Stability of F-box Protein Atrogin-1 is Regulated by p38 Mitogen-activated Protein Kinase Pathway in Cardiac H9c2 Cells

Jun-Jie Li¹, Tian-Peng Zhang¹, Yan Meng², Jie Du³ and Hui-Hua Li²

¹Department of Pathology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, ²The Key Laboratory of Remodeling-related Cardiovascular Diseases, Department of Pathology, School of Basic Medical Sciences, Capital Medical University, Beijing, ³The Key Laboratory of Remodeling-related Cardiovascular Diseases, Beijing Institute of Heart Lung and Blood Vessel Diseases, Beijing Anzhen Hospital Affiliated the Capital Medical University, Beijing

Key Words

Atrogin-1/MAFbx • Ubiquitination • Degradation • p38 MAPK

Abstract

Background: Atrogin-1/MAFbx is a major atrophy-related E3 ubiquitin ligase that functions as a negative regulator of cardiac hypertrophy. The mRNA expression of atrogin-1 is induced by oxidative stress via p38 mitogen-activated protein kinase (p38 MAPK). However, the molecular mechanisms that regulate the stability of atrogin-1 protein remain unclear. **Methods:** 293T and cardiac H9c2 cells were transfected with plasmids as indicated. The *in vivo* and *in vitro* ubiquitination assay and pulse-chase analysis were performed to detect the ubiquitination and stability of atrogin-1. The protein levels were measured by Western blot analysis. **Results:** We found that atrogin-1 underwent ubiquitin-mediated degradation by proteasome. The F-box motif of atrogin-1 and Skp1-Cul1-Roc1-F-box (SCF) complex are required for ubiquitination and degradation of atrogin-1. Furthermore, p38 MAPK signaling plays critical roles in regulating the ubiquitination and degradation of atrogin-1 as well as serum starvation-induced expression of atrogin-1 and reduction of H9c2 cell

size. **Conclusion:** These findings may define a new mechanism for regulating the stability of atrogin-1 partially by p38 MAPK signaling.

Copyright © 2011 S. Karger AG, Basel

Introduction

Ubiquitin E3 ligases are the final components in the multi-enzyme process that provide specificity to ubiquitin conjugation, leading to the covalent modification of proteins with ubiquitin [1, 2]. F-box proteins were first described as components of SCF ubiquitin-ligase (E3) complex that contains four components: Skp1, Cullin 1, Roc1/Rbx1/Hrt1, and an F-box protein [3]. It has been reported that the F-box protein binds specific substrates through protein-protein interaction domains (such as WD repeats and leucine-rich repeats), and it links to the complex by binding Skp1 through the F-box itself. SCF complexes have been shown to play a critical role in targeting the substrates for ubiquitination. Polyubiquitinated substrates are subsequently degraded by the 26S proteasome. There is a wide variety of SCF targets that include cyclins and their inhibitors, transcription factors, and components of different signal transduction pathways [3].

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2011 S. Karger AG, Basel
1015-8987/11/0275-0463\$38.00/0

Accessible online at:
www.karger.com/cpb

Huihua Li
Department of Pathology, School of Basic Medical Sciences
Capital Medical University
No.10 Xitoutiao, You An Men, Beijing 100069 (China)
Tel. +86 10 83911698, Fax +86 10 83911698, E-Mail hhl1935@yahoo.cn

Atrogin-1 (also known as MAFbx) is a muscle-specific F-box containing E3 ubiquitin ligase, which is highly expressed in skeletal and cardiac muscle [4, 5]. It functions as an adaptor that binds to Skp1, Cul1, and Roc1, components of the SCF family of ubiquitin ligases to target substrates for ubiquitin-dependent degradation [4, 6, 7]. Recent studies have demonstrated that atrogin-1 targets several substrates including calcineurin, Myo D, eIF3-f and mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) for proteasome-dependent degradation [6-10]. Atrogin-1 is also known to be elevated in a variety of models of catabolic states, including fasting, diabetes, cancer, heart failure, and sepsis and appears to be essential for the initiation and development of skeletal muscle atrophy [4, 5, 11, 12]. Because the atrogin-1 genes respond to such diverse catabolic conditions, it is important to determine the cellular mechanisms that regulate atrogin-1 expression. Recent studies have shown that TNF- α and hydrogen peroxide (H₂O₂) stimulate atrogin-1 mRNA expression and ubiquitin conjugating activity in skeletal muscle through p38 MAPK signaling pathway [13-15]. However, the molecular mechanisms that regulate the ubiquitination and stability of atrogin-1 at protein level remain unclear. In this study, we demonstrated that atrogin-1 was ubiquitinated and degraded via proteasome. The intact F-box and SCF complex were required for atrogin-1 ubiquitination. Furthermore, the ubiquitin-mediated stability of atrogin-1 protein was regulated by p38 MAPK signaling pathway, and these results were confirmed in cardiac-derived H9c2 cells.

Materials and Methods

Plasmid construction and reagents

The expression vector for Flag-and Myc-atrogin-1 wild-type (Wt), F-box deletion mutant (Δ F-box), His-ubiquitin (His-Ub), T7-Skp1, Myc-Cul1, HA-Roc1, constitutively active form of MKK6 (caMKK6) and dominant negative p38 MAPK (Dnp38 MAPK) have been described previously [6, 16]. The following primary and secondary antibodies were used: anti-Flag (M2, Sigma-Aldrich); anti-atrogin-1 (Santa Cruz); anti-His and anti-GST (Chemicon); and anti-mouse- or anti-rabbit-conjugated antibodies (Cell Signaling). MG-132 (proteasome inhibitor), Cycloheximide (CHX, protein synthesis inhibitor) and SB203580 (p38 MAPK inhibitor) were obtained from Calbiochem. Other reagents were purchased from Sigma -Aldrich.

Cell culture and transfection

293T cells and cardiac H9c2 cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml)

and streptomycin (100 μ g/ml) at 37°C in the presence of 5% CO₂ as described previously [17]. 293T cells were transiently transfected with empty vector, His-Ub, Flag-atrogin-1 Wt and F-box mutant, Dnp38 or caMKK6 as indicated using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cycloheximide (10 μ g/ml), MG132 (20 μ M) and SB203580 (10 μ M) were added to culture medium for incubation as indicated.

Measurement of cell size

H9c2 cells (1 $\times$ 10⁵) were cultured in 60 mm dishes were replenished with FBS-free DMEM and then treated with p38 MAPK inhibitor SB203580 (10 μ M) or transfected with Dnp38 or caMKK6 plasmids for 24 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The relative expression levels of atrogin-1, and p38 MAPK were examined using immunoblotting analysis with an antibody directed against atrogin-1, total and phosphor-p38 MAPK and GAPDH. The morphological changes in the cells were visualized with a phase-contrast microscope and quantitation of H9c2 cell surface area was performed on digitized images using NIH Image software as described previously [6]. One hundred twenty cells in 8-10 fields were examined in 3 independent experiments.

Nickel-agarose chromatography and ubiquitination assays in 293T cells

Cells were transfected with indicated plasmids and lysed in Ni-agarose lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 5 mM imidazole, 0.05% Tween 20, 10 mM of Nethylmaleimide, and complete protease inhibitor). Ubiquitin -conjugated proteins were purified by nickel chromatography (Ni-NTA-agarose, Qiagen) and detected by immunoblotting using anti-Flag or anti-His antibody. Ubiquitination assay *in vivo* was performed as described previously [6].

Immunoprecipitation and immunoblotting

293T cells were cotransfected with plasmids encoding His-ubiquitin (His-Ub), Flag-tagged atrogin-1 wild-type or deletion of F-box mutant (Δ F-box), T7-Skp1, Myc-Cul1, and HA-Roc1 using Lipofectamine 2000 (Invitrogen). Immunoprecipitation and immunoblotting were performed as described previously [6]. Tagged proteins were immunoprecipitated for 1-2 hours at 4°C using protein A/G Plus-agarose (Santa Cruz) and 2 μ g of the appropriate antibody (anti-Flag). The beads were washed and fractionated by 10% SDS-PAGE followed by immunoblotting [6].

In vitro ubiquitination reactions

Skp1, Cul1, and Roc1 immunocomplexes were precipitated from transfected 293T cells with 2 μ g of anti-Flag antibody as described previously [6]. Washed immunocomplexes were added to an ubiquitination reaction (final volume 30 μ l) containing 60ng of E1, 600ng of UbcH3, 10 μ g of Ub in the presence of bacterially purified GST alone or and GST-atrogin-1 (1 μ g). The reaction was stopped and analyzed by SDS-PAGE, followed by immunoblotting with anti-His or anti-Flag antibodies as described previously [6].

Pulse-chase Assays

To analyze the degradation kinetics of atrogin-1, 293T cells were transfected with Flag-atrogin-1 Wt, Flag-atrogin-1 Δ F-box, Dnp38 MAPK, caMKK6 or empty vector as indicated or treated with SB203580. Protein lysates were prepared at indicated time points after addition of CHX (10 μ g/ml). Equal amounts of protein were separated by SDS-PAGE. The levels of atrogin-1 protein were determined by immunoblotting and quantified at indicated time points. The protein levels were normalized to the GAPDH.

Statistical analysis

Data are presented as means $\pm$ SEM. Differences between groups were analyzed for statistical significance using Student's *t* and ANOVA tests. *P* < 0.05 was considered to indicate statistical significance.

Results

Atrogin-1 is ubiquitinated and degraded by the proteasome

We first sought to investigate whether atrogin-1 undergoes ubiquitination *in vivo*. 293T cells were co-transfected with Flag-tagged atrogin-1 (Flag-atrogin-1) and hexahistidine-tagged ubiquitin (His-Ub) expression constructs. Immunoblotting analysis of ubiquitinated proteins purified on nickel resins under denaturing conditions revealed that marked ubiquitination of Flag-atrogin-1 was readily detected in the presence of His-Ub. However, the ubiquitination was not observed when either Flag-atrogin-1 or His-ubiquitin plasmids were omitted from transfection (Fig. 1A). To verify that the endogenous atrogin-1 is ubiquitinated, ubiquitination was performed in H9c2 cells transfected with His-Ub. As shown in Fig. 1B, marked ubiquitination of endogenous atrogin-1 was also detected. These results suggest that atrogin-1 is ubiquitinated *in vivo*.

To assess whether atrogin-1 is degraded by proteasome, 293T cells were co-transfected with the expression vectors encoding Flag-atrogin-1 and His-Ub, and treated with or without proteasome inhibitor MG132. Coimmunoprecipitation analysis of Flag-atrogin-1 showed that MG132 treatment led to accumulation of ubiquitinated atrogin-1 and increase of atrogin-1 protein levels compared with DMSO control (Fig. 2A, compare lane 4 with lane 3). Furthermore, pulse-chase analysis with cycloheximide (CHX) confirmed that Flag-atrogin-1 protein rapidly degraded in 293T cells, whereas treatment of cells with MG132 significantly inhibited this degradation (Fig. 2B). Moreover, overexpression of His-Ub significantly decreased the level of endogenous atrogin-1 proteins from H9c2 cells, whereas treatment with MG132 resulted in

Fig. 1. Atrogin-1 is ubiquitinated *in vivo*. (A) 293T cells were co-transfected with Flag-atrogin-1 (1.5 μ g) and His-Ub (1.5 μ g) plasmids as indicated. Ub-conjugated proteins were purified by nickel chromatography and analyzed by immuno-blotting with anti-Flag. The expression of atrogin-1 in the cell extracts was determined using anti-Flag or anti-GAPDH antibody (bottom). (B) H9c2 cells were transfected with His-Ub (2.0 μ g) plasmids. Immunoprecipitation (IP) was performed with anti-atrogin-1 antibodies and analyzed by immunoblotting with anti-His (top) or anti-atrogin-1 (middle) antibodies. Cell extracts were subjected to immunoblotting using anti-atrogin-1 or GAPDH antibody (bottom).

marked stabilization of atrogin-1 compared with that in the cells without MG132 (Fig. 2C). Collectively, these data suggest that atrogin-1 is an unstable protein that undergoes proteasome-dependent degradation.

Ubiquitination and degradation of atrogin-1 is dependent on its F-box motif

It has been known that ubiquitination and degradation of F-box proteins depend on the integrity of their F-box motifs [4, 5]. We therefore determined whether the intact F-box motif is required for atrogin-1 ubiquitination and degradation. 293T cells were co-transfected with Flag-atrogin-1 wild-type (Wt) or deletion of F-box motif (Δ F-box) mutant together with His-Ub plasmids. As shown in Fig. 3A, ubiquitination of atrogin-1 Wt was detected in the presence of His-Ub, whereas Δ F-box mutant of atrogin-1 markedly abolished this effect (compare lane 3 to 1). Furthermore, CHX chase analysis showed that degradation of Δ F-box mutant was significantly slower than that of atrogin-1 Wt (Fig. 3B). These

Fig. 2. Atrogin-1 is degraded by proteasome. (A) 293T cells were co-transfected with Flag-atrogin-1 (1.5 μ g) and His-Ub (1.5 μ g) plasmids and treated with MG132 (20 μ M) for 4 h before harvesting. Cell extracts were immunoprecipitated with the anti-Flag and immunoblotted with the anti-His (top) or anti-Flag (middle) antibody. Cell extracts were subjected to immunoblotting using anti-Flag or anti-GAPDH antibody (bottom). (B) 293T cells were transfected with Flag-atrogin-1 (1.5 μ g) plasmid and treated with cycloheximide (CHX, 10 μ g/ml) in the presence or absence of MG132 (20 μ M) for 4 h. The protein level of atrogin-1 was determined by immunoblotting using anti-Flag or anti-GAPDH antibodies. (C) H9c2 cells were transfected with His-Ub (2.0 μ g) plasmids and treated with MG132 for an additional 4 h. The expression of proteins in the cell extracts was examined with anti-atrogin-1 (top) or anti-GAPDH (bottom) antibodies. NS: nonspecific band.

findings indicate that F-box motif is required for the efficiency of atrogin-1 ubiquitination and degradation.

Ubiquitination of atrogin-1 within the SCF Complex

Our previous data indicated that *in vitro* recombinant atrogin-1 Wt efficiently binds to Skp1, Cul1 and Roc1 proteins to form a SCF ubiquitin ligase complex [4, 6]. Studies of several F-box proteins including HOS, Skp2, Cdc4p, β Tcrp1 suggest that F-box proteins may undergo ubiquitination within the SCF complex in an autocatalytic manner [18-21]. To examine whether atrogin-1 is ubiquiti-

Fig. 3. The F-box motif is required for efficient ubiquitination and degradation of atrogin-1. (A) 293T cells were transfected with His-Ub (1.5 μ g), Flag-atrogin-1 Wt (1.5 μ g) or deletion of F-box (Δ F-box) (1.5 μ g) expression constructs and treated with MG132 (20 μ M) for 4 h before harvesting. Cell extracts were immunoprecipitated with the anti-Flag and immunoblotted with the anti-His (top) or anti-Flag (middle) antibody. Cell extracts were subjected to immunoblotting using anti-Flag antibody (bottom). (B) CHX chase analysis of degradation of Flag-atrogin-1 proteins (wild type and Δ F-box mutant) expressed in 293T cells. Levels of proteins at the indicated time points after adding CHX (10 μ g/ml) to the cells were analyzed by immunoblotting with anti-Flag or anti-GAPDH antibody (top). The steady state level of atrogin-1 protein was quantified. The values shown are the means $\pm$ S.E.M. from three independent experiments (bottom). NS: nonspecific band. * p < 0.05 vs. atrogin-1 Wt.

nated by SCF complex, 293T cells were co-transfected with expression plasmids encoding His-Ub, Flag-atrogin-1, T7-Skp1, Myc-Cul1, and HA-Roc1. Coimmunoprecipitation analysis of Flag-atrogin-1 showed that SCF (Skp1+Cul1+Roc1) complex significantly promoted atrogin-1 ubiquitination (Fig. 4A). Moreover, using *in vitro* ubiquitination assays, we further confirmed that SCF complex strongly enhanced GST-atrogin-1 ubiquitination but not GST alone in the presence of Ub, E1 and E2 (UBCH3) (Fig. 4B). These results indicate that SCF complex is involved in the regulation of atrogin-1 ubiquitination.

Fig. 4. SCF complex promotes atrogin-1 ubiquitination. (A) 293T cells were co-transfected with expression plasmids encoding His-Ub (1.5μg), Flag-atrogin-1 (1.5μg), T7-Skp1 (1.0 μg), Myc-Cul1 (1.0μg), and HA-Roc1 (1.0μg) as indicated. Cell extracts were immunoprecipitated with the anti-Flag antibody and immunoblotted with the anti-His (top) or anti-Flag (middle) antibody. Cell extracts were subjected to immunoblotting using anti-Flag or anti-GAPDH antibody (bottom). (B) *In vitro* ubiquitination of GST-atrogin-1 Wt and ΔF-box mutant by SCF complex (Skp1-Cul1-Roc1) was evaluated in the presence of Ub, E1 and E2 (UbcH3) as indicated. The samples were analyzed by immunoblotting with an anti-Ub antibody to identify ubiquitinated atrogin-1 (top). GST-atrogin-1 and ΔF-box fusion proteins were analyzed for by blotting with GST antibody (bottom). NS: nonspecific band.

Effect of p38 MAPK signaling on the ubiquitination and degradation of atrogin-1 protein

It is reported that p38 MAPK signaling plays a critical role in regulating atrogin-1 gene expression in response to oxidative stress and TNF-α [22-26], we then evaluated whether p38 MAPK affects the ubiquitination and stability of atrogin-1 at protein level using ubiquitination assays and pulse-chase analysis in 293T cells. We found that treatment with SB203580 or co-transfection with Dnp38 MAPK significantly increased ubiquitination and degradation of atrogin-1, whereas overexpression of caMKK6 resulted in decreased ubiquitination and degradation of atrogin-1 (Fig. 5A and B). We next sought to

Fig. 5. Effects of p38 MAPK on the ubiquitination and degradation of atrogin-1. (A) 293T cells were co-transfected with His-Ub (1.5 μg), Flag-atrogin-1 (1.5μg), Dnp38 MAPK (1.5μg) and caMKK6 (1.5μg) or incubated with SB203580 (10μM) for 24 h. Ubiquitinated atrogin-1 proteins were analyzed by immunoblotting with anti-His antibody (top). The expression of atrogin-1 in the cell extracts was determined using anti-Flag or anti-GAPDH antibody (bottom). (B) 293T cells were cultured and treated as in A. The CHX chase analysis of atrogin-1 proteins were analyzed as in Fig. 3B. The amount of atrogin-1 was quantified and shown as the means± S.E.M. (n=3, bottom). NS: nonspecific band. **p* < 0.05 vs. vector alone. (C) H9c2 cells were treated and co-transfected with indicated plasmids as in A but there is no Flag-atrogin-1 plasmid in all lanes. Cell extracts were immunoprecipitated with anti-atrogin-1 and immunoblotted with the anti-His (top) antibodies. Whole cell extracts were subjected to immunoblotting using anti-atrogin-1 and anti-GAPDH antibody (bottom).

determine whether p38 MAPK signaling is involved in the ubiquitin-mediated degradation of endogenous atrogin-

Fig. 6. The roles of p38 MAPK in atrogin-1 expression and size change in H9c2 cells. (A) H9c2 cells were replenished with FBS-free DMEM and then incubated with 0.1% DMSO or 10 μ M SB203580 or transfected with Dnp38 MAPK (2.0 μ g) or caMKK6 (2.0 μ g) for 24 h. Cell lysates were immunoblotted with anti-atrogin-1, anti-total and phospho-p38 MAPK, or anti-GAPDH antibody. (B) H9c2 cells were cultured and treated as in A. The cell sizes were measured under a phase-contrast microscope (X100) and shown in top panels. The values shown are the means $\pm$ S.E.M. from three independent experiments (bottom). Cell size in nonstarved cells (control) was considered to be 100%. * p < 0.05 vs. Control. # p < 0.01 vs. Control.

1 protein in H9c2 cells (Fig. 5C). Indeed, these results were consistent with the findings observed from 293 cells. Together, these results indicate that ubiquitin-mediated stability of atrogin-1 protein is regulated by p38 MAPK.

Roles of p38 MAPK in atrogin-1 expression and cell size

Previous studies have suggested that serum starvation can be used as a cellular model of the atrophy of skeletal muscle. Therefore, we determined the effects of p38 MAPK on the levels of atrogin-1 protein, p38 MAPK phosphorylation and cell sizes in H9c2 cells treated with SB203580 or transfected with Dnp38 MAPK or caMKK6. After 24 hours of starvation, the expression of atrogin-1 and phosphorylation of p38 MAPK were significantly increased, and the cells size was markedly reduced compared with control (10% serum medium) (Fig. 6A and B, compared lane 2 and lane 1). However, treatment with p38 MAPK inhibitor SB203580 or overexpression of Dnp38 MAPK markedly attenuated

these effects (compared lanes 3 and 4 with lane 2). In contrast, overexpression of caMKK6 further enhanced the levels of atrogin-1 expression, p38 MAPK phosphorylation, and reduction of cell size in response to starvation (Fig. 6A and B, compared lane 5 with lane 2). Collectively, these results indicate that p38 MAPK signaling plays a critical role in regulating the expression of atrogin-1 and cell size in response to starvation.

Discussion

Atrogin-1 contains an F-box-motif and acts as an E3 ligase to regulate the atrophy of a variety of cell types, including skeletal and cardiac muscle cells [4-7, 12]. However, the mechanisms that regulate the stability of atrogin-1 at protein level remain unclear. In this study, we demonstrated that atrogin-1 itself was an unstable protein that underwent ubiquitin-dependent proteasomal degradation. This effect was regulated by the MKK6/p38 MAPK signaling pathway. Moreover, the expression of atrogin-1 and size changes in H9c2 cells induced by serum-free starvation were further enhanced by overexpression of caMKK6 but reversed in cells treated with a p38 MAPK inhibitor or transfected with Dnp38 MAPK. These results indicate that p38 MAPK appears to be essential for the stability of atrogin-1 protein.

Posttranslational modification by ubiquitin targets many proteins for rapid degradation by the 26S proteasome. The small soluble protein ubiquitin is transferred and attached to these target substrate proteins by the sequential action of E1, E2 and E3 enzymes. Among them, E3 ligases play a key role in determining the specificity of substrate recognition and catalysis of ubiquitin transfer [27]. The F-box proteins as E3 ligases serve as receptors for the SCF E3 ligases family, whose members ubiquitinate specifically phosphorylated substrates, play a critical role in cell cycle regulation, signal transduction and transcriptional activation [3, 27]. Previous data also show that F-box proteins undergo ubiquitination and proteasome-dependent degradation in yeast and mammalian cells [18-21], and emerge as an important mechanism underlying rapid switching of different receptors allows utilization of the core SCF ligase for ubiquitination of various protein substrates according to cellular needs [18-21, 28]. In the present study, our results demonstrated that atrogin-1 underwent ubiquitin-mediated proteasomal degradation (Fig. 1 and 3), and the efficiency required the intact F-box and SCF complex activities (Fig. 3 and 4). These observations are consistent

with previous studies in the F-box proteins including HOS, Skp2, Cdc4p and β Tcrp1 [18-21]. Together with these data our findings give more insight into the role of substrate in ubiquitination and degradation of F-box proteins. Ubiquitination and degradation of F-box proteins emerge as an important mechanism underlying rapid switching.

Many studies have demonstrated that muscle atrophy is regulated by both the MAPK and AKT pathways. MAPKs are proline-directed serine/threonine protein kinases that are regulated by extracellular stimuli including growth factors, cytokines, and cellular stress [29]. This superfamily consists of three main protein kinase families: the extracellular signal-regulated protein kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 family of kinases, which regulate many cellular activities, such as cell growth, differentiation and apoptosis [30]. p38 MAPK is activated by the MEK homologs MKK3 and MKK6 in response to a variety of stimuli, including oxidative stress and cytokines tumor necrosis factor- α (TNF- α) [29, 31]. The activation of p38 MAPK signaling leads to phosphorylation of their substrate proteins, and plays important roles in the stabilization of substrate proteins or short lived mRNAs of cytokines and growth factors [32-34]. Moreover, p38 MAPK has been identified as an important mediator of catabolic signaling in skeletal muscle. Recent studies demonstrate that H₂O₂ and TNF- α can stimulate atrogen-1 mRNA expression and ubiquitin conjugating activity in skeletal muscle through the activation of p38 MAPK [13-15]. However, the precise mechanisms of their activation in regulating the stability of atrogen-1 are still unknown. In this study, our results showed that the treatment of cells with p38 MAPK inhibitor SB203580 or overexpression of Dnp38 MAPK markedly increased the ubiquitination and degradation of atrogen-1, whereas forced expression of caMKK6 significantly attenuated these effects inhibited atrogen-1 degradation (Fig. 5). Together, these data indicate that p38 MAPK signaling pathway is required for maintaining the stabilization of atrogen-1 protein.

Several catabolic conditions including fasting can upregulate the levels of p38 MAPK and expression of atrogen-1 in skeletal and cardiac muscles [4-7, 12]. It has also been reported that the expression of atrogen-1 is

increased in cardiac cells cultured in serum-free medium [35, 36]. In this study, we have demonstrated that p38 MAPK plays a critical role in regulating atrogen-1 degradation. However, the regulation of atrogen-1 expression by the p38 MAPK pathway in response to cardiomyocyte atrophy had not been demonstrated. Therefore, we investigated the effect of p38 MAPK on the expression of atrogen-1 and cell size in H9c2 cells. In this study, we observed p38 MAPK activity was increased in serum-starved H9c2 cells, and a p38 MAPK inhibitor or overexpression of Dnp38 MAPK abolished this increase in atrogen-1 in response to serum starvation. In contrast, transfection with caMKK6 further increased starvation-induced response (Fig. 6). Collectively, the present results demonstrate for the first time that atrogen-1 protein is a downstream target of p38 MAPK signaling, and the expression of atrogen-1 protein in atrophic cells is regulated by p38 MAPK. This is consistent with the concept that pathologic elevation of p38 activity thereby increasing atrogen-1 E3 ligase activity, enhancing protein degradation and muscle atrophy. However, future study is required to identify the immediate target of p38 kinase activity, which promotes ubiquitination and degradation of atrogen-1.

In conclusion, the current study demonstrates that atrogen-1 stability is regulated via the ubiquitin-proteasome pathway. The F-box motif of atrogen-1 and the activity of SCF complex are required for ubiquitination and degradation of atrogen-1. Activation of p38 MAPK protects atrogen-1 from ubiquitin-mediated degradation and leads to reduced cell size, whereas inhibition of p38 MAPK reversed these effects. Together, our findings suggest that atrogen-1 stability and its-mediated atrophy observed in H9c2 cells is regulated by p38 MAPK. These findings advance our understanding of the post-translational modification of atrogen-1 that regulates cardiac atrophy, and suggest potential targets for therapeutic intervention.

Acknowledgements

This work was supported by grants from China Natural Science Foundation (H. Li., 81025001, 30971097 and 20070023022).

References

- 1 Thompson SJ, Loftus LT, Ashley MD, Meller R: Ubiquitin-proteasome system as a modulator of cell fate. *Curr Opin Pharmacol* 2008;8:90-95.
- 2 Pickart CM, Eddins MJ: Ubiquitin: Structures, functions, mechanisms. *Biochim Biophys Acta* 2004;1695:55-72.
- 3 Strack P, Caligiuri M, Pelletier M, Boisclair M, Theodoras A, Beer-Romero P, Glass S, Parsons T, Copeland RA, Auger KR, Benfield P, Brizuela L, Rolfe M:

- Scf(beta-trcp) and phosphorylation dependent ubiquitination of i kappa b alpha catalyzed by ubc3 and ubc4. *Oncogene* 2000;19:3529-3536.
- 4 Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD, Glass DJ: Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 2001;294:1704-1708.
- 5 Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL: Atrogin-1, a muscle-specific f-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA* 2001;98:14440-14445.
- 6 Li HH, Kedar V, Zhang C, McDonough H, Arya R, Wang DZ, Patterson C: Atrogin-1/muscle atrophy f-box inhibits calcineurin-dependent cardiac hypertrophy by participating in an scf ubiquitin ligase complex. *J Clin Invest* 2004;114:1058-1071.
- 7 Li HH, Willis MS, Lockyer P, Miller N, McDonough H, Glass DJ, Patterson C: Atrogin-1 inhibits akt-dependent cardiac hypertrophy in mice via ubiquitin-dependent coactivation of forkhead proteins. *J Clin Invest* 2007;117:3211-3223.
- 8 Csibi A, Leibovitch MP, Cornille K, Tintignac LA, Leibovitch SA: Mafbx/atrogin-1 controls the activity of the initiation factor eif3-f in skeletal muscle atrophy by targeting multiple c-terminal lysines. *J Biol Chem* 2009;284:4413-4421.
- 9 Tintignac LA, Lagirand J, Batonnet S, Sirri V, Leibovitch MP, Leibovitch SA: Degradation of myod mediated by the scf (mafbx) ubiquitin ligase. *J Biol Chem* 2005;280:2847-2856.
- 10 Xie P, Guo S, Fan Y, Zhang H, Gu D, Li H: Atrogin-1/mafbx enhances simulated ischemia/reperfusion-induced apoptosis in cardiomyocytes through degradation of mapk phosphatase-1 and sustained jnk activation. *J Biol Chem* 2009;284:5488-5496.
- 11 Adams V, Linke A, Wisloff U, Doring C, Erbs S, Krankel N, Witt CC, Labeit S, Muller-Werdan U, Schuler G, Hambrecht R: Myocardial expression of murf-1 and mafb after induction of chronic heart failure: Effect on myocardial contractility. *Cardiovasc Res* 2007;73:120-129.
- 12 Glass DJ: Skeletal muscle hypertrophy and atrophy signaling pathways. *Int J Biochem Cell Biol* 2005;37:1974-1984.
- 13 Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, Reid MB: Tnf-alpha acts via p38 mapk to stimulate expression of the ubiquitin ligase atrogin1/mafbx in skeletal muscle. *Faseb J* 2005;19:362-370.
- 14 Li YP, Chen Y, Li AS, Reid MB: Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific e2 and e3 proteins in skeletal muscle myotubes. *Am J Physiol Cell Physiol* 2003;285:C806-812.
- 15 Li YP, Lecker SH, Chen Y, Waddell ID, Goldberg AL, Reid MB: Tnf-alpha increases ubiquitin-conjugating activity in skeletal muscle by up-regulating ubch2/e220k. *Faseb J* 2003;17:1048-1057.
- 16 Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Glembotski CC: Mkk6 activates myocardial cell nf-kappab and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. *J Biol Chem* 1998;273:8232-8239.
- 17 Fan Y, Xie P, Zhang T, Zhang H, Gu D, She M, Li H: Regulation of the stability and transcriptional activity of nfatc4 by ubiquitination. *FEBS Lett* 2008;582:4008-4014.
- 18 Li Y, Gazdoui S, Pan ZQ, Fuchs SY: Stability of homologue of slimb f-box protein is regulated by availability of its substrate. *J Biol Chem* 2004;279:11074-11080.
- 19 Wirbelauer C, Sutterluty H, Blondel M, Gstaiger M, Peter M, Reymond F, Krek W: The f-box protein skp2 is a ubiquitylation target of a cull-based core ubiquitin ligase complex: Evidence for a role of cull in the suppression of skp2 expression in quiescent fibroblasts. *Embo J* 2000;19:5362-5375.
- 20 Kamura T, Brower CS, Conaway RC, Conaway JW: A molecular basis for stabilization of the von hippel-lindau (vhl) tumor suppressor protein by components of the vhl ubiquitin ligase. *J Biol Chem* 2002;277:30388-30393.
- 21 Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L, Pagano M: Control of meiotic and mitotic progression by the f box protein beta-trcp1 in vivo. *Dev Cell* 2003;4:799-812.
- 22 Chen Q, Olashaw N, Wu J: Participation of reactive oxygen species in the lysophosphatidic acid-stimulated mitogen-activated protein kinase kinase activation pathway. *J Biol Chem* 1995;270:28499-28502.
- 23 Bhat NR, Zhang P: Hydrogen peroxide activation of multiple mitogen-activated protein kinases in an oligodendrocyte cell line: Role of extracellular signal-regulated kinase in hydrogen peroxide-induced cell death. *J Neurochem* 1999;72:112-119.
- 24 Dickinson RJ, Keyse SM: Diverse physiological functions for dual-specificity map kinase phosphatases. *J Cell Sci* 2006;119:4607-4615.
- 25 Keyse SM: Dual-specificity map kinase phosphatases (mkps) and cancer. *Cancer Metastasis Rev* 2008;27:253-261.
- 26 Owens DM, Keyse SM: Differential regulation of map kinase signalling by dual-specificity protein phosphatases. *Oncogene* 2007;26:3203-3213.
- 27 Cardozo T, Pagano M: The scf ubiquitin ligase: Insights into a molecular machine. *Nat Rev Mol Cell Biol* 2004;5:739-751.
- 28 Zhou P, Howley PM: Ubiquitination and degradation of the substrate recognition subunits of scf ubiquitin-protein ligases. *Mol Cell* 1998;2:571-580.
- 29 Michel MC, Li Y, Heusch G: Mitogen-activated protein kinases in the heart. *Naunyn Schmiedebergs Arch Pharmacol* 2001;363:245-266.
- 30 Baines CP, Molkentin JD: Stress signaling pathways that modulate cardiac myocyte apoptosis. *J Mol Cell Cardiol* 2005;38:47-62.
- 31 Obata T, Brown GE, Yaffe MB: Map kinase pathways activated by stress: The p38 mapk pathway. *Crit Care Med* 2000;28:N67-77.
- 32 Pages G, Berra E, Milanini J, Levy AP, Pouyssegur J: Stress-activated protein kinases (jnk and p38/hog) are essential for vascular endothelial growth factor mrna stability. *J Biol Chem* 2000;275:26484-26491.
- 33 Chen CY, Gherzi R, Andersen JS, Gaietta G, Jurchott K, Royer HD, Mann M, Karin M: Nucleolin and yb-1 are required for jnk-mediated interleukin-2 mrna stabilization during t-cell activation. *Genes Dev* 2000;14:1236-1248.
- 34 Briata P, Forcales SV, Ponassi M, Corte G, Chen CY, Karin M, Puri PL, Gherzi R: P38-dependent phosphorylation of the mrna decay-promoting factor ksrp controls the stability of select myogenic transcripts. *Mol Cell* 2005;20:891-903.
- 35 Schulze PC, Fang J, Kassik KA, Gannon J, Cupesi M, MacGillivray C, Lee RT, Rosenthal N: Transgenic overexpression of locally acting insulin-like growth factor-1 inhibits ubiquitin-mediated muscle atrophy in chronic left-ventricular dysfunction. *Circ Res* 2005;97:418-426.
- 36 Skurk C, Izumiya Y, Maatz H, Razeghi P, Shiojima I, Sandri M, Sato K, Zeng L, Schiekofe S, Pimentel D, Lecker S, Taegtmeier H, Goldberg AL, Walsh K: The foxo3a transcription factor regulates cardiac myocyte size downstream of akt signaling. *J Biol Chem* 2005;280:20814-20823.