Hydrogen sulfide attenuates doxorubicin-induced cardiotoxicity by inhibition of the p38 MAPK pathway in H9c2 cells

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Abstract. We previously demonstrated the protective effect of hydrogen sulfide (H_2S) against doxorubicin (DOX)-induced cardiotoxicity through inhibition of endoplasmic reticulum stress. The aim of the present study was to explore the role of p38 mitogen-activated protein kinase (MAPK) in DOX-induced cardiotoxicity and ascertain whether exogenous H_2S protects DOX-induced injury by inhibiting p38 MAPK in cardiomyoblasts (H9c2). We observed that exposure of H9c2 cells to 5 μM DOX not only markedly induced injuries, including cytotoxicity, apoptosis, overproduction of reactive oxygen species (ROS) and dissipation of mitochondrial membrane potential (MMP), but also enhanced the expression level of phosphorylated (p)-p38 MAPK. The DOX-induced increase in expression of p-p38 MAPK was significantly attenuated by pretreatment of H9c2 cells with either 400 μM sodium hydrogen sulfide (NaHS) (a donor of H_2S) or 1,000 μM N-acetyl-L-cysteine (NAC, an ROS scavenger) prior to exposure to DOX. Pretreatment with either 400 μM NaHS or 3 μM SB203580, a selective inhibitor of p38 MAPK, ameliorated DOX-induced cardiomyocyte injuries, as evidenced by an increase in cell viability, and decreases in the number of apoptotic cells, ROS generation as well as dissipation of MMP. In conclusion, the findings of the present study demonstrated that the activation of p38 MAPK contributes to DOX-induced injuries, including cytotoxicity, apoptosis, mitochondrial damage and oxidative stress in H9c2 cells. We also provide novel evidence that exogenous H_2S protects H9c2 cells against DOX-induced cardiotoxicity by inhibition of the p38 MAPK pathway.

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Introduction

To date, doxorubicin (DOX) remains one of the most widely used antitumor agents (1). It is a valuable component of various chemotherapeutic regimens used to treat breast and small-cell lung carcinoma as well as leukemia. However, its clinical use is limited by severe, dose-dependent acute and chronic cardiotoxicity (2,3), which may ultimately lead to severe and irreversible cardiomyopathy (4). The cause of DOX-induced cardiotoxicity is multifactorial, even though most of DOX-elicited cardiac effects are contributed to reactive oxygen species (ROS) generation, which ultimately leads to cardiomyocyte apoptosis (5,6). The signal transduction pathway that links DOX-induced oxidative stress and cardiac injury is a topic of strong current interest. Increasing evidence reveals that p38 mitogen-activated protein kinase (MAPK), one of the members of the MAPK family, may play an important role in DOX-induced cardiotoxicity (7,8).

p38 MAPK is activated by cellular stress and is thought to participate in cardiomyocyte apoptosis and cardiac pathologies (9,10). In isolated cardiomyocytes, DOX induces activation of p38 MAPK, followed by activation of pro-apoptotic protein Bax (11). In a previous *in vivo* study, multiple treatments of DOX to rats resulted in a persistent increase in the phosphorylation of p38 MAPK (12). The results of these studies (11,12) suggested that the p38 MAPK pathway may be involved in DOX-induced cardiotoxicity. However, the role of the p38 MAPK pathway in DOX-induced cardiotoxicity is not completely understood.

More recently, we demonstrated that hydrogen sulfide (H_2S) protects against DOX-induced cardiotoxicity through inhibition of endoplasmic reticulum (ER) stress and oxidative stress (13). H_2S, a well-known toxic gas with the characteristic smell of rotten eggs, has been qualified as the third gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO) (14,15). Accumulating evidence has shown that H_2S plays an important physiologic and pathophysiological role in regulating cardiovascular function. Exogenous H_2S can attenuate myocardial necrosis and rescue contractile activity in isoproterenol-stimulated rat hearts (16). H_2S also ameliorates hyperhomocysteinemia-induced myocardial tissue damage by inhibiting ER stress-associated apoptosis in rats (17). Recently, we demonstrated that exogenous H_2S not

only protects H9c2 cells from hypoxia-induced injury (18), but also inhibits hypoxia-induced activation of p38 MAPK (19). Kang *et al* (7) also reported that metallothionein (MT) which functions as an antioxidant can suppress DOX-induced apoptosis by inhibition of p38 MAPK. Based on our recent studies (18,19) and a previous one (20), we speculate that inhibition of the p38 MAPK pathway may contribute to the protective effect of exogenous H_2S against DOX-induced cardiotoxicity.

To test this hypothesis, in the present study, H9c2 cells were treated with 5 μM DOX to establish a chemotherapy-induced cardiotoxicity model (13). We then explored i) the effect of DOX on the phosphorylation of p38 MAPK; ii) the effect of exogenous H_2S on DOX-induced increase in the activation of p38 MAPK; iii) the role of the activation of p38 MAPK in DOX-induced cardiomyocyte insults; and iv) whether exogenous H_2S protects H9c2 cells against DOX-induced cardiotoxicity by inhibiting the p38 MAPK pathway.

Materials and methods

Materials. Sodium hydrogen sulfide (NaHS), SB203580, DOX, dichlorofluorescein diacetate (DCFH-DA) and Hoechst 33258 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Lab (Japan). JC-1 was purchased from Molecular Probes. DMEM-F12 medium and fetal bovine serum (FBS) were purchased from Gibco-BRL. H9c2 cells were obtained from the Sun Yat-sen University Experimental Animal Center.

Cell culture and treatments. H9c2 cells were cultured in DMEM-F12 medium supplemented with 10% FBS at 37˚C under an atmosphere of 5% CO_2. To explore the protective effects of H_2S on DOX-induced injury, H9c2 cells were pretreated with NaHS (a well-known H_2S donor) for 30 min prior to DOX treatment. To further determine whether the protective effects of H_2S were associated with the inhibition of p38 MAPK activity, H9c2 cells were pretreated with SB203580, a selective inhibitor of p38 MAPK, for 60 min prior to DOX treatment.

Cell viability assay. After H9c2 cells cultured in 96-well plates received different treatments, 10 μl CCK-8 solution was added to each well at a 1/10 dilution, followed by a further 2-h incubation in the incubator. Absorbance was measured at 450 nm with a microplate reader (Multiskan MK3 Microplate Reader, Thermo Fisher Scientific, Inc., USA). The mean optical density (OD) of 5 wells in the indicated groups was used to calculate the percentage of cell viability according to the formula:

Percentage of cell viability = OD treatment group/OD control group x 100%.

Experiments were performed in triplicate.

Hoechst 33258 nuclear staining to assess apoptosis. Apoptosis was analyzed by fluorescence microscopy with the chromatin dye Hoechst 33258. H9c2 cells were analyzed 24 h following the indicated treatments. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. Following 3 washes with PBS, cells were stained with 5 mg/l Hoechst 33258 for 5 min, washed briefly with PBS and air-dried again. The cells were visualized under a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan). Apoptotic cells showed condensed, fractured or distorted nuclei, viable cells displayed normal nuclear size and uniform fluorescence.

Measurement of intracellular ROS generation. Intracellular ROS generation was determined by the oxidative conversion of cell-permeable DCFH-DA to fluorescent DCF. H9c2 cells were cultured on a slide in DMEM-F12 medium. After the indicated treatments, slides were washed twice with PBS. DCFH-DA solution in serum-free medium was added at a concentration of 10 μM and co-incubated with H9c2 cells at 37˚C for 60 min. The slides were washed 3 times, and DCF fluorescence was measured over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The mean fluorescence intensity (MFI) from 4 random fields was analyzed using ImageJ 1.41o software (National Institutes of Health, USA) and the MFI of DCF was used to indicate the amount of ROS.

Assessment of MMP. Mitochondrial membrane potential (MMP) was assessed using the fluorescent indicator 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1; Molecular Probes). The DOX-treated cells were incubated with 1 mg/ml JC-1 for 30 min at 37˚C and visualized using a fluorescence microscope. A shift from red to green fluorescence indicates a loss of MMP, which was assessed by obtaining multiple merged images and using MetaMorph software to count cells that fluoresced red or green. Data are expressed as the percentage of cells that had undergone mitochondrial membrane transition (i.e., gained green fluorescence).

Western blot assay. After different treatments, H9c2 cells were harvested and lysed, and the homogenate was centrifuged at 12,000 rpm for 10 min at 4˚C. The total protein in the supernatant was quantitated with a BCA protein assay kit. Total protein (30 μg from each sample) was separated by 12% SDS-PAGE. The protein in the gel was transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% free-fat milk in TBS-T for 1 h at room temperature, and then incubated with primary antibodies specific to p38 MAPK and phosphorylated (p)-p38 MAPK (1:4,000), caspase-3 (1:2,000) (Cell Signaling Technology, Inc., Beverly, MA, USA), or GAPDH with gentle agitation at 4˚C overnight and subsequently incubated with the secondary antibodies for 1.5 h at room temperature. Following 3 washes with TBS-T, membranes were developed using enhanced chemiluminescence and exposed to X-ray films. To quantify protein expression, the X-ray films were scanned and analyzed with ImageJ 1.41o software.

Statistical analysis. All data are presented as means ± standard error (SE). Differences between groups were analyzed by one-way analysis of variance (ANOVA) with SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistical significant difference.

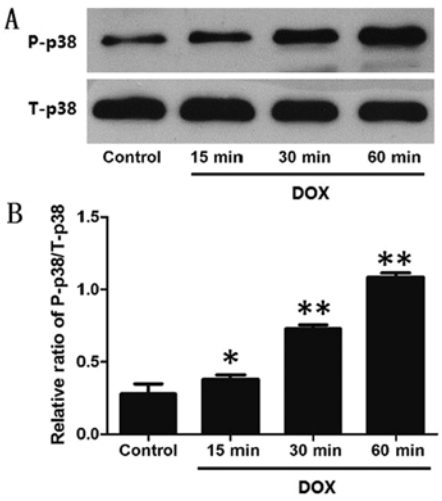

Figure 1. DOX time-dependently increases the expression of phosphorylated (P)-p38 MAPK in H9c2 cells. H9c2 cells were treated with 5 μM for the indicated times. (A) Expression of p38 MAPK was assessed by western blot analysis and (B) quantified by densitometric analysis with ImageJ 1.41o software. Data are shown as the means ± SE (n=3). *P<0.05, **P<0.01 vs. the control group. T, total. DOX, doxorubicin.

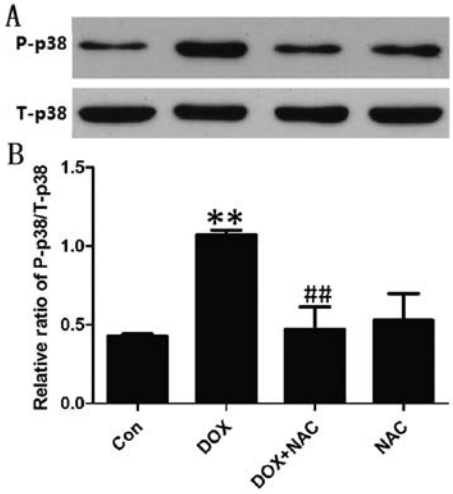

Figure 3. NAC suppresses DOX-induced activation of p38 MAPK in H9c2 cells. H9c2 cells were treated with 5 μmol/l DOX for 60 min and either treated or pretreated with 1,000 μmol/l NAC for 60 min. (A) Western blot analysis was applied to detect change in expression of total (T)-p38 MAPK and phosphorylated (P)-p38 MAPK. (B) Densitometric analysis of the results in A. The data are presented as the means ± SE (n=3). **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DOX treatment group. DOX, doxorubicin.

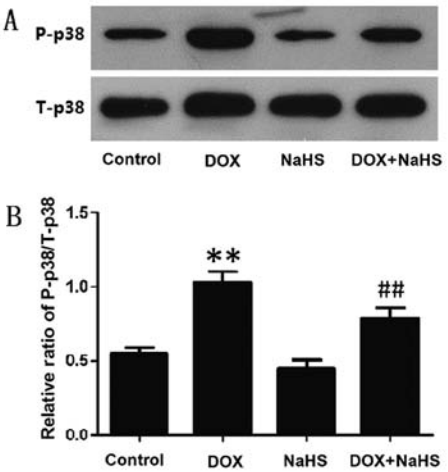

Figure 2. Exogenous H_2S attenuates DOX-induced expression of phosphorylated (P)-p38 MAPK in H9c2 cells. H9c2 cells were treated with 5 μmol/l DOX for 60 min and either untreated or pretreated with 400 μmol/l NaHS for 30 min prior to DOX exposure. (A) Expression of p38 MAPK was examined by western blot analysis and (B) quantified by densitometric analysis with ImageJ 1.41o software. Data are shown as the means ± SE (n=3). **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DOX-treated group. T, total; DOX, doxorubicin.

Results

DOX increases the expression of phosphorylated p38 MAPK in H9c2 cells. To test the effect of DOX on activation of p38 MAPK, H9c2 cells were treated with 5 μM DOX for the indicated times (15, 30 and 60 min). As shown in Fig. 1, DOX time-dependently enhanced the expression level of p-p38 MAPK. However, DOX at 5 μM alone did not induce significant changes in expression of total (t) p38 MAPK.

Exogenous H_2S inhibits DOX-induced increase in expression of phosphorylated p38 MAPK in H9c2 cells. Western blot analysis showed that exposure of H9c2 cells to 5 μM DOX for 60 min markedly enhanced the expression level of p-p38 MAPK (Fig. 2A and B). This increased expression of p-p38 MAPK was attenuated by pretreatment of cells with 400 μM NaHS (a donor of H_2S) for 30 min prior to exposure to 5 μM DOX. NaHS at 400 μM alone did not alter the basal expression level of p-p38 MAPK in H9c2 cells (Fig. 2A and B).

NAC suppresses the DOX-induced activation of p38 MAPK in H9c2 cells. To confirm whether the inhibitory effect of NaHS on the DOX-induced increase in expression of p-p38 MAPK is associated with its antioxidation, H9c2 cells were pretreated with 1,000 μM NAC (ROS scavenger) for 60 min before exposure to 5 μM DOX. As shown in Fig. 3, similar to the inhibitory effect of NaHS pretreatment, the pretreatment of cells with NAC for 60 min markedly depressed the increased expression of p-p38 MAPK induced by 5 μM DOX for 60 min. NAC at 1,000 μM did not significantly change the basal level of p-p38 MAPK expression. The results revealed that an antioxidant effect contributed to the inhibitory effect of H_2S on the DOX-induced increased expression of p-p38 MAPK.

Exogenous H_2S and p38 MAPK inhibitor attenuate DOX-induced cytotoxicity in H9c2 cells. As presented in Fig. 4, exposure of H9c2 cells to DOX at 5 μM for 24 h induced marked cytotoxicity, leading to a decrease in cell viability. However, pretreatment of cells with 400 μM NaHS for 30 min prior to exposure to DOX significantly ameliorated the DOX-induced cytotoxicity, as evidenced by an increase in cell viability. In order to examine whether the activation of p38 MAPK is involved in DOX-induced cytotoxicity, H9c2 cells were pretreated with 3 μM SB203580, a selective inhibitor of p38 MAPK, for 60 min before exposure to 5 μM

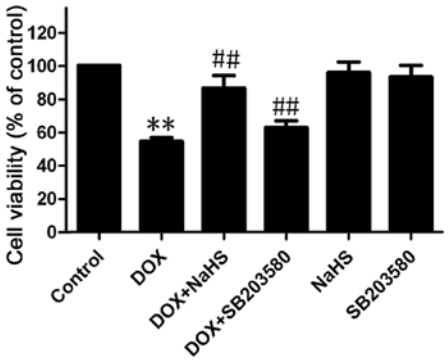

Figure 4. Exogenous H_2S and p38 MAPK inhibitor protect H9c2 cells against DOX-induced cytotoxicity. H9c2 cells were treated with 5 μmol/l DOX for 24 h in the absence of or pretreated with 400 μM NaHS for 30 min or 3 μM SB203580 for 60 min before DOX treatment. Cell viability was measured using the CCK-8 assay. Data are shown as the means ± SE (n=3). **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DOX treatment group. DOX, doxorubicin.

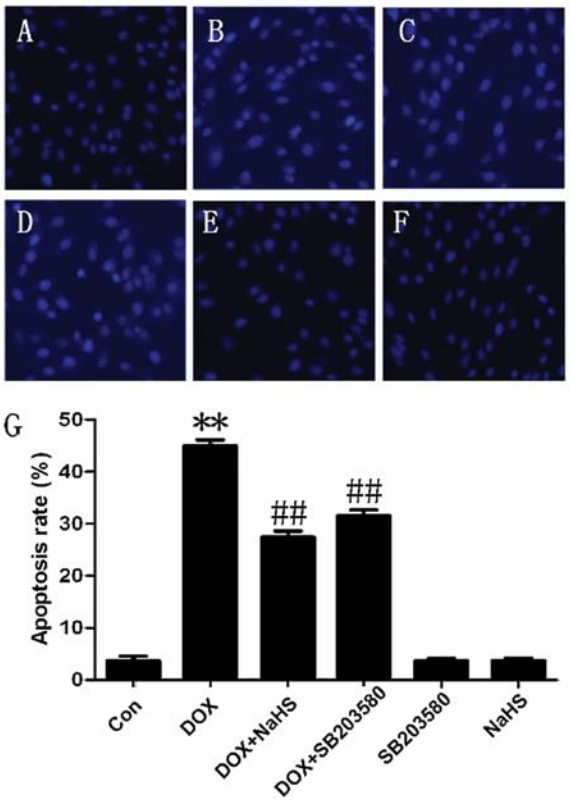

Figure 5. Exogenous H_2S and p38 MAPK inhibitor reduce DOX-induced apoptosis in H9c2 cells. (A-F) Hoechst 33258 nuclear staining followed by fluorescence imaging to observe cell apoptosis. (A) Control group; H9c2 cells were (B) exposed to 5 μM DOX for 24 h; (C) pretreated with 400 μM NaHS for 30 min prior to exposure to 5 μM DOX for 24 h; (D) treated with 3 μM SB203580 for 60 min followed by exposure to 5 μM DOX for 24 h; (E) treated with 400 μM NaHS for 30 min followed by 24 h culture; (F) treated with 3 μM SB203580 for 60 min followed by 24-h culture. (G) The apoptotic rate was analyzed with a cell counter and ImageJ 1.41 software. **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DOX treatment group. DOX, doxorubicin.

DOX for 24 h. Pretreatment with SB203580 had a similar cytoprotective effect as H_2S against DOX-induced cytotoxicity, suggesting involvement of the activation of p38 MAPK

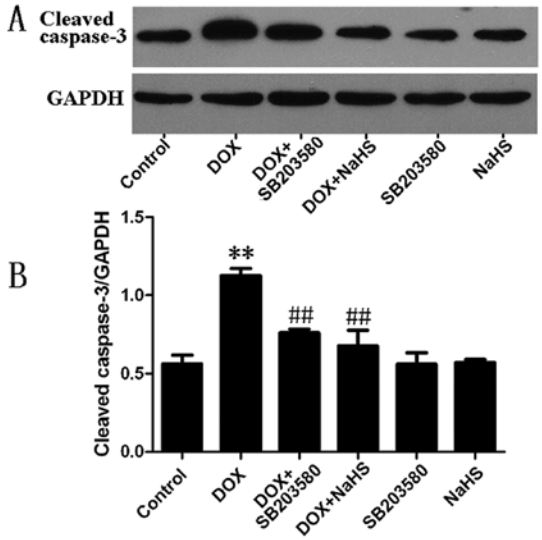

Figure 6. Exogenous H_2S and p38 MAPK inhibitor suppress the DOX-induced increase in the expression of cleaved caspase-3 in H9c2 cells. H9c2 cells were treated with 5 μmol/l DOX for 12 h in the absence of pretreated with 400 μM NaHS for 30 min or 3 μM SB203580 for 60 min prior to DOX exposure. (A) Expression of cleaved caspase-3 was assessed by western blot assay. (B) The data in (A) were quantified by densitometric analysis with ImageJ 1.41 software. Data are shown as the means ± SE (n=3). **P<0.01 vs. the control group; $^{\#\#}$P<0.01 vs. the DOX-treated group. DOX, doxorubicin.

in DOX-induced cytotoxicity in H9c2 cells. Alone NaHS or SB203580 did not alter cell viability in the H9c2 cells (Fig. 4).

Exogenous H_2S and p38 MAPK inhibitor reduce DOX-induced apoptosis in H9c2 cells. We further observed the effects of both NaHS and p38 MAPK inhibitor on DOX-induced apoptosis. As shown in Fig. 5B, H9c2 cells treated with 5 μM for 24 h exhibited typical characteristics of apoptosis, including condensation of chromatin, shrinkage of nuclei and apoptotic bodies. However, pretreatment of cells with 400 μM NaHS for 30 min before DOX exposure obviously decreased the DOX-induced increased number of cells with nuclear condensation and fragmentation (Fig. 5C). NaHS alone did not markedly alter cell morphology or the percentage of apoptotic H9c2 cells (Fig. 5F and G). In addition, the western blot analysis (Fig. 6A and B) revealed that exposure of cells to 5 μM DOX for 12 h considerably upregulated the expression level of cleaved caspase-3 which is thought to be one of the main effectors of apoptosis (21); this effect was dramatically suppressed by pretreatment of cells with 400 μM NaHS for 30 min (Fig. 6A and B). NaHS alone did not affect the basal expression of cleaved caspase-3 in H9c2 cells. The above findings indicate that exogenous H_2S protects H9c2 cells against DOX-induced apoptosis.

To ascertain whether the activation of p38 MAPK is implicated in DOX-induced apoptosis, H9c2 cells were pretreated with 3 μM SB203580 for 60 min followed by exposure to 5 μM DOX for 24 or 12 h (for examining cleaved caspase-3 expression). The results showed that pretreatment with SB203580 attenuated not only the DOX-induced increased number of apoptotic H9c2 cells (Fig. 5D and G), but also the expression level of cleaved caspase-3 induced by DOX (Fig. 6). Alone, SB203580 did not induce H9c2 cell apoptosis

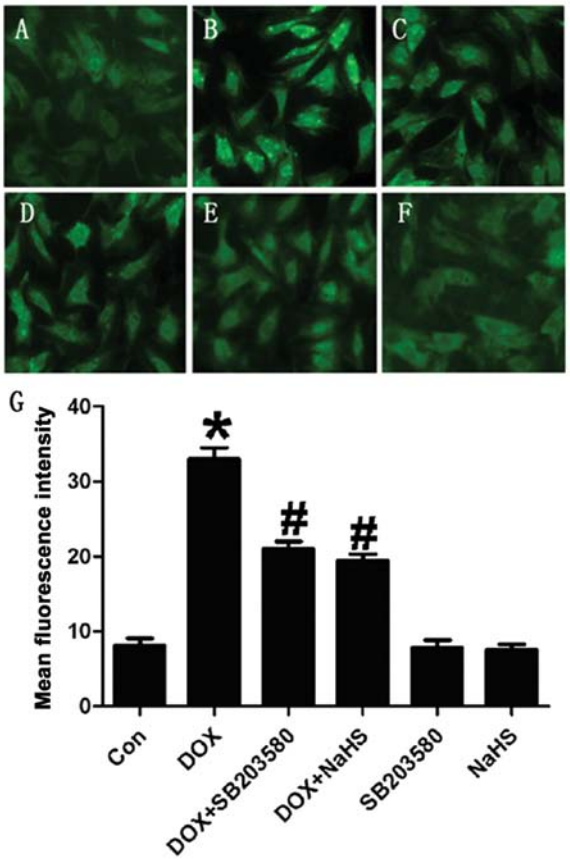

Figure 7. Exogenous H_2S and p38 MAPK inhibitor decrease DOX-induced ROS accumulation in H9c2 cells. (A-F) After the indicated treatments, intracellular ROS generation was measured by DCFH-DA staining followed by photofluorography. (A) Control group; H9c2 cells were (B) exposed to 5 μM DOX for 24 h; (C) pretreated with 400 μM NaHS for 30 min before exposure to 5 μM DOX for 24 h; (D) pretreated with 3 μM SB203580 for 60 min before exposure to 5 μM DOX for 24 h; (E) treated with 400 μM NaHS for 30 min followed by 24-h culture; (F) treated with 3 μM SB203580 for 60 min followed by a 24-h culture. (G) Quantitative analysis of the MFI of DCF in A-F with Image J 1.41o software. Data are shown as means ± SE (n=5). *P<0.05 vs. control group; $^{\#}$P<0.05 vs. the DOX-treated group. DOX, doxorubicin; MFI, mean fluorescence intensity.

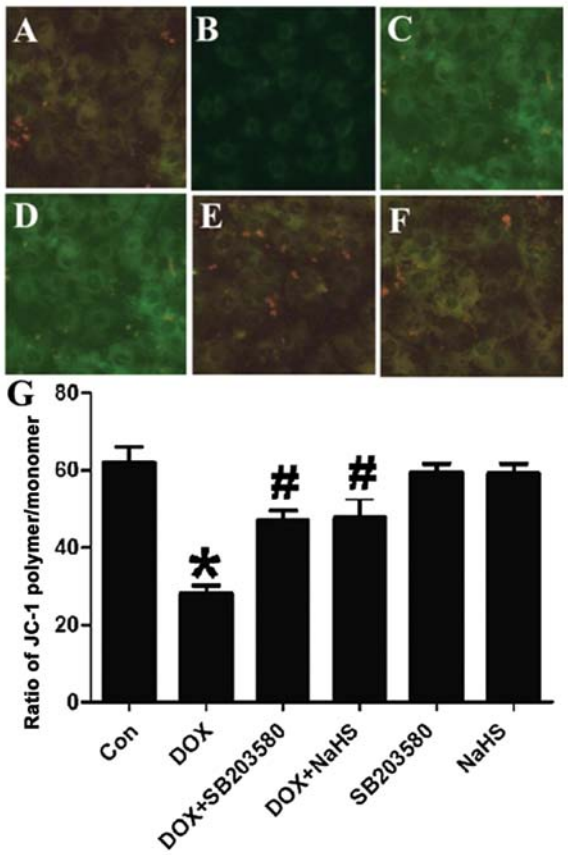

Figure 8. Exogenous H_2S and p38 MAPK inhibitor ameliorate DOX-induced MMP loss in H9c2 cells. (A-F) After the indicated treatments, MMP was measured by JC-1 staining followed by photofluorography. (A) Control group; H9c2 cells were (B) treated with 5 μM DOX for 24 h; (C) pretreated with 400 μM NaHS for 30 min prior to exposure to 5 μM DOX for 24 h; (D) pretreated with 3 μM SB203580 for 60 min prior to exposure to 5 μM DOX for 24 h; (E) treated with 400 μM NaHS for 30 min followed by a 24-h culture; (F) treated with 3 μM SB203580 for 60 min followed by a 24-h culture. (G) Quantitative analysis of the MFI of JC-1 in A-F with Image J 1.41o software. Data are shown as means ± SE, n=5. *P<0.05 vs. the control group, $^{\#}$P<0.05 vs. the DOX-treated group. DOX, doxorubicin; MFI, mean fluorescence intensity.

or a change in the basal expression of cleaved caspase-3. Our findings suggest that the p38 MAPK pathway participates in the DOX-induced apoptosis of H9c2 cells.

Exogenous H_2S and p38 MAPK inhibitor induce DOX-induced oxidative stress in H9c2 cells. Previous studies have shown that oxidative stress plays a pivotal role in DOX-induced cardiotoxicity (5,6,13). Thus, we investigated the effects of H_2S and SB203580 on DOX-induced ROS generation in H9c2 cells. As shown in Fig. 7, exposure of cells to 5 μM DOX for 24 h markedly enhanced ROS generation. The increased ROS generation was attenuated by pretreatment of cells with 400 μM NaHS for 30 min before DOX exposure, suggesting that exogenous H_2S protects H9c2 cells against DOX-induced oxidative stress. To explore whether the activation of p38 MAPK contributes to the DOX-induced overproduction of ROS, H9c2 cells were preconditioned with 3 μM SB203580 for 60 min prior to exposure to DOX. The preconditioning with SB203580 significantly decreased the DOX-induced increase in ROS generation, indicating the involvement of the p38 MAPK pathway in DOX-induced oxidative stress. NaHS at 400 μM or SB203580 at 3 μM alone did not alter basal ROS generation (Fig. 7).

Exogenous H_2S and p38 MAPK inhibitor block the DOX-induced loss of MMP in H9c2 cells. As it has been demonstrated that ROS-elicited mitochondrial insult participates in DOX-induced cardiotoxicity (20), we investigated the effects of both H_2S and p38 MAPK inhibitor on the DOX-induced dissipation of MMP. As shown in Fig. 8, after H9c2 cells were subjected to 5 μM DOX for 24 h, mitochondria were markedly damaged, leading to a decrease in the uptake of JC-1, indicating a loss of MMP (Fig. 8B). Notably, the loss of MMP was ameliorated by pretreatment with 400 μM NaHS for 30 min (Fig. 8D and G). Similarly, pretreatment of H9c2 cells with 3 μM SB203580 for 60 min before exposure to DOX also blocked the DOX-induced dissipation of MMP (Fig. 8C and G). These data suggest that both exogenous H_2S and p38 MAPK inhibitor protect H9c2 cells against DOX-induced mitochondrial damage.

Discussion

Although intensive studies on DOX-induced cardiotoxicity have continued for decades, the underlying mechanisms have not been fully elucidated. Accumulating evidence supports the notion that free radical-induced oxidative stress and cardiomyocyte death by apoptosis and necrosis are the key contributors to DOX-induced cardiotoxicity. In agreement with previous studies (5-7,11-13,20,22-24), in the present study, we observed that exposure of H9c2 cells to DOX markedly induced cellular injuries, including a decrease in cell viability, increases in cell apoptosis, expression of cleaved caspase-3 and ROS generation as well as dissipation of MMP. We further confirmed that the activation of p38 MAPK plays a pivotal role in DOX-induced cardiotoxicity. This is supported by the findings of this study that i) treatment with 5 μM DOX time-dependently upregulated the expression of p-p38 MAPK; ii) pretreatment of H9c2 cells with SB203580, a specific inhibitor of p38 MAPK, dramatically attenuated DOX-induced cytotoxicity, leading to an increase in cell viability; iii) pretreatment with SB203580 markedly reduced the increased number of apoptotic cells and expression level of cleaved caspase-3 induced by DOX; iv) pretreatment with SB203580 obviously decreased DOX-induced ROS generation; v) pretreatment with SB203580 considerably ameliorate DOX-induced dissipation of MMP. All of these results demonstrated the functional significance of the activation of p38 MAPK in DOX-induced cardiotoxicity.

The p38 MAPK is a subfamily of the MAPK superfamily. This subfamily is composed of p38α, p38β, p38γ and p38δ (25-27). p38 MAPK has been shown to be a pivotal group of signal molecules that response to environmental stress in various cell types. In cardiomyocytes, p38 MAPK is implicated in the onset of apoptosis in ischemia-reperfusion-injured hearts (28,29). Particularly, transfection experiments using primary cultures of neonatal rat cardiomyocytes further demonstrate the involvement of p38α in myocyte apoptosis (30). Recently, the roles of p38 MAPK in DOX-induced cardiotoxicity have received attention (6-8,11,12,31). Poizat *et al* (31) reported that DOX activates p38α and p38β which are implicated in the phosphorylation of the transcriptional co-activator p300, which parallels the DOX-induced apoptosis of primary neonatal cardiomyocytes. In transgenic mice containing high levels of cardiac metallothionein (MT) and neonatal mouse cardiomyocytes, the activation of p38 MAPK has also been shown to participate in DOX-induced cardiomyocyte apoptosis (7). These previous studies (7,31) support the findings of the present study.

Since the common observation is that cardiomyocyte apoptosis contributes to DOX-induced cardiotoxicity, therefore, in the present study, we investigated the possible roles of p38 MAPK in DOX-induced apoptosis. Based on the results obtained from the recent study and a previous observation (31), the following mechanism may be responsible, at least partly, for the roles of p38 MAPK in DOX-induced cardiac apoptosis. Firstly, the activation of p38 MAPK induces accumulation of ROS which was attenuated by pretreatment with SB203580, a specific inhibitor of p38 MAPK. Secondly, p38 MAPK enhanced the activation of cleaved caspase-3, one of the apoptotic effectors. Third, p38 MAPK induced dissipation of MMP. Fourth, p38 MAPK activates p300 (31), which triggers cardiomyocyte apoptosis. Since SB203580 used in the present study acts as a specific inhibitor of p38α and p38β, but not p38γ and p38δ, the exact roles of the different subunits of p38 MAPK require further study. Noteworthy, the results of the present study provide novel evidence that an interaction between ROS and p38 MAPK exists in DOX-treated H9c2 cells, as SB203580 pretreatment attenuated DOX-induced ROS production, whereas the pretreatment of H9c2 cells with NAC (ROS scavenger) prior to DOX exposure reduced the expression level of p-p38 MAPK. These results are comparable with our previous study (32). More understanding of the role of the interaction between ROS and p38 MAPK in DOX-induced cardiotoxicity may aid in the treatment and prevention of cardiac injury.

Another important novel finding of the present study was that exogenous H_2S protects against the DOX-induced cardiotoxicity by inhibition of the p38 MAPK pathway in H9c2 cells. Increasing evidence has shown that H_2S is cardioprotective (13,16-18,33,34). Recently, we showed that exogenous H_2S offers protection against chemical hypoxia-induced injury by its antioxidant effect and upregulation of heat shock protein 90 (HSP90) expression in H9c2 cells (18,33). Importantly, our more recent study demonstrated that DOX inhibits the expression and activity of cystathionine-γ-lyase (CSE), an H_2S synthase, and that exogenous H_2S prevents DOX-induced cardiotoxicity by inhibiting ER stress and oxidative stress (13). Furthermore, we found that the inhibitory effect of exogenous H_2S on the activation of p38 MAPK was induced by chemical hypoxia in PC12 cells (19). The results of our recent studies (19) suggest that inhibition of p38 MAPK may contribute to the protective effect of exogenous H_2S against DOX-induced cardiotoxicity. The findings of the present study support this hypothesis. We found that pretreatment of H9c2 cells with NaHS (a donor of H_2S) prior to exposure to DOX significantly attenuated the DOX-induced increase in expression of p-p38 MAPK. In addition, pretreatment with NaHS had a similar cardioprotective effect as SB203580 (an inhibitor of p38 MAPK) against DOX-induced cardiotoxicity, as evidenced by an increase in cell viability, and decreases in the number of apoptotic cells, the expression of cleaved caspase-3, dissipation of MMP and ROS accumulation. To explore the mechanism responsible for the inhibitory effect of exogenous H_2S on DOX-induced activation of p38 MAPK, H9c2 cells were pretreated with NAC, an ROS scavenger, before DOX exposure. Similar to exogenous H_2S, NAC pretreatment also markedly inhibited the increased expression of p-p38 MAPK, suggesting that the inhibitory effect of exogenous H_2S on the activation of p38 MAPK may be associated with its antioxidation. Our results are comparable with a previous study that MT having an antioxidative effect decreased DOX-induced cardiomyocyte apoptosis through inhibition of p38 MAPK (7).

In conclusion, in DOX-treated H9c2 cardiac cells, we demonstrated for the first time that exogenous H_2S protects against cardiotoxicity by inhibiting the activation of p38 MAPK induced by DOX treatment. Investigation of the potential of H_2S to protect against DOX-induced cardiotoxicity

may lead to the development of novel approaches to this clinical problem.

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