Astragalus polysaccharide improves cardiac function in doxorubicin-induced cardiomyopathy through ROS-p38 signaling

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Abstract: Doxorubicin (DOX) is widely used as an antitumor agent, but it is significantly challenged by clinical workers due to the severe and acute cardiotoxicity. Astragalus polysaccharide (APS) is characterized by anti-inflammation and anti-oxidant features. In the current study, we explored the effects and specific mechanisms of APS on DOX-induced cardiomyopathy in mouse primary myocardial cells. To explore the effect of DOX on ROS production, DHE staining and flow cytometry analysis were used in primary cardiomyocytes treated with 1 μM DOX for 24 h. MTT assay was applied to determine the effect of DOX on cell viability. The effects of DOX on rat cardiomyocytes apoptosis by Hoechst staining and annexin V-PI staining, while caspase3 activity was determined using an assay kit. Two-dimensional echocardiography of rats was performed to determine left ventricular fraction and relative wall thickness. Activation of p38 and Akt was analyzed using western blot. ROS production was significantly enhanced by DOX stimulation in primary cardiomyocytes. DOX reduced rat cardiomyocytes viability in a time- and dose-dependent manner. DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3. Cardiac function was significantly impaired by enhanced p38 activation. APS treatment reduced DOX-induced rat cardiomyocytes apoptosis by decreasing ROS production. To conclude, APS reduced DOX-induced cell apoptosis and ROS production by reduced activation of p38 signaling pathway.

Keywords: APS, DOX, cardiac cell apoptosis, ROS, p38 signaling

Introduction

Doxorubicin (DOX) is widely used as an antitumor agent in a variety of cancers due to the antineoplastic and antibiotic characteristics [1, 2]. However, DOX is significantly challenged by clinical workers due to the severe and acute cardiotoxicity that finally leads to the irreversible chronic cardiomyopathy and heart failure. In tons of animal studies, DOX induced cardiomyopathy (DIC) has been widely reported [3]. But the specific mechanism is still not clearly elucidated. Studies indicate that multifactors and mechanisms are involved in the pathology [3]. Among these factors, oxidative stress may play a key role in DIC. Contractile dysfunction and rhythm disturbances of DIC result in the congestive heart failure in a dose dependent manner [1]. And progression of heart failure mainly involves the death of cardiac myocytes and non-myocyte myocardial cells as well as myofibril loss and fibrosis [4, 5]. Moreover, activation of matrix metalloproteinases (MMPs) induces extracellular matrix (ECM) degradation thereby increasing the collagen synthesis [5]. In previous studies, erythropoietin and antioxidants treatment demonstrates strong inhibiting capabilities of DIC-related apoptosis [6]. Since DIC is a major public health problem, necessary treatment methods should be explored.

In clinical practices, many approaches have been applied to reduce the severe side effects. However, there are still many problems [7]. Through DNA intercalation and Topoisomerase II inhibition, DOX can significantly kill cancer cells. However, in the heart failure, DOX has been showed to enhance oxidative stress by increased reactive oxygen species (ROS), such as O₂⁻, OH · and H₂O₂ [8]. From this perspective, cardiac myocytes death can be avoided by blocking the cardiomyocyte death pathways,
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which may improve DOX-induced side effects. And tons of studies find that supplementation of antioxidants in animals significantly improves DOX-induced heart failure [3]. However, clinical trials with vitamin E, an antioxidant, did not demonstrate obvious improvement [9]. Therefore, further study is needed to explore DOX induced cardiomyocyte death from the perspective of antioxidant-based therapy.

Astragalus polysaccharide (APS) is the major monomer that is extracted from Huang qi (Radix Astragali seu Hedysari). APS has long been used as an anti-inflammation and anti-oxidant herbal prescription in traditional Chinese medicine [10, 11]. In previous studies, APS has been widely reported to possess potent immunomodulatory activity [12]. One of the mechanisms of APS-related immunomodulatory activity is due to its anti-oxidative and anti-inflammatory capabilities. In the current study, we explored the effects and specific mechanisms of APS on DIC in mouse primary myocardial cells.

Materials and methods

Primary cardiomyocyte culture

Primary cardiomyocytes from rat neonatal hearts were isolated as described [13]. Animal protocol was approved by the Jinling Hospital. Briefly, hearts were isolated and digested with collagenase type II (Worthington) solution. After digestion, the cells were preplated for 2 hr to collect cardiomyocytes. Then, the attached cells were considered as non-myocytes and discarded. And the unattached cells were primarily cardiomyocytes.

Study protocols -in vivo

Eight-week-old male Sprague-Dawley rats were obtained from the fourth affiliated hospital of Harbin medical University. Then, the SD rats were injected with 3 mg/kg DOX (n = 4) or saline (n = 4) through the tail vein weekly for 5 weeks [14]. After injection with DOX or saline, the rats were explored respectively at week 0, 2 weeks and 4 weeks. The cardiac function was evaluated with echocardiography. After that, the hearts were excised and the proteins were extracted.

Two-dimensional echocardiography

Two-dimensional echocardiography was performed with certain modifications according to the previously reported methods [15]. On the day of evaluation, sodium pentobarbital (50 mg/kg) was used to anesthesize the rats. After shaving the chest, two-dimensional echocardiography was conducted using the echocardiographic systems (model SSD-900; Aloka, Tokyo) and 7.5 MHz probe (UST-987-7.5, Aloka). The M-mode echocardiograms of the left ventricle (LV) were determined at the papillary muscle level. All the index, including end-diastolic posterior wall thickness, end-diastolic and end-systolic internal diameters of the LV, were explored by a single observer. The follow formular was applied to determine the relative wall thickness (RWT):

\[ RWT = 2 \times \frac{LVPWTd}{LVDd} \]

LVPWTd refers to an end-diastolic posterior wall thickness of LV, and LVDd means an end-diastolic internal diameter of LV.

The formular was applied to determin fractional shortening (FS):

\[ FS = \frac{LVDd - LVDs}{LVDd} \]

LVDs equals to an end-systolic internal diameter of the LV.

Dimethyl thiazolyl diphenyl tetrazolium (MTT) assay

Cell viability was determined by a colormetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, sigma). To determine the impacts of DOX on cell viability, primary cardiomyocytes were cultured at approximately 70% confluency and starved in serum-free DMEM (SF-DMEM) (Life Technologies, Inc.) overnight. Then, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM DOX was preincubated with primary cardiomyocytes for 24 h. After drug treatment, the cells were cultured in fresh medium including 0.5 mg/mL MTT for 4 h. Then, DMSO was added into the wells to dissolve the blue formazan products and the density was determined spectrophotometrically at a wavelength of 550 nm. Besides, the cells were preincubated with 1 μM DOX for 8, 16, 24, 48 h and cell viability was determined in the same method as described. Each experiment was independently performed at least 3 times.
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**Hoechst 33258 staining**

Primary cardiomyocytes (1 × 10⁵ cells per well) were cultured in six-well tissue culture plates. The cells were incubated in serum-free DMEM medium for 16 h at 70-80% confluence. Then, 1 μM DOX was added to the fresh medium and preincubated with the cells for 48 h. After DOX treatment, the medium was removed, and the cells were washed three times with cold PBS and then fixed with 4% formaldehyde (Zhongshan Technology) in PBS for 20 min at room temperature. Then, the cells were washed three times with cold PBS and stained with Hoechst 33258 (10 μg/ml) (50 μL/slides) (Sigma) for 5 min. After staining, the cells were further rinsed with cold PBS and examined under fluorescence microscope.

**Apoptosis assay**

To detect the effects of DOX on primary cardiomyocytes apoptosis, the cells were treated with 1 μM DOX for 24 h with or without preincubation of 1 μM APS. After DOX treatment, the cells were washed with cold PBS for three times. Then, flow cytometry was used to determine cell apoptosis with an Annexin-V FITC-PI Apoptosis Kit (Invitrogen, Carlsbad, CA). In summary, cells were washed with 1 × PBS for three times and suspended at 2-3 × 10⁵ cells/mL in 1 × Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, which were then incubated at room temperature for 15 minutes in the dark. The cells without any treatment were used as internal control. After incubation, the cells were filtered by a filter screen and the cells were analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ) within 1 h of staining.

**Determination and quantification of ROS**

Cells were cultured on slides in six-well chamber at 60% confluence. Two days later, the slides were washed with cold PBS for three times. And then the slides were treated with 5 μM DHE (Vigorous Biotechnology Beijing Co., Ltd) in serum-free DMEM F-12 medium for 30 min at 37°C in darkness. The cells were fixed in 4% paraformaldehyde for 30 min at RT. The slides were washed with cold PBS for three times and mounted. Immunofluorescence images were captured by fluorescence microscopy. To quantify the intracellular ROS, relative fluorescence intensities were analyzed with flow cytometry (Becton-Dickinson) in the primary cardiomyocytes.

**Measurement of caspase-3 activities**

To measure the changes of caspase-3 activity, an assay kit (Medical & Biological Laboratory, Nagoya) was applied (22) through exploring the cleavage of the substrate 7-amino-4-trifluoromethyl coumarin conjugated to Asp-Glu-Val-Asp (DEVD-AFC). Firstly, 5 × 10⁵ cardiomyocyte cells were harvested and treated with the substrate for 1 h at 37°C. Then, the intensity of fluorescence was determined using a spectrophotometer (Bio Lumin 960; Molecular Dynamics, Sunnyvale, CA, USA). The activity was determined in comparison with the control.

**Protein extraction, western blotting and antibodies**

Cellular proteins were extracted using RIPA buffer (SolarBio, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl 1% (v/v) NP-40, 0.1%(w/v) SDS) with 1% (v/v) PMSF (SolarBio), 0.3% (v/v) protease inhibitor (Sigma) and 0.1% (v/v) phosphorylated protease inhibitor (Sigma). Lysates were collected for total protein after centrifugation. BCA protein assay kit (Pierce) was used to determine the protein concentration. 15 μg of protein was separated on an SDS-PAGE gel (10% (v/v) polyacrylamide) and transferred onto a PVDF membrane. After blocking with 8% (w/v) milk in PBST for 2 hr at room temperature, the membranes were then incubated with primary antibodies against GAPDH, cleaved-caspase3, p-Akt, p-p38, Akt and p38 (Cell Signaling) overnight at 4°C. Then, the membranes were washed with TBST for three times. And the membranes were incubated in HRP-conjugated goat anti-rabbit or HRP-conjugated mouse anti-goat IgG (Abmart, all at a 1:5000 dilution) for 2 hr at room temperature and then washed. Enhanced chemiluminescence (Millipore) was used to determine the protein concentrations according to the manufacturer’s recommendations. The relative contents of protein was normalized against GAPDH.

**Statistical analysis**

Data were presented as mean ± SE from 3 independent experiments. Statistical analysis
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was carried out with Student’s t test. P < 0.05 was considered as statistically significant difference.

Results

**DOX significantly enhanced ROS production in primary rat cardiomyocytes**

To explore the effect of DOX on ROS production, primary cardiomyocytes from rat neonatal hearts were treated with 1 μM DOX for 24 h. DHE staining showed that DOX significantly enhanced ROS production (Figure 1A). Furthermore, flow cytometry analysis revealed that DOX increased more than 2 fold of ROS production compared with control (Figure 1B). These data indicated that ROS production was significantly enhanced by DOX stimulation in primary cardiomyocytes.

**DOX inhibited primary rat cardiomyocytes viability in a time- and dose-dependent manner**

To determine the effect of DOX on cell viability, rat cardiomyocytes were preincubated with 1 μM DOX for 8, 16, 24, 48 hrs. As shown in Figure 2A, preincubation with DOX decreased cell viability by 32% and 45% at 24 h and 48 h, respectively. Meanwhile, when the cells were preincubated with 1 nM, 10 nM, 100 nM, and 1 μM DOX for 24 hrs, cell viability was reduced by 30% and 74% at 100 nM, and 1 μM, respectively (Figure 3B). These results demonstrated that DOX reduced rat cardiomyocytes viability in a time- and dose-dependent manner.
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DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3

We next observed the effects of DOX on rat cardiomyocytes apoptosis by Hoechst staining. As shown in Figure 3A, cell apoptosis was obvious in rat cardiomyocytes that were treated with 1 μM DOX for 24 hrs when compared with the control (Figure 3A). Furthermore, rat cardiomyocytes apoptosis was also analyzed using Annexin V-PI staining. As analyzed by flowcytometry, rat cardiomyocytes apoptosis was significantly enhanced by 1 μM DOX treatment, which was increased by nearly two fold (Figure 3C). Furthermore, the activity of caspase-3 in DOX-treated rat cardiomyocytes was significantly increased over untreated cells at 16 h and 24 h (Figure 3D). Together these data indicated that DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3.

DOX decreased cardiac function with enhanced p38 activation

In rats with DOX, FS was significantly decreased at age 14 weeks (2 weeks after the end of DOX-administration) and was further markedly diminished at age 16 weeks (Figure 4A). The changes in RWT of the LV, as assessed by echocardiography, are shown in Figure 4B. In rats with DOX, RWT was significantly decreased at age 14 weeks and was still further reduced at age 16 weeks (Figure 3B). Furthermore, we also examined the energy metabolism status in rats with DOX or saline. As shown in Figure 3C, when rats were injected with 3 mg/kg DOX (n = 4) or saline (n = 4) through the tail vein for 5 weeks, caspase3 was significantly activated at 16 weeks (Figure 4C). And the activation of p38 was significantly enhanced. Interestingly, the phosphorylation of Akt was obviously enhanced.
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which suggested a potential stress response thereby protecting the heart (Figure 4C). These data indicated that cardiac function was significantly impaired by enhanced p38 activation.

APS treatment reduced DOX-induced rat cardiomyocytes apoptosis by decreasing ROS production

To explore the protective role of APS on DOX-induced ROS production, rat cardiomyocytes were preincubated with or without preincubation of 1 µM APS and ROS contents were determined using DHE staining. As shown in Figure 5A, preincubation with 1 µM APS significantly reduced DOX-induced ROS contents. Meanwhile, flow cytometry was applied to determine apoptotic cells. As shown in Figure 5B, cell apoptosis rate was significantly reduced when rat cardiomyocytes were pretreatment with 1 µM APS. Furthermore, in previous report, it has been suggested that p38 MAPKs and p-Akt are involved in cell apoptosis [16]. Thus, we also tested caspase-3, p38 and Akt activation in rat cardiomyocytes treated by DOX with or without preincubation of 1 µM APS. The results found

Figure 5. APS reduced DOX-induced rat cardiomyocytes apoptosis by decreasing p38 activation. A. Preincubation with 1 µM APS significantly reduced DOX-induced ROS contents. B. Cell apoptosis rate was significantly reduced when rat cardiomyocytes were pretreatment with 1 µM APS as analyzed by flow cytometry. C. Western blot analysis was applied to explore caspase-3, p38 and Akt activation in rat cardiomyocytes treated by DOX with or without preincubation of 1 µM APS. Data represent the means ± SEM, n = 3 independent experiments. *P<0.05, **P<0.01, versus control. †P<0.05, ††P<0.01 versus APS+DOX.
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that APS preincubation significantly reduced DOX-induced caspase3 activation. In comparison, p38 phosphorylation was significantly reduced with preincubation of 1 µM APS. These data indicated that APS reduced DOX-induced rat cardiomyocytes apoptosis by decreasing p38 activation.

Discussion

In previous studies, apoptosis was significantly induced in animal models and patients of heart failure [17, 18]. Dox was formally used as an anti-tumor agent in clinical practices. However, the cardiac toxicity significantly restricted its promotion and application. In the present study, we found that DOX significantly induced ROS production and cell apoptosis in cardiomyocytes. Meanwhile, the activity of caspase-3 was significantly increased. In the rats injected with DOX, the left ventricular function had significantly impaired and the RWT was obviously reduced. These data indicated that DOX induced cardiomyocytes apoptosis mainly by increased ROS production and activation of caspase3. And it is thus suggested that apoptosis played a key role in the progression of myocardial dysfunction induced by DOX.

Oxidative stress are proved to be widely involved in cellular apoptosis [19]. Upregulation of ROS usually leads to abnormal intracellular signaling pathways and then triggers cell apoptosis [20]. Consequently, reduced ROS production prompts cell survival in a variety of cells [21]. We showed that DOX stimulated ROS formation thereby enhancing cell apoptosis. Then, we explored several different cellular signaling pathways that are suggested to be stimulated by ROS. PI3K/Akt signaling pathway was proved to significantly enhanced cell survival. In this study, when rat cardiomyocytes were treated with DOX, Akt was obviously activated, which may indicate the stress-response effect of cardiomyocytes. This result is in consistent with other studies, which all prove the stress-response effect of cardiomyocyte after abnormal stimulation [22, 23]. MAPKs are also key regulators in cell survival, which mainly include p38, ERK, and JNK [24]. In this study, we choose p38 as a representative signaling effecter in MAPK signaling pathway. Our data found that phosphorylation of p38 was significantly enhanced, which indicated impaired cell survival. And we also tested cell viability with MTT assay, which indicated the DOX impaired cell viability in a time- and dose- dependent manner. These data indicated that DOX reduced cardiomyocytes viability through the ROS-p38-pathway.

Astragalus polysaccharide (APS) is an active component extracted from traditional Chinese medicine, which is widely used in a variety of disease models, such as diabetes, cancer, renal failure and so on. Previous studies have demonstrated the antioxidant, antihypertensive, and immunomodulatory characteristics [25, 26]. In this study, we found that preincubation with APS significantly decreased DOX-induced ROS production. Consequently, decreased oxidative stress by lowering ROS production significantly inhibited cell apoptosis. APS was found to inhibit cell apoptosis by scavenging ROS and decreasing mitochondrial permeability transition. In line with the study, we demonstrated that DOX induced ROS production and APS treatment demonstrated obvious protective effects by reducing caspase3 activation [18]. Several studies have indicated that APS can inhibit ROS-p38 activation in advanced glycation end product-stimulated macrophages [27]. Similar to these findings, our studies found that APS reduced DOX-induced cell apoptosis by reduced activation of p38 signaling pathway.

In summary, DOX induced cardiomyocytes apoptosis mainly by increased ROS production and activation of caspase3, which then play key roles in DOX-induced cardiomyopathy. Furthermore, APS worked as an anti-oxidant and anti-inflammatory agent, which significantly decreased ROS production and prevented cardiac injury.

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Disclosure of conflict of interest

None.

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