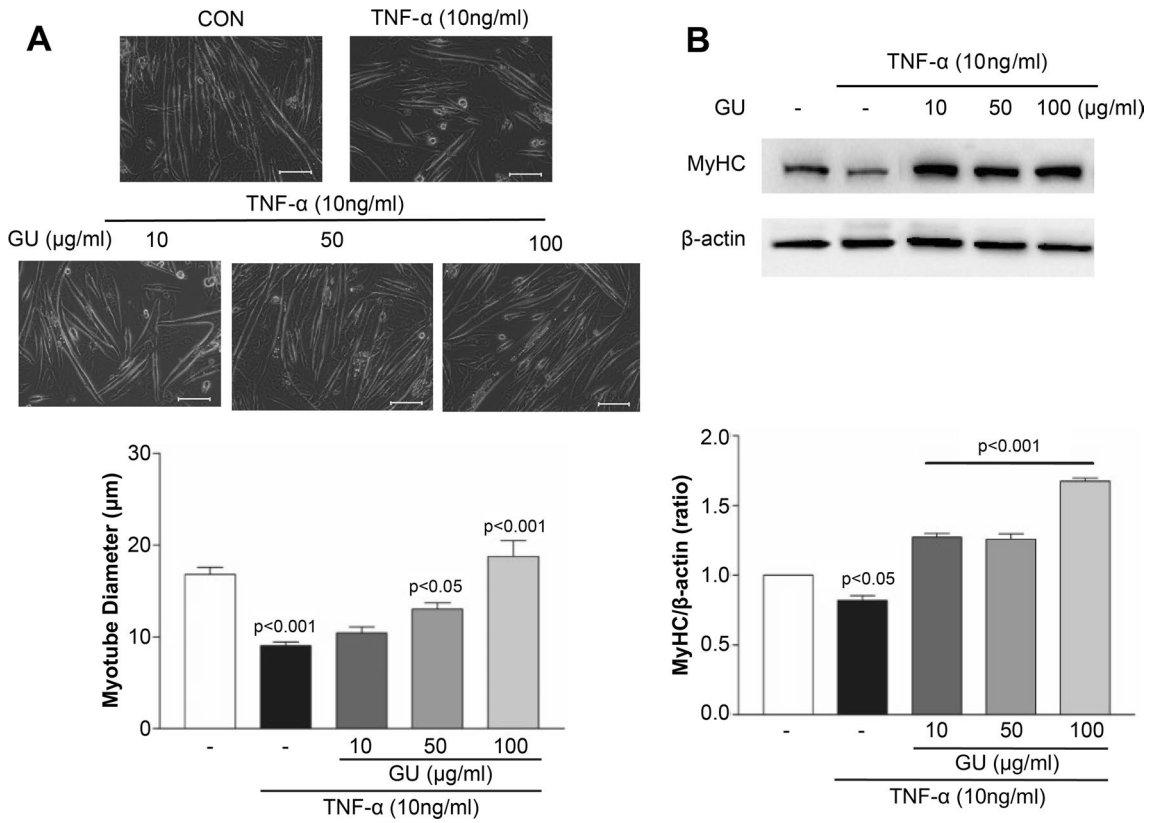


Fig. 2 Effect of *Glycyrrhiza uralensis* on myotube differentiation in tumor necrosis factor (TNF)-α-stimulated C2C12 cells. Cells were induced to differentiation in differentiation media (DM) for 5 days. GU was administrated alone for 24 h and then re-plated with TNF-α (10 ng/mL) in DM. After incubation for 1 h, myotube formation was quantified using a microscope (**A**) and the differentiation protein marker myosin heavy chain (**B**). Data are presented as the means ± standard error of the means (SEMs) of three independent experiments. Scale bar: 100 μm

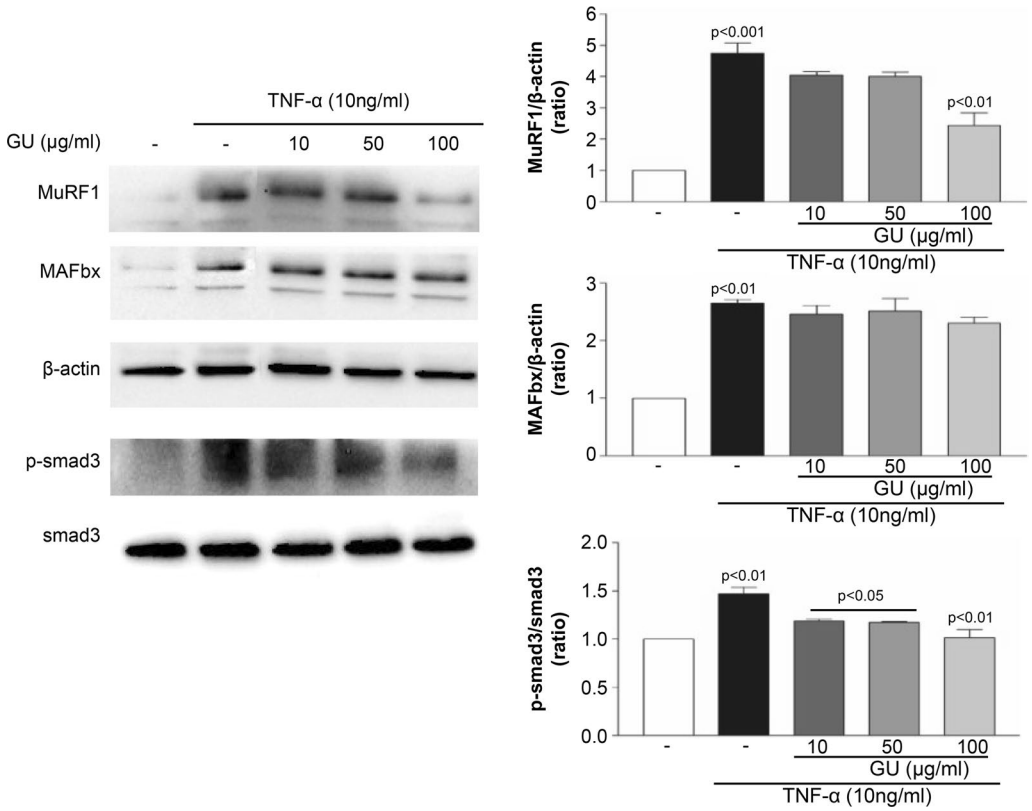


Fig. 3 *Glycyrrhiza uralensis* reduced protein levels of atrophy markers and E3 ligase in C2C12 myotubes. Myotubes were induced for 5 days and treated with TNF-α to establish a model of atrophy. C2C12 cells were treated in the presence or absence of TNF-α (10 ng/mL) and GU (10, 50, and 100 μg/mL) for 24 h. The expression levels of muscle atrophy F-box, muscle RING-finger protein-1, and Smad3 proteins were measured by western blot analysis. Data are presented as the means ± standard error of the means (SEMs) of three independent experiments

activates nuclear factor kappa B (NF-κB), which, in turn, translocates into the nucleus, exacerbating multiple diseases, including immune disorders and arthritis. In this study, we aimed to examine whether C2C12 cells regulate the intranuclear translocation of NF-κB through TNF-α. After treating C2C12 cells with TNF-α and different concentrations of GU, NF-κB protein expression was examined in nuclear and cytosolic cell fractions. The results showed that NF-κB expression in the nucleus was increased three fold by TNF-α, but reduced when treated with GU, at all concentrations (Fig. 4A). The NF-κB expression in the cytosolic fraction, which was reduced to half by TNF-α, was elevated by GU, confirming that GU regulates NF-κB (Fig. 4B).

C2C12 cells were treated with GU (10, 50, and 100 μg/mL) in the presence or absence of TNF-α (10 ng/mL), and cell lysates were prepared for western blot analysis. Levels of nuclear (A) or cytosolic (B) phospho-p65 were normalized to total expression of β-actin and lamin B, respectively. Representative band images from individual experiments are shown in the figure. Data are presented as the means ± standard error of the means (SEMs) of three independent experiments.

GU regulates ROS signaling in C2C12 cells

Nrf2 is a transcription factor for antioxidant genes. It has been reported to regulate the expression of antioxidant enzymes in response to the loss of muscle cells because of increased oxidative stress caused by aging [30]. Kelch-like ECH-associated protein 1 (KEAP1) is involved in the degradation of Nrf2 [31]. We treated C2C12 cells with 10, 50, and 100 μg/mL of GU extract and examined Nrf2 protein expression in the nuclear and cytosolic cell fractions (Fig. 5). The results indicated that Nrf2 expression in the nucleus increased in a dose-dependent manner by 1.5–2.5 times compared to the control group and decreased in the cytosolic fraction. HO-1 expression was upregulated in a dose-dependent manner by approximately 2–3 times in the GU-treated groups, and we speculate that Nrf2

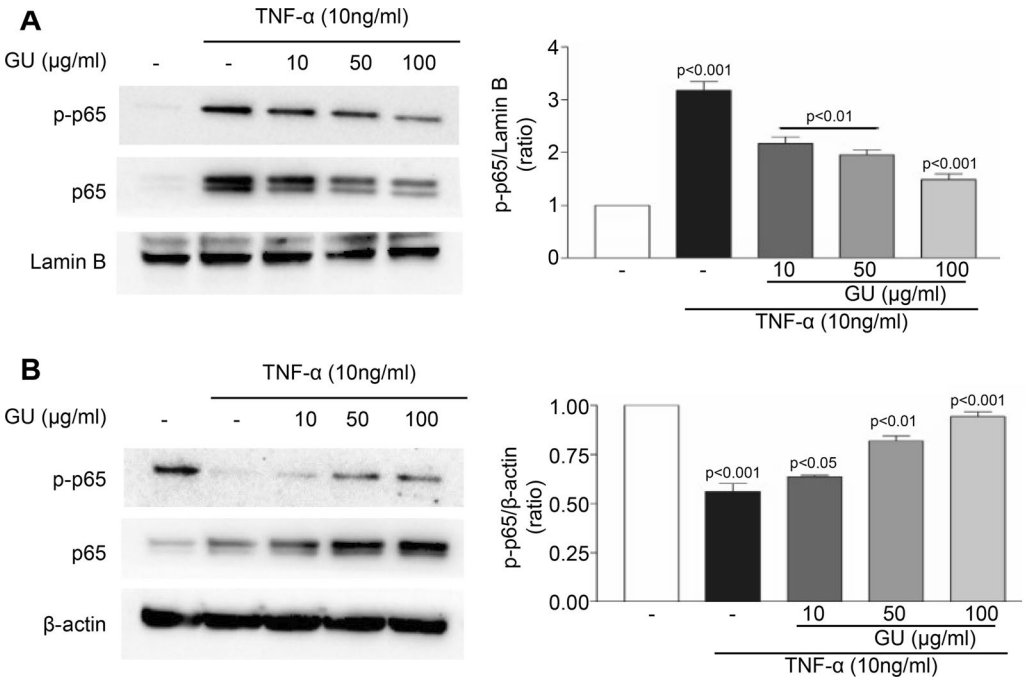


Fig. 4 *Glycyrrhiza uralensis* regulated inflammation-related kinases in atrophy-induced C2C12 cells. C2C12 cells were treated with GU (10, 50, and 100 μg/mL) in the presence or absence of TNF-α (10 ng/mL), and cell lysates were prepared for western blot analysis. Levels of nuclear (**A**) or cytosolic (**B**) phospho-p65 were normalized to total expression of β-actin and lamin B, respectively. Representative band images from individual experiments are shown in the figure. Data are presented as the means ± standard error of the means (SEMs) of three independent experiments

translocated into the nucleus bound to the antioxidant response element to regulate the expression of anti-inflammatory and antioxidant-related proteins, such as HO-1 (Fig. 5). These results suggest that GU suppresses muscle atrophy by regulating the oxidative stress induced by TNF-α.

Cells were incubated with GU (10, 50, and 100 μg/mL) for 30 min; total and nuclear proteins were then harvested from the cells. Cell lysates were prepared for western blot analysis. Nuclear Nrf2 expression in C2C12 cells was normalized to lamin B, and the expression levels of other proteins were normalized to β-actin. Data are presented as the means ± standard error of the means (SEMs) of three independent experiments.

GU protects against the apoptosis of myotubes

The expression and activity of caspases 3 and 7, the hallmarks of proapoptotic signaling, were measured using western blot and MUSE analysis. Cleaved caspase-3/7 expression increased in a dose-dependent manner by 1.3 times when treated with TNF-α and decreased with GU treatment; the reductions were comparable to the control group at concentrations of 50 and 100 μg/mL (Fig. 6A). We examined the phosphorylation of phosphoinositide 3-kinases (PI3K) and protein kinase B (Akt), which are involved in cellular apoptosis, leading to muscle atrophy. Phosphorylation increased in the control and GU-treated groups, showing that GU regulates TNF-α-induced apoptosis of muscle cells (Fig. 6A). Caspase-3/7 activities were examined using MUSE analysis. The results confirmed that caspase-3/7 activities were reduced in the GU-treated groups, similar to the results of protein expression (Fig. 6B).

C2C12 cells were plated onto 6-well plates at a density of 5×10^5 cells/well. After treatment with different concentrations (10, 50 and 100 μg/mL) of GU or 10 ng/mL of TNF-α for 12 or 24 h, the expression levels of cleaved caspase-3, -7, phosphoinositide 3-kinases (PI3K), phospho (p)-PI3K, protein kinase B (Akt), and p-Akt proteins were measured using western blot analysis (A). Each protein expression was normalized to total expression of β-actin and total form of PI3K and Akt. Caspase-3/7 activity was quantified by using MUSE analysis (B). Data are presented as the means ± standard error of the means (SEMs) of three independent experiments.

Discussion

The present study suggests that *G. uralensis* prevents skeletal muscle atrophy induced by inflammation or oxidative stress. We also examined the candidate mechanisms of muscle atrophy prevention by GU; it regulates inflammation-related proteins and antioxidant markers,

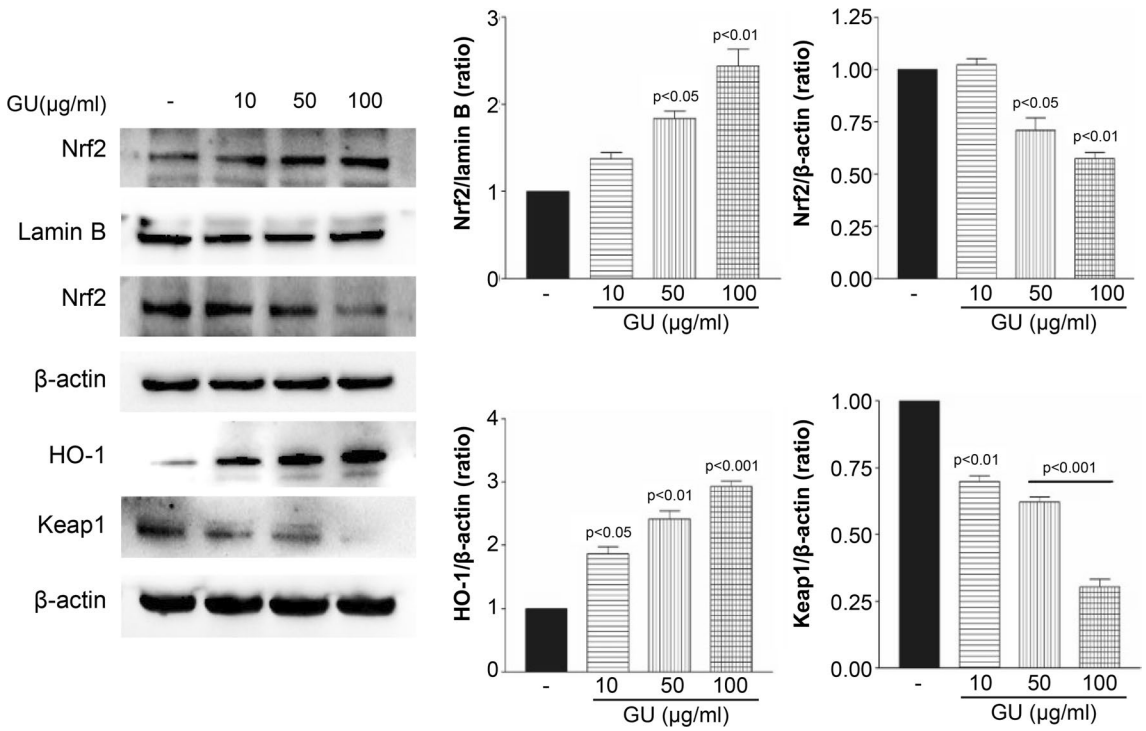


Fig. 5 *Glycyrrhiza uralensis* increased Nrf2 and heme oxygenase-1 protein levels in C2C12 cells. Cells were incubated with GU (10, 50, and 100 μg/mL) for 30 min; total and nuclear proteins were then harvested from the cells. Cell lysates were prepared for western blot analysis. Nuclear Nrf2 expression in C2C12 cells was normalized to lamin B, and the expression levels of other proteins were normalized to β-actin. Data are presented as the means ± standard error of the means (SEMs) of three independent experiments

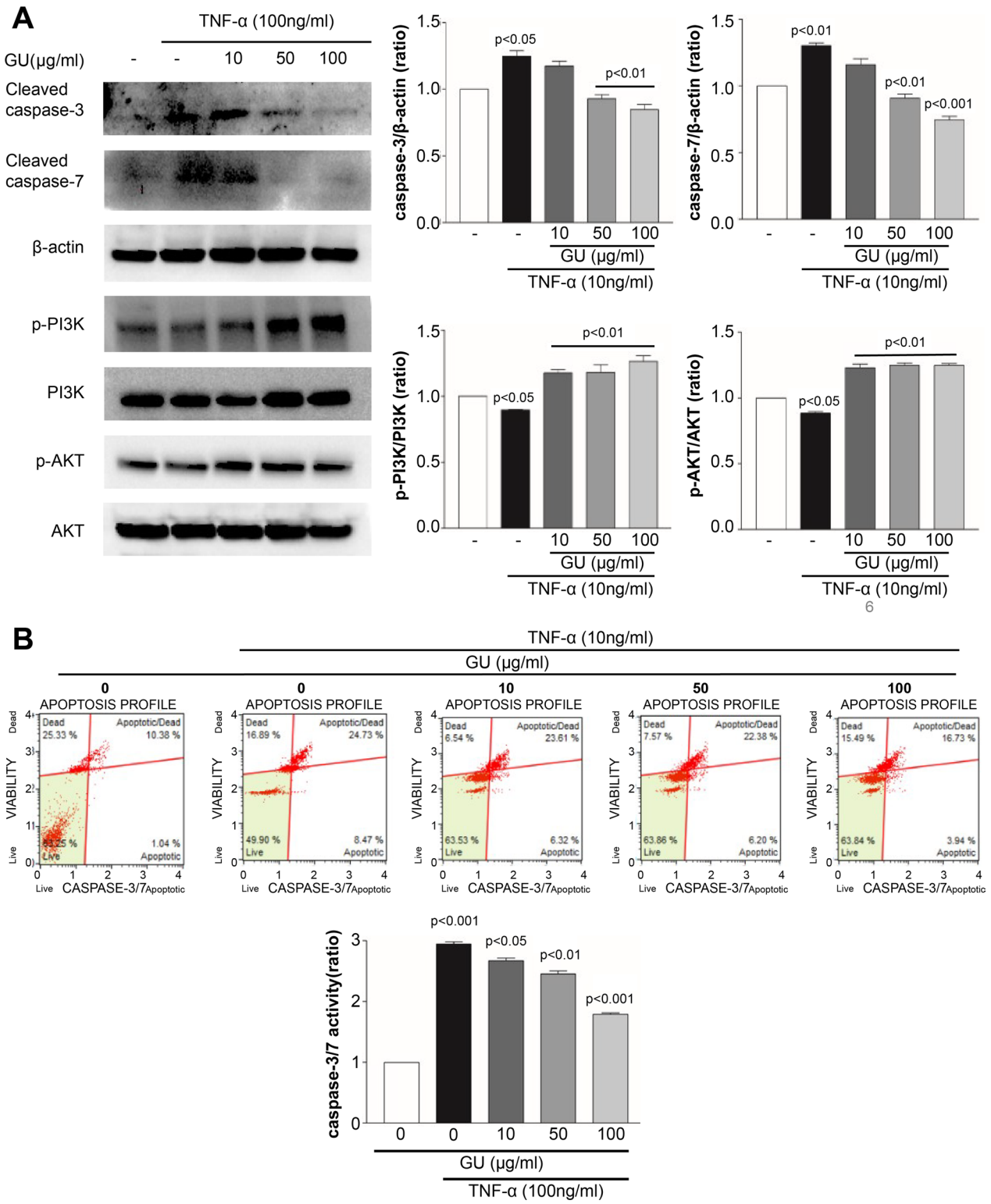
which accumulate in the aged or obese and protect muscle cells from apoptosis.

Muscle atrophy is caused by numerous factors. The most common cause is the accumulation of ROS and inflammatory reactions caused by aging. The persistence of these reactions leads to apoptosis. Inflammation and oxidative stress activate the ubiquitin–proteasome pathway by promoting ROS formation. This increases proteolysis and reduces myosin expression, which ultimately causes muscle atrophy [32–35]. C2C12 cells are a murine myoblast cell line derived from satellite cells [36]. They are commonly used as in vitro models for muscle regeneration because they can transition from a proliferative phase into differentiated myofibers. In this study, we established a TNF-α-induced inflammatory and oxidative muscle atrophy model. Differentiation was induced for five days using the differentiation media (DM). A group of cells was treated with both TNF-α and GU; this group showed lesser myotube injury than the group treated with TNF-α alone. GU is expected to prevent muscle injury and improve myotube differentiation by increasing the expression of muscle-specific MyHC protein. Previous studies have investigated the moderation of muscle atrophy by GU; however, no study has thus examined the biomarkers and mechanisms of muscle differentiation in muscle hypertrophy. Therefore, studies examining GU and relevant muscle differentiation mechanisms are needed.

Ubiquitin is a short peptide that can conjugate to specific substrates. Conjugation of ubiquitin to protein

(See figure on next page.)

Fig. 6 Protective effect of *Glycyrrhiza uralensis* on tumor necrosis factor (TNF)-α-induced C2C12 cell death. C2C12 cells were plated onto 6-well plates at a density of 5×10^5 cells/well. After treatment with different concentrations (10, 50 and 100 μg/mL) of GU or 10 ng/mL of TNF-α for 12 or 24 h, the expression levels of cleaved caspase-3, -7, phosphoinositide 3-kinases (PI3K), phospho (p)-PI3K, protein kinase B (Akt), and p-Akt proteins were measured using western blot analysis (**A**). Each protein expression was normalized to total expression of β-actin and total form of PI3K and Akt. Caspase-3/7 activity was quantified by using MUSE analysis (**B**). Data are presented as the means ± standard error of the means (SEMs) of three independent experiments



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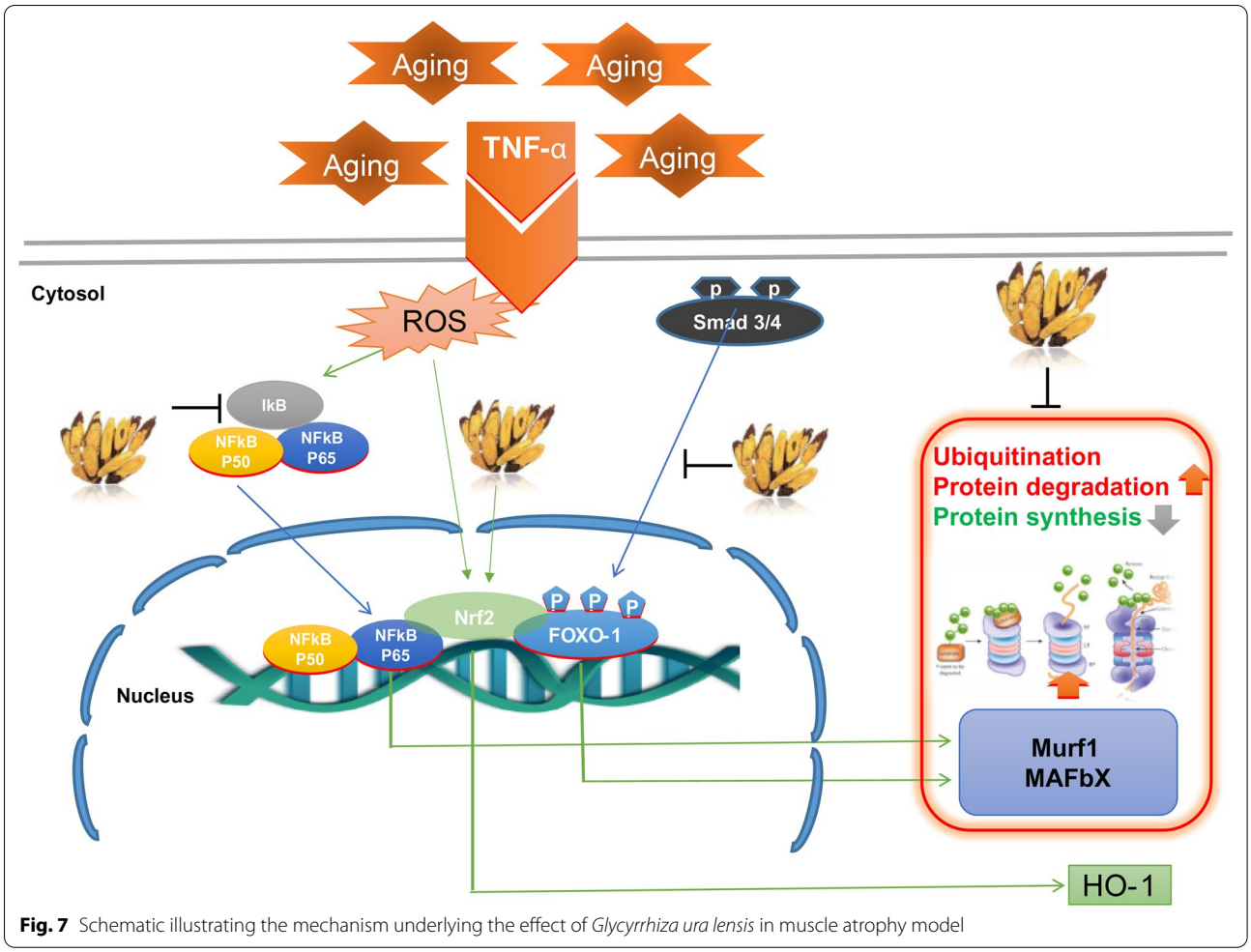

Fig. 7 Schematic illustrating the mechanism underlying the effect of *Glycyrrhiza ura lensis* in muscle atrophy model

occurs in a series of steps involving several enzymes: ubiquitin-activating enzyme (E1), ubiquitin carrier (E2), and ubiquitin-protein ligase (E3). Two of these are increased during muscle atrophy, MuRF1 and MAFbx or atrogin-1 [37, 38]. Isoflavones and phytochemicals, such as psoralen and resveratrol, have been derived from soybean and natural products, respectively. They have been reported to reduce the expression of MuRF1 and MAFbx in a TNF-α-induced muscle atrophy model [15, 39, 40]. Smad (Sma-and Mad-related proteins) 2/3 are downstream signaling molecules for transforming growth factor beta (TGF-β) and myostatin. The TGF-β/Smad3 pathway plays a negative role in regulating muscle mass. The activation of Samd3 promotes the expression of forkhead box protein O1 (FoxO1) and FoxO3, which leads to the expression of muscle atrophy markers [41]. In this study, GU suppressed muscle atrophy by regulating the expression of MuRF1 and MAFbx and decreasing smad3 phosphorylation. In a previous study, NF-κB was shown to augment the expression of several proteins of the ubiquitin–proteasome system that are involved in the degradation of muscle fibers during muscle atrophy. We postulated that NF-κB expression may be associated with MAFbx and MuRF1 expression, which involves a proteasome mechanism [12]. The inflammatory cytokine TNF-α directly hinders skeletal muscle atrophy through the NF-κB pathway [42]. In our examination following TNF-α treatment in the cytosolic and nuclear cell fractions, p-p65 expression reduced in a dose-dependent manner in the cytosolic fraction and increased in a dose-dependent manner in the nuclear fraction, as expected. Excess ROS stress can inhibit muscle protein synthesis and increase protein breakdown, which causes muscle atrophy [43]. ROS is related to the canonical activation of Nrf2, a transcription factor with an antioxidant mechanism that prevents binding to its repressor KEAP1. Moreover, recent studies have demonstrated Nrf2 expression in C2C12 cells [31]. In our study, GU increased Nrf2 expression, which was accompanied by an increase in HO-1 expression in the nuclear fraction. These results suggest that GU can regulate inflammatory and oxidative stress-induced muscle atrophy.

The early stage of apoptosis involves death-inducing signals, such as ROS and nitrogen species. Extrinsic or ligand-induced apoptosis through the TNF receptor superfamily causes the activation of caspase-8, an initiator caspase, and subsequently caspase-3 and caspase-7. This pathway has been demonstrated to be involved in muscle atrophy. Activated caspase-3 is involved in the induction of apoptosis in myotubes [44–46]. The PI3K/Akt signaling pathway has been reported to play a critical role in the inhibition of apoptosis [47]. In this study, we found that TNF-α suppressed PI3K and Akt phosphorylation, and GU restored the activation of PI3K/Akt. Yuan et al. reported that arsenic induces apoptosis in C2C12 myoblasts through ROS-induced mitochondrial dysfunction and inactivation of the Akt pathway. Panax ginseng recovered muscle atrophy through AMPK and PI3K signaling [48–50]. The effect of GU requires further study using specific inhibitors and other related mechanisms. (Fig. 7).

Abbreviations

GU: Glycyrrhiza uralensis; TNF: Tumor necrosis factor; Nrf2: Erythroid 2-related factor 2; IL: Interleukin; FA: Formic acid; DMSO: Dimethyl sulfoxide; DMEM: Dulbecco's Modified Eagle's Medium; FBS: Fetal bovine serum; HPLC: High-performance liquid chromatography; LC–MS: Liquid chromatography-mass spectroscopy; HPLC–ESI–MS: High-performance liquid chromatography electrospray ionization mass spectrometry; ACN: Acetonitrile; MyHCs: Myosin heavy chains; MAFbx: Muscle atrophy F-box; MuRF1: Muscle RING-finger protein-1; HO: Heme oxygenase; SEM: Standard error of the mean; KEAP1: Kelch-like ECH-associated protein 1; PI3K: Phosphoinositide 3-kinases; DM: Differentiation media; TGF-β: Transforming growth factor beta; NF-κB: Nuclear factor kappa B; ROS: Reactive oxygen species; E1: Ubiquitin-activating enzyme; E2: Ubiquitin carrier; E3: Ubiquitin-protein ligase.

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Institutional review board statement

Not applicable.

Authors' contributions

Conceptualization, JH; methodology, JWC, HHLL, GJY, IWC and SYC; software, JWC, HHLL, and GJY; validation, JWC and CHC; formal analysis, JWC and HHLL; investigation, JH, JWC, and SYC; data curation, JH, JWC, and CHC; writing—original draft preparation, JH, SYC, and JWC; writing—review and editing, JH, SYC, and JWC; visualization, CHC and GJY; supervision, JH. All authors read and approved the final manuscript.

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Not applicable.

Availability of data and materials

The data presented in this study are available from the corresponding authors on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

Informed consent

Not applicable.

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