Hydrogen sulfide attenuates doxorubicin-induced cardiotoxicity by inhibiting the expression of peroxiredoxin III in H9c2 cells

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Abstract. Doxorubicin (DOX) is a widely used chemotherapeutic agent, which can give rise to severe cardiotoxicity, limiting its clinical use. Preliminary evidence suggests that hydrogen sulfide (H_2S) may exert protective effects on DOX-induced cardiotoxicity. Therefore, the aim of the present study was to investigate whether peroxiredoxin III is involved in the cardioprotection of H_2S against DOX-induced cardiotoxicity. The results demonstrated that DOX not only markedly induced injuries, including cytotoxicity and apoptosis, it also increased the expression levels of peroxiredoxin III. Notably, pretreatment with sodium hydrosulfide significantly attenuated the DOX-induced decrease in cell viability and increase in apoptosis, and also reversed the increased expression levels of peroxiredoxin III in H9c2 cardiomyocytes. In addition, pretreatment of the H9c2 cells with N-acetyl-L-cysteine, a scavenger of reactive oxygen species, prior to exposure to DOX markedly decreased the expression levels of peroxiredoxin III. In conclusion, the results of the present study suggested that exogenous H_2S attenuates DOX-induced cardiotoxicity by inhibiting the expression of peroxiredoxin III in H9c2 cells. In the present study, the apoptosis of H9c2 cardiomyocytes was assessed using an methyl thiazolyl tetrazolium assay and Hoechst staining. The levels of Prx III and cystathionine-γ-lyase were examined by western blotting.

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Introduction

Doxorubicin (DOX) is an anthracycline antibiotic, which is used to treat various types of neoplastic disease in humans (1). However, the use of DOX clinically is limited by severe toxic side-effects on the heart, which can lead to dilated cardiomyopathy and congestive heart failure (2). Several studies have shown that the production of reactive oxygen species (ROS) is implicated in DOX cardiotoxicity, which eventually leads to endothelial dysfunction (3,4) and cardiomyocyte apoptosis (5). A number of pharmacological interventions have been suggested as therapies to inhibit DOX-induced cardiotoxicity (6-8).

Hydrogen sulfide (H_2S) has long bee considered a toxic gas, however, it has now been qualified as the third gasotransmitter, along with nitric oxide and carbon monoxide, exerting various effects in the cardiovascular system (9-11). H_2S has been shown to protect the heart from myocardial ischemia-reperfusion injury in various studies (12,13). Our previous study demonstrated that increased endogenous H_2S generation in the early reperfusion phase is important in ischemia preconditioning (IPC)-elicited protection in isolated hearts (11).

Various antioxidant signaling pathways protect or regulate the response of cells to oxidative stress (14,15). Several antioxidants, including peroxiredoxins (Prxs), are oxidized in cells treated with ROS. Prxs are a recently characterized family of antioxidant enzymes, which control the expression levels of cytokine-induced peroxide and mediate signal transduction in mammalian cells (16). Multiple mammalian Prxs (I-VI) often coexist in the same cell in various intracellular locations and function as scavengers of cellular H_2O_2, which are released following stimulation with growth factors during proliferation, apoptosis or oxidative stress (17,18). Furthermore, the expression levels of Prxs are high in the heart and there has been

increasing interest in their importance in the cardiac response to oxidative stress (19). Notably, Prx III has a protective role in cisplatin- and gentamicin-induced apoptosis through a mitochondria-dependent signaling pathway (20). The over-expression of Prx III protects the mouse myocardium from infarction (21). By contrast, the depletion of Prx III results in increased intracellular expression levels of H_2O_2 and sensitizes cells to apoptotic signaling (22).

In the present study, H9c2 cells were treated with $5\,\mu$M DOX to establish a chemotherapy-induced cardiotoxicity model (6). This model was then used to investigate whether DOX induces the expression of Prx III in the H9c2 cells, and to examine the role of Prx III in the protective effect of H_2S against DOX-induced injury in H9c2 cells.

Materials and methods

Materials. Methyl thiazolyl tetrazolium (MTT), Hoechst 33258, DOX, sodium hydrosulfide (NaHS) and N-acetyl-L-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cell culture medium components were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), unless otherwise specified. The H9c2 cardiomyocytes were purchased from the Shanghai Cell Library of China, which were originally obtained from the American Type Culture Collection (Manassas, VA, USA).

Cell culture. The H9c2 cardiomyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM; Beyotime Institute of Biotechnology, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Beyotime Institute of Biotechnology), 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO_2 at 37˚C, and the cells were passaged every 2 days. The H9c2 cardiomyocytes were seeded at a density of $2x10^6$ cells/dish into 100 mm dishes containing 10% FBS, and incubated at 37˚C for 24 h, following which the medium was replaced with 0.5% DMEM supplemented with FBS for 24 h starvation.

MTT assay. The MTT assay, which is a standard method used to assess the viability of cells, was performed in the present study. Prior to each experiment, the H9c2 cardiomyocytes ($5x10^3$ cells/well) were seeded into 96-well microtitre plates. Following incubation with NAC for 60 min and/or NaHS for 30 min at 37˚C, the cells were treated with 5 μM DOX for a further 24 h. Subsequently, 10 μl MTT solution was added to each of the wells, and the plates were incubated for 4 h at 37˚C. The absorbance was then measured at 470 nm using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), and used to calculate the relative ratio of cell viability. Three independent experiments were performed for each experimental condition.

Assessment of cardiomyocyte cell apoptosis. The levels of apoptosis were analyzed using fluorescence microscopy following staining of the cells with Hoechst 33258 chromatin dye. Following the treatments described above, the cells were fixed with ice-cold 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) at room temperature for 20 min. Non-specific binding was blocked using 5% normal goat serum (Sigma-Aldrich) in 0.01 M PBS containing 0.3% Triton X-100. The cells were then washed twice with PBS and incubated with 10 μg/ml Hoechst 33258 for 10 min at room temperature in the dark. The cells were visualized under a fluorescence microscope (BX50-FLA; Olympus Corporation, Tokyo, Japan). Apoptotic cells exhibited condensed, fractured or distorted nuclei, whereas viable cells exhibited normal nuclear size and uniform fluorescence. The apoptotic rate was analyzed with a cell counter in Image J 1.4 software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. The cells were homogenized directly using cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) and the lysates were centrifuged at 12,000 x g for 10 min at 4˚C. Protein concentrations were determined with the use of a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The extracted proteins (30 μg) were mixed with 5% SDS sample buffer (Beyotime Institute of Biotechnology) prior to being boiled at 100˚C for 7 min and separated by electrophoresis on a 10% SDS-PAGE. Following electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Beyotime Institute of Biotechnology). The membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T), containing 5% non-fat dried milk for 2 h at room temperature with agitation. Following blocking, the membranes were incubated with the following antibodies: Rabbit anti-Prx III poly-clonal antibody (cat. no. ab73349; 1:400; Abcam, Cambridge, UK; 1:400, rabbit anti-cystathionine-γ-lyase (CSE) monoclonal antibody (cat. no. 12217-1-AP; 1:1,000; Proteintech Group, Inc., Chicago, IL, USA) and GAPDH (cat. no. AG019; dilution 1:1,000; Beyotime Institute of Biotechnology (Shanghai, China). Subsequently, the membranes were incubated with 5% milk or bovine serum albumin overnight at 4˚C. Primary antibody was removed by washing the membranes three times in TBS-T, and incubated for 2 h with the appropriate horseradish perox-idase-conjugated secondary antibody (cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology). Following three washes in TBS-T, the antigen-antibody bands were detected using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) and quantified using a densitometry soft-ware program (Quantity One; Bio-Rad Laboratories, Hemel Hempstead, UK) and GAPDH was used as a loading control.

Statistical analysis. The results of the present study are presented as the mean ± standard error of the mean. Statistical analysis was performed using Student's t-test or one-way analysis of vari-ance with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

DOX increases the expression levels of Prx III in H9c2 cells. in a time-dependent manner. To examine the effect of DOX on the expression levels of Prx III, the H9c2 cells were treated with 5 μM DOX for the indicated time periods (0, 3, 6, 12 and 24 h). The results of the western blot analysis demonstrated that DOX increased the expression levels in of Prx III a time-dependent manner (Fig. 1).
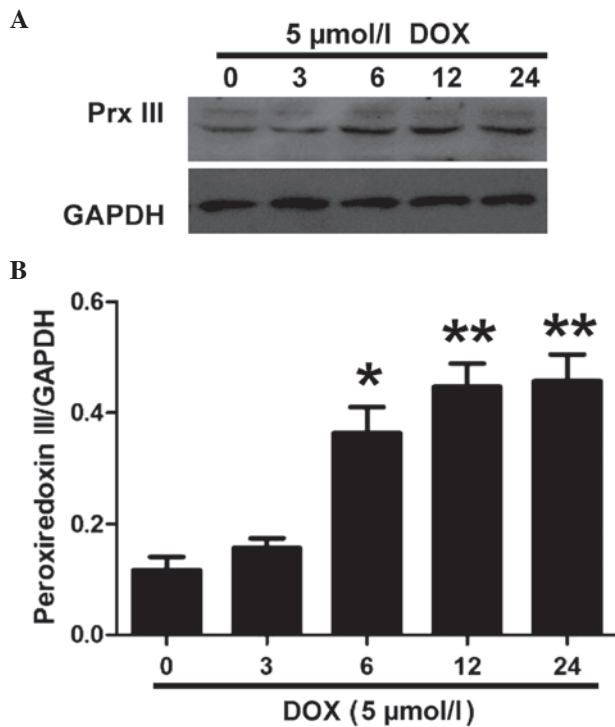
A

5 µmol/l DOX

0　3　6　12　24

Prx III

GAPDH

B

Figure 1. DOX increases the expression levels of of Prx III in H9c2 cells. (A) Expression levels of of Prx III were analyzed using immunoblotting. (B) Relative expression levels of Prx III were determined using blot densitometry. The data are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01 vs. control (0 μmol/l) group. DOX, doxorubicin; Prx, peroxiredoxin.

A

5 µmol/L DOX
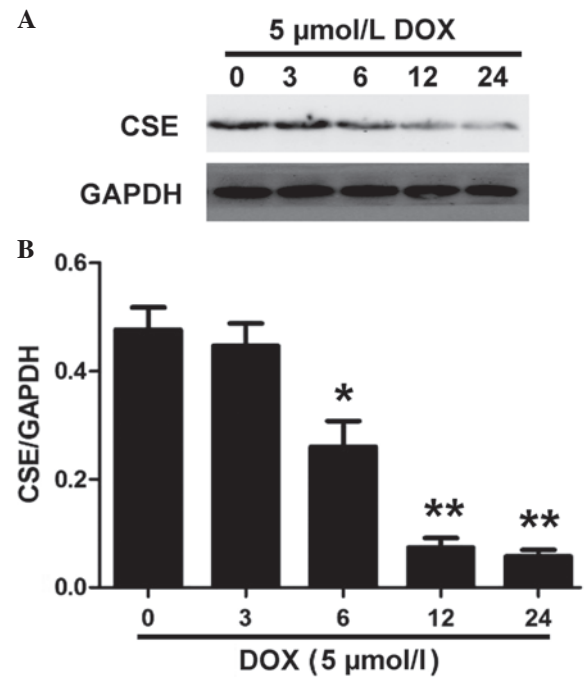
0　3　6　12　24

CSE

GAPDH

B

Figure 2. Inhibitory effects of DOX on the expression levels of CSE in H9c2 cells. (A) Expression levels of CSE were analyzed using immunoblotting. (B) Relative expression levels of CSE were determined using blot densitometry. Data are presented as the mean ± standard error of the mean (n=3). *P<0.05 and **P<0.01 vs. control (0 μmol/l) group. DOX, doxorubicin; CSE, choreoathetosis/spasticity, episodic.

DOX inhibits the expression and activity of CSE in H9c2 cells. CSE is the enzyme responsible for endogenous H_2S generation in H9c2 cells (23). Western blot analysis was performed to evaluate whether DOX decreases endogenous H_2S production by inhibiting the expression of CSE. As shown in Fig. 2, treatment with 5 μM DOX for the indicated time periods (0, 3, 6, 12 and 24 h) caused a significant downregulation in the expression of CSE in the H9c2 cells. These results suggested that DOX induced CSE inhibition in the H9c2 cells, which contributed to a decrease in endogenous H_2S production.

Exogenous H_2S inhibits DOX-induced expression of Prx III in H9c2 cells. To determine whether the protective effect of H_2S against the toxicity induced by DOX was associated with the inhibition of Prx III, the effects of NaHS on the expression levels of Prx III induced by DOX were evaluated. Pretreatment of the H9c2 cells with 100 μmol/l NaHS (a donor of H_2S) for 30 min prior to exposure to 5 μmol/l DOX for 24 h significantly inhibited the DOX-induced overexpression of Prx III (Fig. 3). NaHS did not affect the basal expression levels of Prx III in the H9c2 cells when treated alone. These results suggested that the inhibition of Prx III may be involved in the cryoprotective effects of H_2S from DOX-induced cytotoxicity in H9c2 cells.

NAC reverses the DOX-induced decrease of Prx III in H9c2 cells. To determine whether the inhibitory effect of NaHS on the DOX-induced increased expression of Prx III was associated with its antioxidant properties, the H9c2 cells were pretreated with 1,000 μM NAC, a ROS scavenger, for 60 min,

A

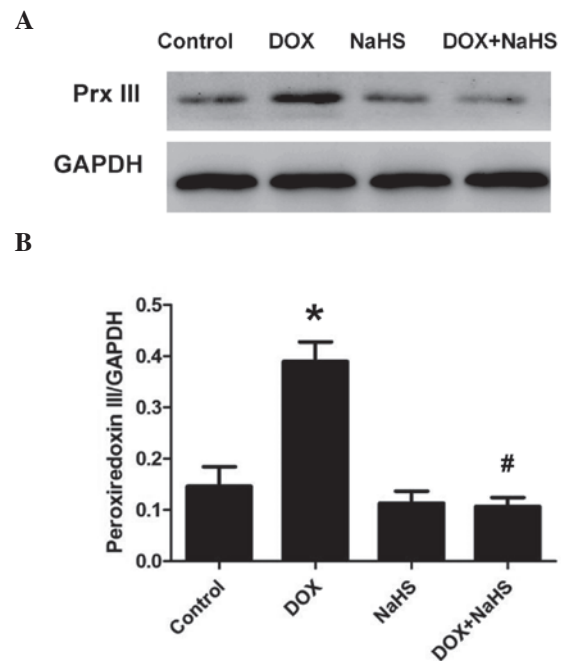Control　DOX　NaHS　DOX+NaHS

Prx III

GAPDH

B

Figure 3. Exogenous H_2S decreases the DOX-induced expression levels of Prx III in H9c2 cells. (A) Expression levels of Prx III were analyzed using immunoblotting. (B) Relative expression levels of Prx III were determined using blot densitometry. The data are presented as the mean ± standard error of the mean (n=3). *P<0.05, vs. control group; #P<0.05, vs. DOX-treated group. DOX, doxorubicin; H_2S, hydrogen sulfide; Prx, peroxiredoxin.

following which the cells were exposed to 5 μM DOX for 24 h. As shown in Fig. 4, similar to the inhibitory effect of pretreatment of cells with NaHS, pretreatment of the H9c2 cells with
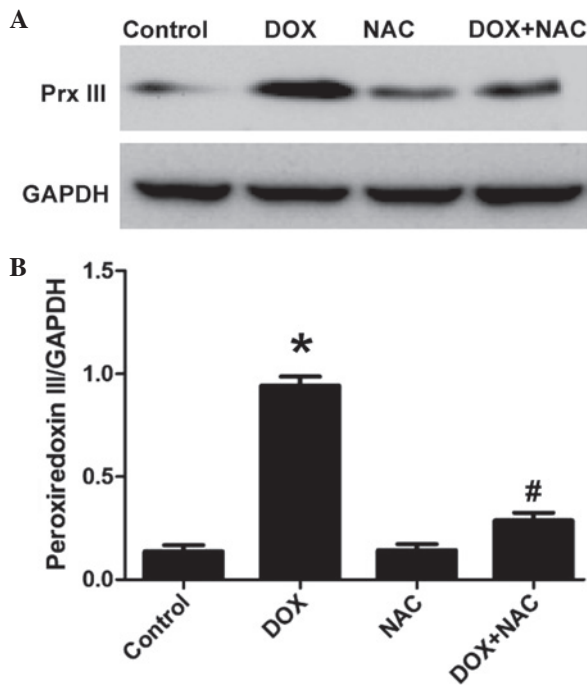
A

B

Figure 4. Effect of NAC on the expression of Prx III induced by DOX in H9c2 cells. (A) Expression levels of Prx III were analyzed using immunoblotting. (B) Relative levels of Prx III were determined using blot densitometry. The data are presented as the mean ± standard error of the mean (n=3). *P<0.05, vs. control group; $^\#$P<0.05, vs. DOX-treated group; DOX, doxorubicin; NAC, N-acetyl-L-cysteine; Prx, peroxiredoxin.

NAC for 60 min significantly decreased the expression levels of Prx III. These results suggested that the inhibitory properties of H_2S on the DOX-induced expression of Prx III involved the contribution of an antioxidant effect.

Exogenous H_2S inhibits DOX-induced cytotoxicity. As shown in Fig. 5, exposure of the H9c2 cells to DOX at 5 μM for 24 h induced significant cytotoxicity, resulting in a reduction in the viability of the cells. However, pretreatment of the cells with 100 μM NaHS for 30 min prior to exposure to DOX significantly reduced the effects of DOX-induced cytotoxicity, which was demonstrated by an increase in the viability of the cells. Pretreatment of the cells with NAC had a similar protective effect as H_2S against DOX-induced cytotoxicity, also suggesting the involvement of an antioxidant effect against the cytotoxicity induced by DOX. Treatment with either NaHS or NAC alone did not affect the viability of the H9c2 cells.

Exogenous H_2S reduces DOX-induced apoptosis in H9c2 cells. The effects of NaHS and NAC on DOX-induced apoptosis were further investigated in the present study. As shown in Fig. 6, the H9c2 cells treated with 5 μM DOX for 24 h exhibited characteristics typical of apoptosis, including the condensation of chromatin, nuclear shrinkage and the presence of apoptotic bodies. However, pretreatment of the H9c2 cells with 100 μM NaHS for 30 min prior to DOX exposure significantly decreased the DOX-induced increase in cells exhibiting nuclear condensation and fragmentation. In addition, the H9c2 cells were preconditioned with the ROS scavenger, NAC (1,000 μM) prior to exposure to DOX. The results demonstrated that pretreatment
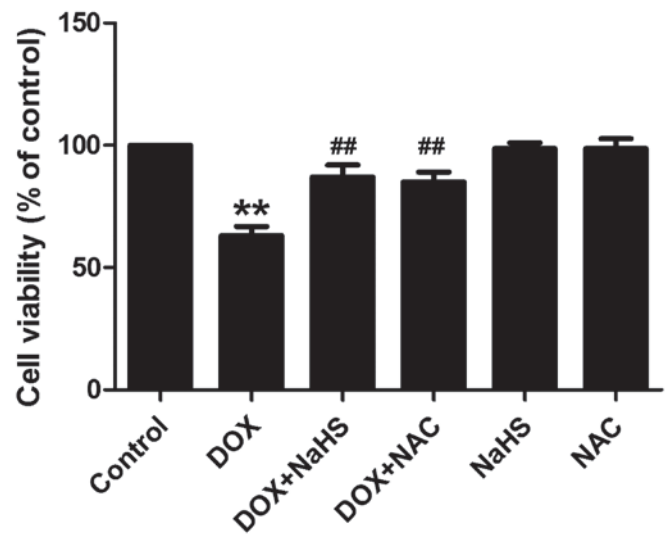

Figure 5. Exogenous H_2S and NAC protect H9c2 cells against DOX-induced cytotoxicity. The viability of the cells in the different treatment groups were measured using a methyl thiazolyl tetrazolium assay. The data are presented as the mean ± standard error of the mean (n=3). **P<0.01, vs. control group; $^{\#\#}$P<0.01, vs. DOX-treated group; DOX, doxorubicin; NAC, N-acetyl-L-cysteine; NaHS, sodium hydrosulfide.

of the cells with NAC significantly attenuated DOX-induced H9c2 cell apoptosis. Treatment with NaHS or NAC alone did not have a marked effect on the morphology of the cells, or on the number of apoptotic H9c2 cells identified. These findings suggested that antioxidant properties are involved in, and contribute to, the inhibitory effects of H_2S on the DOX-induced apoptosis of H9c2 cells.

Discussion

Several studies have demonstrated that the major molecular mechanism underlying DOX-induced cardiotoxicity are free radical-induced oxidative stress and cardiomyocyte death caused by apoptosis and necrosis (6,24). Concordant with previous studies (24,25), the present study demonstrated that exposure of H9c2 cells to DOX markedly increased cellular injury, decreased cell viability, increased cell apoptosis and increased the expression levels of Prx III.

A previous study demonstrated the cardioprotective effects of H_2S in animal models of disease (26). Treatment with H_2S significantly reduced myocardial infarct size, improved regional left ventricular function, and increased endothelium-dependent and endothelium-independent microvascular reactivity in a porcine model of myocardial ischemia-reperfusion (27). In addition, H_2S has been observed to attenuate myocardial necrosis and apoptosis (28). Endogenous H_2S has been associated with cardioprotection, enabled via metabolic inhibition preconditioning in rat ventricular myocytes (29). The inhibition of the production of endogenous H_2S by its synthesis inhibitor, DL-propargylglycine, has been observed to inhibit the protective effects of IPC in isolated hearts and cardiomyocytes (30). In the present study, H9c2 cells were used to investigate the effects of DOX on endogenous H_2S generation. The results demonstrated that exposure of the H9c2 cells to DOX led to a significant decrease in H_2S generation.
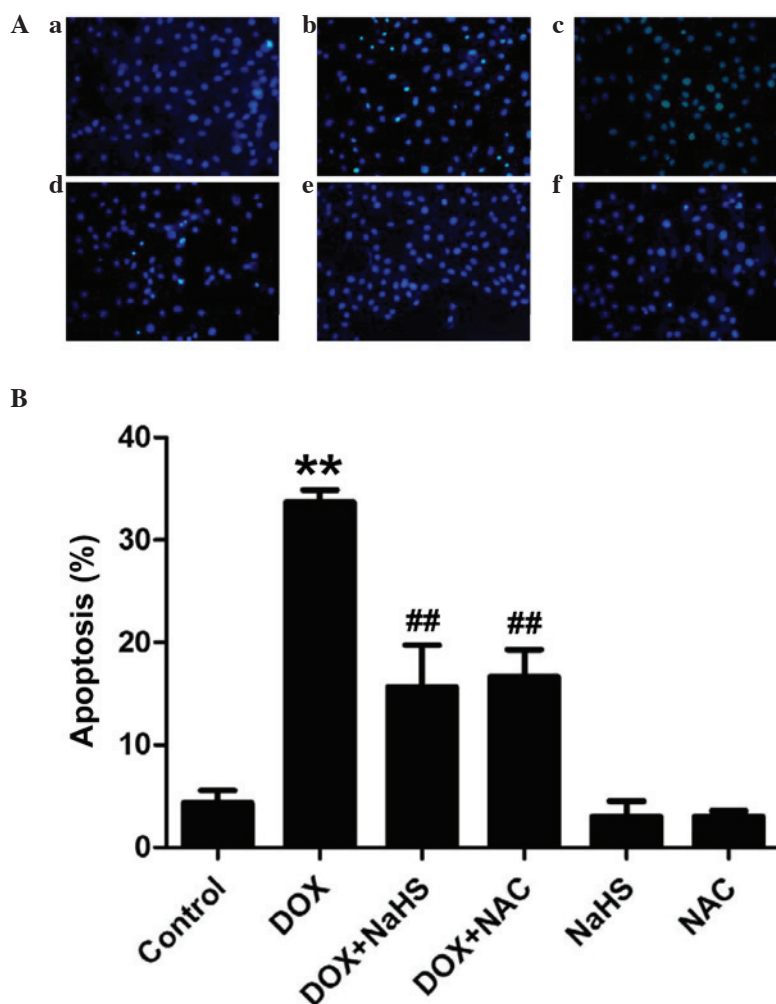
Figure 6. Exogenous H_2S and NAC reduce DOX-induced apoptosis in H9c2 cells. (A) Hoechst 33258 nuclear staining followed by fluorescence imaging was used to observe cell apoptosis. (a) Control group; (b) DOX group; (c) NaHS+DOX group; (d) NAC+DOX group; (e) NaHS group; (f) NAC group (magnification, x40). (B) Apoptotic rates were analyzed using a cell counter and ImageJ 1.41 software. The data are presented as the mean ± standard error of the mean (n=3). [**]$P<0.01$, vs. control group; [##]$P<0.01$, vs. DOX-treated group. DOX, doxorubicin; NAC, N-acetyl-L-cysteine; NaHS, sodium hydrosulfide.

Prx III is a mitochondrial antioxidant protein and member of the Prx family, which is capable of catalyzing H_2O_2 reduction (31). In addition, the overexpression of Prx III has been demonstrated to protect neurons against oxidative stress-induced cell death (32). These beneficial effects make Prx III a valuable potential candidate for the treatment of left ventricular systolic dysfunction following myocardial infarction, in which the production of ROS has been observed to be increased in the mitochondria (21). Although several previous studies have shown the beneficial effects of antioxidants in heart failure (33,34), investigations have not been performed to specifically examine the role of Prx III in DOX-induced cytotoxicity. The results of the present study demonstrated that the expression levels of Prx III were significantly increased in the DOX-induced H9c2 cell injury model. Similarly, an increase of Prx III was previously reported by Xi *et al* (35), and nitrate treatment restored the expression of Prx III. In the present study, the results demonstrated that the Prx III expression levels of Prx III increased following DOX treatment, and exogenous H_2S preconditioning suppressed the expression of Prx III, markedly attenuating DOX-induced apoptosis.

In previous years, several studies have demonstrated that ROS are important in the pathogenesis of cardiac failure (36-38). In addition, antioxidants have been reported to exert protective effects against heart failure (21). Oxidative stress is a primary mechanism by which DOX-induced cardiomyocyte injury (24). Notably, the present study demonstrated that oxidative stress was involved in DOX-induced cell injury, and further examined whether the activation of Prx III by DOX was due to the induction of ROS. It was shown that pre-treatment of the H9c2 cells with the NAC ROS scavenger significantly decreased DOX-induced expression levels of Prx III. Collectively, these results supported the hypothesis that the induction of ROS by DOX activates Prx III in H9c2 cells.

In conclusion, the principal finding of the present study was that H_2S inhibited DOX-induced apoptosis in the H9c2 cells, and its effects may involve inhibition of the ROS-mediated expression of Prx III. The results of the present study not only improved current understanding of the mechanisms underlying H_2S-mediated anti-apoptosis in cardiomyocytes, but they also provide valuable evidence in support of the potential use of H_2S as a treatment for cardiovascular diseases.

Acknowledgements

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