Atrogin-1 ubiquitin ligase is upregulated by doxorubicin via p38-MAP kinase in cardiac myocytes

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Aims Doxorubicin (DOX) is one of the most effective anti-neoplastic agents; however, its clinical use is limited by drug-induced cardiomyopathy. The molecular mechanisms responsible for this toxicity remain to be fully addressed. In the present study, we investigated the involvement of atrogin-1, one of the muscle-specific ubiquitin ligases, in DOX-induced cardiotoxicity.

Methods and results This method involved intraperitoneal administration of DOX-induced atrogin-1 in the hearts and skeletal muscles of C57BL/6 mice. Consistently, atrogin-1 mRNA was upregulated with DOX treatment in cultured rat neonatal cardiomyocytes. Adenoviral transfer of atrogin-1 induced a reduction in cell size that was ameliorated by the ubiquitin proteasome inhibitor, MG-132. The transduction of constitutively active Akt (caAkt), a serine/threonine protein kinase, inhibited the DOX-mediated induction of atrogin-1. The phosphorylation status of Akt and its downstream target, FOXO, was not affected by DOX. DOX treatment did not activate the atrogin-1 promoter that contains FOXO-binding sites, suggesting that DOX induced atrogin-1 without modulating the Akt/FOXO pathway; importantly, DOX activated p38-mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK). Furthermore, pharmacological inhibition of p38-MAPK, but not JNK, abrogated DOX-mediated induction of atrogin-1. Finally, adenoviral transfer of caAkt inhibited the DOX-induced p38-MAPK activation.

Conclusions DOX induces atrogin-1 through a p38-MAPK-dependent pathway in cardiac myocytes. Constitutive activation of Akt negatively regulates DOX-mediated atrogin-1 induction by inhibiting p38-MAPK activity as a novel mechanism.

1. Introduction

Doxorubicin (DOX) is an anti-tumour agent of the anthracycline family, widely used in the treatment of solid tumours and haematologic malignancies. Though DOX is one of the most effective anti-neoplastic agent, its clinical use is limited by its cardiotoxicity.1 DOX induces severe myocardial injury, resulting in cardiomyopathy. A number of efforts have been made to clarify the pathophysiological mechanisms for DOX-induced cardiotoxicity; however, it remains to be determined how DOX treatment leads to cellular damage, especially in the heart.

In eukaryotic cells, ubiquitin-proteasome system (UPS) is responsible for the maintenance of cellular homeostasis through the degradation of a wide range of proteins.2,3 The proteolytic reactions by UPS are highly specific for the target proteins and the activities of UPS are finely regulated. Recent studies implicate that the disturbance of UPS is closely associated with cardiac diseases.4,5 Interestingly, DOX activates the UPS in cardiomyocytes both in vitro and in vivo,6 although it remains to be elucidated how UPS is activated in response to DOX.

Atrogin-1, a muscle-specific ubiquitin ligase, plays critical roles in muscular atrophy.7 In the pathological process of muscle atrophy, including disuse atrophy and steroid-induced atrophy, atrogin-1 is upregulated and promotes protein degradation.8 Atrogin-1 is one of the target genes of FOXO transcriptional factors, forkhead family transcription factors.9–11 Importantly, activation of serine-threonine kinase Akt/protein kinase B negatively regulates the expression of atrogin-1 through FOXO pathways.12,13 Akt phosphorylates FOXO transcription factors, leading to the exclusion of phosphorylated FOXO proteins from the nucleus, with the inhibition of their transcriptional functions as a result. FOXO-mediated induction of atrogin-1 plays an important role in determining the cell size in cardiac myocytes.14

In the present study, we addressed the hypothesis that atrogin-1 is associated with DOX-induced cardiotoxicity. Here, we demonstrated that atrogin-1 was induced by DOX.
in cardiac myocytes. Furthermore, the DOX-induced upregulation of atrogin-1 was mediated by p38 mitogen activated protein kinase (MAPK), which was suppressed by activation of Akt. This study proposes a novel molecular mechanism for cardiotoxicity of DOX.

2. Materials and methods

2.1 Reagents and antibodies

DOX was kindly provided by Kyowa Hakko, Tokyo, Japan. MG-132 and SP600125 were purchased from Calbiochem, La Jolla, CA, USA. SB202190 and U0126 were the products from ALEXIX, San Diego CA, USA and Cell Signaling Technology, Danvers, MA, USA, respectively. Insulin-transferrin-sodium selenite (ITS) was purchased from Roche, Mannheim, Germany.

Antibodies against Akt, c-Jun N-terminal kinase (JNK), p38-MAPK and ERK1/2, and their phospho-specific antibodies were purchased from Cell Signaling Technology. Anti-phospho-FOXO3 antibody and anti-FOXO3 antibody were obtained from Upstate, Lake Placid, NY, USA. Anti-sarcomeric α-actinin antibody and anti-FLAG M5 antibody were from Sigma, Saint Louis, MO, USA. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Chemicon, Temecula, CA, USA. Horseradish peroxidase-conjugated anti-rabbit IgG antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody, and anti-ubiquitin antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Alexa Fluor 546-conjugated anti-mouse IgG antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody were the products of Molecular Probes, Eugene, OR, USA.

2.2 Cell culture

Primary cardiomyocyte cultures were prepared according to the procedure described previously. Briefly, cardiomyocytes were isolated by collagenase and trypsin digestion from the ventricles of one-day-old Wistar rats. Cultures were enriched with cardiac myocytes by plating for 60 min to eliminate the fibroblasts. Non-attached cells were used as cardiac myocytes. More than 90% cells were identified as cardiac myocytes, assessed by immunostaining with anti-sarcomeric α-actinin antibody (data not shown). Cardiomyocytes were maintained in Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F12) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were treated with DOX for 24 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay system (Promega).

2.3 Animal study

Male C57BL/6 mice (seven-week-old) were intraperitoneally injected with DOX (15 mg/kg body weight) in order to induce cardiotoxicity, according to the previous report. Forty-eight hours after injection, total RNA was prepared from various kinds of tissues and northern blot analyses were performed. All experimental procedures were performed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.4 Northern blot analysis

Northern blot analyses were performed as described previously. In brief, 10 μg of total RNA was electrophoresed in 1.2% agarose-formaldehyde gels. The gels were transferred to nylon membranes (Hybond-N+, Amersham Bioscience, Arlington Heights, IL, USA), and the membranes were UV-irradiated to crosslink RNA with the membrane. The probes were labelled with α-32P-dCTP by a random priming method according to the manufacturer's protocol (Megaprima DNA Labelling Systems, Amersham Bioscience). Hybridization was carried out in QuickHyb Hybridization Solution (Stratagene, La Jolla, CA, USA). The band intensity was normalized with that of GAPDH. Intensities of bands were quantitatively analysed by Scion Image (Scion Corporation, Frederick, MD, USA).

2.5 Immunoblot analysis

Immunoblot analyses were performed as described previously. Briefly, cells were treated with DOX under various conditions. After washing with phosphate-buffered saline (PBS), cell lysates were prepared by directly adding the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) sample solution. Proteins were separated by SDS–PAGE and transferred onto polyvinylidene difluoride membranes. After blocking, membranes were probed with the first antibody. Horseradish peroxidase-conjugated antibody was used as secondary antibody. All the first antibodies were diluted 1:1000 and secondary antibodies 1:2000. The ECL system (Santa Cruz Biotechnology) was used for detection. Intensities of bands were quantitatively analysed by Scion Image (Scion Corporation).

2.6 Promoter assays

The DNA fragment of the −3.5 kb promoter region of atrogin-1 gene was amplified by PCR using genomic DNA from Wistar rat as a template and was cloned into pGL3-basic (Promega, Madison, WI, USA). The reporter plasmid with the −3.5 kb promoter region of atrogin-1 gene including unique KpnI and BglII sites was incorporated at the 5’ and 3’ ends of the sequence, respectively, to simplify directional cloning into KpnI and BglII sites in the reporter plasmid, pGL3-basic. Primers used in the present study are as follows: (forward) 5’-GGG GTA CCC TTC TCC AGG CCA GTA GGT G-3’; (reverse) 5’-GGGA AGA TCT GGG TAC AGC ATC CAG ATC AGC AGG-3’. Plasmids were transfected with the FuGENE™ 6 transfection reagent (Roche Applied Science) according to the manufacturer’s protocol. Briefly, cardiomyocytes were cultured in 12-well plates and were transfected with 0.3 μg of reporter luciferase plasmid of atrogin-1 gene promoter construct and 0.2 μg of the control Renilla plasmid (pRL-TK-luc; Promega). After culturing in DMEM/F12 containing 1% FBS for 24 h, cardiomyocytes were treated with DOX for 24 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay system (Promega).

2.7 Immunofluorescent microscopy

Immunofluorescent microscopic analyses were performed as described previously. Briefly, cells were fixed with 3.7% formaldehyde in PBS for 15 min at room temperature and stained with anti-α-actinin or anti-FLAG antibody diluted 1:100. Alexa Fluor 546-conjugated anti-mouse IgG antibody, diluted 1:200, was used as a secondary antibody. Cells were examined by Olympus IX70 microscope.

2.8 Adenoviral vector construction and infection

DNA fragment of atrogin-1 cDNA, tagged with FLAG, was amplified by PCR, using cDNA from Wistar rat cardiomyocytes as a template. The primer sequences were: (forward) 5’-ATG GAC TAC AAA GAC GAT GAC GAC AAG TGG CCG TTC TCT GGG CAG-3’; (reverse) 5’-TGA ACA AAT TGA TAA AGT CCT TGA GGG GAG GGG-3’. Plasmids were transfected into 293 cells using calcium phosphate precipitation. Cell supernatants were collected 72 h after transfection. After purification of adenoviral particles, the adenoviral stock was titrated and stored at −80°C. The adenovirus titre was determined by a standard adenovirus plaque assay. Cardiomyocytes were infected with the adenovirus at a multiplicity of infection of 100 and were cultured for 24 h. The infected cells were then harvested and analysed by immunoblot and immunofluorescent analyses.
The generation of recombinant adenoviral vectors driven by the cytomegalovirus promoter was carried out through homologous recombination between pJM17 and the shuttle plasmid co-transfected in HEK293 cells as described previously. Viral vectors were purified by ultracentrifugation in the presence of caesium chloride. Adenovirus vector containing the β-galactosidase (β-gal) gene was used as a control vector. The construction of the adenoviral vector expressing constitutively active Akt (caAkt) was described previously.

In this study, cardiac myocytes were transfected with adenovirus vectors at MOI 5. In order to estimate the transfection efficiency, we infected cultured cardiac myocytes with adenovirus vector expressing β-galactosidase (β-gal) at MOI 5. Forty-eight hours after transfection, β-gal staining was performed and β-gal-positive cells were counted. As a result, we confirmed that more than 90% of cardiac myocytes were infected at MOI 5 (data not shown).

2.9 Statistical analysis

Statistical significance was analysed by t-test or by Mann–Whitney U test. Each value was expressed as mean ± SEM. Difference was considered statistically significant when the calculated P-value was <0.05.

3. Results

3.1 Doxorubicin induced atrogin-1 in the hearts and the skeletal muscles

In order to address pathological activation of UPS in the hearts, we examined the effects of DOX on the cardiac expression of a muscle-specific ubiquitin ligase, atrogin-1. Since atrogin-1 is expressed specifically in the striated muscles, we analysed whether DOX induces atrogin-1 in a tissue-specific manner. Mice were treated with DOX and total RNA was prepared from hearts, skeletal muscles, brains, kidneys, and livers. Northern blot analyses revealed that DOX-mediated induction of atrogin-1 was observed exclusively in the hearts and skeletal muscles (Figure 1A and B). Mouse atrogin-1 transcripts exhibited two bands in vivo, as described previously. To confirm the results from northern blot analyses, real time–PCR was performed. Consistently, atrogin-1 mRNA was upregulated by DOX in the hearts and skeletal muscles (Supplementary material online, Figure S1). We also examined the expression of MuRF-1, another muscle-specific ubiquitin ligase, which was upregulated by DOX in the skeletal muscles, but not in the hearts (Supplementary material online, Figure S2). Although DOX-induced ubiquitin ligases, such as atrogin-1 and MuRF-1, are associated with skeletal muscle atrophy (Supplementary material online, Figure S3), we focused on the induction of atrogin-1 in cardiomyocytes, considering the clinical importance of DOX-mediated cardiotoxicity. Indeed, we confirmed that the DOX treatment, according to the protocol of this study, resulted in the decrease in the heart-weight-to-tibia-length ratio two weeks after injection (Supplementary material online, Table S1).

To examine whether atrogin-1 is induced by DOX in cardiac myocytes, rat cardiomyocytes, cultured in the medium containing 1% FBS, were treated with DOX for 24 h and the expression of atrogin-1 was analysed by northern blotting (Figure 1C and D). In cultured rat cardiomyocytes, atrogin-1 transcript showed a single band that corresponds to the high molecular weight of the two bands, observed in the in vivo mouse experiments. Consistent with the in vivo results, atrogin-1 mRNA was remarkably increased by DOX treatment in cultured cardiomyocytes. In this study, we examined the effects of DOX in the low FBS medium, containing 1% FBS, in order to minimize the hypertrophic effects of FBS. DOX-induced upregulation of atrogin-1 was also examined in the medium containing 5% FBS (Supplementary material online, Figure S4).

To address the differences in the atrogin-1 bands, obtained by northern blotting between mouse in vivo studies and rat in vitro studies, we performed the northern blot analyses using total RNA prepared from neonatal mouse hearts. Mouse atrogin-1 showed two bands, while rat atrogin-1 mainly showed the larger one (Supplementary material online, Figure S5). Therefore, it is likely that the difference in atrogin-1 bands is derived from species differences.
3.2 Adenoviral overexpression of atrogin-1 induced cardiomyocyte atrophy with reduction in cell surface area

In order to examine the effects of DOX on cardiomyocyte morphology, cultured cardiac myocytes were treated with DOX and stained with anti-α-actinin antibody (Figure 2A and B). DOX treatment induced cardiomyocyte atrophy with the reduction of cell surface area. Importantly, DOX-induced atrophy was ameliorated by MG-132, a proteasome inhibitor, suggesting that UPS is involved in DOX-induced atrophy.

Since atrogin-1 is induced by DOX, we examined whether atrogin-1 induction contributes to cardiomyocyte atrophy. Cardiac myocytes were transfected with adenovirus vector expressing atrogin-1 and cultured for 24 h, followed by the immunostaining with anti-sarcromeric α-actinin antibody. The expression of exogenous atrogin-1 was confirmed by the immunoblotting with anti-FLAG antibody (Supplementary material online, Figure S6A and B). Similar to DOX treatment, adenoviral transfection of atrogin-1 resulted in the reduction of cell surface area, compared with β-gal, a control (Figure 2C and D). Moreover, MG-132 prevented the morphological changes induced by atrogin-1 overexpression. We also examined the localization of atrogin-1 overexpressed by the adenovirus vector (Supplementary material online, Figure S6C). In the presence of MG-132, atrogin-1 was localized on the sarcromeric structures as reported previously, while diffusely distributed in the absence of MG-132. Moreover, MG-132 treatment increased the expression level of atrogin-1 protein (Supplementary material online, Figure S6D). These results suggest that atrogin-1-mediated proteasome activity modulates the localization and expression of this protein.

3.3 Constitutively active Akt inhibited doxorubicin-mediated induction of atrogin-1 independent of FOXO transcription factors

Atrogin-1 expression is negatively regulated by Akt in cardiac and skeletal muscles. Thus, we examined the effects of adenoviral vectors expressing caAkt on DOX-mediated upregulation of atrogin-1. As shown in Figure 3A and B, atrogin-1 was upregulated in cardiac myocytes transfected with β-gal, a control, while transfection of caAkt suppressed atrogin-1 induction mediated by DOX.

FOXO transcription factors mediate the Akt-dependent downregulation of atrogin-1 expression. Therefore, to examine whether DOX induces atrogin-1 through FOXOs, we analysed the effects of DOX on the phosphorylation status of FOXO3 transcriptional factor, an Akt-mediated modulator of atrogin-1 transcription (Figure 3C). Cells were treated with DOX for 24 h, and immunoblot analyses were performed using phospho-FOXO3-specific antibody. DOX did not affect FOXO3 phosphorylation, whereas ITS treatment—a positive control—upregulated phospho-FOXO3 accompanied by a small upregulation of total FOXO3. Furthermore, to examine whether DOX regulates the transcriptional activity of FOXOs, the promoter analyses were performed by using the reporter constructs of −3.5 kb atrogin-1

![Figure 2 Atrogin-1 overexpression induced cardiomyocyte atrophy with decreased cell surface area. (A, B) Cardiac myocytes were pretreated with (-) or without (--) 2 μM proteasome inhibitor MG-132 for 30 min and then treated with DOX (0.1 μM) in the presence or absence of MG-132 for 24 h. After 24 h cultivation, cells were stained with anti-sarcromeric α-actinin antibody. (A) Representative results were shown. (B) Quantitative analyses of cell surface area. Data are shown as mean ± SEM, n = 100 cells for each culture. Statistical significance was analysed by t-test. *P < 0.05, **P < 0.01. Experiments were repeated three times with similar results. (C, D) Cardiomyocytes were infected with adenovirus-expressing atrogin-1 or β-galactosidase (β-gal) at MOI 5 for 18 h. The culture media containing virus vectors were removed and changed to the medium with (+) or without (--) 2 μM protease inhibitor MG-132. After 6 h of cultivation, cells were stained with anti-sarcromeric α-actinin antibody. (C) Representative results were shown. (D) Quantitative analyses of cell surface area. Data are shown as mean ± SEM, n = 100 cells for each culture. Statistical significance was analysed by t-test. *P < 0.05, **P < 0.01. Experiments were repeated three times with similar results.](image-url)
The membrane was reprobed with anti-total-Akt antibody. Band intensities for phospho-Akt were normalized with those for total-Akt. The results from four independent experiments were quantitatively analysed. *P < 0.05, **P < 0.01. (C) Cardiac myocytes were treated with 0.1 μM DOX or 5 μg/mL insulin-transferrin-sodium selenite (ITS) for 24 h. Cell lysates were prepared and immunoblotted with anti-phospho-FOXO3 antibody. Band intensities for phospho-FOXO3 were normalized with those for total-FOXO3. The results from four independent experiments were quantitatively analysed. *P < 0.05 vs. DOX (−), ITS (−). (D) Cardiac myocytes were treated with 0.1 μM DOX for 24 h. Cell lysates were prepared and immunoblotted with anti-phospho-Akt antibody. The membrane was reprobed with anti-total-Akt antibody. Band intensities for phospho-Akt were normalized with those for total-Akt. The results from four independent experiments were quantitatively analysed. Data are shown as mean ± SEM. Statistical significance was analysed by t-test.

gene promoter, which contains FOXO binding sites. After transfection, cells were exposed to DOX for 24 h, and luciferase activity was measured (Figure 4). Serum starvation enhanced atrogin-1 promoter activity, as reported previously, because serum starvation inactivates Akt, resulting in the activation of FOXO. In contrast, DOX did not activate the −3.5 kb promoter of atrogin-1 gene including FOXO-binding sites. Collectively, these data indicate that atrogin-1 was upregulated by DOX in an Akt-sensitive but FOXO-independent pathway.

3.4 Activation of p38-mitogen-activated protein kinase is required for atrogin-1 induction by doxorubicin in cardiac myocytes

To explore the molecular mechanisms responsible for atrogin-1 induction in the cardiomyocytes, we examined the DOX-induced activation of other signalling pathways, such as MAPK family. As shown in Figure 5, p38-MAPK and JNK, but not extracellular signal regulated protein kinase (ERK) 1/2, were activated by DOX in cardiomyocytes.

To reveal whether p38-MAPK or JNK is involved in atrogin-1 expression, we tested the effects of protein kinase inhibitors, SB202190, SP600125, and U0126 for inhibiting p38-MAPK, JNK, and ERK1/2, respectively. Cardiomyocytes were pretreated with these protein kinase inhibitors for 30 min, followed by DOX treatment in the presence of these inhibitors. Twenty-four hours after DOX treatment, cells were harvested and the expression of atrogin-1 was analysed by northern blotting. As shown in Figure 6, DOX-mediated induction of atrogin-1 was abrogated by SB202190, suggesting that p38-MAPK is required for DOX-induced upregulation of atrogin-1. To exclude the possibility that SB202190 inhibits atrogin-1 induction by modulating cardiotoxicity by DOX, we examined the effects of SB202190 on DOX-induced cytotoxicity by MTS assay. As a result, it was found that DOX showed no effect on cell viability in the presence of SB202190 (data not shown). Unexpectedly, the inhibition of JNK and ERK1/2 augmented atrogin-1 induction by DOX.

3.5 Constitutive activation of Akt inhibited p38-mitogen-activated protein kinase activity in cardiac myocytes

Since caAkt inhibited atrogin-1 induction, we examined the crosstalk between Akt and p38-MAPK, by analysing the effects of adenoviral transfection of caAkt on p38-MAPK phosphorylation (Figure 7). The phosphorylation of p38-MAPK by DOX was suppressed in cardiac myocytes transfected with adenovirus vector expressing caAkt, but not those with β-gal. We reproducibly observed that total p38-MAPK was slightly downregulated in caAkt-transfected cells, while the band intensities of GAPDH, an intrinsic marker, were not changed. The mechanism for Akt-mediated
downregulation of p38-MAPK protein remains to be elucidated; it might be possible that activation of p38-MAPK might increase its protein stability.

4. Discussion

In the present study, we demonstrated that atrogin-1, a muscle-specific ubiquitin ligase, was induced by DOX in the hearts and skeletal muscles, but not in non-muscle organs. Induction of atrogin-1 was observed in cultured cardiac myocytes, and the overexpression of atrogin-1 reduced cardiomyocyte size in vitro. Constitutive activation of Akt inhibited DOX-mediated induction of atrogin-1, but DOX induced atrogin-1 independently of FOXO pathway. Importantly, DOX activated p38-MAPK, whose inhibition abrogated the atrogin-1 induction. Finally, caAkt resulted in the inactivation of p38-MAPK. These findings suggest that DOX induced atrogin-1 through p38-MAPK pathway and contributed towards muscle atrophy in cardiac myocytes.

It has been reported that DOX activates UPS in cardiac myocytes. The transgenic mice overexpressing the surrogate substrate for UPS exhibited the enhanced activities of UPS in response to DOX treatment. DOX-mediated activation of UPS contributes to the downregulation of cardiac-specific genes, in cultured cardiac myocytes. Collectively, it is strongly suggested that activation of UPS is involved in DOX toxicity. And this is the first proposal that atrogin-1 is a candidate ubiquitin ligase that may play an important role in DOX-induced cardiotoxicity.

Throughout this study, there seems to be some difference in atrogin-1 induction by DOX, although atrogin-1 was reproducibly upregulated by DOX both in vitro and in vivo. Since we showed the results as fold induction of untreated samples, the different induction may be explained by the variation in basal expression level of atrogin-1, which has tendency to be influenced by cardiomyocyte preparation. Moreover, we confirmed the induction of atrogin-1 by real time RT-PCR, supporting the reliability of the northern blot analyses data.

In IGF-1 signalling, Akt negatively regulates atrogin-1 induction through FOXO transcriptional factors in cardiac and skeletal muscles. However, though the constitutive activation of Akt inhibited DOX-mediated induction of atrogin-1, FOXO phosphorylation was not affected by DOX in cultured cardiomyocytes. Moreover, the activity of atrogin-1 gene promoter with FOXO binding sites was not affected by DOX treatment. Thus, our data suggest that atrogin-1 was upregulated by DOX through an Akt-sensitive but FOXO-independent pathway in cardiac myocytes.

Interestingly, MuRF-1 was upregulated in skeletal muscles by DOX, but not in cardiac muscles. The difference in MuRF-1 expression between skeletal and cardiac muscles might propose the possibility that atrogin-1 expression might be regulated in skeletal muscles through distinct mechanisms from cardiomyocytes. Since MuRF-1 is a downstream target of FOXO transcriptional factors, these data...
by experiments were repeated four times. Statistical significance was analysed phospho-p38-MAPK and GAPDH were measured with densitometry. The downregulates p38-MAPK signalling in endothelial cells. In endothelial growth factor (VEGF)-mediated activation of Akt presented here, it has been reported that vascular endothelial crosstalk between Akt and p38-MAPK in cardiomyocytes demonstrated that caAkt negatively regulates p38-MAPK p38-MAPK and Akt has not been examined. Here, we have C2C12 myotubes, although the relation between stimulates the expression of atrogin-1 via p38-MAPK in its negative regulation of atrogin-1. support our findings that FOXO activity is not activated in cardiac myocytes by DOX and that DOX-mediated induction of atrogin-1 in cardiac myocytes is not related with the FOXO-dependent pathway. Finally, we demonstrated that DOX-induced activation of p38-MAPK was required for atrogin-1 upregulation. The involvement of p38-MAPK in atrogin-1 induction is also observed in skeletal muscles. Tumour necrosis factor-α stimulates the expression of atrogin-1 via p38-MAPK in C2C12 myotubes, although the relation between p38-MAPK and Akt has not been examined. Here, we have demonstrated that caAkt negatively regulates p38-MAPK activity, proposing a novel mechanism to explain Akt-mediated atrogin-1 upregulation. Consistent with the crosstalk between Akt and p38-MAPK in cardiomyocytes presented here, it has been reported that vascular endothelial growth factor (VEGF)-mediated activation of Akt downregulates p38-MAPK signalling in endothelial cells. In endothelial cells, VEGF stimulation causes MEKK3 phosphorylation via Akt, which is associated with decreased MEKK3 kinase activity and downregulation of MKK3/6 and p38-MAPK activities. Further studies would be required to address the involvement of MEKK3 phosphorylation by Akt in its negative regulation of atrogin-1. In this study, we propose that atrogin-1 is a novel player responsible for DOX-mediated cardiomyopathy. In this context, it should be noted that elevated myocardial Akt signalling ameliorates DOX-induced congestive heart failure and heart growth in vivo. Considering that atrogin-1 is a key molecule of muscle atrophy, as presented here, Akt might prevent DOX-induced cardiac dysfunction by inhibiting atrogin-1 induction in vivo. Indeed, the hearts from DOX-treated rats showed the atrophic phenotype with the reduction in heart weight, compared with those from non-treated rats, and the elevation of Akt activities ameliorates DOX-mediated reduction in heart weight. In conclusion, DOX upregulates atrogin-1 through p38-MAPK pathway in cardiac myocytes, leading to cardiomyocyte atrophy. Akt negatively regulates atrogin-1 expression by inhibiting p38-MAPK activity, as a novel mechanism. DOX-mediated induction of atrogin-1 could be a critical event for the onset of DOX cardiomyopathy.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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