Catalpol ameliorates doxorubicin-induced inflammation and oxidative stress in H9C2 cells through PPAR-γ activation

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Abstract. Drug-induced cardiomyopathy is a severe disease that leads to refractory heart disease at late stages, with increasing detrimental effects. DOX-induced cell damage is primarily induced via cellular oxidative stress. The present study investigated the effects of catalpol on doxorubicin (DOX)-induced H9C2 cardiomyocyte inflammation and oxidative stress. The Cell Counting Kit-8 assay was performed to detect cell viability, and western blotting was performed to detect the expression of peroxisome proliferator-activated receptor (PPAR)-γ in H9C2 cells. The expression levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were measured using ELISAs. Furthermore, the oxidative stress kit was used to detect the levels of malondialdehyde, superoxide dismutase and glutathione peroxidase. A reactive oxygen species (ROS) kit and DCF-DA staining were used to detect ROS levels. The results indicated that DOX treatment inhibited H9C2 cell expression of PPAR-γ and decreased H9C2 cell viability. Various concentrations of catalpol exhibited a less potent effect on H9C2 cell viability compared with DOX; however, catalpol increased the viability of DOX-induced H9C2 cells. Catalpol treatment also significantly decreased the expression levels of inflammatory factors (TNF-α, IL-1β and IL-6) in DOX-induced H9C2 cells, which was reversed by transfections with short hairpin RNA targeting PPAR-γ. Results from the present study indicated that catalpol ameliorated DOX-induced inflammation and oxidative stress in H9C2 cardiomyoblasts by activating PPAR-γ.

Introduction

Doxorubicin (DOX) is a highly effective and widely used chemotherapeutic agent for the treatment of human tumors, including solid tumors and hematological malignancies (1,2). However, the severe cardiotoxic side effects associated with DOX have limited its application in the clinic (1). DOX-induced cardiomyopathy may be caused by a variety of factors, including oxidative stress (3); however, the exact mechanisms underlying DOX-induced cardiomyopathy are not completely understood. Cancer chemotherapy-associated heart disease is a main cause of the increased mortality of cancer survivors (4,5); therefore, investigating the mechanisms of action of anthracyclines, including DOX, is important for the treatment of tumors and cardiovascular diseases.

As the main active ingredient in Rehmannia, catalpol belongs to the class of iridoid monosaccharides (6). Previous studies have demonstrated that catalpol exhibits a variety of biological activities, including anti-inflammatory (7-9), antioxidant (10,11), antiapoptotic (12,13) and hypoglycemic (14,15) effects. Previous studies investigating catalpol have primarily focused on its protective effects in the nervous system, and research into the effects of the compound on the cardiovascular system is in the early stages (16-18). Previous studies have reported that catalpol displays protective effects on cardiomyocytes at the cellular level, protecting against myocardial injury through antioxidation and ameliorating cardiac dysfunction in rat models (19,20). However, further studies are required to verify the cardiovascular protective effects of catalpol.

Peroxisome proliferator-activated receptor (PPAR) is a ligand-inducible transcription factor that is a member of the type II nuclear receptor superfamily. PPAR is primarily expressed in vascular smooth muscle cells, where it activates receptors, prevents proliferation and migration of vascular smooth muscle cells, weakens vascular remodeling, exerts anti-inflammatory and antiproliferative effects, and protects against pulmonary hypertension (21,22). It has also been reported that PPAR-γ receptors are also involved in the development of a variety of cardiovascular diseases. Moreover, PPAR-γ displays a range of physiological effects, including anti-inflammatory and antiatherosclerotic activities, and improving left ventricular remodeling (23,24). PPAR-γ also displays a protective effect against kidney and brain tissue ischemia-reperfusion injury (25,26); however, whether PPAR-γ displays a protective effect against myocardial ischemia-reperfusion injury has not been previously reported.
The present study aimed to investigate the protective effects of catalpol against DOX-induced inflammation and oxidative stress in H9C2 cardiomyoblasts, and to explore the possible mechanisms associated with PPAR-γ. The results of the present study may provide a theoretical basis for the treatment of DOX-induced inflammation and oxidative stress in H9C2 cardiomyoblasts.

Materials and methods

Cell lines and reagents. H9C2 cells were obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 2 ml glutamine, 100 U/ml penicillin and streptomycin at 37˚C with 5% CO₂. Catalpol (purity >98.0%) and DOX were purchased from the National Institute for the Control of Pharmaceutical and Biological Products. H9C2 cells were cultured with various concentrations of DOX (0, 0.1, 1 and 10 µM) for 12 and 24 h at 37˚C. The same cells were also treated with various concentrations of catalpol (0, 10, 20, 40 and 80 µM) for 24 h at 37˚C.

Cell Counting Kit-8 (CCK-8) assay. H9C2 cells were seeded (0.5x10⁵ cells/ml) into 96-well plates and incubated with various concentrations of DOX (0, 0.1, 1 and 10 µM) for 12 and 24 h at 37˚C (0 µM as control group). Subsequently, 10 µl CCK-8 reagent (Roche Diagnostics) was added to each well according to the manufacturer's protocol and incubated for 2 h at 37˚C. The optical density of each well was measured at a wavelength of 490 nm using a microplate reader.

To confirm the effect of catalpol on H9C2 cells, H9C2 cells were cultured with various concentrations of catalpol (0, 10, 20, 40 and 80 µmol/l) for 24 h at 37˚C (0 µM as control group). Then, to confirm the effect of DOX-induced H9C2 cardiomyocytes, catalpol administration in each dose group (10, 20, 40 and 80 µmol/l) after H9C2 cells treatment with DOX (1 µM) for 24 h at 37˚C, respectively. According to the aforementioned method, 10 µl CCK-8 regent was added to each well in accordance with the manufacturer's protocol and incubated for 2 h at 37˚C. The optical density of each well was measured at a wavelength of 490 nm using a microplate reader.

Reverse transcription-quantitative PCR (RT-qPCR). H9C2 cell total RNA was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and stored at -40˚C. Total RNA samples were thawed on ice and were reverse transcribed into cDNA using the PrimeScript RT reagent (Takara Bio, Inc.), according to the manufacturer's protocol. Subsequently, qPCR was performed using SYBR Green Master Mix I (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primers were used for qPCR: PPAR-γ forward 5'-AAAGC AACCTTGCTACAAGC-3', reverse 5'-TCTGTAGATGGTCCTCC TGCA-3'; GAPDH, forward 5'-CCAAGGGGTGCTCTTCTTTT C-3', reverse 5'-CGTGGTGAGTGACCTACTGG-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 5 min; followed by 45 cycles of amplification, including denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and a final extension at 72˚C for 10 min. mRNA expression levels were quantified using the 2⁻ΔΔCq method and normalized to the internal reference gene GAPDH (27).

ELISA. Detection of reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels in the H9C2 cell culture medium were measured by ELISA using commercial ELISA kits for Reactive Oxygen Species (ROS) Assay Kit 520 nm (cat. no. 88-5930; Thermo Fisher Scientific, Inc.), MDA ELISA kit (cat. no. AMS.EL-0060; Whuan Boster Biological Technology, Ltd.), SOD Human ELISA kit (cat. no. BMS222; Thermo Fisher Scientific, Inc.) and High Throughput Glutathione Peroxidase Assay kit (cat. no. 7512-100-K; R&D Systems, Inc.). The expression levels of tumor necrosis factor (TNF)-α (cat. no. ADI-901-099; NeoBioscience Technology Co., Ltd.), interleukin (IL)-1β (cat. no. EHC002b.48; NeoBioscience Technology Co., Ltd.)
and IL-6 (cat. no. ADI-901-033; NeoBioscience Technology Co., Ltd.) were detected using ELISA kits according to the manufacturer’s protocol. The levels were quantified using Multiskan Mk3 microplate reader (Thermo Fisher Scientific, Inc.) to detect the concentration.

**DCF-DA staining.** H9C2 cells were seeded into 6-well plates, incubated for 24 h, washed twice with Earle’s Balanced Salt Solution and subsequently incubated with 25 µM DCFH-DA for 30 min at 37°C. Following the incubation, cells were washed twice with sugar Earle’s solution. Fluorescence intensity was analyzed using a fluorescence reader (Fluoroscan Ascent FL; Thermo Labsystems) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The images were obtained via confocal laser scanning microscopy (Olympus FV500; Olympus Corporation). Images were analyzed using ImageJ software (version 1.45; National Institutes of Health).

**Statistical analysis.** Data are expressed as the mean ± SD. All experiments were performed in triplicate. Statistical analyses were performed using SPSS (version 11.5; SPSS, Inc.) and GraphPad Prism (version 5; GraphPad Software, Inc.) software. Comparisons among groups were determined using one-way ANOVA followed by Tukey’s or Dunnett’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**DOX inhibits the expression of PPAR-γ.** Following treatment with various concentrations of DOX (0, 0.1, 1 and 10 µM) for 12 and 24 h, the protein expression levels of PPAR-γ in H9C2 cells were detected by western blotting. PPAR-γ expression levels decreased with increasing DOX concentrations (Fig. 1A). The results indicated that DOX inhibited the expression of PPAR-γ in a dose-dependent manner.

**DOX affects H9C2 cell viability.** Results from the CCK-8 assay indicated that the concentration of DOX (0.1 µM) had no significant effect on the viability of H9C2 cells, and that various concentrations of DOX (1 and 10 µM) significantly decreased H9C2 cell viability compared with the control group (P<0.05; Fig. 1B). As the IC_{50} of DOX at 24 h was ~1 µM, this concentration and duration were used for subsequent experiments.

**Effect of catalpol on H9C2 cell viability.** The results of the CCK-8 assay also indicated that H9C2 cell viability was not significantly altered following treatment with various concentrations of catalpol (0, 10, and 20 µM) for 24 h, but 40 and 80 µM catalpol significantly decreased H9C2 cell viability compared with the untreated control group (Fig. 2A).

**Effect of catalpol on DOX-induced reductions to H9C2 cell viability.** DOX treatment reduced viability, but co-treatment with catalpol reduced these effects. Additionally, H9C2 cell viability was significantly increased in the 10 µM catalpol+1 µM DOX group, the 20 µM catalpol+1 µM DOX group and the 40 µM catalpol+1 µM DOX group when compared with the 0 µM catalpol+1 µM DOX group (P<0.05; Fig. 2B). The results indicated that catalpol (10, 20 and 40 µM) attenuated DOX-induced effects on H9C2 cell viability and
80 µM catalpol significantly decreased H9C2 cell viability. Furthermore, 20 µM catalpol increased H9C2 cell viability to the highest level compared with other catalpol concentrations. Therefore, 20 µM catalpol was used for subsequent experimentation.

Catalpol serves a role in reducing DOX-induced cell damage via PPAR-γ. The expression of PPAR-γ was detected by western blotting. PPAR-γ expression levels in the DOX group were significantly lower compared with the control group; however, catalpol co-treatment increased PPAR-γ expression levels in the DOX group (Fig. 2C). The results indicated that catalpol serves a role in reducing DOX-induced cell damage via PPAR-γ.

Catalpol reduces DOX-induced inflammation and oxidative stress in H9C2 cells. The transfection efficiency of shRNA-PPAR-γ was detected by western blotting and RT-qPCR (Fig. 3A and B, respectively). The results suggested that shRNA-PPAR-γ-1 and shRNA-PPAR-γ-2 significantly decreased PPAR-γ expression levels compared with the control group; however, the inhibitory effects of shRNA-PPAR-γ-1 were superior compared with shRNA-PPAR-γ-2 in H9C2 cells and was used in subsequent experiments.

The results of ELISA revealed that concentrations of the inflammatory factors TNF-α, IL-1β and IL-6 in the DOX group was significantly higher compared with the control group, and catalpol treatment significantly downregulated DOX-induced inflammatory factor expression (Fig. 4A). Furthermore, the expression of TNF-α, IL-1β and IL-6 in the 20 µM catalpol+1 µM DOX+ shRNA-PPAR-γ group was significantly upregulated compared with the 20 µM catalpol+1 µM DOX+ shRNA-NC group (Fig. 4A). Subsequently, the levels of oxidative stress-associated factors were examined. The levels of ROS and MDA were significantly increased in the DOX group compared with the control group, and catalpol co-treatment significantly downregulated DOX-induced ROS and MDA expression. The levels of ROS and MDA in the 20 µM
catalpol+1 µM DOX+ shRNA-PPAR-γ group were significantly upregulated compared with the 20 µM catalpol+1 µM DOX+shRNA-NC group. SOD and GSH-Px displayed the opposite trend to ROS and MDA (Fig. 4B).

To further investigate ROS expression levels, DCF-DA staining was performed to detect the expression of ROS in H9C2 cells (Fig. 5). The fluorescence intensity in normal cardiomyocytes was generally weak with low ROS content. Following treatment with DOX for 24 h, the fluorescence intensity of the myocardial cells was enhanced and the fluorescence value was notably increased compared with the control group, indicating that the intracellular ROS content had increased. Compared with the DOX group, catalpol treatment significantly reduced the intracellular fluorescence intensity, the fluorescence value and the ROS content of H9C2 cells. However, the addition of shRNA-PPAR-γ significantly reversed catalpol-mediated downregulation of intracellular ROS content. These results indicated that catalpol reversed DOX-induced ROS production. Collectively, the results suggested that catalpol reversed DOX-induced inflammation and oxidative stress in H9C2 cells through PPAR-γ activation.

Discussion

Drug-induced cardiomyopathy is a severe disease that occurs independent of other cardiovascular risk factors, but is a widespread side effect of a number of therapeutic agents, such as Daunorubicin, DOX and Paclitaxel (28-30). As it is difficult to identify symptoms and signs during the early stages of the disease, drug-induced cardiomyopathy often leads to refractory heart disease in the late stages, with increasing detrimental effects (31,32). DOX-induced cardiomyopathy is the most common type of drug-induced heart disease (33). DOX-induced damage to the heart has significantly limited its therapeutic use in the clinic (34). The main mechanism of action of DOX is inhibition of DNA double-strand break, DNA replication and DNA transcription by topoisomerase 2 inhibition. DOX also directly embeds into the DNA, induces reactive oxygen species production and regulates the binding of histone DNA (35,36). Furthermore, DOX sequesters iron ions to produce free radicals, which in turn trigger the initiation of apoptotic factor expression and promotes cell death (37). Therefore, DOX induces cardiomyocyte damage when it destroys tumor cells (4). Early myocardial damage manifests as myocarditis and arrhythmia; however, when DOX reaches a specific dose, irreversible myocardial expansion occurs, which may eventually develop into heart failure (38). Although numerous efforts have been made to reduce the toxicity of DOX, no significant progress in the prevention and treatment of DOX-induced toxicity has been reported. Therefore, the development of novel therapeutic agents and strategies for the prevention of DOX-induced myocardial damage is important.

**Rehmannia glutinosa** is one of the most commonly used Traditional Chinese Medicine, and it has been reported to lower blood sugar, regulate immunity, enhance hematopoietic functions and inhibit tumors. *R. glutinosa* also displays antiaging properties by exerting protective effects on the cardiovascular and vascular systems. Catalpol, an iridoid glycoside isolated from the roots of *R. glutinosa*, has been reported to display neuroprotective effects (38,39). Previous studies have also demonstrated the cardioprotective and anti-inflammatory properties of catalpol, including apoptosis inhibition, reduced neuronal death and promotion of differentiation (40,41). In a mouse model of lipopolysaccharide-induced acute lung injury, catalpol prevents injury by inhibiting TNF-α, IL-1β and IL-6 expression (42). However, the mechanisms underlying...
the effects of catalpol on inflammation are not completely understood.

DOX has a potent toxic effect on cardiomyocytes and can alter cell morphology, induce cell death and promote apoptosis through a series of molecular mechanisms (2,43,44). Therefore, identifying whether catalpol can attenuate the effects of DOX on myocardial cell survival is important. In the present study, H9C2 cell viability was significantly reduced in the DOX...
group compared with the control group, which indicated that DOX displayed an inhibitory effect on cardiomyocyte survival. Furthermore, compared with the DOX group, H9C2 cells treated with catalpol displayed significantly increased cell viability, suggesting that catalpol attenuated the inhibitory effects of DOX on myocardial cell survival. The results indicated that the 20 µM catalpol group displayed the optimal protective effect, which suggested that catalpol reduced DOX-induced cardiomyocyte damage.

A previous study has reported that catalpol displays potent antioxidant effects, and DOX-induced cell damage is primarily induced via cellular oxidative stress (45). The initiation of oxidative stress in cardiomyocytes increases intracellular oxygen free radical production, and damages cells by attacking cell membranes and the mitochondria (46). Catalpol can reduce the generation of oxygen free radicals to decrease cell damage (47). The present study demonstrated that DOX increased the toxicity of cardiomyocytes, and reduced the ability of cells to resist oxidation. Our results indicating that catalpol reduced cardiomyocyte toxicity compared with the DOX group.

The inflammatory response is a defensive response of the body to damaging factors, involving several types of cells and cytokines, such as white blood cells, neutrophils, TNF-α, IL-1β and IL-6 (48,49). An increase in inflammatory cytokine levels is a sign of an inflammatory reaction in the body, which can induce the adhesion and migration of neutrophils and vascular endothelial cells, as well as the accumulation of neutrophils in myocardial tissue, the release of lysosomal enzymes and myocardial cell damage (50). TNF-α induces inflammation by activating inflammatory cells, including neutrophils, which mediate damage. TNF-α also displays direct cytotoxic effects, leading to alterations in the myocardial calcium balance and excitation-contraction coupling, as well as inducing apoptosis (51). A previous study has demonstrated that the mechanism underlying DOX-induced myocardial injury is complex (52). DOX damages myocardial tissue by increasing the expression of inflammatory factors, including TNF-α, IL-1β and IL-6 (53). Similarly, TNF-α, IL-1β, IL-6 and other inflammatory factors are involved in the process of isoproterenol-induced myocardial injury (54). In the present study, the expression of TNF-α, IL-1β and IL-6 in the DOX group was significantly increased compared with the control group, which was consistent with the results of previous studies. The expression of inflammatory factors in the catalpol co-treatment group was significantly decreased, indicating that catalpol effectively prevented the DOX-induced inflammatory reaction in cardiomyocytes by inhibiting the release of inflammatory factors, thereby exerting a protective effect against myocardial injury.

To identify the possible mechanism underlying the anti-inflammatory activity of catalpol in cardiomyocytes, the present study focused on the role of PPAR-γ, as it has been reported that PPAR-γ receptors are also involved in the development of a variety of cardiovascular diseases, including inflammation, atherosclerosis and left ventricular remodeling (55-57). The present study demonstrated that catalpol acted to significantly increase PPAR-γ expression. Furthermore, to verify the effect of catalpol on PPAR-γ expression, H9C2 cells were treated with shRNA-PPAR-γ and 20 µM catalpol. The results suggested that PPAR-γ protein expression was inhibited at the transcriptional level after the addition of shRNA-PPAR-γ, and catalpol downregulated DOX-induced proinflammatory cytokine production. Collectively, the results indicated that catalpol played a role in DOX-induced cell damage by regulating PPAR-γ expression.

In conclusion, results from the present study indicated that DOX inhibited the expression of PPAR-γ and decreased H9C2 cardiomyocyte cell viability. Catalpol alleviated DOX-induced damage to H9C2 cardiomyocytes, potentially by reducing oxidative stress in cardiomyocytes. Therefore, the present study suggested that catalpol reversed DOX-induced H9C2 cardiomyocyte inflammation and oxidative stress by increasing PPAR-γ expression.
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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

YJ designed the study, collected and analyzed the data, and drafted the manuscript. QZ conceived the study, contributed to the study design and critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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