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Astragalus polysaccharide, a component of traditional Chinese medicine, inhibits muscle cell atrophy (cachexia) in an *in vivo* and *in vitro* rat model of chronic renal failure by activating the ubiquitin-proteasome pathway

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Abstract. The present study aimed to determine the effect of Astragalus polysaccharide (APS) in an *in vivo* and *in vitro* rat model of muscle atrophy (cachexia) caused by chronic renal failure (CRF), along with the potential corresponding roles of atroglin-1 and the ubiquitin-proteasome pathway. A rat model of CRF was established using subtotal bilateral nephrectomy. It was observed by reverse transcription-quantitative polymerase chain reaction and western blot analysis that APS and the specific inhibitor of nuclear factor (NF)-κB, pyrrolidine dithiocarbamate (PDTC), significantly reduced the expression of atrogin-1, ubiquitin and the NF-κB subunit p65 mRNA in rat skeletal muscle *in vivo* and *in vitro,* respectively (P<0.05). NF-κB and PDTC also markedly reduced the expression of atrogin-1, ubiquitin and p65 protein. In addition, cultured rat myoblasts pretreated with tumor necrosis factor (TNF)-α exhibited significantly reduced expression of atrogin-1, ubiquitin and p65 mRNA *in vitro* (P<0.05). Fluorescence microscopy was subsequently used to evaluate TNF-α-treated myoblasts administered with APS or PDTC, whereby no evidence of muscle cell atrophy was observed in cells treated with APS. These data suggest that APS may delay muscle cell atrophy associated with cachexia in CRF by targeting atrogin-1 and the ubiquitin-proteasome pathway.

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Key words: Astragalus polysaccharide, atrogin-1, cachexia, chronic renal failure, muscle atrophy, ubiquitin, ubiquitin-proteasome pathway

Introduction

Chronic renal disease, resulting in chronic renal failure (CRF), is associated with a number of systemic effects, including malnutrition and muscle wasting (cachexia) (1,2). CRF is becoming a major medical issue not only due to an increasing incidence of tumor malignancy, but also regarding the human and economic cost for health systems. CRF affects about 10% of the general adult population worldwide, and is complicated by sepsis and cardiovascular disease, mostly in parallel (2-5).

Treatment strategies for CRF include low protein diets and the use of ketoacid analogs, however their associated side effects, such as a probable increase in proteinuria and functional impairment (6), may lead to further renal tubular damage occurring, particularly in pregnant patients with severe eGFR reduction at baseline (7). In traditional Chinese medicine, *Huang qi* (*Radix Astragali seu Hedysari*) is used to treat CRF (8) and it has been previously demonstrated in a rat model of diabetic nephropathy that the primary active ingredient of *Huang qi,* Astragalus polysaccharide (APS), may improve renal function (9). However, the underlying mechanisms regarding the effects of APS in CRF remain unknown.

The ubiquitin-proteasome pathway (UPP) is the principal mechanism for protein catabolism within mammalian cells (10). The UPP pathway consists of three enzymatic components, E1, E2 and E3 ubiquitin-protein ligases, of which E3 ubiquitin-protein ligase is considered to be the key enzyme that is recruited to catalyze ubiquitin transfer to a substrate protein (10). In C57/BL6 mice with radiation-induced cell damage, it has been demonstrated that the UPP is activated in cachexia and that the nuclear factor (NF)-κB pathway is activated in the kidneys and cachexic muscle tissue (10,11).

It has been demonstrated that expression of atrogin-1, a key muscle-specific ligase, increase during muscle atrophy and in the early stages of CRF when renal cell atrophy occurs (12). In addition, previous results in mice have indicated that reduced levels of atrogin-1 may confer resistance to muscle

atrophy following muscle denervation (13). However, the roles of atrogin-1 and its regulatory pathways in cachexia remain unknown.

The present study aimed to evaluate the effects of APS on muscle cell atrophy in a rat model of CRF *in vivo* and *in vitro*, principally by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The current study also investigated the potential corresponding roles of atroglin-1 and the UPP.

Materials and methods

Reagents. The ketoacid analog ketosteril (KT; as compound α-ketoacid tablets) was purchased from Fresenius Kabi Asia-Pacific, Ltd. (Hong Kong SAR, China). APS, tumor necrosis factor (TNF)-α and the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Other common laboratory reagents were purchased as reagent-grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Animal experiments. A total of 32 male Sprague-Dawley rats (7-8 weeks old, 250-300 g) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The animal experiments in the current study were approved by the Shanghai Animal Care and Use Committee, Shanghai Institute of Materia Medica, Chinese Academy of Sciences [Certificate number, SCXK (Shanghai) 2002-0010; Shanghai, China]. Rats were housed at a temperature of 22±1˚C and humidity of 55±5%. They were fed once a day and housed under a 12 h light/dark cycle with free access to water. Animals fasted for 12 h before sacrifice.

After a 3-day adaptation period, rats were divided into four groups (n=8 rats in each group). Three groups underwent 5/6 subtotal nephrectomy to establish a rat model of CRF-induced cachexia, as previously described (14). Of the three CRF rat groups, one group received treatment with APS (intraperitoneally, 3 g/kg/day) for 6 weeks and one group received treatment with KT (intravenously, 0.14 g/ml suspension, 1 ml/200 g/day) for 4 weeks. The third group received treatment with saline solution (intraperitoneally, 3 g/kg/day). Rats in the fourth group acted as a negative control without any renal damage but receiving saline solution (intraperitoneally, 3 g/kg/day). Following treatments for 6 weeks, femur skeletal muscle tissues from mice under sodium pentobarbital anesthesia (intraperitoneal, 40 mg/kg) were collected and stored at -80˚C until use for further biochemistry analysis.

Malnutrition (cachexia) model in rat L6 myoblasts. Rat L6 myoblasts were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (cat. no. 15240096; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. An L6 muscle cell malnutrition model was induced by pretreatment with TNF-α (10 ng/ml) for 24 h at 37˚C, as described previously (15).

After washing twice with PBS solution (0.01 M) twice for 2 min, $1.2x10^6$ L6 cells were treated with APS (15 mg/l) or PDTC (50 μmol/l) or PBS as a control for 48 h following induction of malnutrition at 37˚C. Ten randomly selected fields were observed using an inverted microscope (Olympus Corporation, Tokyo, Japan) and the transaction diameter values of all four groups were measured prior to cell harvesting, as previously described (16).

Small interfering (si)-RNA knockdown experiments in vitro. L6 myoblasts treated with APS or PDTC, or PBS as a control, as described above, were seeded into 6-well plates in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic solution (cat. no. 15240096; Invitrogen; Thermo Fisher Scientific, Inc.) at a density of $3x10^5$ cells/well and incubated for 1 day at 37˚C. Cells in each well were then transfected with a full-length atrogin-1-siRNA (5'-CTACGTAGTAAGGCTG TTG-3') transfection mix (100 μl Lipofectamine 2000 in a final volume of 1 ml cell medium; Shanghai GeneChem Co., Ltd., Shanghai, China) for 72 h at 37˚C, according to the manufacturer's protocol, and incubated for 72 h at 37˚C. All knockdown experiments were performed in triplicate.

RNA isolation and RT-qPCR. Total RNA from tissues was isolated using a UNIQ-10 column and a TRIzol total RNA isolation kit (both from Sangon Biotech Co., Ltd., Shanghai, China) after fully grinding on ice for 10 min. Total RNA from cells was also isolated using a UNIQ-10 column and a Trizol total RNA isolation kit. Reverse transcription was performed using 1 μg total RNA in a reaction volume of 20 μl with cloned AMV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 2 μl cDNA was used for qPCR using a Takara Ex Taq RT-PCR Version 2.1 kit (Takara Bio, Inc., Otsu, Japan). The following gene-specific PCR primers (Sangon Biotech Co., Ltd.) were used: atrogin-1 were forward, 5'-AGCTTGTGCGATGTTACCA-3' and reverse, 5'-GGTGA AAGTGAGACGGAGCA-3'; ubiquitin were forward, 5'-TCA GATGTGGAGAAAGGAGGG-3' and reverse, 5'-GTTGAG CCGGCTGAGTTGAT-3'; β-actin were forward, 5'-CATTG CTGACAGGATGCAG-3' and reverse, 5'-CTGCTGGAA GGTGGACAGTGA-3'. PCR signals were detected with a DNA Engine Opticon® 2 Continuous Fluorescence Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). PCR conditions were as follows: An annealing temperature of 60˚C, followed by 40 cycles of 94˚C for 20 sec, 58˚C for 20 sec and 72˚C for 20 sec. Melt curve analysis and electrophoresis in 2% agar were performed in three replicates to evaluate the purity of PCR products. Negative control reactions (no template DNA) were included to monitor potential contamination of reagents. Relative amounts of atrogin-1 and ubiquitin mRNA were normalized to that of β-actin using the $2^{-\Delta\Delta Cq}$ method (17).

Protein isolation and western blot analysis. The concentrations of the protein extracts obtained from rat skeletal muscle tissue and L6 cells were determined using a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Protein lysates (30 μg) were then separated on a 10% SDS-PAGE gel followed by transfer

to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Western blot analysis was performed according to a standard protocol (18) using primary antibodies against atrogin-1 (cat. no. ab74023, 1:10,000; Abcam, Cambridge, UK), ubiquitin (cat. no. sc-4316, 1:1,000) and NF-κB subunit p65 (cat. no. c-20, 1:2,000; both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and GAPDH (cat. no. A3853, 1:10,000; Sigma-Aldrich; Merck KGaA). Horseradish peroxidase conjugated mouse anti-rabbit IgG (sc-2357, 1:5,000; Santa Cruz Biotechnology, Inc.) was used as a secondary antibody. Resulting protein signals were detected using an enhanced chemiluminescence system (EMD Millipore, Billerica, MA, USA) and data was analyzed using the Stata 7.0 software package (StataCorp LLC, College Station, TX, USA). Three replicates were performed for each experiment.

Statistical analysis. Data are expressed as the mean ± standard deviation. Differences between groups were assessed using the Student's t-test and one-way analysis of variance followed by the Tukey's post hoc test in SPSS 19.0 (IBM SPSS, Armonk, NY, USA). Differences were considered to be statistically significant when P<0.05.

Results

APS reduces atrogin-1 and ubiquitin expression in vivo. Western blot analysis indicated that expression of atrogin-1 and ubiquitin protein was markedly decreased in CRF rats treated with KT (intravenously, 0.14 g/ml suspension; 1 ml/200 g/day) or APS (intraperitoneally, 3 g/kg/day), compared with untreated CRF rats (Fig. 1A). Results from RT-qPCR indicated that levels of atrogin-1 (Fig. 1B) and ubiquitin (Fig. 1C) mRNA in CRF rats treated with KT were significantly decreased relative to untreated CRF rats (P<0.05). Treatment with APS also reversed the rise in astrogin-1 and ubiquitin protein expression (Fig. 1A), and significantly reversed the elevated levels of atrogin-1 and ubiquitin (both P<0.05; Fig. 1B and C) in CRF rats. In comparison to the normal control group, CRF rats treated with KT or APS, exhibited significantly increased expression of atrogin-1 and ubiquitin mRNA (P<0.05; Fig. 1B and C).

APS reduces atrogin-1 and ubiquitin expression in vitro. A state of cell malnutrition (cachexia) was established *in vitro* by pretreating rat L6 myoblasts with TNF-α (10 ng/ml). Atrogin-1-siRNA was also used to inhibit atrogin-1 expression. Efficiency of atrogin-1-siRNA transfection has been confirmed by western blot analysis and RT-qPCR (data not shown). Results indicated that the elevated levels of atrogin-1 and ubiquitin observed in TNF-α treated L6 myoblasts were reversed following administration of APS (Fig. 2A). NF-κB subunit p65 was also measured, and a lower level of protein expression was observed in L6 myoblasts following TNF-α + APS and TNF-α + atrogin-1-siRNA treatment compared with TNF-α treatment alone (Fig. 2A). Furthermore, RT-qPCR demonstrated that the elevated levels of atrogin-1 (Fig. 2B) and ubiquitin (Fig. 2C) mRNA induced by TNF-α were significantly reversed 48 h following administration of APS (P<0.05). In comparison to the normal control group, TNF-α + APS treated L6 myoblasts and TNF-α + atrogin-1-siRNA-treated L6 myoblasts exhibited significantly increased expression of atrogin-1 and ubiquitin mRNA (P<0.05; Fig. 2B and C).

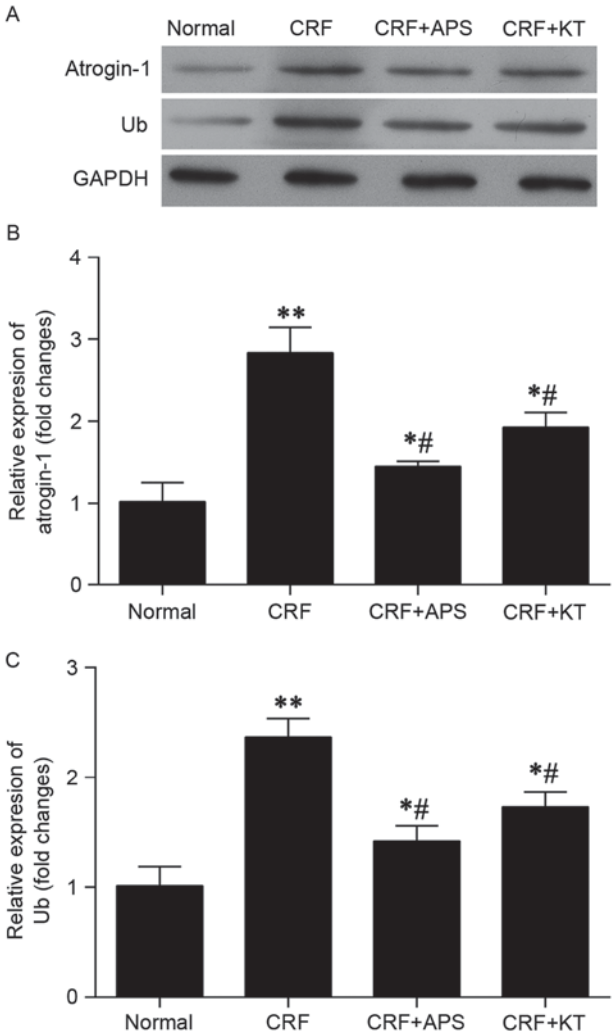

Figure 1. APS decreased the mRNA and protein expression of atrogin-1 and Ub in rat skeletal muscle cells. (A) Protein expression of atrogin-1 and Ub. Expression levels normalized to that of GAPDH. In CRF rats, APS treatment reversed upregulation in atrogin-1 and Ub. Treatment with KT had similar effects. Levels of (B) atrogin-1 and (C) Ub mRNA were determined by reverse transcription-quantitative polymerase chain reaction and normalized to β-actin expression. In CRF rats, APS significantly reduced the elevated expression of atrogin-1 and Ub mRNA. Data are expressed as the mean ± standard deviation (n=8). *P<0.05, **P<0.01 vs. normal (non-CRF) group; #P<0.05 vs. CRF group. APS, Astragalus polysaccharide; CRF, chronic renal failure; Ub, ubiquitin; KT, ketosteril.

APS inhibits cell atrophy in vitro. It was observed by fluorescence microscopy that L6 myoblasts treated with TNF-α alone were atrophic, while cell sizes in the TNF-α + APS treatment group and TNF-α + atrogin-1-siRNA appeared unaffected, compared with normal control cells (Fig. 3A). In addition, relative to the TNF-α treatment group, the transverse diameters of the L6 myoblasts in the APS + TNF-α and atrogin-1-siRNA + TNF-α groups were significantly increased (P<0.05; Fig. 3B). TNF-α + APS treated L6 myoblasts and atrogin-1-siRNA + TNF-α treated L6 myoblasts had significantly decreased transverse diameters compared with the control group, ~70 and 90% of the control transverse diameter, respectively (both P<0.05; Fig. 3B).

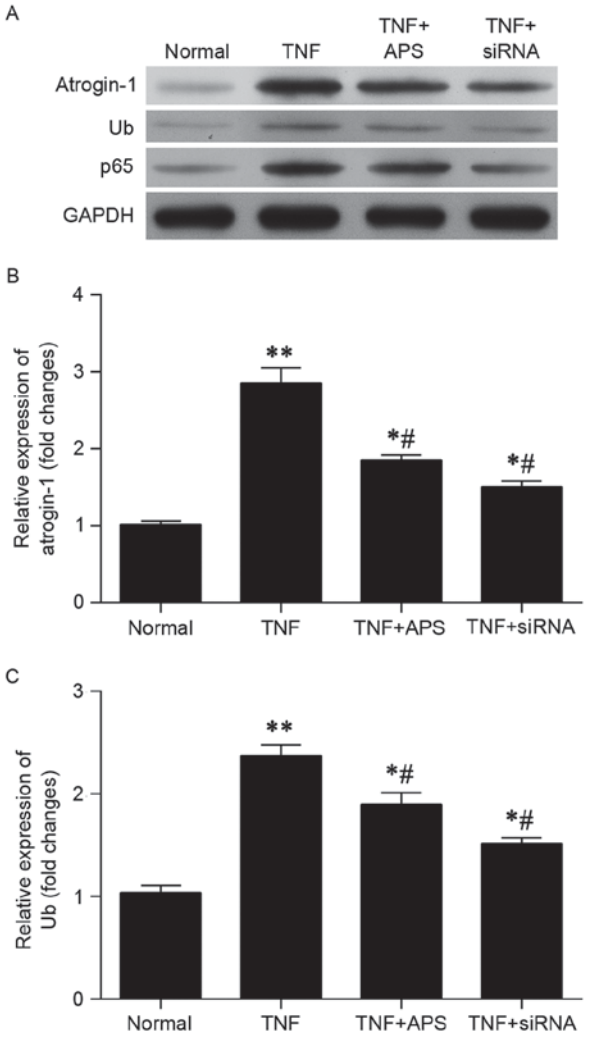

Figure 2. APS decreased the mRNA and protein expression of atrogin-1 and Ub in rat L6 myoblasts. (A) Protein expression of atrogin-1 and Ub. Expression levels normalized to that of GAPDH. In malnourished (TNF-α-treated) L6 myoblasts, APS treatment reversed upregulation of atrogin-1, Ub and p65. Levels of (B) atrogin-1 and (C) Ub mRNA were determined by reverse transcription-quantitative polymerase chain reaction and normalized to the expression of β-actin. APS significantly reduced the elevated expression of atrogin-1 and Ub mRNA induced by TNF-α. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. normal group; $^{\#}$P<0.05 vs. TNF-α group. APS, Astragalus polysaccharide; Ub, ubiquitin; TNF-α, tumor necrosis factor-α; siRNA, small interfering RNA; p65, NF-κB subunit.

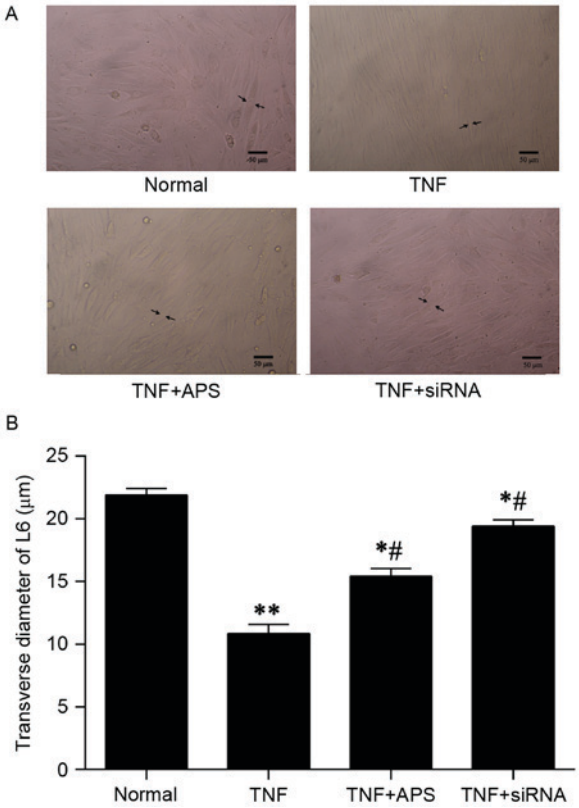

Figure 3. APS reduced muscle cell atrophy induced by TNF-α-mediated malnutrition (cachexia). (A) Relative to normal control cells, rat L7 myoblasts treated with TNF-α alone were atrophic, while myoblasts in the APS + TNF-α group were not reduced in size. Arrows indicate the region used for diameter measurement. Scale bars, 50 μm. (B) Relative to the TNF-α group, the cell transverse diameters in the APS + TNF-α and atrogin-1-siRNA + TNF-α groups were significantly increased. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. normal group; $^{\#}$P<0.05 vs. TNF-α group. APS, Astragalus polysaccharide; TNF-α, tumor necrosis factor-α; siRNA, small interfering RNA.

PDTC reduces atrogin-1 and ubiquitin expression in vitro. At the protein level, it was observed that the elevated levels of atrogin-1, ubiquitin and p65 induced by TNF-α were markedly reduced by PDTC (Fig. 4A). Similarly, analysis of mRNA expression indicated that upregulation of atrogin-1 (Fig. 4B) and ubiquitin (Fig. 4C) mediated by TNF-α was significantly inhibited 48 h following administration of PDTC. In comparison with the normal control group, the TNF + PDTF group exhibited significantly increased expression of atrogin-1 and ubiquitin mRNA (both P<0.05).

PDTC prevents cell atrophy in vitro. Inverted fluorescent microscopy demonstrated that L6 rat myoblasts treated with TNF-α alone were atrophic compared with normal control cells, while cell sizes in the TNF-α + PDTC treatment group appeared unaffected (Fig. 5A). In addition, relative to the TNF-α treatment group, the transverse diameters of the L6 myoblasts in the PDTC + TNF-α group were significantly increased. In comparison with the normal control group, TNF+PDTF group had a significantly reduced transverse diameter (~90% relative to control group; P<0.05; Fig. 5B).

Discussion

The results of the current study indicated that APS, a component of traditional Chinese medicine, may protect muscle cells *in vivo* and *in vitro* from atrophy associated with CRF (cachexia). Other studies have reported that traditional Chinese medicine have beneficial effect on treating CRF (19,20). Li *et al* (21) reported that icariin-treated human umbilical cord mesenchymal stem cells could improve kidney function via reduced inflammatory responses and oxidative damage in CRF rats. Zhang *et al* (22) indicated that Shenkang granules ameliorate renal injury in a rat model of CRF through preventing the accumulation of extracellular matrix, by decreasing the expression of collagen I and III and inhibiting the expression of matrix metalloproteinases-2 and -9 in the renal tissue. In the present study, it was observed that APS reduced the expression of atrogin-1 and ubiquitin *in vivo* and reversed muscle cell atrophy following TNF-α pretreatment, while the NF-κB inhibitor

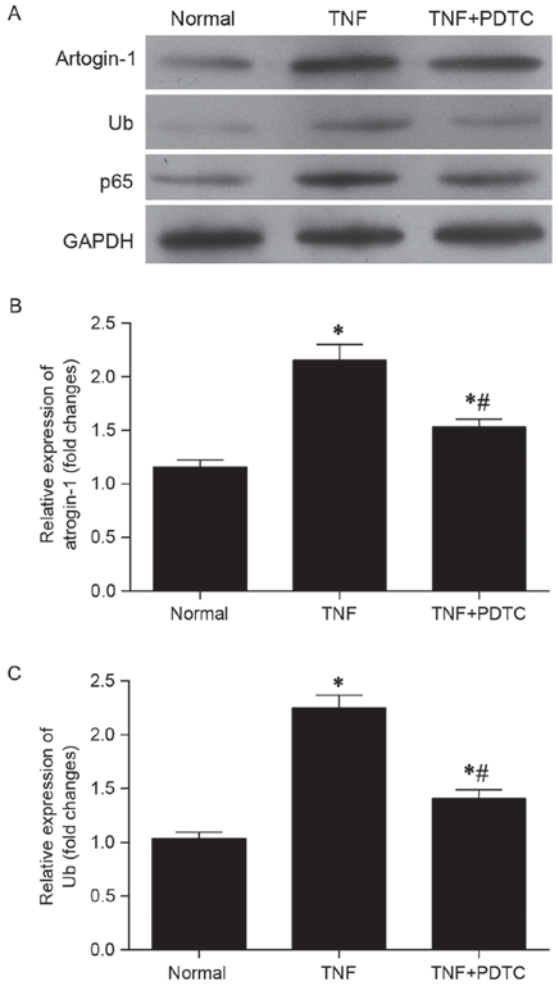

Figure 4. PDTC treatment reduced the expression of atrogin-1, Ub and p65 in rat L6 myoblasts *in vitro*. (A) At the protein level, PDTC treatment decreased upregulation of atrogin-1, Ub and p65 induced by TNF-α pretreatment. At the mRNA level, upregulation of (B) atrogin-1 and (C) Ub following TNF-α pretreatment was inhibited by PDTC. Data are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. normal group; $^{\#}$P<0.05 vs. TNF-α group. PDTC, pyrrolidine dithiocarbamate; TNF-α, tumor necrosis factor-α; Ub, ubiquitin; p65, NF-κB subunit.

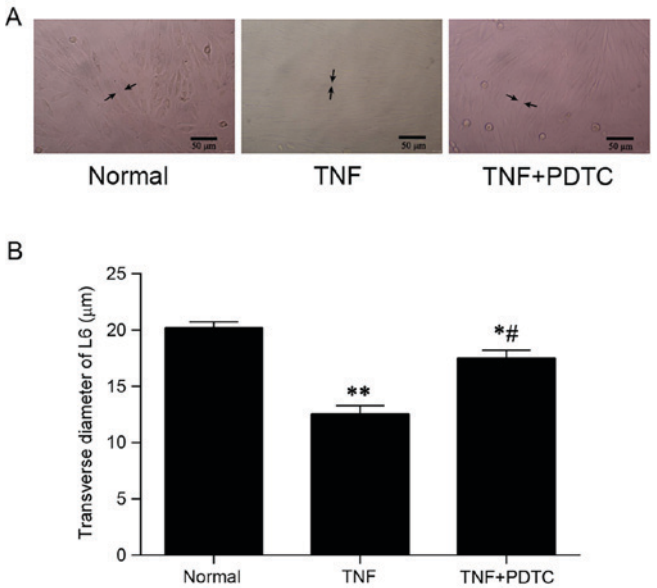

Figure 5. PDTC reduced muscle cell atrophy induced by TNF-α-mediated malnutrition (cachexia). (A) Relative to control cells, rat L7 myoblasts treated with TNF-α alone were atrophic, while myoblasts in the PDTC + TNF-α group were not reduced in size. Arrows indicate the region used for diameter measurement. Scale bars, 50 μm. (B) Relative to the TNF-α group, the cell transverse diameters in the PDTC + TNF-α group were significantly increased. Data are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. normal group; $^{\#}$P<0.05 vs. TNF-α group; PDTC, pyrrolidine dithiocarbamate; TNF-α, tumor necrosis factor-α.

PDTC had similar effects *in vitro*. These results suggest that APS may target atrogin-1 through inhibitory effects on the NF-κB pathway, leading to reductions in ubiquitin expression and reduced muscle cell atrophy. In addition, compared with other investigations of traditional Chinese medicine in CRF, the protective effect was via a different mechanism, suggesting a combined therapeutic strategy for CRF may be effective.

Previous results support the use of the subtotal nephrectomy CRF rat model, in which an impairment of glomerular filtration rate and disturbances in calcium and phosphate metabolism have been observed (23). Clinical research has also indicated that cell atrophy is prevalent in patients with CRF and is associated with the progression of renal failure (1,2). One of the classic treatments for CRF is the use of ketoacid analogs, and the current study compared the administration of APS and the use of ketoacid analog KT. A similar protective effect was observed in both groups, supporting the proposal that APS may be used to treat CRF.

CRF is often associated with cachexia. It has been demonstrated that serum obtained from patients with CRF activates the UPP by a currently unknown mechanism (10,24). The UPP and protein degradation is the primary mechanism by which NF-κB signaling is regulated within skeletal muscle during CRF (10,25,26). Furthermore, recent *in vitro* studies, animal models and human studies have indicated that upregulation of NF-κB has a pathogenic role in mediating chronic inflammation during chronic renal disease (25,27). In the present study, a state of cell malnutrition (cachexia) was established by pretreating rat L6 myoblasts with TNF-α. The results indicated that APS protected cells from atrophy related to CRF (cachexia) via reducing the expression of atrogin-1 and ubiquitin. This effect was similar to direct inhibition of atrogin-1 using atrogin-1 siRNA.

Of particular relevance to the present study are the recent findings that a combination of APS and another traditional Chinese medicine, rhein, may alleviate the pathologies of CRF, including functional damage to the glomeruli, interstitial inflammation and the apoptosis of renal tubular cells (8).

Further clinical studies are warranted to confirm the preliminary findings obtained from the *in vitro* and *in vivo* models in the current study. In future studies, the limitations of the present study need to be resolved, including side-effect and dose-dependency evaluation, as well as time point studies. It may also be useful to identify and develop components of the UPP as serological markers in CRF patients, particularly those being treated with traditional Chinese medicines such as APS, since these will be important for developing therapeutic strategies for patients with CRF.

In conclusion, APS may delay the progression of muscle cell atrophy associated with malnutrition in CRF, possibly by targeting the UPP and its downstream effector atrogin-1.

Acknowledgements

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