Ononin Alleviates Doxorubicin-Induced Cardiotoxicity by Inhibiting ER Stress Through Activation of SIRT3

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Abstract

Introduction

Doxorubicin (DOX) is a powerful anthracycline antineoplastic drug, but the clinical application of DOX is seriously limited by its dose-dependent cardiotoxicity. Ononin is a natural isoflavone glycoside and plays a key role in modulating apoptosis related signaling pathways. The aim of this study was to assess the possible cardioprotective effects of Ononin in DOX-induced cardiotoxicity and the underlying molecular mechanisms.

Materials and methods

Wistar rats were treated with normal saline, DOX with or without Ononin. After the last administration, cardiac function was evaluated by echocardiography. Rats were then sacrificed for histological and TUNEL analyses, with immunological detection for β -actinin, Bax, Bcl-2, GRP78, CHOP and SIRT3. An enzyme-linked immunosorbent assay was performed to assess the myocardial injury markers. H9C2 cells were treated with vehicle, DOX with or without Ononin. Then, 3-TYP was used to find out the relationship between ER stress and SIRT3.

Results

Ononin treatment ameliorated DOX-induced myocardial injury as demonstrated by echocardiography. Ononin partially restored DOX-induced cardiac dysfunction, both LVEF and LVFS were increased under the cotreatment of Ononin. Ononin also inhibited DOX-induced ER stress and apoptosis in rat cardiomyocytes and H9C2 cells. DOX group had a higher Bax/Bcl-2 ratio, GRP78 and CHOP expression than control group, but Ononin treatment improved these results. This effect was associated with SIRT3 activity, moreover, selective inhibition of SIRT3 blocked the protective effects of Ononin.

Conclusion

In the present study, we tested the hypothesis that Ononin may protect against DOX-induced cardiomyopathy through ER stress both in vitro and in vivo. Ononin is able to protect against DOX-induced cardiotoxicity by inhibiting ER stress and apoptosis, this effect may via stimulation of the SIRT3 pathway.

Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is a commonly used and highly effective antineoplastic agent used for the treatment of several forms of cancers[1]. Unfortunately, the scope of dox-therapy is significantly limited by a delayed and progressive cardiomyopathy[2], which is dose-related, cumulative,

and essentially irreversible[3]. Chronically, DOX can result in irreversible heart failure manifesting as dilated cardiomyopathy and refractory congestive cardiac failure. The mechanisms of DOX-induced cardiotoxicity have not been fully elucidated. An excessive reactive oxygen species (ROS) generation, lipid peroxidation and endoplasmic reticulum-mediated apoptosis have all been linked to DOX-induced cardiotoxicity[4–6]. Recent studies have demonstrated that a chemical endoplasmic reticulum chaperone could obliterate DOX-induced cardiac dysfunction[7]. Some treatments may alleviate DOX-induced cardiotoxicity by inhibiting endoplasmic reticulum-mediated apoptosis[8, 9]. Further investigation of the molecular pathways responsible for Dox-induced cardiotoxicity is needed to identify effective interventions.

Flavonoids are natural polyphenolic compounds, typically known for their antioxidant activity[10]. They are also emerging as a notable choice for their multilevel, multitarget, and integrated intervention effects for cancer treatment[11]. Ononin, formononetin-7-O- β -D-glucoside, is one type of flavonoid found in many functional foods and plants, especially rich in Chinese herbal medicines such as Astragali Radix, Glycyrrhizae Radix, Puerariae Radix and Codonopsis Radix[12]. Ononin has benefit on ameliorating the obesity-provoked metabolic injury through the inflammation inhibition through its anti-inflammatory activities[13].

Although the interplay between anthracyclins and the lipidome is not fully understood, the changes in the lipidome may affect the internalization and the mechanism of action of the drug to induce cell death, in which ER stress plays a key role. With The lipophilic and amphiphilic nature of the DOX, cardiomyocytes often accumulate more DOX. Relieving the effect of ER stress can delay the development of dox-induced cardiotoxicity to a certain extent. SIRT3 is a class III histone deacetylase in mitochondria. Recent studies have demonstrated that GADD45 β -I could attenuate apoptosis via Sirt3-mediated inhibition of ER stress in osteoarthritis chondrocyte[14] and SIRT3 overexpression maintained mitochondrial integrity and prevented DOX-mediated cardiomyocyte death[15, 16]. However, whether Ononin could protect DOX-induced cardiotoxicity is not yet clear, and the underlying mechanisms need to be elucidated. To examine the preventive effect of Ononin and further determine its specific mechanisms, we performed an in vitro investigation and an in vivo study in rats with DOX-induced cardiotoxicity.

Materials And Methods

Ethics statement

All animal experiments were approved by Shaoxing People's Hospital Ethics Committee and performed according to the guidelines from the National Institutes of Health. All efforts were made to minimize suffering.

DOX-induced cardiac injury in rats

24 male Wistar rats (8-weeks-old, 250 g $\pm$ 10 g), were purchased from the Experimental Animal Center of Basic Medicine, Zhejiang Chinese Medical University, China. Before being assigned to the experimental

groups, rats were allowed to adapt to the environment in a light-controlled room (12 h light/dark cycle) where they had free access to standard chow and water for 7 days. All rats were randomly divided into 4 groups: control (CON), DOX, DOX + Ononin30 (Cat. HY-N0270, MedChemExpress, USA), and DOX + Ononin60 according to previous research[17]. Animals in the DOX, DOX + Ononin30 and DOX + Ononin60 groups were administered 2.5 mg/kg DOX (Cat.HY-15142A, MedChemExpress, USA) via the tail vein once a week for 6 weeks (total dose: 15 mg/kg) to induce cardiac injury. Animals in the DOX + Ononin30 and DOX + Ononin60 groups were intragastrically injected with Ononin (30 mg/kg/day or 60 mg/kg/day, respectively) before receiving the above DOX regimen. The CON group received an equivalent saline solution by gavage for 6 weeks (Fig. 1A). The dosage of DOX was chosen based on previous studies and our preliminary work. The chemical structures of DOX and Ononin were shown in Fig. 1B and C.

At the end of the study, body weight was measured following anesthesia. After euthanasia, blood was collected. All hearts were weighed and cut into several parts for further investigation.

Echocardiography

At the end of the 6-week DOX treatment, two-dimensional M-mode echocardiography was performed using the Philips iE33 system (Philips Medical, Best, Netherlands) equipped with an s5-1 probe (12–14 MHz) to evaluate rat cardiac function. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were measured and calculated with Philips QLab 9 post-processing software.

Measurements of LDH

Cardiac marker enzymes including LDH was measured in serum using commercially available kits (Cat. A020-1-2, Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocol.

Histological analysis

After the rats were sacrificed, the myocardial tissues were immediately separated and fixed in 10 % neutral buffered formalin for 24 h. To evaluate cardiac morphology and fibrosis, the tissue sections were respectively stained with hematoxylin and eosin (H&E), Masson's trichrome stain, and wheat germ agglutinin (WGA, Cat.L4895, Sigma Aldrich, USA) according to standard protocols. Subsequently, the sections were imaged using a Leica DM3000 microscope (Leica, Wetzlar, Germany) and Nikon Eclipse Ti-U fluorescence microscope (Minato-ku, Tokyo, Japan).

Immunohistochemistry (IHC) staining

Immunohistochemistry was used to visualize the expression of GRP78. Histologic sections were dehydrated with a series of alcohol and xylene gradients. Then, 3% H₂O₂ was used to quench endogenous peroxidases followed by treatment with citrate buffer for 15 min at 95 °C. The tissue sections were incubated with anti-GRP78 or anti-SIRT3 at 4 °C overnight. The next day, specimens were

stained with the secondary antibody for 30 min and 3,3'-diaminobenzidine (DAB) for 5 min at 37 °C. Finally, images were taken using a Leica DM3000 microscope.

TdT-mediated dUTP nick end-labeling (TUNEL) staining

Cardiomyocyte apoptosis was examined with TUNEL staining, using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) following the manufacturer's protocol. Results were imaged under a fluorescence microscope (Minato-ku, Tokyo, Japan).

Cell culture and treatment

The H9C2 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and applied in our experiments. All of these cells were cultured in DMEM (Sigma, USA) supplemented with 10 % FBS (Gibco, USA) and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco), and maintained at 37 °C in a humidified incubator with 5% CO₂. H9C2 cells were treated with Ononin (0.5µM) for 24h and then exposed in DOX (1µM) for 24h. In a separate experiment, 3-TYP (SIRT3 inhibitor, 1µM, Cat.HY-108331, MCE) was used 12h before adding DOX. The dosage of 3-TYP was based on previous studies[18].

Cell viability assay

Cell viability was examined using the CCK-8 assay kit (Cat. HY-K0301, MedChemExpress, USA) according to the manufacturer's instructions. The results were read with microplate reader at 450 nm.

Western blot

Heart tissues or H9C2 cells were lysed with RIPA lysis buffer (Beyotime, China) containing protease inhibitors and phosphatase inhibitor (Mce, China). The extracted proteins were separated by 10 % polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, MA). The membranes were blocked for 60 min with 5% skim milk at room temperature and incubated overnight with primary antibodies against GAPDH, Bcl-2, Bax, GRP78, CHOP, Sirt3 (all obtained from Abcam) at 4 °C. Next day, the membranes were incubated with the corresponding secondary antibody for 1 h. Subsequently, the membranes were visualized using a ECL chemiluminescence detection kit and protein bands were analyzed by Quantity One.

Statistical analysis

All data were presented as the mean ± standard error of the mean (SEM). Statistical significance was assessed by one-way analysis of variance (ANOVA) with a Bonferroni post hoc test for multiple comparisons, using GraphPad Software. P < 0.05 was considered statistically significant.

Results

General features of rats and Echocardiography

Before DOX injection, each group had a similar mean body weight. After DOX administration, as shown in Fig. 1D and E, the DOX group exhibited significantly decreased body weight and heart weight compared with the CON group. With the treatment of Ononin, the rats in DOX + Ononin30 and DOX + Ononin60 groups obtained a less weight loss. Echocardiography was performed to assess the cardiac function of rats (Fig. 1F). LVEF and LVFS were markedly decreased in the DOX group compared to CON group. Compared to DOX group, LVEF and LVFS were increased under the cotreatment of Ononin (Fig. 1G and H). As shown in Fig. 1I, the LDH level in DOX group was higher than CON group and DOX + Ononin60 group. Taken together, these results illustrate that Ononin can prevent DOX-induced LV dysfunction in vivo.

Pretreatment with Ononin prevented DOX-induced cardiac tissue damage in rats

H&E staining, Masson staining and WGA staining were used in order to compare the protective effect of Ononin on myocardial tissue. H&E staining of the rat cardiac tissue revealed that DOX caused significant myocardial injury. There were more disordered or even vacuolization and broken myofibers among fibers, which was clearly reduced by Ononin pre-treatment groups (Fig. 2A). Masson staining results showed that myocardial fibrosis was significantly increased in the DOX group compared to DOX + Ononin groups and CON group (Fig. 2B and D). Furthermore, WGA staining indicated that Ononin treatment abolished DOX caused cardiac hypertrophy (Fig. 2C and E). Overall, these results illustrate that Ononin exhibits a protective role in DOX-induced cardiac tissue damage.

Ononin inhibited DOX-induced cardiomyocyte apoptosis in rats and H9C2 cells

Apoptosis plays a key role in the pathophysiology of DOX-induced myocardial injury. The TUNEL assay was used to investigate the effect of Ononin on cardiomyocyte apoptosis. A significant increase in the ratio of TUNEL-positive cells was observed in the rat heart tissues in DOX group. In Ononin groups, the ratio of TUNEL-positive cells significantly decreased compared with DOX group (Fig. 3A). These findings were in agreement with Western blot analysis. Western blot analysis in rats revealed that the ratio of Bax/Bcl-2 was increased in the DOX group compared with the CON group. DOX + Ononin groups showed a lower ratio of Bax/Bcl-2 compared with DOX group (Fig. 3B). We verified the effect of Ononin on DOX-induced apoptosis in H9C2 cells. CCK-8 assay was performed in H9C2 cells treated with DOX and Ononin, and their viability was evaluated. According to the CCK-8 assay result, pretreatment with Ononin increased the cells viability, 0.5 μ M Ononin prevented DOX-induced cell viability reduce (Fig. 3C). Furthermore, Ononin pretreatment prevented DOX-stimulated increase in Bax/Bcl-2 ratio, which was same as vivo findings (Fig. 3D). In a word, our results indicate that Ononin attenuates DOX-induced apoptosis in rats and H9C2 cells.

Ononin inhibited DOX-induced ER stress in rats and H9C2 cells

Endoplasmic reticulum stress can cause cardiomyocyte apoptosis. Western blot was performed to investigate the effect of Ononin on ER stress. In vitro experiments, DOX treatment increased the GRP78 and CHOP relative protein expression while the pretreatment of Ononin prevented these (Fig. 4A). Immunohistochemistry staining revealed the same result that DOX increased the express of GRP78 in heart tissue and DOX + Ononin groups showed a lower level of GRP78 than DOX group (Fig. 4B and C). In vivo experiment, H9C2 cells showed a high GRP78 and CHOP expression level than CON in DOX group, however pretreatment of Ononin made this situation better (Fig. 4D). Taken together, these results indicate that Ononin inhibits DOX-induced activation of the ER stress in rats and H9C2 cells.

Ononin upregulated SIRT3 protein expression and inhibited DOX-induced ER stress by SIRT3

To investigate the mechanisms underlying Ononin-induced protective effects, we detected the changes of SIRT3 protein in rat heart tissue and in H9C2 cells. The Western blot showed that under the intervention of DOX, less SIRT3 was expressed, however, the expression of SIRT3 was increased by Ononin treatment (Fig. 5A). Rat heart tissue sections also showed the same trend (Fig. 5B). And then, to confirm whether activation of SIRT3 contributes to the Ononin-induced protection, ER stress was determined after a selective SIRT3 inhibitor (3-TYP) used. DOX downregulated SIRT3 protein expression in vivo and in vitro, which was prevented by co-administration of Ononin. After treatment of 3-TYP, compared with the DOX-treated and DOX + Ononin-treated cells, the expression of GRP78 and CHOP were increased meanwhile the ratio of Bax/Bcl-2 was increased (Fig. 5C and D). In summary, these results suggest that Ononin suppresses DOX-induced ER stress by stimulating SIRT3 pathway, and high level of active SIRT3 caused by Ononin could protect cells from apoptosis.

Discussion

Ononin is a natural plant compound with a 17- β -estradiol-like chemical structure and is widely found in Fabaceae/Leguminosae family like soybeans, soy foods, and legumes[19]. Many Chinese herbal medicines and Chinese medicine prescription, such as Astragalus mongholicus, Qishen Yiqi dripping pills and Dangguibuxue decoction, also contain a lot of Ononin[20–22]. Ononin has benefit on promoting skin growth, scavenging oxygen free radicals and protecting ischemia-reperfusion injury through its multiple physiological functions, such as antioxidant, antimicrobial and anti-inflammatory activities[23–26]. Recent studies indicate that Ononin could regular MAPK and NF- κ B signaling pathways to alleviate apoptosis in rheumatoid arthritis[27]. In view of its so many important physiological functions, we speculated that it had a special effect in DOX-induced cell apoptosis. Finally, our experimental results confirmed that Ononin can alleviate DOX-induced cardiomyocyte apoptosis in rats and H9C2 cells.

To determine the specific mechanism of Ononin in regulating apoptosis we began our exploration with multiple directions and finally focused on ER stress. It has been demonstrated that it plays an important role in doxorubicin-induced cardiotoxicity and is almost related to mitochondria[28]. GRP78 and CHOP are two biomarkers of ER stress. previous studies indicated that cells that inhibit CHOP expression can resist apoptosis caused by ER stress[29]. ER stress participate DOX-induced cardiovascular diseases in

various pathological processes[30]. The ER firstly triggers the unfolded protein reaction to maintain the homeostasis but more intense stimulation resulting in ER stress[31]. In our experimental results, Ononin has the ability to reduce the express of GRP78 and CHOP. This confirmed that Ononin can alleviate DOX-induced ER stress to decrease the level of apoptosis in rats and H9C2 cells.

As a widely used and effective anticancer drugs, the clinical application of DOX is seriously limited by its dose-dependent cardiotoxicity[32]. Cumulative evidence has suggested that doxorubicin could induce cardiotoxicity through multiple and indistinct mechanisms, such as oxidative stress and ER stress[33]. Recently it was reported that DOX inhibited SIRT3 expression and that SIRT3 overexpression prevented DOX-induced cardiomyocyte death[16]. However, whether SIRT3 can affect DOX-induced ER stress in is not clear. As the primary mitochondrial deacetylase, SIRT3 regulates the functions of mitochondria. SIRT3 activate SOD2, which in turn reduce ROS and oxidative stress[34, 35]. A recent study about SIRT3 indicated that ER stress could be inhibited by SIRT3 to reduce cell apoptosis[14]. In our results, high SIRT3 level in Ononin treatment group may reduce GRP78 and CHOP, but these benefits may also be obtained by Ononin through other pathways. The treatment of SIRT3 inhibitor 3-TYP partially restored the expression of GRP78 and CHOP. These results indicate that the activity of SIRT3 is indeed related to ER stress.

Conclusion

In our study, Ononin alleviated DOX-induced apoptosis by increasing the expression of SIRT3. First, we demonstrated that Ononin pretreatment could prevent DOX-induced cardiac injury in vivo. Moreover, experiments in vitro showed that DOX induced a high ER stress and high apoptosis level. Ononin pretreatment suppressed all these adverse effects of DOX but SIRT3 inhibitor 3-TYP can partly suppress Ononin-induced protective. However, our study had several limitations. First, the effects of SIRT3 inhibition on Ononin activity were only evaluated in vitro. Second, 3-TYP may not be as precise as genetic intervention. Finally, we still need a SIRT3-overexpress cell to investigate deeper mechanism. It requires much more further studies to dig out how the Ononin and SIRT3 protect DOX-treated cardiomyocytes.

In summary, our study demonstrated that Ononin treatment alleviates DOX-induced cardiomyocytes apoptosis. The cardioprotective effects of Ononin were mediated by activation of SIRT3 and consequent suppression of the ER stress. These findings suggest that drugs that target the SIRT3 might have therapeutic potential in alleviating DOX-related cardiotoxicity. Chinese herbal medicines and Chinese medicine prescription containing Ononin might also relieve the cardiotoxicity of DOX during chemotherapy.

Declarations

Author Contributions

Shimin Sun carried out most of the experiments described in this report under experimental planning and guidance from Hangyuan Guo and Jufang Chi. Shimin Sun and Wenqiang Lu performed the experiments with H9C2 cells. Zhenzhu Sun and Jingfan Weng performed the experiments in rats. Shimin Sun, Jingfan Weng, Qi Yang and Xingxiao Huang contributed to the planning and preparation of the manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

Figure 1

General features of rats and Echocardiography. (A) Protocol for animal experiments in our study. The chemical structures of DOX (B) and Ononin were exhibited (C). Body weight (D) and heart weight (E) of rats at the end of our study were measured. (F) Echocardiographic images of rats at the end of our animal study. The LVEF (G) and LVFS (H) were calculated to assess cardiac function. The cardiac marker

enzymes LDH (l) was measured to evaluate cardiac injury under relevant stimulation. (n = 6, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group).

Figure 2

Pretreatment with Ononin prevented DOX-induced cardiac tissue damage in rats. H&E(A), Masson's trichrome stain (B,D) and WGA stain (C,E) showing the difference in myocardial damage in each group. (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group).

Figure 3

Ononin inhibited DOX-induced cardiomyocyte apoptosis in rats and H9C2 cells. (A) TUNEL assays showed the apoptosis of heart tissues in each group (bar =100 μm). (B) Heart protein expression of Bax and Bcl-2 was estimated by western blot and quantified (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group). (C) Results of the CCK-8 assay in H9C2 cells CCK-8 assay exposed to different concentrations of DOX and Ononin for 24 h (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group). (D) Protein expression

of Bax and Bcl-2 was estimated by western blot and quantified (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group).

Figure 4

Ononin inhibited DOX-induced ER stress in rats and H9C2 cells. (A) Protein expression of GRP78 and CHOP in rats were estimated by western blot and quantified (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group). (B,C) The expression of GRP78 was visualized using immunohistochemistry in each group

(bar =100 μm). (D) Protein expression of GRP78 and CHOP in H9C2 cells were estimated by western blot and quantified (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group).

Figure 5

Ononin upregulated SIRT3 protein expression and inhibited DOX-induced ER stress by SIRT3. (A)Protein expression of SIRT3 in rats were estimated by western blot and quantified (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group). (B) The expression of SIRT3 was visualized using immunohistochemistry in

each group (bar =100 μ m). (C) Protein expression of SIRT3 in H9C2 cells was estimated by western blot and quantified (n = 3, *P < 0.05 vs. CON group, #P < 0.05 vs. DOX group). (D,E) Protein expression of Bax, Bcl-2, GRP78 and CHOP were estimated by western blot and quantified (n = 3, *P < 0.05 vs. DOX+Ononin group)