Ginsenoside Rg1 prevents starvation-induced muscle protein degradation via regulation of AKT/mTOR/FoxO signaling in C2C12 myotubes

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Abstract. Skeletal muscle atrophy is often caused by catabolic conditions including fasting, disuse, aging and chronic diseases, such as chronic obstructive pulmonary disease. Atrophy occurs when the protein degradation rate exceeds the rate of protein synthesis. Therefore, maintaining a balance between the synthesis and degradation of protein in muscle cells is a major way to prevent skeletal muscle atrophy. Ginsenoside Rg1 (Rg1) is a primary active ingredient in *Panax ginseng*, which is considered to be one of the most valuable herbs in traditional Chinese medicine. In the current study, Rg1 was observed to inhibit the expression of MuRF-1 and atrogin-1 in C2C12 muscle cells in a starvation model. Rg1 also activated the phosphorylation of mammalian target of rapamycin (mTOR), protein kinase B (AKT), and forkhead transcription factor O, subtypes 1 and 3a. This phosphorylation was inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor. These data suggest that Rg1 may participate in the regulation of the balance between protein synthesis and degradation, and that the function of Rg1 is associated with the AKT/mTOR/FoxO signaling pathway.

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Introduction

Muscle atrophy is characterized by an increase in protein degradation and reduction in protein synthesis. It is associated with a number of human diseases and catabolic conditions, including fasting, disuse, aging, cancer, neuromuscular diseases, stroke, chronic obstructive pulmonary disease, chronic heart failure, HIV-acquired immunodeficiency syndrome and sepsis (1-5). Muscle volume shrinking and muscle weakness induced by muscular dystrophy typically disrupt and adversely affect the life of patients (3,6-8). Therefore, it is essential to understand the mechanism by which skeletal muscle atrophy is regulated.

A large amount of protein hydrolysis has previously been identified to occur during muscle atrophy and the ubiquitin-proteasome system (UPS) has been demonstrated to be involved in this process (9-14). In this system, the proteins are first conjugated into multiple molecules of ubiquitin. The 26S proteasome then recognizes and degrades the ubiquitinated proteins (15,16). Multiple enzymes regulate the protein ubiquitination; these include E1, the ubiquitin-activating enzyme, E2, the ubiquitin conjugating enzyme and E3 ubiquitin ligases (17,18). E3 ubiquitin ligases serve a major role in the specificity of protein degradation, because the specific binding between the protein substrate and E3 occurs prior to the reaction with ubiquitin (18,19).

It has been demonstrated that the insulin-signaling pathway is involved in the inhibition of UPS (20). In this pathway, the phosphorylation of insulin receptor substrate is stimulated when insulin binds its receptor. Phosphatidylinositol 3-kinase (PI3K), an intracellular intermediate, is recruited to phosphorylate a serine/threonine kinase, protein kinase B (AKT) during this process. AKT then phosphorylates the forkhead box class O transcription factors, subtype 1 and 3a (FoxO1 and FoxO3a), which prevents the translocation of these factors into the nucleus from the cytoplasm. Subsequently, the expression of two muscle-specific E3 ubiquitin ligases, muscle atrophy F-box (atrogin-1/MAFbx) and muscle ring finger protein (MuRF-1) is inhibited (21-23).

Accumulating evidence suggests that the PI3K/AKT-dependent signaling pathway of mammalian target of rapamycin

(mTOR) serves a key role in protein synthesis (24-26). Activation of mTOR enhances the activity of eukaryotic initiation factor 4E-binding protein 1 and ribosomal protein S6 kinase and, ultimately, increases protein synthesis (11,26-28).

One of the most valuable herbs in traditional Chinese medicine is Panax ginseng. The main bioactive components in *P. ginseng* are considered to be ginsenosides, a class of steroidal glycosides. The ginsenoside ingredients in *P. ginseng* vary, depending on the seasons, extraction methods and cultivating soils (29,30). Ginsenoside Rg1 is one of the main ingredients in *P. ginseng*. It has been demonstrated that ginsenoside Rg1 has an anti-inflammatory effect on human skeletal muscle during exercise (31). Yu *et al* (32) have also demonstrated that the antioxidant defense system against exercise-induced oxidative stress is strengthened by oral supplements of Rg1 in rat skeletal muscle. However, the function of Rg1 in the resistance to muscle atrophy remains unclear (33-38).

In the present study, Rg1 was observed to increase the viability of C2C12 muscle cells following starvation by inhibiting the expression of MuRF-1 and atrogin-1. PI3K dependent phosphorylation of AKT/FoxO and mTOR was involved in this process, indicating that Rg1 may resist muscle atrophy by balancing the protein degradation and protein synthesis pathways.

Materials and methods

Cell culture. C2C12 mouse myoblast cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 μg streptomycin and 100 units penicillin (all Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA). The cells were maintained in the growing medium at 37°C in a humidified 5% CO_2 atmosphere. At confluence, myoblasts were induced to fuse by changing the medium to medium supplemented with 2% horse serum (NQBB International Biological Corp., Hong Kong, China). The cells were maintained in 2% horse serum before the experiments. In starvation studies, the medium of the differentiated myotubes was replaced with serum-free medium for 48 h of incubation.

MTT cell activity assay. C2C12 cells were cultured in 96-well plates (5x10⁴ cells/plate) and were incubated for 24 h in DMEM containing 0.1% FBS. After differentiation of the C2C12 cell line to form myotubes, control group cells did not receive any further treatment in addition to the incubation in medium supplemented with horse serum medium for 48 h. Following treatment with Rg1 (10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mM; Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) for 48 h, the starvation model was induced by incubation of the cells with serum-free basal medium for 48 h. Myotubes were incubated with MTT solution (Invitrogen; Thermo Fisher Scientific, Inc.) for 4 h at 37°C. Non-reduced MTT was removed by aspiration and the formazan crystals were dissolved in dimethyl sulfoxide (150 μl/well) for 30 min at 37°C. The formazan was quantified using spectroscopy using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 490 nm.

Western blot analysis. To assess the expression and phosphorylation levels of AKT, mTOR, FoxO1 and Fox3a, and the expression of atrogin-1 and MuRF-1, C2C12 cells were cultured in 6-well plates (1x10⁶ cells/plate) after the differentiation of the C2C12 cell line to form myotubes. For the specific inhibitor experiments, following pretreatment with the PI3K inhibitor, LY294002 (Merck KGaA, Darmstadt, Germany), at 25 μM for 30 min, the myotubes were cultured in serum-free medium in the absence or presence of Rg1 (10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mM) for 5, 10, 30, 60 min and 24 h at 37°C in a humidified 5% CO_2 atmosphere. For immunoblotting, cells were washed with ice-cold phosphate-buffered saline twice. Subsequently, the cells were immersed in 1 ml precooled RIPA solution and PMSF (both Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was added into the RIPA solution, at a final concentration of 1 mM, for protein extraction. The pyrolysis liquid was transferred to an EP tube that was precooled on ice for 30 min. Following centrifugation at 13,000 x *g* for 10 min (4°C), the quantity of protein in the supernatants was detected using a bicinchoninic acid protein assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The lysates were collected 60 min later for western blot analysis.

The protein samples were separated by SDS-PAGE (6-8%) and were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The amount of protein used per lane was 20 μg. Following blocking with 5% non-fat milk for 1 h at room temperature, the membranes were incubated with primary antibodies specific for AKT (9272), mTOR (2972), FoxO1 (2880), FoxO3a (2497), phospho-AKT Ser473 (p-AKT; 4051), phospho-mTOR Ser2448 (p-mTOR; 2971), phospho-FoxO1 thr24 (p-FoxO1; 9464), phospho-FoxO3a thr32 (p-FoxO3a; 9464; all Cell Signaling Technology, Inc., Danvers, MA, USA), atrogin-1 (ab74023), MuRF-1 (ab172479; both Abcam, Shanghai, China) and β-actin (141205; ZSGB-BIO Technology Co. Ltd. Beijing China) overnight at 4°C. All primary antibodies were used at a dilution of 1:1,000. Blots underwent a total of three 8-min washes with Tris-buffered saline with 0.1% Tween-20 and were then incubated with a secondary, immunoglobulin G antibody (1:8,000; 14708; Cell Signaling Technology, Inc.) at room temperature for 1 h. The membranes were washed as described above and the bands were scanned using the Tanon 5500 fully automatic digital gel image analysis system (Tanon Science & Technology Co., Ltd., Shanghai, China).

Statistical analysis. All data are expressed as the mean ± standard deviation. Statistical analyses were performed using analysis of variance and Tukey's post hoc tests using Sigma-Plot version 10.0 software (Systat Software, Inc., San Jose, CA, USA) and $P<0.05$ was considered to represent a statistically significant difference.

Results

Ginsenoside Rg1 increases the viability of mouse C2C12 myoblast cells in the starvation model. The viability of myoblast C2C12 cells decreases when they are cultured during starvation (39). The results of the present study are consistent with this, as a reduction in the viability of the cells cultured in serum-free medium was observed compared with that in the
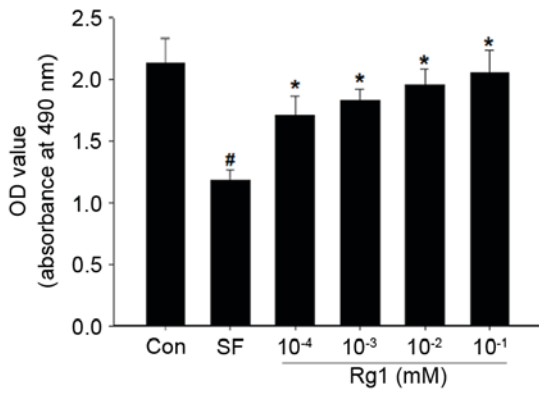
Figure 1. Effect of Rg1 on cell viability. On day 4, differentiated C2C12 myotubes were incubated for 48 h in serum-free Dulbecco's modified Eagle medium. Differences in their viability, following a MTT assay were measured vs. an untreated control. Treatment with 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} mM Rg1 for 48 h resulted in a dose-dependent enhancement of cell viability. Results are presented as mean ± standard deviation (n=5). #P<0.05 vs. the Con group; *P<0.05 vs. the SF group. Rg1, ginsenoside Rg1; OD, optical density; Con, control; SF, serum free.
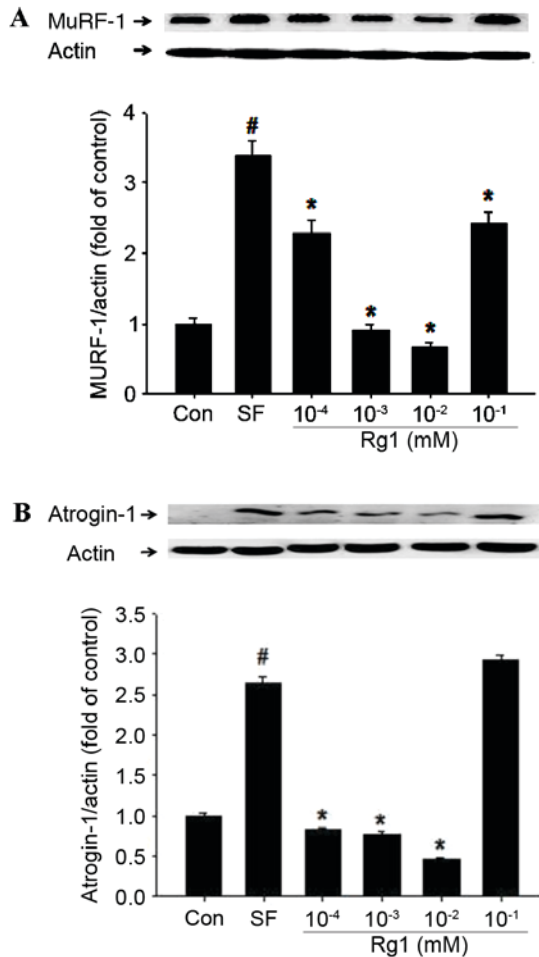

Figure 2. Effect of an increasing dose of Rg1 on the protein expression level of (A) MuRF1 and (B) atrogin-1 in serum-starved C2C12 cells. A significant dose-dependent suppressive effect on the expression of atrogin-1 and MuRF1 was observed following treatment with 10^{-4}, 10^{-3} and 10^{-2} mM Rg1. When 10^{-1} mM Rg1 was used, no significant effect on the expression of atrogin-1 observed. Results are presented as means ± standard deviation, (n=6 for each group). #P<0.05 vs. the Con group; *P<0.05 vs. the SF group by analysis of variance and Tukey's post hoc tests. Rg1, ginsenoside Rg1; MuRF1, muscle ring finger protein; atrogin-1, muscle atrophy F-box; Con, control; SF, serum free; Actin, β-actin.
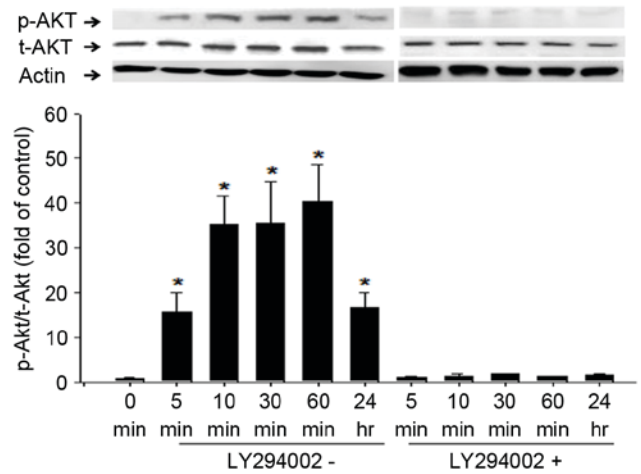

Figure 3. Rg1-induced phosphorylation of AKT in C2C12 cells over 24 h. Differentiated C2C12 cells were cultured with serum-free basal medium for 12 h prior to treatment with 10^{-4} mM Rg1 in the presence or absence of LY294002, a PI3K inhibitor. Cells were collected 5, 10, 30 and 60 min and 24 h later for western blot analysis of AKT phosphorylation at Ser473. The western blot analysis results are presented using representative images and the ratio of p-AKT to t-AKT levels at different time points is indicated using combined quantitative data. Results are presented as means ± standard deviation, (n=4 for each group). *P<0.01 vs. 0 min by analysis of variance and Tukey post hoc tests. AKT, protein kinase B; p-, phosphorylated; t-, total; Rg1, ginsenoside Rg1; PI3K, phosphatidylinositol 3-kinase; Ser, Serine; Actin, β-actin.

control group (Fig. 1). However, treatment with Rg1 increased the viability of the starved C2C12 cells in a dose-dependent manner. Treatment with 10^{-1} mM Rg1 restored the viability of cells to that of the cells in the normal medium. The results of the present study suggest that ginsenoside Rg1 promoted the viability of mouse myoblast cells in the starvation model.

Rg1 inhibits the expression of atrogin-1 and MuRF-1 in C2C12 cells in the starvation model. In order to assess how Rg1 increased the viability of C2C12 cells in the starvation model, the present study examined its effects on the expression of MuRF1 and atrogin-1 (Fig. 2). The results indicated that treatment with 10^{-2} mM Rg1 had the largest significant inhibitory effect on atrogin-1 and MuRF1 expression in C2C12 cells (P<0.05). However, significantly reduced expression was also observed in the 10^{-4} and 10^{-3} mM Rg1 treatment groups (P<0.05). The expression of atrogin-1 and MuRF1 was less strongly inhibited by treatment with 10^{-1} mM Rg1. Therefore, 10^{-2} mM was considered to be the optimal dose of Rg1 for inhibiting the expression of atrogin-1 and MuRF1 and increasing the viability of C2C12 cells in the starvation model.

Rg1 stimulates PI3K-dependent phosphorylation of AKT and FoxO in C2C12 cells in the starvation model. The inhibition of atrogin-1 and MuRF1 expression is primarily caused by the phosphorylation of the transcription factors, AKT and FoxO (40). The present study therefore assessed whether Rg1 is able to change the phosphorylation levels of AKT and FoxO. The cells were treated with Rg1 at various time points (0, 5, 10, 30, and 60 min and 24 h) for western blot analysis of targeted protein phosphorylations. Changes in cellular AKT phosphorylation appeared as early as 5 min and reached a peak at 60 min
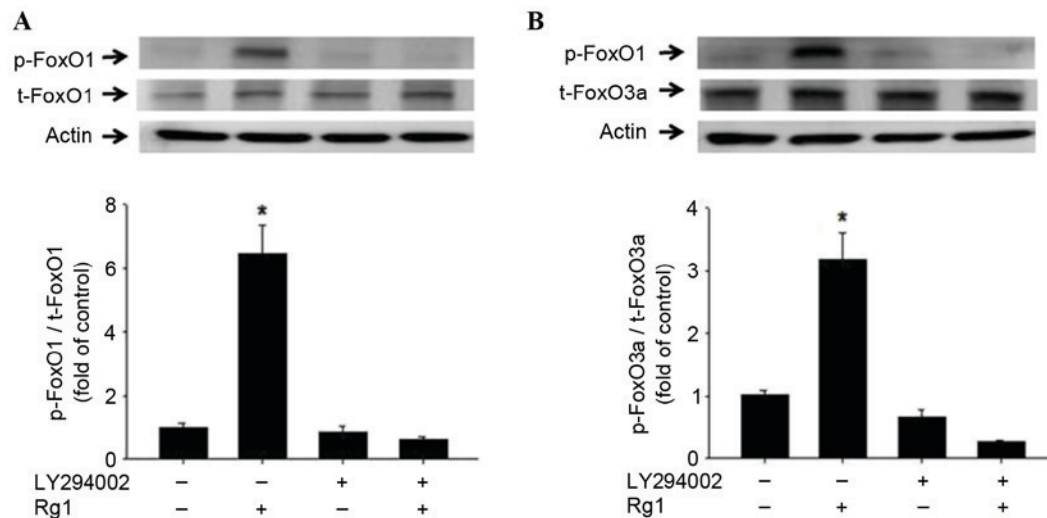
Figure 4. Phosphorylation of FoxO1 and FoxO3a in C2C12 cells following Rg1 treatment. Levels of (A) FoxO1 and (B) FoxO3a phosphorylation in cultured C2C12 cells, incubated in the presence of 10^{-2} mM Rg1 and/or the PI3K inhibitor LY294002. LY294002 was added 30 min prior to the addition of 10^{-2} mM Rg1. Western blot analysis results are presented using representative images and the p-FoxO1:t-FoxO1 and p-FoxO3a:t-FoxO3a ratios are demonstrated using combined quantitative data. Results are presented as the mean ± standard deviation of four experiments. *$P<0.05$ vs. Rg1 group by analysis of variance and Tukey's post hoc tests. FoxO1, forkhead transcription factor O, subtype 1; FoxO3a, forkhead transcription factor O, subtype 3a; Rg1, ginsenoside Rg1; PI3K, phosphatidylinositol 3-kinase; LY294002, inhibitor of PI3K; p-, phosphorylated; t-, total; Actin, β-actin.
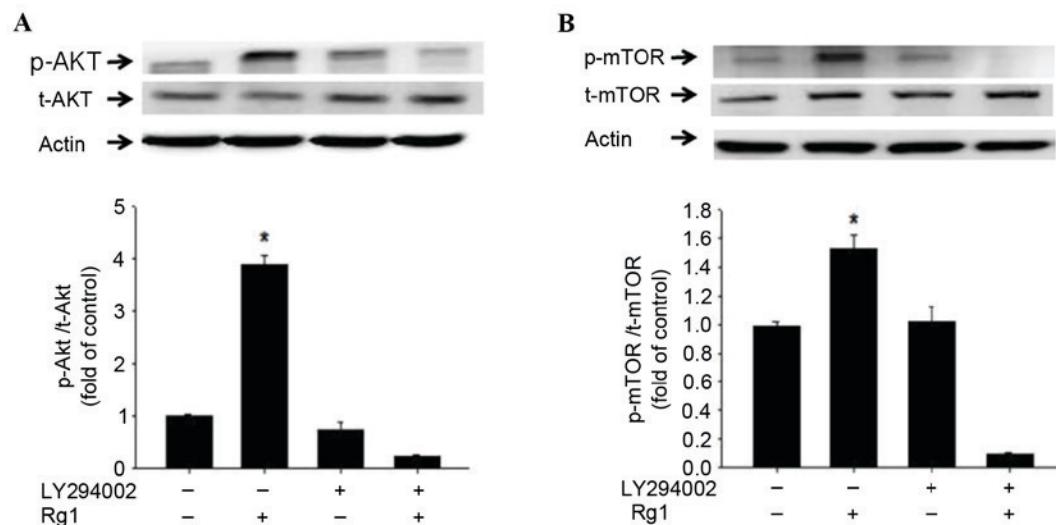

Figure 5. Effect of Rg1 on the phosphorylation of AKT and mTOR in C2C12 cells. Phosphorylation of (A) AKT and (B) mTOR following 12 h culture in serum-free basal medium. Cells were incubated in the presence of 10^{-2} mM Rg1 and/or the PI3K inhibitor LY294002. LY294002 was added 30 min prior to treatment with Rg1. The lysates were collected 60 min later for western blot analysis. Western blot analysis results are presented using representative images and the p-Akt:t-Akt and the p-mTOR:t-mTOR ratios are demonstrated using combined quantitative data. Results are presented as mean ± standard deviation (n=4 for each group). *$P<0.05$ vs. Rg1 group by analysis of variance and Tukey's post hoc tests. Rg1, ginsenoside Rg1; AKT, protein kinase B; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; LY294002, inhibitor of PI3K; p-, phosphorylated; t-, total; Actin, β-actin.

after the intervention before decreasing. PI3K inhibition by LY294002 completely abolished this Rg1-induced phosphorylation at the indicated time points. The results demonstrated that the phosphorylation levels of AKT, FoxO1 and FoxO3a were all increased following the treatment of C2C12 cells in serum-free medium with 10^{-2} mM Rg1, whereas the expression levels of AKT, FoxO1 and FoxO3a were not changed by treatment with Rg1 (Figs. 3 and 4).

The phosphorylation of AKT and FoxO is known to be regulated by PI3K (4,41). Therefore, the present study investigated the effects of LY294002, an inhibitor of PI3K. C2C12 cells in serum-free medium were treated with Rg1 and LY294002

individually and together. The Rg1-induced phosphorylation levels of AKT, FoxO1 and FoxO3a were significantly impaired by treatment with LY294002 (P<0.05; Figs. 3 and 4). These results indicate that Rg1 promoted the phosphorylation of AKT, FoxO1 and FoxO3a and was dependent on PI3K activity.

Rg1 activates the PI3K-dependent phosphorylation of mTOR in C2C12 cells of the starvation model. As a downstream molecule of AKT, mTOR serves a key role in protein synthesis (42-44). The results of the present study demonstrated that Rg1 upregulated the phosphorylation of mTOR in C2C12 cells in the starvation model. The PI3K inhibitor,

LY294002 was observed to inhibit the mTOR phosphorylation induced by Rg1 (Fig. 5). This indicates that Rg1 promoted the PI3K-dependent phosphorylation of mTOR in C2C12 cells in the starvation model.

Discussion

Rg1 has been indicated to have an effect on the function of human diseases via anti-inflammatory and antioxidant defense systems (45-55). The present study demonstrated that Rg1 increased the viability of muscle cells within a starvation model, by inhibiting protein degradation via the AKT/FoxO pathway and promoting protein synthesis by the mTOR pathway.

The viability of muscle cells is a key factor for resistance to muscle atrophy. When the rate of protein degradation exceeds the rate of protein synthesis in adult tissues, muscle atrophy occurs. The ubiquitin-proteasome pathway is one of most important protein degradation pathways, which is activated during muscle atrophy and contributes to the loss of muscle mass. The ubiquitin proteasome system is controlled by the modulation of rate-limiting enzyme expression in proteolytic systems, including atrogin-1/MAFbx and MuRF-1 (21,56-60).

Atrogin-1/MAFbx and MuRF-1 knockout mice have been demonstrated to be resistant to muscle atrophy induced by denervation (61). MuRF-1 knockout mice are also resistant to dexamethasone-induced muscle atrophy (62), while knockdown of atrogin-1 spares muscle mass in fasting animal models (63). Furthermore, MuRF1 ubiquitinates a number of structural proteins in the muscle including actin (64), myosin heavy chains (65,66), troponin I (67), myosin binding protein C and myosin light chains 1 and 2 (68). Atrogin-1 promotes degradation of MyoD, a key muscle transcription factor and eukaryotic translation initiation factor 3, subunit F, an important activator of protein synthesis (69,70). Therefore, high expression of atrogin-1/MAFbx and MuRF-1 may be crucial factors in muscle atrophy.

The insulin-AKT pathway negatively regulates FoxO transcription factors, which. were the first to be identified as critical for the process of atrophy (9). It has been indicated that hypertrophy may be induced in myotubes by the activation of protein synthesis through the AKT/mTOR pathway (21).

Cell apoptosis is closely related to muscle protein degradation in cells, and previous reports have indicated that apoptosis signaling is essential, and precedes protein degradation, in wasting skeletal muscle during catabolic conditions (40,71). These previous reports highlight that an apoptotic signal is necessary for the activation of skeletal muscle protein degradation. Activation of muscle protein hydrolysis resulting in muscle atrophy is a complex process, and it has been demonstrated that there are various mechanisms involved, including mechanisms related to cell apoptosis signaling molecules (40). A review article by Argilés *et al* (40) also demonstrated that the inhibition of apoptosis is able to inhibit protein degradation. In the condition of health, skeletal muscle protein metabolism, protein synthesis and protein decomposition does not require caspase-3 activated protein hydrolysis. In fact, an *in vitro* experiment demonstrated that using the specific compound,

Ac-DEVD-CHO (a caspase-3 inhibitor), did not inhibit the degradation of proteins of the basement membrane (71). However, in the case of catabolism, muscle fibers in excess protein degradation may use the above inhibitors to block the degradation of protein (71). This conclusion is also supported by experimental acute induced diabetes (71). In view of the above, under the condition of catabolism, excessive protein degradation is related to the activation of apoptotic protease caspase-3 (40). As previously demonstrated, the inhibition of apoptosis may provide a potential target for a drug for the treatment of muscular dystrophy (71). This will be the focus of future research.

In conclusion, the current study observed that Rg1 treatment has an inhibitory effect on Atrogin-1/MAFbx and MuRF1 translation via the activation of Akt, mTOR and FoxO phosphorylation, which prevents starvation-induced muscle cell death. The present study therefore provides a theoretical basis for the use of Rg1 to treat muscle atrophy in a clinical setting.

Acknowledgements

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