Atrogin-1/MAFbx Enhances Simulated Ischemia/Reperfusion-induced Apoptosis in Cardiomyocytes through Degradation of MAPK Phosphatase-1 and Sustained JNK Activation*

Ping Xie1, Shubin Guo1, Yongna Fan1, Hua Zhang1, Dongfeng Gu1, and Huihua Li1

From the 1Department of Pathology and National Laboratory of Medical Molecular Biology, Institute of Basic Medical Sciences, and the 2Department of Acute Medicine, Peking Union Medical Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, 5 Dong Dan San Tiao, Beijing 100005, China, and the 4Division of Population Genetics and Prevention, Cardiovascular Institute and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, 167 Beili Shi Road, Beijing 100037, China

Atrogin-1/MAFbx is a major atrophy-related E3 ubiquitin ligase that is expressed specifically in striated muscle. Although the contribution of atrogin-1 to cardiac and muscle hypertrophy/atrophy has been examined extensively, it remains unclear whether atrogin-1 plays an essential role in the simulated ischemia/reperfusion-induced apoptosis of primary cardiomyocytes. Here we showed that atrogin-1 markedly enhanced ischemia/reperfusion-induced apoptosis in cardiomyocytes via activation of JNK signaling. Overexpression of atrogin-1 increased phosphorylation of JNK and c-Jun and decreased phosphorylation of Foxo3a. In addition, atrogin-1 decreased Bcl-2, increased Bax, and enhanced the activation of caspases. Furthermore, JNK inhibitor SP600125 markedly blocked the effect of atrogin-1 on cell apoptosis and the expression of apoptotic-related proteins and caspases. Importantly, atrogin-1 induced sustained activation of JNK through a mechanism that involved degradation of MAPK phosphatase-1 (MKP-1) protein. Atrogin-1 interacted with and triggered MKP-1 for ubiquitin-mediated degradation. In contrast, proteasome inhibitors markedly blocked the degradation of MKP-1. Taken together, these results demonstrate that atrogin-1 promotes degradation of MKP-1 through the ubiquitin-proteasome pathway, thereby leading to persistent activation of JNK signaling and further cardiomyocyte apoptosis following ischemia/reperfusion injury.

Apoptosis in cardiac myocytes appears to be an early event and is well recognized to be responsible for myocardial infarction following ischemia/reperfusion (I/R) injury. However, the intracellular signaling pathways that are involved in stimulus recognition and progression to apoptosis in cardiomyocytes following I/R injury remain to be elucidated. It is reported that activation of the mitogen-activated protein kinase (MAPK) signaling cascades plays an essential role in this progress (1–3). It is believed that activation of p38 is initiated by ischemia and sustained during reperfusion, whereas sustained activation of JNK occurs only during reperfusion and is involved in apoptosis (1, 2). Although I/R is found to be the activator of apoptosis via JNK activation, the mechanism by which I/R prolongs the JNK activation is not fully understood.

MAPKs become activated by dual phosphorylation on Ser/Thr and Tyr residues of TEY sites within the activation loop, whereas dephosphorylation of these residues by MAPK phosphatases (MKPs) terminates such activation (4–8). Thus, MKPs play an important role in negatively regulating MAPK signaling. MKPs constitute a family of 11 dual-specificity phosphatases that exhibit differential specificity toward MAPK substrates. Among these phosphatases, MKP-1(also known as DUSP1) is identified as the first member of this family (9–12) and preferentially dephosphorylates and inactivates both JNK and p38 (13–15). Recent studies have shown that MKP-1-deficient macrophage cells exhibit prolonged JNK activation as well as enhanced production of tumor necrosis factor-α and interleukin-6 compared with wild-type cells (16–18). Several observations demonstrate that MKP-1 is overexpressed in a number of cell types, and its overexpression has been shown to protect cells from apoptosis induced by I/R injury, cisplatin, ethanol, and H2O2 through inactivation of JNK (14, 15, 19, 20). Taken together, these data demonstrate that MKP-1 plays a role in the regulation of stress-responsive JNK-mediated apoptosis.

MKP-1 has been reported to be a labile protein whose stabilization can be enhanced by proteasome inhibitors (21). Choi et al. reported that activation of PKCδ triggers MKP-1 degradation via the ubiquitin-proteasome pathway (22). Furthermore, MKP-1 DEF motif is necessary for active ERK2 binding to initiate site-specific phosphorylation and serves as an essential recognition domain for the Skp1/Cul1/Skp2 (SCF^{Skp2}) ubiquitin-ligase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interference RNA; MEK, MAPK/ERK kinase.
Atrogin-1 Induces Cardiomyocyte Apoptosis via MKP-1

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—The plasmids Myc-atrogin-1, His-ubiquitin, and GST-atrogin-1 have been described as previously (31). Anti-FLAG antibody (M2, Sigma-Aldrich), anti-Myc (clone 9E10), and MKP-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Akt, anti-phospho-Akt (Ser-473), anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-JNK1/2, anti-phospho-JNK1/2, anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-cleaved caspase-9, anti-Foxo3a, anti-phospho-Foxo3a, anti-GAPDH and anti-mouse or anti-rabbit-conjugated antibodies were from Cell Signaling Technology. SP600125, U0126, SB203580, wortmannin, and MG132 were purchased from Calbiochem.

**Cell Culture and Adenovirus Infection**—H9c2 cells were obtained from ATCC and cultured in Dulbecco’s modified Eagle’s medium. All media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In some experiments, cells were pre-treated with MG132 (20 µM) for 4 h before harvesting. Neonatal rat cardiomyocytes were isolated by enzymatic dissociation of 1- to 2-day-old neonatal rat hearts as described previously (30). Recombinant adenoviruses, including Ad-control, Ad-atrogin-1, siRNA-control, and siRNA-atrogin-1 were generated as described previously (30). Twenty-four hours after plating, cells were infected with adenovirus vectors containing Ad-atrogin-1 or siRNA-atrogin-1 for 24 h before I/R treatment (30).

**Simulated I/R Protocol**—After 24 h of adenovirus infection, the cells were subjected to simulated ischemia for 1 h by replacing the cell medium with an “ischemia buffer” that contained...
Atrogin-1 Induces Cardiomyocyte Apoptosis via MKP-1

![Graphs and Data](https://example.com/graphs)

**Figure 2. Effect of atrogin-1 overexpression on phosphorylation of AKT and MAPKs.** A–D, cardiomyocytes were infected and treated as in Fig. 1A. Representative Western blots showed expression levels of total and phospho-AKT, total and phospho-JNK1/2, total and phospho-ERK1/2, and total and phospho-p38 (left panel) in atrogin-1 or vector-expressing cardiomyocytes. The intensity of protein bands was quantified and is shown as the ratio of phosphorylated protein/total protein after normalized by control (GAPDH). Results are expressed as means ± S.E. for three independent experiments. *, p < 0.01; **, p < 0.001 versus Ad-control.

118 mM NaCl, 24 mM NaHCO₃, 1.0 mM NaH₂PO₄, 2.5 mM CaCl₂·2H₂O, 1.2 mM MgCl₂, 20 mM sodium lactate, 16 mM KCl, 10 mM 2-deoxyglucose (pH 6.2) as reported previously (32, 33). The cells were incubated in 5% CO₂ incubator (37 °C) adjusting 1% O₂ during the entire I/R period. Reperfusion was accomplished by replacing the ischemic buffer with normal cell medium under normoxia conditions.

**Treatment of Cells with Inhibitors**—To evaluate the involvement of signaling pathways in atrogin-1-induced apoptosis, cardiomyocytes infected with Ad-atrogin-1 or Ad-control were pretreated for 30 min with SB203580 (p38 inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), U0126 (MEK1/2 inhibitor, 2.5 μM), and wortmannin (phosphatidylinositol 3-kinase inhibitor, 200 nM) and then subjected to the I/R protocol described above.

**Cell Viability and TUNEL Assays**—Cell viability was determined by Trypan blue exclusion assay (33). Apoptosis was analyzed by TUNEL staining as reported previously (33). Cells were counterstained with 4′,6-diamidino-2-phenylindole and finally examined by fluorescent microscopy. The number of TUNEL-positive cells was analyzed using National Institutes of Health Image software.

Western Blot Analysis—Protein samples were prepared from cultured neonatal cardiomyocytes, H9c2 cells using extraction buffer as described previously (30). The membranes were incubated with primary antibodies as indicated overnight and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. The blots were developed using a chemiluminescent system, and the bands were scanned, and densitometry analysis was performed with Gel-pro 4.5 Analyzer (Media Cybernetics).

**Immunoprecipitations and GST Pulldown Assays**—Immunoprecipitations were performed as described (30). Briefly, H9c2 cells were cotransfected with His-atrogin-1. Endogenous MKP-1 proteins were immunoprecipitated for 2 h at 4 °C with anti-MKP-1 antibody. The beads were washed and analyzed by immunoblotting with anti-Myc or anti-MKP-1 antibodies. GST fusion protein was prepared, and GST pulldowns assays were performed as described previously (30). Briefly, H9c2 cells were lysed for 30 min in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM MgCl₂, 0.5% Triton X-100, 5 mM dithiothreitol, 10 mM NaF, and protease inhibitors. Lysates were precleared with GST beads for 1 h and incubated with GST or GST-atrogin-1 fusion proteins for 1 h at 4 °C. Protein-bound GST beads were washed with lysis buffer and analyzed by Western blot with anti-MKP-1 antibody.

**Ubiquitination Assays**—H9c2 cells were transfected expression vectors containing His-Ub and Myc-atrogin-1 using Lipofectamine 2000. Cell lysates were immunoprecipitated with anti-MKP-1 antibody, and analyzed by Western blot using anti-His, anti-MKP-1 or anti-Myc antibodies as previously described (30).

**Statistical Analysis**—Data are presented as means ± S.E. Differences between groups were evaluated for statistical significance using Student’s t test. p < 0.05 was regarded as significant.

**RESULTS**

**Atrogin-1 Enhances I/R-induced Apoptosis in Neonatal Cardiomyocytes**—To investigate the role of atrogin-1 in I/R-induced apoptosis, neonatal cardiomyocytes were infected with Ad-atrogin-1 or Ad-control for 24 h and then were exposed to 1 h of simulated ischemia followed by 24 h reperfusion. As
Atrogin-1 Induces Cardiomyocyte Apoptosis via MKP-1

Effect of Atrogin-1 on the Level of Akt, JNK1/2, ERK1/2 and p38 Phosphorylation—To elucidate the signaling pathways involved in atrogin-1-induced cell apoptosis, we first assessed the kinetics of Akt, JNK1/2, ERK1/2, and p38 phosphorylation in cardiomyocytes during I/R stimulation. As shown in Fig. 2 (A, C and D), the level of Akt, ERK1/2, and p38 phosphorylation in Ad-atrogin-1 infection was equivalent to that of the control following reperfusion. In contrast, there was a significant increase in the level of JNK1/2 phosphorylation in Ad-atrogin-1 infection and reached a maximum at 24 h of reperfusion as compared with the control (Fig. 2B). These results demonstrate that atrogin-1 selectively up-regulates the activation of JNK1/2 in cardiomyocytes after I/R injury.

Atrogin-1 Enhances I/R-induced Apoptosis via JNK1/2 Signaling Pathway—To determine the relationship between atrogin-1-induced apoptosis and activation of MAPKs and Akt, cardiomyocytes were treated with p38 inhibitor SB203580, JNK inhibitor SP600125, MEK1/2 inhibitor U0126, and phosphatidylinositol 3-kinase inhibitor wortmannin. The results in Fig. 3A showed that these inhibitors could completely block the phosphorylation of p38, JNK1/2, ERK1/2, and Akt. Interestingly, JNK inhibition with SP600125 only significantly increased the percentage of viable cells and decreased the number of TUNEL-positive cells in atrogin-1 infection as compared with the control (Fig. 3B and C), suggesting that the JNK pathway is the major factor of atrogin-1-induced apoptosis in cardiomyocytes after I/R injury.

atrogin-1 Down-regulates Foxo3a Phosphorylation—Our recent data demonstrate that atrogin-1 decreases Foxo3a phos-
related proteins and caspases, cardiomyocytes were infected with adenovirus vector or atrogin-1 and treated without or with JNK inhibitor SP600125. The protein levels of atrogin-1, JNK-1/2, Bcl-2, Bax, cleaved caspase-9, and caspase-3 were examined as in A. A representative blot is shown for each condition. These results demonstrate that JNK activation by atrogin-1 functions as an upstream regulator of Bcl-2 and Bax, thereby leading to caspases activation and cell apoptosis.

**Atrogin-1 Down-regulates the Level of MKP-1 Protein**—Because MKP-1 plays a negative regulator of JNK activation, we then determine whether sustained activation of JNK1/2 induced by atrogin-1 is associated with down-regulation of MKP-1 in cardiomyocytes following I/R injury. As shown in Fig. 6A, the level of MKP-1 was significantly increased after 1 h of reperfusion in both atrogin-1 and control groups. However, MKP-1 protein in atrogin-1 infection substantially decreased after 2 h of reperfusion as compared with the control. In contrast, knockdown of endogenous atrogin-1 markedly resulted in increased level of MKP-1 in cardiomyocytes following I/R injury. As shown in Fig. 6B, the increase of MKP-1 protein level as compared with siRNA-control and siRNA-atrogin-1 groups was statistically significant. These results indicate that atrogin-1 participates in the down-regulation of MKP-1 in cardiomyocytes after I/R injury.

**Atrogin-1 Interacts with and Decreases Endogenous MKP-1 in H9c2 Cells**—We next assessed whether atrogin-1 interacts with endogenous MKP-1 in rat cardiac myoblast H9c2 cells by immunoprecipitation assays. As shown in Fig. 7A, MKP-1 was detected in the Myc immune complex precipitated with a specific rabbit Myc antibody, whereas no MKP-1 was found in the immune complex precipitated with a nonspecific rabbit IgG. Furthermore, endogenous MKP-1 in H9c2 cells was pulled down by GST-atrogin-1 fusion protein purified from bacteria but not GST alone (Fig. 7B). Thus, these results suggest that atrogin-1 directly associates with MKP-1.

**Atrogin-1 Modulates the Expression of Bcl-2, Bax, and Cleaved Caspases through JNK Signaling**—To investigate whether atrogin-1 could regulate the expression of apoptosis-related proteins and caspases, cardiomyocytes were infected with Ad-atrogin-1 or siRNA-atrogin-1 and stimulated with I/R. As shown in Fig. 5A and B, overexpression of atrogin-1 markedly decreased the level of Bcl-2 protein, and increased the level of Bax protein, cleaved caspase-9 and caspase-3. In contrast, knockdown of atrogin-1 had an opposite effect. These results indicate that atrogin-1 is involved in the regulation of Bcl-2, Bax, and caspases implicated in cardiomyocyte apoptosis.

Because the expression pattern of MKP-1 protein is inversely correlated with atrogin-1 in cardiomyocytes during reperfusion (Fig. 6), we sought to determine whether atrogin-1 is involved in MKP-1 phosphorylation and positively regulates Foxo3a activity with lysine 63-linked ubiquitin chains. Thus, we next examined whether atrogin-1 also affects Foxo3a phosphorylation in cardiomyocytes after I/R. Consistent with our previous data (31), atrogin-1 overexpression resulted in markedly decrease of Foxo3a phosphorylation, whereas depletion of atrogin-1 by siRNA had an opposite effect (Fig. 4, C and D). These results indicate that atrogin-1 also enhances Foxo3a activation.

**Atrogin-1 Modulates the Expression of Bcl-2, Bax, and Cleaved Caspases through JNK Signaling**—To investigate whether atrogin-1 could regulate the expression of apoptosis-related proteins and caspases, cardiomyocytes were infected with Ad-atrogin-1 or siRNA-atrogin-1 and stimulated with I/R. As shown in Fig. 5A and B, overexpression of atrogin-1 markedly decreased the level of Bcl-2 protein, and increased the level of Bax protein, cleaved caspase-9 and caspase-3. In contrast, knockdown of atrogin-1 had an opposite effect. These results indicate that atrogin-1 is involved in the regulation of Bcl-2, Bax, and caspases implicated in cardiomyocyte apoptosis.

To explore the mechanisms for atrogin-1 regulating the expression of Bcl-2 and Bax in cardiomyocytes following I/R, we first examined the relationship between atrogin-1 and Bcl-2 or Bax. Coimmunoprecipitation assays demonstrated that atrogin-1 did not directly interact with Bcl-2 or Bax in vivo (data not shown), indicating that Bcl-2 and Bax are not the targets of atrogin-1 in this assay. We therefore tested the involvement of JNK signaling using JNK-specific inhibitor SP600125. As shown in Fig. 5 (A and B), JNK inhibition with SP600125 eventually attenuated JNK phosphorylation and markedly blocked the effect of atrogin-1 on the expression of Bcl-2, Bax, cleaved caspase-9, and caspase-3 compared with the control. These results demonstrate that JNK activation by atrogin-1 functions as an upstream regulator of Bcl-2 and Bax, thereby leading to caspases activation and cell apoptosis.

**Atrogin-1 Down-regulates the Level of MKP-1 Protein**—Because MKP-1 plays a negative regulator of JNK activation, we then determine whether sustained activation of JNK1/2 induced by atrogin-1 is associated with down-regulation of MKP-1 in cardiomyocytes following I/R injury. As shown in Fig. 6A, the level of MKP-1 was significantly increased after 1 h of reperfusion in both atrogin-1 and control groups. However, MKP-1 protein in atrogin-1 infection substantially decreased after 2 h of reperfusion as compared with the control. In contrast, knockdown of endogenous atrogin-1 markedly resulted in increased level of MKP-1 in cardiomyocytes following I/R injury.

**Atrogin-1 Interacts with and Decreases Endogenous MKP-1 in H9c2 Cells**—We next assessed whether atrogin-1 interacts with endogenous MKP-1 in rat cardiac myoblast H9c2 cells by immunoprecipitation assays. As shown in Fig. 7A, MKP-1 was detected in the Myc immune complex precipitated with a specific rabbit Myc antibody, whereas no MKP-1 was found in the immune complex precipitated with a nonspecific rabbit IgG. Furthermore, endogenous MKP-1 in H9c2 cells was pulled down by GST-atrogin-1 fusion protein purified from bacteria but not GST alone (Fig. 7B). Thus, these results suggest that atrogin-1 directly associates with MKP-1 in vivo and in vitro.

Because the expression pattern of MKP-1 protein is inversely correlated with atrogin-1 in cardiomyocytes during reperfusion (Fig. 6), we sought to determine whether atrogin-1 is involved in MKP-1 phosphorylation and positively regulates Foxo3a activity with lysine 63-linked ubiquitin chains. Thus, we next examined whether atrogin-1 also affects Foxo3a phosphorylation in cardiomyocytes after I/R. Consistent with our previous data (31), atrogin-1 overexpression resulted in markedly decrease of Foxo3a phosphorylation, whereas depletion of atrogin-1 by siRNA had an opposite effect (Fig. 4, C and D). These results indicate that atrogin-1 also enhances Foxo3a activation.

**Atrogin-1 Modulates the Expression of Bcl-2, Bax, and Cleaved Caspases through JNK Signaling**—To investigate whether atrogin-1 could regulate the expression of apoptosis-related proteins and caspases, cardiomyocytes were infected with Ad-atrogin-1 or siRNA-atrogin-1 and stimulated with I/R. As shown in Fig. 5A and B, overexpression of atrogin-1 markedly decreased the level of Bcl-2 protein, and increased the level of Bax protein, cleaved caspase-9 and caspase-3. In contrast, knockdown of atrogin-1 had an opposite effect. These results indicate that atrogin-1 is involved in the regulation of Bcl-2, Bax, and caspases implicated in cardiomyocyte apoptosis.

To explore the mechanisms for atrogin-1 regulating the expression of Bcl-2 and Bax in cardiomyocytes following I/R, we first examined the relationship between atrogin-1 and Bcl-2 or Bax. Coimmunoprecipitation assays demonstrated that atrogin-1 did not directly interact with Bcl-2 or Bax in vivo (data not shown), indicating that Bcl-2 and Bax are not the targets of atrogin-1 in this assay. We therefore tested the involvement of JNK signaling using JNK-specific inhibitor SP600125. As shown in Fig. 5 (A and B), JNK inhibition with SP600125 eventually attenuated JNK phosphorylation and markedly blocked the effect of atrogin-1 on the expression of Bcl-2, Bax, cleaved caspase-9, and caspase-3 compared with the control. These results demonstrate that JNK activation by atrogin-1 functions as an upstream regulator of Bcl-2 and Bax, thereby leading to caspases activation and cell apoptosis.

**Atrogin-1 Down-regulates the Level of MKP-1 Protein**—Because MKP-1 plays a negative regulator of JNK activation, we then determine whether sustained activation of JNK1/2 induced by atrogin-1 is associated with down-regulation of MKP-1 in cardiomyocytes following I/R injury. As shown in Fig. 6A, the level of MKP-1 was significantly increased after 1 h of reperfusion in both atrogin-1 and control groups. However, MKP-1 protein in atrogin-1 infection substantially decreased after 2 h of reperfusion as compared with the control. In contrast, knockdown of endogenous atrogin-1 markedly resulted in increased level of MKP-1 in cardiomyocytes following I/R injury.

**Atrogin-1 Interacts with and Decreases Endogenous MKP-1 in H9c2 Cells**—We next assessed whether atrogin-1 interacts with endogenous MKP-1 in rat cardiac myoblast H9c2 cells by immunoprecipitation assays. As shown in Fig. 7A, MKP-1 was detected in the Myc immune complex precipitated with a specific rabbit Myc antibody, whereas no MKP-1 was found in the immune complex precipitated with a nonspecific rabbit IgG. Furthermore, endogenous MKP-1 in H9c2 cells was pulled down by GST-atrogin-1 fusion protein purified from bacteria but not GST alone (Fig. 7B). Thus, these results suggest that atrogin-1 directly associates with MKP-1 in vivo and in vitro.

Because the expression pattern of MKP-1 protein is inversely correlated with atrogin-1 in cardiomyocytes during reperfusion (Fig. 6), we sought to determine whether atrogin-1 is involved in MKP-1 phosphorylation and positively regulates Foxo3a activity with lysine 63-linked ubiquitin chains. Thus, we next examined whether atrogin-1 also affects Foxo3a phosphorylation in cardiomyocytes after I/R. Consistent with our previous data (31), atrogin-1 overexpression resulted in markedly decrease of Foxo3a phosphorylation, whereas depletion of atrogin-1 by siRNA had an opposite effect (Fig. 4, C and D). These results indicate that atrogin-1 also enhances Foxo3a activation.
in the down-regulation of endogenous MKP-1 protein in H9c2 cells. Indeed, overexpression of atrogin-1 caused a markedly decrease of endogenous MKP-1 protein and increase of JNK1/2 phosphorylation in a dose-dependent manner (Fig. 7C). In contrast, knockdown of endogenous atrogin-1 by siRNA resulted in an increase of endogenous MKP-1 protein and down-regulation of JNK1/2 phosphorylation (Fig. 7D). However, MKP-2 and MKP-3 were not affected markedly by atrogin-1 expression (data not shown), excluding the involvement of MKP-2 and MKP-3 in atrogin-1-induced JNK activation. Taken together, these findings demonstrate that atrogin-1 specifically down-regulates MKP-1 protein level.

Atrogin-1 Promotes the Ubiquitination and Degradation of Endogenous MKP-1—It has been recently shown that the MKP-1 protein is targeted for proteasomal degradation in breast cancer cell line MCF-7 and HT22 cells (15, 22). To determine that the down-regulation of MKP-1 protein by atrogin-1 is mediated through ubiquitin-proteasome degradation, we compared the half-life of endogenous MKP-1 protein in cells transfected with empty vector or atrogin-1. As shown in Fig. 8A, ectopic expression of atrogin-1 resulted in a rapid decrease in the MKP-1 protein as compared with vector control, and this effect was abolished completely by the proteasome inhibitor MG132 (Fig. 8B), indicating that atrogin-1 targets MKP-1 protein for proteasome degradation. Because MKP-1 has been identified as a ubiquitination target in MCF-7 and HT22 cells (15, 22), we examined whether atrogin-1 as an E3 ligase affects ubiquitination of MKP-1 in H9c2 cells. Indeed, ubiquitination of MKP-1 was confirmed and enhanced markedly by the transfection of atrogin-1 in H9c2 cells (Fig. 8C). Moreover, ubiquitination of MKP-1 was enhanced further by MG132 (Fig. 8D). Taken together, these results demonstrate that atrogin-1 promotes the ubiquitination and proteasome degradation of MKP-1 protein.

DISCUSSION

Atrogin-1 (also known as muscle atrophy F-box) is first identified as a crucial participant and is induced early during the atrophy process (25, 26). Mice lacking atrogin-1 are resistant to muscle atrophy following denervation (26), indicating that atrogin-1 targets key muscle protein(s) for destruction, although the identity of these component(s) is still unclear. Atrogin-1 is a cardiac- and skeletal muscle-specific F-box protein that binds to Skp1, Cul1, and Roc1, the common components of SCF ubiquitin ligase complexes (26, 30). We previously found that atrogin-1 inhibits pathologic and physiological hypertrophy in vivo and in vitro (30, 31). Furthermore, atrogin-1 mediates part of the effects of statin in muscle atrophy (16). These observations suggest that atrogin-1 may be a critical mediator of the heart and muscle atrophy. However, the role of atrogin-1 in cardiomyocyte apoptosis after I/R injury remains unknown. In the present study, we demonstrate that atrogin-1 enhances I/R-induced apoptosis in cardiomyocytes. This augmentation is associated with the reduced expression of MKP-1 and the sustained activation of JNK signaling. This study also provides evidence that atrogin-1 down-regulates the level of MKP-1 protein through ubiquitination-mediated proteasomal degradation.

Previous studies have shown that JNK is activated in response to various stress and mitogenic stimuli and plays an important role in apoptosis in the heart and cultured cardiomyocytes (1, 2). JNK was shown to be activated by I/R injury in transgenic hearts (34). Furthermore, JNK inhibition has been recently reported to actually protect cardiac myocytes from I/R-induced apoptosis (35–37). Therefore, activation of JNK plays a critical role in controlling cell apoptosis and survival. In the present study, we found that atrogin-1-induced apoptosis of cardiomyocytes was associated with the increase of JNK phosphorylation. Moreover, treatment with SP600125, a specific inhibitor of JNK, markedly decreased apoptotic cells in atrogin-1 infection (Fig. 3). These results suggest that atrogin-1-induced apoptosis in cardiomyocytes selectively relies upon the activation of JNK signaling.

It may be significant that JNK is markedly enhanced by atrogin-1 overexpression following I/R injury, because JNK and its downstream targets are known to play a key role in mitochondrial-driven apoptosis of cardiomyocytes. First, stimulation of JNK activity induces apoptosis through a mechanism that involves c-Jun activation (38). Indeed, we found that atrogin-1 expression significantly increased the level of c-Jun phosphorylation (Fig. 4). Second, there has been much evidence reporting that the JNK pathway controls the activity and expression of several members of the Bcl-2 family proteins involving the...
Atrogin-1 Induces Cardiomyocyte Apoptosis via MKP-1

**FIGURE 8.** Atrogin-1 promotes the ubiquitination and degradation of the MKP-1 protein. A, H9c2 cells were transfected with vector or Myc-atrogin-1 plasmids and treated with cycloheximide (CHX) for the time points indicated. Equal amounts of total cell protein were subjected to Western blotting using anti-MKP-1 and anti-Myc antibodies. The intensities of MKP-1 band were measured (lower panel). B, H9c2 cells were transfected with vector or Myc-atrogin-1 and treated without or with MG132 (20 μM) for 4 h before harvesting. Cell lysates were analyzed as in A. C, H9c2 cells were transfected with plasmids His-Ub and increasing amounts of Myc-atrogin-1. Twenty-four hours after transfection, whole cell lysates were prepared and MKP-1 protein was precipitated with anti-MKP-1. The presence of ubiquitin-conjugated MKP-1 in the immunocomplex was detected by Western blotting with anti-His (upper panel). The same membrane was reprobed with anti-MKP-1 to assess the amount of precipitated MKP-1 (middle panel). Whole cell lysates were analyzed as in A (lower panel). D, H9c2 cells were transfected with plasmids His-Ub and Myc-atrogin-1. Twenty-four hours after transfection, cells were treated with proteasome inhibitor MG132 for 4 h. The formation of MKP-1 polyubiquitination was detected as in C.

Atrogin-1, resulting in cell death through the release of cytochrome c (42–44). Moreover, Bax was shown to bind to Bcl-2 and demonstrate that the apoptosis-modulating properties of atrogin-1 are mediated by the activation of JNK and its downstream effectors, including c-Jun, Bcl-2, and Bax in cardiomyocytes following I/R injury.

Although atrogin-1 is suggested to markedly contribute to I/R-induced apoptosis via JNK activation in cardiomyocytes mentioned above, there may be other factors that play a role in atrogin-1-induced apoptosis beyond this mechanism. FOXO3a is a member of the forkhead transcription factor family and plays an important role in cell cycle arrest and apoptosis of various cell lines (47). Foxo proteins have been shown to be involved in the transactivation of Bim and FasL, which induce cell death through mitochondria- and death receptor-dependent mechanisms. Our previous data have shown that Foxo3a is a target of atrogin-1 in Akt-induced cardiac hypertrophy, and atrogin-1 positively regulates Foxo3a activation through lysine-63-linked ubiquitination (30), suggesting Foxo3a may participate atrogin-1-induced apoptosis in cardiomyocytes. Indeed, our data showed that atrogin-1 down-regulated Foxo3a phosphorylation in cardiomyocytes following I/R injury. These results indicate that Foxo3a, in part, involves in atrogin-1-induced apoptosis in cardiomyocytes.

There are 11 members of the MKP family that have unique and overlapping substrate specificity intrinsic mitochondria apoptotic pathway (41, 43–45). Activation of JNK signaling can trigger Bcl-2 down-regulation through proteasome degradation (39) and increase Bax expression through p53 in several cell types (40, 41), thereby leading to cytochrome c release from mitochondria and caspase-3 activation (42–44). Moreover, Bax was shown to bind to Bcl-2 and Bcl-XL, resulting in cell death through the release of cytochrome c and Apaf-1 from mitochondria and caspase-3 activation (42–44). Consistent with these data, our results showed that atrogin-1 overexpression markedly decreased the level of Bcl-2, increased the expression of Bax and the activation of caspases. Importantly, treatment with JNK inhibitor SP600125 markedly abolished the effects of atrogin-1 on the alteration of Bcl-2, Bax, cleaved caspases (Fig. 5, A and B), indicating that the atrogin-1-mediated JNK activation is a major upstream regulator of Bcl-2 and Bax expression. Collectively, these results demonstrate that the apoptosis-modulating properties of atrogin-1 are mediated by the activation of JNK and its downstream effectors, toward MAPKs. MKP-1 is identified as the first member of this family and is inducible in response to stress (9–12). It has been shown that overexpression of MKP-1 can inhibit apoptosis in several cell lines, and this effect was mainly associated with its ability to inactivate JNK or p38 (13, 14, 48). Importantly, MKP-1 has been shown to be targeted for ubiquitin-mediated proteasomal degradation, whereas the degradation of MKP-1 protein is attenuated by proteasome inhibitors (21). Recent studies suggested that degradation of MKP-1 was triggered by ERK and PKCδ signaling pathways through activation of the ubiquitin-proteasome pathway, which contributes to the sustained activation of ERK1/2 and JNK1/2, thereby providing a positive-feedback mechanism (22, 23). Venugopal et al. (20) demonstrated that ethanol causes the sustained activation of JNK and apoptosis in hepatocytes via the enhancement of MKP-1 degradation. More importantly, F-box protein Skp2, a vital E3 ligase, promotes MKP-1 polyubiquitination and subsequent destruction via the 26 S proteasome (23). These reports suggest to us that MKP-1 degradation might play a role in atrogin-1-induced sus-
Atrogin-1 induces cardiomyocyte apoptosis via MKP-1

*Acknowledgments—We are grateful to Dr. Cam Patterson and Dr. Mingpeng She for helpful discussions about the results.*

**REFERENCES**

1. Michel, M. C., Li, Y., and Heusch, G. (2001) *Naunyn-Schmiedebergs Arch. Pharmacol.* **363**, 245–266
2. Armstrong, S. C. (2004) *Cardiovasc. Res.* **61**, 427–436
3. Baines, C. P., and Molkentin, J. D. (2005) *J. Mol. Cell. Cardiol.* **38**, 47–62
4. Chen, Q., Olashaw, N., and Wu, J. (1995) *J. Biol. Chem.* **270**, 28499–28502
5. Bhat, N. R., and Zhang, P. (1999) *J. Neurochem.* **72**, 112–119
6. Dickinson, R. J., and Keyse, S. M. (2006) *J. Cell Sci.* **119**, 4607–4615
7. Keyse, S. M. (2008) *Cancer Metastasis Rev.* **27**, 253–261
8. Owens, D. M., and Keyse, S. M. (2007) *Oncogene* **26**, 3203–3213
9. Charles, C. H., Abler, A. S., and Lau, L. F. (1992) *Oncogene* **7**, 187–190
10. Lau, L. F., and Nathans, D. (1985) *EMBO J.* **4**, 3145–3151
11. Emslie, E. A., Jones, T. A., Sheer, D., and Keyse, S. M. (1994) *Hum Genet* **93**, 513–516
12. Keyse, S. M., and Emslie, E. A. (1992) *Nature* **359**, 644–647
13. Li, J., Gorospe, M., Hutter, D., Barnes, I., Keyse, S. M., and Liu, Y. (2001) *Mol. Cell. Biol.* **21**, 8213–8224
14. Franklin, C. C., and Kraft, A. S. (1997) *J. Biol. Chem.* **272**, 16917–16923
15. Zhou, J. Y., Liu, Y., and Wu, G. S. (2006) *Cancer Res.* **66**, 4888–4894
16. Hanai, J., Cao, P., Tanksale, P., Imamura, S., Koshimizu, E., Zhao, J., Kishi, S., Yamashita, M., Phillips, P. S., Sukhatme, V. P., and Lecker, S. H. (2007) *J. Clin. Invest.* **117**, 3940–3951
17. Hammer, M., Mages, J., Dietrich, H., Servatius, A., Howells, N., Cato, A. C., and Lang, R. (2006) *J. Exp. Med.* **203**, 15–20
18. Salojin, K. V., Owusu, I. B., Millercg, K. A., Potter, M., Platt, K. A., and Oravec, T. (2006) *J. Immunol.* **176**, 1899–1907
19. Sanchez-Perez, J., Martinez-Gomara, M., Williams, D., Keyse, S. M., and Perona, R. (2000) *Oncogene* **19**, 5412–5512
20. Venugopal, S. K., Chen, J., Zhang, X., Clemens, D., Follenzi, A., and Zern, M. A. (2007) *J. Biol. Chem.* **282**, 31900–31908
21. Brondello, J. M., Pouyssegur, J., and McKenzie, F. R. (1999) *Science* **286**, 2514–2517
22. Choi, B. H., Hur, E. M., Lee, J. H., Jun, D. J., and Kim, K. T. (2006) *J. Cell Sci.* **119**, 1329–1340
23. Lin, Y. W., and Yang, J. L. (2006) *J. Biol. Chem.* **281**, 915–926
24. Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K., Nunee, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E., Dharmarajan, K., Pan, Z. Q., Valenzuela, D. M., DeChiara, T. M., Stitt, T. N., Yancopoulos, G. D., and Glass, D. J. (2001) *Science* **294**, 1704–1708
25. Lecker, S. H., Jagoe, R. T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S. R., Mitch, W. E., and Goldberg, A. L. (2004) *FASEB J.* **18**, 39–51
26. Gomes, M. D., Lecker, S. H., Jagoe, R. T., Navon, A., and Goldberg, A. L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 14440–14445
27. Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker, S. H., and Goldberg, A. L. (2004) *Cell* **117**, 399–412
28. Li, Y. P., Chen, Y., John, I., Moylan, J., Jin, B., Mann, D. L., and Reid, M. B. (2005) *FASEB J.* **19**, 362–370
29. Adams, V., Linke, A., Wisloff, U., Doring, C., Erbs, S., Krankel, N., Witt, C. C., Labeit, S., Muller-Werdan, U., Schuler, G., and Hambrecht, R. (2007) *Cardiovasc. Res.* **73**, 120–129
30. Li, H. H., Kedar, V., Zhang, C., McDonough, H., Arya, R., Wang, D. Z., and Patterson, C. (2004) *J. Clin. Invest.* **114**, 1058–1071
31. Li, H. H., Willis, M. S., Lockyer, P., Miller, N., McDonough, H., Glass, D. J., and Patterson, C. (2007) *J. Clin. Investig.* **117**, 3211–3223
32. Engelbrecht, A. M., Niesler, C., Page, C., and Lochner, A. (2004) *Basic Res. Cardiol.* **99**, 338–350
33. Das, A., Smolenski, A., Lohmann, S. M., and Kukreja, R. C. (2006) *J. Biol. Chem.* **281**, 38644–38652
34. He, H., Li, H. L., Lin, A., and Gottlieb, R. A. (1999) *Cell Death Differ.* **6**, 987–991
35. Hreniak, D., Garay, M., Gaarde, W., Monia, B. P., McKay, R. A., and Cioffi, C. C. (2001) *Mol. Pharmacol.* **59**, 867–874
36. Ferrandri, C., Ballerio, R., Giaillard, P., Giachetti, C., Carboni, S., Vitte, P. A., Gotteland, J. P., and Cirillo, R. (2004) *Br. J. Pharmacol.* **142**, 953–960
37. Milano, G., Morel, S., Bonny, C., Samaja, M., von Segesser, L. K., Nicod, P., and Vassalli, G. (2007) *Annu. Rev. Physiol.* **69**, H1828–H1835
38. Aoki, H., Kang, P. M., Hampe, J., Yoshimura, K., Noma, T., Matsuoka, M., and Izumo, S. (2002) *J. Biol. Chem.* **277**, 10244–10250
39. Thompson, S. J., Loftus, L. T., Ashley, M. D., and Meller, R. (2008) *Curr. Opin. Pharmacol.* **8**, 90–95
40. Assimakopoulos, M., Kondylis, M., Gatzounis, G., Maraziotis, T., and Varahki, I. (2007) *BMC Cancer* **7**, 202
41. Zhang, L., Xing, D., Liu, L., Gao, X., and Chen, M. (2007) *Cell Cycle* **6**, 1479–1486
42. Xifro, X., Falluel-Morel, A., Minano, A., Aubert, N., Fado, R., Malagalda,
Atrogin-1 Induces Cardiomyocyte Apoptosis via MKP-1

C., Vaudry, D., Vaudry, H., Gonzalez, B., and Rodriguez-Alvarez, J. (2006) J. Biol. Chem. 281, 6801–6812

43. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. (1999) Mol. Cell. Biol. 19, 8469–8478

44. Lei, K., Nimnual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D., and Davis, R. J. (2002) Mol. Cell. Biol. 22, 4929–4942

45. Le-Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F. X., Green, D. R., and Karin, M. (1999) Mol. Cell. Biol. 19, 751–763

46. Capano, M., and Crompton, M. (2006) Biochem. J. 395, 57–64

47. Huang, H., and Tindall, D. J. (2007) J. Cell Sci. 120, 2479–2487

48. Wu, J. J., and Bennett, A. M. (2005) J. Biol. Chem. 280, 16461–16466