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Research Article

Astragalus Polysaccharide Suppresses Doxorubicin-Induced Cardiotoxicity by Regulating the PI3k/Akt and p38MAPK Pathways

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Background. Doxorubicin, a potent chemotherapeutic agent, is associated with acute and chronic cardiotoxicity, which is cumulatively dose-dependent. *Astragalus* polysaccharide (APS), the extract of *Astragalus membranaceus* with strong antitumor and antiglomerulonephritis activity, can effectively alleviate inflammation. However, whether APS could ameliorate chemotherapy-induced cardiotoxicity is not understood. Here, we investigated the protective effects of APS on doxorubicin-induced cardiotoxicity and elucidated the underlying mechanisms of the protective effects of APS. *Methods.* We analyzed myocardial injury in cancer patients who underwent doxorubicin chemotherapy and generated a doxorubicin-induced neonatal rat cardiomyocyte injury model and a mouse heart failure model. Echocardiography, reactive oxygen species (ROS) production, TUNEL, DNA laddering, and Western blotting were performed to observe cell survival, oxidative stress, and inflammatory signal pathways in cardiomyocytes. *Results.* Treatment of patients with the chemotherapeutic drug doxorubicin led to heart dysfunction. Doxorubicin reduced cardiomyocyte viability and induced C57BL/6J mouse heart failure with concurrent elevated ROS generation and apoptosis, which, however, was attenuated by APS treatment. In addition, there was profound inhibition of p38MAPK and activation of Akt after APS treatment. *Conclusions.* These results demonstrate that APS could suppress oxidative stress and apoptosis, ameliorating doxorubicin-mediated cardiotoxicity by regulating the PI3k/Akt and p38MAPK pathways.

1. Introduction

Anthracyclines such as doxorubicin, daunomycin, epirubicin, and idarubicin are anticancer chemotherapeutic drugs that are widely used in clinical cancer treatment [1]. Unfortunately, these types of drugs are associated with dose-dependent acute or chronic cardiotoxicity, which is characterized by hypotension, tachycardia, arrhythmia, transient depression of left ventricular function, and even refractory late-onset cardiomyopathy [2]. Because of such negative effects, their usage is limited despite their potent and effective functions in treating cancer. Doxorubicin has been postulated to induce cardiotoxicity because of its free radical-induced mitochondrial damage [3]. Given a series of studies reporting that reactive oxygen species (ROS) scavengers fail to effectively prevent cardiac toxicity, this hypothesis is not sufficient for explaining the cardiac toxicity caused by doxorubicin [4]. Many studies have demonstrated that doxorubicin induces DNA damage, inhibits DNA and protein synthesis, promotes myofiber degeneration, suppresses the transcription of specific genes, and results in cardiomyocyte apoptosis through a caspase-3-dependent mechanism [5].

In addition, we used LY294002 (2 μM) [15], a PI3K inhibitor, to inhibit Akt phosphorylation. As expected, LY294002 significantly suppressed the elevated Akt phosphorylation caused by APS pretreatment (Figure 5(e)). Accordingly, DNA laddering showed that the APS-mediated protection of doxorubicin-treated NRVMs could be abrogated by LY294002 (Figure 5(f)). In summary, in vitro and in vivo results indicate that APS exerts its cardioprotective effects against doxorubicin-induced cardiomyocyte injury by regulating the p38MAPK and Akt pathways.

5. Discussion

5.1. Doxorubicin Triggers Cardiotoxicity In Vitro and In Vivo. The anthracycline drug doxorubicin remains one of the most potent active antineoplastic agents used for the treatment of solid tumors and hematologic malignancies [16]. However, acute and chronic cardiotoxicity limit its clinical use. It has been suggested that doxorubicin could cause severe cardiomyopathy and heart failure.

In this study, we collected the clinical information of 206 cancer patients who had no history of cardiovascular disease and performed a retrospective analysis. Approximately 30.6% of patients who had a normal ECG before chemotherapy and an abnormal ECG after chemotherapy had a decreased LVEF with a cumulative dose of 450–600 mg/m^2. Our results are similar to the retrospective analysis of three trails performed by Swain et al., who reported that the estimated percentage of patients receiving doxorubicin-based treatment at a cumulative dose of 550 mg/m^2 who had doxorubicin-induced heart injury is 26% [17].

In vitro and in vivo experiments have been previously performed using cardiomyocyte cell lines such as H9c2, neonatal rat ventricular myocytes, mice, and rats to investigate doxorubicin-induced cardiomyocyte injury. According to previous research, doxorubicin could induce cardiomyocyte loss and heart dysfunction in a dose-dependent manner [18, 19], which was confirmed by our study. Thus, considering potent effects for the treatment of solid tumors and hematologic malignancies [20–22], we should focus considerable attention on lessening DOX-induced cardiotoxicity, reducing the doxorubicin limit for clinical usage.

5.2. APS Protects Cardiomyocytes from Doxorubicin-Induced Oxidative Stress and Apoptosis. APS has long been known to act as an effective traditional medicine for enhancing immunity, inhibiting tumor growth, and alleviating inflammation-induced artery endothelium cell injury and atherosclerosis [9, 23]. Among these functions, APS has been most widely studied, mainly for its immunopotentiating properties such as stimulating immunocyte proliferation and cytokine production to defend against cancer [9]. Additionally, previous studies have demonstrated that APS may be used as a potent protective medicine for addressing heart diseases such as myocardial hypertrophy and heart failure [7]. Many Chinese patent medicines derived from APS are prescribed to patients with cardiac disease. Clinical studies have also indicated that APS could counteract the side effects of chemotherapeutic drugs, such as remarkably mitigating the degree of myelosuppression in cancer patients [11]. However, few groups have investigated whether APS could provide benefit for hearts damaged by doxorubicin treatment. In this study, we verified that APS markedly reduces doxorubicin-induced cardiomyocyte injury via the suppression of oxidative stress and apoptosis.

5.3. APS Suppresses Cardiomyocyte Apoptosis via Activation of the PI3K/Akt Signaling Pathway. Apoptosis is important for the development of most organs and adult tissue homeostasis and remodeling. However, inexorable loss of terminally differentiated cardiomyocytes followed by their replacement with fibrotic tissue is a hallmark of the transition from cardiac hypertrophy to functionally decompensated heart failure [24], thus playing a pivotal role in the development of various heart diseases.

Previous studies have shown that the PI3K/Akt signaling pathway provides an essential cell survival signal in cardiomyocytes [25]. In this study, we found that APS could activate the PI3K/Akt signaling pathway and reduce doxorubicin-induced cardiomyocyte apoptosis. Doxorubicin induced an increase in Akt phosphorylation, which is similar to findings by Swain et al. [17, 26], and considered a compensatory protective upregulation. However, our in vitro experiments showed that, after APS treatment, phosphorylated Akt was dramatically decreased. To explain the different phenomenon, we analyzed the time course of doxorubicin-induced Akt phosphorylation in NRVMs. Western blotting showed that phosphorylated Akt was elevated after doxorubicin treatment within a short period, which was reduced after 48 h. These results strongly support the explanation that doxorubicin treatment could induce a compensatory Akt phosphorylation increase within a short period [17, 27]. After APS pretreatment, phosphorylated Akt dramatically increased as compared with the DOX group, whereas proapoptotic proteins, such as activated caspase 3 and caspase 9, were decreased in vitro and in vivo, indicating that APS could effectively suppress apoptosis by activating the Akt pathway.

5.4. APS Impairs Doxorubicin-Induced ROS Generation and p38MAPK Activation in Cardiomyocytes. Myocardial oxidative stress has been shown to lead to ventricular dilatation in humans and animal heart failure models [28]. Doxorubicin has been suggested to induce the activation of p38MAPK, leading to inflammatory reactions and cell injury [29]. Consequently, reducing oxidative stress by lowering ROS production is crucial for the management of doxorubicin-induced cardiotoxicity. In this study, we found that doxorubicin led to increased ROS generation and p38MAPK activation, which was largely decreased by treatment with APS. Given that oxidative stress could activate p38MAPK, APS could effectively impair p38MAPK phosphorylation and attenuate cell inflammation, which suppresses cardiomyocyte apoptosis and injury in vivo and in vitro.

5.5. The Clinical Relevance of Heart Failure and APS Treatment. In this study, we demonstrated that pretreatment with APS suppresses the doxorubicin-induced apoptosis of

2.7. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) and Hoechst 33342 Staining of Heart Cryosections and Cultured Cardiac Myocytes. Nuclear fragmentation was detected by TUNEL staining with an apoptosis detection kit (Roche) or by incubating fixed cells in 10 mM Hoechst 33342 as previously described [13]. Cells (500–700) in 10 randomly chosen fields from each dish were counted to determine the percentage of apoptotic nuclei. Each data point indicates results from 1600 to 2000 cells from 4 independent experiments [13].

2.8. Isolation and Culture of Rat Cardiac Myocytes. Neonatal rat ventricular myocytes (NRVMs) were isolated from 1–3-day-old Sprague Dawley rats via combined trypsin and collagenase type II digestion [13]. The cardiac myocytes were plated at a density of 6.6×10^4 cells/cm^2 in DMEM supplemented with 10% FBS in the presence of 0.1 mM 5-bromo-2-deoxyuridine.

2.9. Western Blotting Analysis. Cell lysates were analyzed by SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked with 8% bovine serum albumin for 2 h and incubated with specific antibodies for 2 h. After 5 washes in TBST (TBS containing 0.1% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in TBST for 1 h. Bands were detected by chemiluminescence detection reagents. Blot densitometry was performed, and bands were analyzed with ImageJ software.

2.10. Cell Viability Assay. Cell viability was examined by the MTT assay according to the instructions of the manufacturer. NRVMs (5000 cells/well) were plated in 24-well plates. NRVMs were pretreated with APS for 1 h and then treated with the indicated concentrations of DOX for 24 h. All assays were performed in triplicate. The cells were incubated with 0.5 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyltetrazolium bromide for 4 h, and the absorbance at 490 nm was measured as previously described [13]. The MTT kit was purchased from Roche Applied Science (Indianapolis, IN).

2.11. In Situ Detection of Reactive Oxygen Species (ROS). To evaluate heart ROS production in situ, frozen, unfixed, whole heart cross-sections or living NRVMs were stained with 10 μmol/L DHE (Sigma) for 30 min in a dark humidified chamber at 37°C. ROS generation was indicated by red fluorescence and visualized with fluorescence microscopy.

2.12. DNA Laddering. Cells were lysed in lysis buffer (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 10 mM EDTA, 0.4% SDS, and 100 g/mL protease K) and incubated at 50°C for 5 h with gentle agitation. DNA was then extracted with phenol/$CHCl_3$/isoamyl alcohol and $CHCl_3$/isoamyl alcohol. DNA fragmentation was detected by loading 10 μg of total DNA into a 2% agarose gel; the gel was run in Tris-acetate-EDTA buffer and visualized by ethidium bromide staining [13].

3. Statistical Analysis

All statistical calculations were performed using GraphPad Prism 5 software. The data are expressed as the mean ± SEM. Student's t-test was used to compare two conditions, and one-way ANOVA with Bonferroni correction was used for multiple comparisons. Probability values less than 0.05 were considered significant.

4. Results

4.1. Doxorubicin Treatment Leads to Increased Levels of Serum Myocardial Enzymes Accompanied by Heart Dysfunction in Cancer Patients. Clinical data from 206 cancer patients who had no comorbidities, such as diabetes, hypertension, and ischemic heart disease, and underwent initial doxorubicin-based chemotherapy (a cumulative dose of 450–600 mg/m^2) were retrospectively analyzed. Fifteen patients who had no history of cardiovascular disease but an abnormal electrocardiograph (ECG) before chemotherapy were excluded from the database. A total of 63 patients (30.6% of total patients) who had a normal ECG before chemotherapy and an abnormal ECG after chemotherapy had a normal baseline LVEF 65.1 ± 4.5% and a final value less than 53.2 ± 7.4% after doxorubicin therapy (Figure 1(a)). Serial determinations of cardiac enzymes (cTNT, CK-MB) were performed. After chemotherapy, the median serum cTNT level increased from 0.04 ng/mL (75%—CI: 0.025–0.072 ng/mL) to 0.15 ng/mL (75%—CI: 0.13–0.21 ng/mL) for 63 patients (Figure 1(b)), and the median serum CK-MB level was elevated from 2.1 ng/mL (75%—CI: 1.29–2.49 ng/mL) to 5.73 ng/mL (75%—CI: 5.17–6.28 ng/mL) (Figure 1(c)). The average serum cTNT and CK-MB levels exceeded the threshold for normal values (0.1 and 5.0 ng/mL, resp.) [14].

4.2. Doxorubicin Induces Cardiomyocyte Injury by Promoting Oxidative Stress and Apoptosis. Cell viability assays were performed using doxorubicin-treated NRVMs. As shown in Figure 2(a), doxorubicin treatment reduced cell viability in a dose-dependent manner compared with the control; 0.1 μM doxorubicin treatment led to 20% decreased cell viability. Similarly, TUNEL assay (Figure 2(b)) verified that doxorubicin triggered apoptosis in a dose-dependent manner. Moreover, to explore the underlying mechanisms of cardiotoxicity induced by doxorubicin, cell survival and apoptosis-related signaling proteins such as caspase 3 and p38MAPK were detected. The levels of active (cleaved) caspase 3 and phosphorylated p38MAPK were significantly increased in a concentration-dependent manner in cardiomyocytes treated for 24 h with doxorubicin (Figure 2(c)). Moreover, doxorubicin led to elevated ROS generation in the hearts of C57BL/6J mice and cultured NRVMs (Figure 3(a)). These results indicate that doxorubicin could induce cardiotoxicity via oxidative stress and apoptosis.

4.3. APS Reverses Doxorubicin-Induced Oxidative Stress and Apoptosis in Cultured Primary Neonatal Rat Ventricular Myocytes. As shown in Figure 3(a), NRVMs pretreated with 50 μg/mL APS attenuated doxorubicin-induced ROS generation.

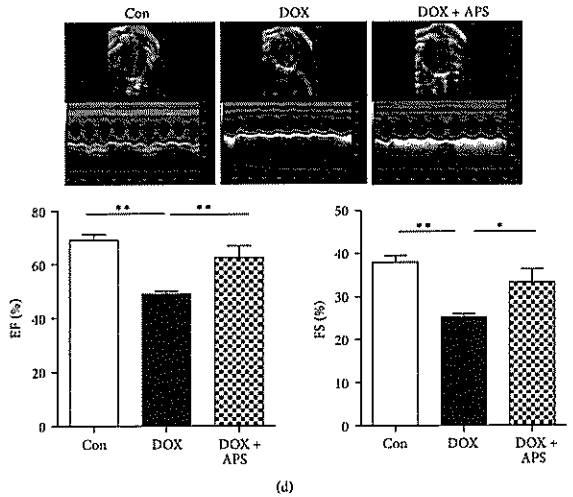

FIGURE 4: APS ameliorates doxorubicin-induced cardiotoxicity and apoptosis in vivo. (a) H&E staining of normal mice (Con) and mice with doxorubicin-induced heart failure (HF) ($n = 5$). (b) Representative TUNEL staining of apoptotic cells in normal and doxorubicin-induced heart injury samples. Red staining indicates TUNEL-positive cells ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$). (c) Western blotting and average data for caspase 3, caspase 9, and Bcl2 in sham, doxorubicin-induced heart injury mice (DOX), and mice with APS pretreatment followed by doxorubicin treatment (APS + DOX) ($n = 15$, $^{**}P < 0.01$, $^{***}P < 0.001$). (d) Mouse heart function 5 days after doxorubicin injection as shown by fractional shortening % (FS %) and ejection fraction % (EF %) ($n = 8$, $^{*}P < 0.05$, $^{**}P < 0.01$).

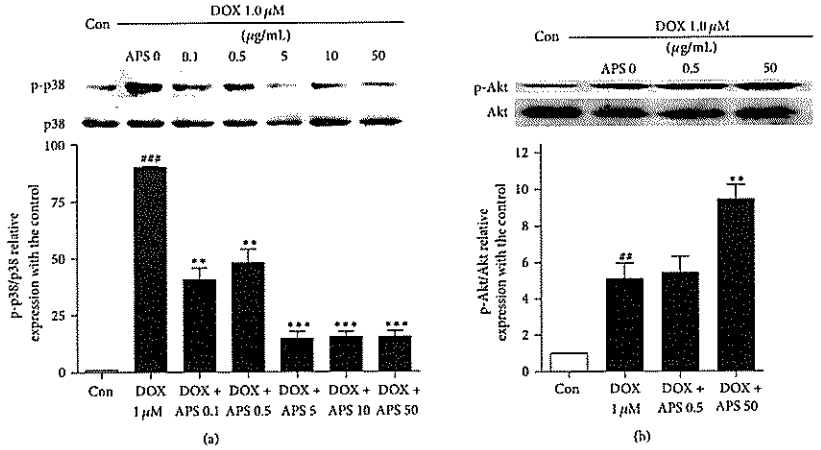

FIGURE 5: Continued.

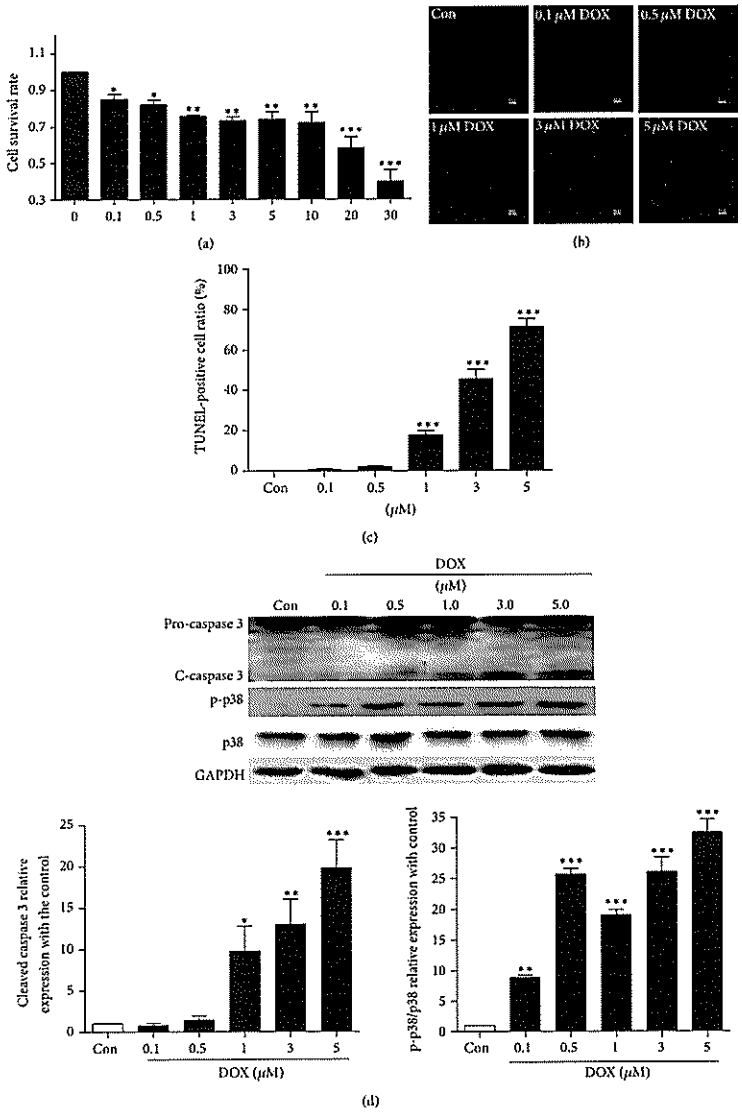

FIGURE 2: Doxorubicin induces cell injury by promoting apoptosis in cardiomyocytes. (a) The cell viability NRVMs cultured with different concentrations of doxorubicin (0, 0.1, 0.5, 1, 3, 5, 10, 20, and 30 μM) for 24 h as demonstrated by MTT assay ($n = 4$). (b) Representative TUNEL staining of NRVMs cultured with different concentrations of doxorubicin (0, 0.1, 0.5, 1, 3, and 5 μM) for 24 h. (c) Average TUNEL staining data ($n = 4$). (d) Dose response of activated (cleaved) caspase 3 and phosphorylated p38MAPK as assayed by Western blotting for NRVMs treated with 0.1–5.0 μM doxorubicin for 24 h ($n = 3$) ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the control group).