Antioxidant Effects of Hydrogen Sulfide on Left Ventricular Remodeling in Smoking Rats AreMediated via PI3K/Akt-Dependent Activation of Nrf2

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ABSTRACT

There is growing evidence that oxidative stress plays critical roles in the pathogenesis of cardiac remodeling. In the present study, we established a rat model of passive smoking and investigated the antioxidant effects of hydrogen sulfide (H2S) on smoking-induced left ventricular remodeling. Cardiac structure and function were evaluated using 2-dimensional echocardiography. Myocardial fibrosis was detected by Masson’s trichrome staining and immunohistochemistry. Oxidative stress was assessed by measuring malondialdehyde levels, superoxide dismutase and glutathione peroxidase activities, and reactive oxygen species generation in the myocardium. Neonatal rat cardiomyocytes transfected with specific siRNA and exposed to cigarette smoke condensate and H2S donor sodium hydrosulfide were used to confirm the involvement of Nrf2 and PI3K/Akt signaling in the antioxidant effects of H2S. Our results indicated that H2S could protect against left ventricular remodeling in smoking rats via attenuation of oxidative stress. Moreover, H2S was also found to increase the phosphorylation of Akt and GSK3β and decrease the nuclear expression of Fyn, which consequently leads to nuclear translocation of Nrf2 and elevated expression of HO-1 and NQO1. In conclusion, H2S may exert antioxidant effects on left ventricular remodeling in smoking rats via PI3K/Akt-dependent activation of Nrf2 signaling.

Key words: hydrogen sulfide; left ventricular remodeling; smoking; oxidative stress; PI3K/Akt; Nrf2

Cardiac remodeling, defined as a physiological or pathological process which is clinically manifested as changes in size, shape, and function of the heart in response to cardiac injury or increased load, is critically involved in the development and progression of heart failure (Cohn et al., 2000). The pathophysiological features of remodeling include cardiac hypertrophy, interstitial fibrosis, ventricular dilatation, contractile dysfunction, and cardiomyocyte apoptosis (Koitabashi and Kass, 2011). Over the past few decades, clinical and experimental studies have provided substantial evidence that oxidative stress, defined as an excess production of reactive oxygen species (ROS) relative to antioxidant defence, plays critical roles in the pathogenesis of ventricular remodeling (Hori and Nishida, 2009; Nabeebacuss et al., 2011; Takimoto and Kass, 2007; Tsutsui et al., 2011). In our previous studies, we established a rat model of passive smoking and found that chronic exposure to cigarette smoke could eventually lead to left ventricular remodeling and dysfunction (Zhou et al., 2012, 2013).

Hydrogen sulfide (H2S) is an endogenously generated gaseous signaling molecule with diverse physiological functions. In the cardiovascular system, H2S is produced in the myocardium, fibroblasts, and blood vessels from L-cysteine by the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). There is growing evidence that H2S can exert cardioprotective effects by neutralizing ROS, inhibiting leukocyte-endothelial cell interactions, promoting vascular smooth muscle relaxation, reducing apoptotic cell death, and modulating mitochondrial respiration (Liu et al., 2012; Pan et al., 2012). In the present study, we aimed to investigate the antioxidant effects of H2S on left ventricular remodeling in smoking rats and explore the potential molecular mechanisms involved.
MATERIALS AND METHODS

Animal model and grouping. All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Soochow University. Male Sprague Dawley rats weighing 200–250 g were obtained from the Experimental Animal Center of Soochow University and were housed in a room at 22 ± 2°C and 50 ± 5% relative humidity with an alternating 12-h light/dark cycle for 4 months. The rats were randomly assigned into 4 groups: CS group (n = 10; exposed to cigarette smoke at the rate of 40 cigarettes/day as previously described) (Zhou et al., 2012), NaHS group (n = 10; intraperitoneally administered with H2S donor NaHS at a dose of 14 μmol/kg/day), CS + NaHS group (n = 10; exposed to cigarette smoke and administered with NaHS), and control group (n = 10; neither exposed to cigarette smoke nor treated with NaHS).

Cell culture and treatment. Primary cultures of neonatal rat cardiomyocytes (NRCMs) were prepared as previously described (Zhou et al., 2010). The antioxidant effect of H2S was evaluated by pretreatment of NRCMs with 100 μM NaHS for 30 min prior to exposure to 10% cigarette smoke condensate (CSC) for 24 h. CSC was purchased from Murty Pharmaceuticals (Lexington, Kentucky) and was prepared from Kentucky standard cigarettes using a smoking machine designed for Federal Trade Commission testing.

Measurement of H2S content. After 4 months of cigarette smoke exposure, the plasma and myocardial levels of H2S were determined by the Methylene Blue method. This method is based on the reaction of sulfide with N,N-dimethyl-p-phenylenediamine, in a ferric chloride catalyzed reaction with a 1:2 stoichiometric ratio to give the Methylene Blue dye, which is detected spectrophotometrically.

Echocardiographic study. Left ventricular structure and function were evaluated by 2-dimensional echocardiography. Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and left ventricular posterior wall thickness (LVPWT) were measured from the parasternal long-axis view. Left ventricular fractional shortening (LVEF) were determined to assess left ventricular systolic function. The mitral peak flow velocities at early diastole and atrial contraction were recorded by pulsed Doppler technique and the E/A ratio was calculated to reflect left ventricular diastolic function. All measurements were averaged for 3 consecutive cardiac cycles by an experienced technician who was blinded to study grouping.

Histopathology and immunohistochemistry. Left ventricular tissue was surgically removed, fixed in 10% buffered formalin, embedded in paraffin, and sliced into 5-μm-thick sections. The slides were then stained with Masson's trichrome and collagen volume fractions (CVFs) were measured using an image analysis software (Image-Pro Plus, Media Cybernetics). The expression of types I and III collagen was examined by immunohistochemistry. Briefly, left ventricular tissue sections were deparaffinized with xylene, rehydrated with graded ethanol, and treated with 3% hydrogen peroxide to quench endogenous peroxidase, followed by blocking with 10% goat serum for 30 min. The sections were then incubated with rabbit anti-rat collagen I and III antibodies (Santa Cruz Biotechnology, Santa Cruz, California) at 4°C overnight. After washing with PBS, the slides were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) at room temperature for 30 min. Finally, the sections were exposed to diaminobenzidine peroxidase substrate for 5 min and counterstained with Mayer's hematoxylin.

Measurement of oxidative stress. Oxidative stress was evaluated by detecting malondialdehyde (MDA) levels, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, and ROS generation in the myocardium according to the instructions of detection kits (Jiancheng Biotech, Nanjing, China).

Electrophoretic mobility shift assay. Nuclear protein extracts were prepared from myocardial tissue using NE-PER extraction kit (Thermo Scientific, Rockford, Illinois). Equal amounts of nuclear protein were incubated with biotin-labeled oligonucleotide probes of antioxidant responsive element (ARE) as previously described (Zhou et al., 2015). The DNA-protein complexes were separated on a non-denaturing polyacrylamide gel and then transferred onto a nylon membrane. The transferred DNA was cross-linked to the membrane, incubated with horseradish peroxidase-conjugated streptavidin, and visualized by enhanced chemiluminescence.

siRNA transfection. NRCMs were seeded into 6-well plates (1 × 10^5 cells/well) for 24 h and then transfected with Nrf2 or PI3K-specific siRNA using Lipofectamine 2000 reagents (Invitrogen). Subsequently, the cells were exposed to 10% CSC for 24 h after incubation with or without NaHS (100 μM) for 30 min. In this study, nonspecific siRNA was used to determine the efficiency of Nrf2 or PI3K siRNA transfection.

PI3K activity assay. PI3K activity was measured using a commercial ELISA kit (Echelon Biosciences) according to the manufacturer's instructions. In this method, PI3K activity was assessed by detecting the conversion of PI(4,5)P2 into PI(3,4,5)P3.

Western blotting. Rats were sacrificed by carbon dioxide asphyxiation, and left ventricular tissues were removed and homogenized in ice-cold lysis buffer. Protein concentrations were quantified with the BCA protein assay kit (Thermo Scientific). Equal amounts of protein (50 μg) were separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and blocked with 5% nonfat milk. The membranes were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-CSE, anti-Nrf2, anti-HO-1, anti-NQO1, anti-Akt, anti-phospho-Akt (Ser473), anti-GSK3β, anti-phospho-GSK3β (Ser9), and anti-Fyn (Santa Cruz Biotechnology). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences).

Statistical analysis. Data are expressed as mean ± SD, and differences between groups were analyzed using analysis of variance with SPSS version 15.0 (SPSS, Chicago, Illinois). Post hoc analysis was used if the analysis of variance was significant. A value of P < 0.05 was considered statistically significant.

RESULTS

As shown in Figure 1, H2S levels in plasma and myocardial tissue were reduced in the smoking rats and elevated after treatment with NaHS. The protein expression of CSE was down-regulated in the CS group compared with that in the
control group. However, NaHS administration was not associated with significant changes in CSE expression.

The echocardiographic data are shown in Figure 2. LVEDD and LVESD were significantly increased in the CS group and decreased in the CS + NaHS group. LVEF and LVFS, indicators of left ventricular systolic function, were found to be remarkably lower in the CS group, whereas NaHS treatment could improve left ventricular systolic function in the CS + NaHS group. In addition, there were no significant differences in LVPWT and E/A ratio among these groups.

Myocardial fibrosis was detected by Masson’s trichrome staining and immunohistochemistry (Fig. 3). CVF was found to be significantly increased in the CS group and decreased in the CS + NaHS group. Furthermore, types I and III collagen expression were remarkably up-regulated in the smoking rats and down-regulated after administration of NaHS.

Oxidative stress was assessed by measuring MDA levels, SOD and GSH-Px activities, and ROS generation in the myocardium (Fig. 4). There were marked increases in MDA levels and decreases in SOD and GSH-Px activities in smoking rats, whereas NaHS treatment was associated with decreased MDA levels and increased SOD and GSH-Px activities. Moreover, myocardial ROS production was significantly elevated in the CS group and reduced in the CS + NaHS group.

The activity of Nrf2 was determined by Western blotting and electrophoretic mobility shift assay (Fig. 5). Nrf2 protein accumulated in the nucleus of cardiomyocytes and Nrf2-ARE binding activity was significantly enhanced in the myocardium of smoking rats following administration of NaHS. Consequently, the protein expression of 2 downstream targets of Nrf2, HO-1, and NQO1, was remarkably increased in the CS + NaHS group compared with that in the CS group.

To confirm whether H2S inhibits smoking-induced oxidative stress in an Nrf2-dependent manner, we transfected NRCMs with Nrf2-specific siRNA and then subjected them to CSC. We found that Nrf2 siRNA-transfected cells exposed to CSC and NaHS exhibited reduced Nrf2 nuclear expression and increased ROS production compared with cells not transfected with Nrf2 siRNA (Fig. 6).

The activity of PI3K was measured by ELISA and the phosphorylation of Akt was detected by Western blotting (Fig. 7). Our results showed that PI3K activity and Akt phosphorylation were significantly decreased in the CS group and increased in the CS + NaHS group, suggesting that PI3K/Akt signaling may be involved in the cardioprotective effects of H2S.

To determine whether H2S activates Nrf2 signaling via PI3K/Akt-dependent pathway, we transfected NRCMs with PI3K-specific siRNA and then subjected them to CSC. We found that PI3K siRNA-transfected cells that had been exposed to CSC and NaHS exhibited reduced Akt phosphorylation and Nrf2 nuclear expression and elevated ROS production compared with cells that had not been transfected with PI3K siRNA (Fig. 8).

Phosphorylated Akt can inactivate GSK3β by phosphorylating its Ser9 residue, resulting in inactivation of Fyn kinase which relieves ubiquitination-mediated Nrf2 suppression and thereby reinforces cell defence mechanism. In this study, we
found that H₂S could increase the phosphorylation of Akt and GSK3β and consequently decrease the nuclear localization of Fyn in NRCMs exposed to CSC. Furthermore, Akt-specific siRNA-transfected NRCMs exposed to CSC and NaHS exhibited reduced GSK3β phosphorylation and elevated Fyn nuclear expression (Fig. 9).

DISCUSSION

In the present study, we established a rat model of passive smoking and found that cigarette smoke exposure could lead to cardiac enlargement, myocardial fibrosis, and left ventricular systolic dysfunction. NaHS treatment was found to improve left ventricular function and attenuate left ventricular remodeling.
in smoking rats. In addition, our findings indicated that endoge-
ous H₂S generation and CSE protein expression were signifi-
cantly decreased in smoking rats, whereas exogenous
administration of NaHS increased H₂S contents in both plasma
and myocardial tissue.

It has been well documented that oxidative stress plays crit-
ical roles in the pathophysiology of cardiac remodeling (Hori
and Nishida, 2009; Nabeebaccus et al., 2011; Takimoto and Kass,
2007; Tsutsui et al., 2011). ROS directly impair contractile func-
tion by modifying proteins central to excitation-contraction
coupling. Moreover, ROS activate a variety of hypertrophy
signaling kinases and transcription factors and mediate
cardiomyocyte apoptosis. They also stimulate cardiac fibroblast
proliferation and activate the matrix metalloproteinases, lead-
ing to the extracellular matrix remodeling. These cellular events
are involved in the development and progression of maladap-
tive cardiac remodeling and failure. In the present study, our
findings suggested that ROS generation was significantly
increased a smoking rat model. H₂S was found to reduce smok-
ing-induced oxidative stress, which might be an important
protective mechanism against left ventricular remodeling in
smoking rats.
Nrf2, a member of the NF-E2 family of nuclear basic leucine zipper transcription factors, regulates the gene expression of several antioxidative enzymes by binding to ARE (Nguyen et al., 2009). Under physiological conditions, Nrf2 locates in the cytoplasm and binds to Keap1 which mediates a rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome. During oxidative stress, Nrf2 is free from Keap1 and translocates into the nucleus to bind to ARE in the promoters of genes encoding antioxidant enzymes (Kaspar et al., 2009; McMahon et al., 2003). In the present study, our findings revealed that H2S could increase the binding activity of Nrf2-ARE and up-regulate the protein expression of antioxidant enzymes HO-1 and NQO1, FIG. 7. PI3K activity in myocardial tissue was measured by ELISA (A). Representative immunoblots and densitometric analysis of Akt phosphorylation in myocardium (B). *P < 0.05, versus Control; **P < 0.05, versus CS (n=5).

FIG. 8. PI3K activity in NRCMs was detected by ELISA (A). Western blot analysis of Akt phosphorylation in NRCMs (B). Nrf2 protein expression was increased in cells treated with CSC and NaHS and decreased in PI3K siRNA-transfected cells exposed to CSC and NaHS (C). PI3K siRNA-transfected cells pre-conditioned with NaHS prior to CSC exposure exhibited increased ROS production (D). NRCMs, neonatal rat cardiomyocytes; CSC, cigarette smoke condensate; ROS, reactive oxygen species. *P < 0.05, versus cells treated with normal culture medium; **P < 0.05, versus cells treated with CSC; #P < 0.05, versus cells treated with CSC and NaHS (n=5).

FIG. 9. Western blot analysis of GSK3β phosphorylation (A) and Fyn nuclear expression (B) in NRCMs. NaHS significantly increased phosphorylation levels of GSK3β and decreased nuclear localization of Fyn in NRCMs exposed to CSC. Akt siRNA-transfected NRCMs exposed to CSC and NaHS exhibited reduced GSK3β phosphorylation and elevated Fyn nuclear expression. NRCMs, neonatal rat cardiomyocytes; CSC, cigarette smoke condensate. *P < 0.05, versus cells treated with normal culture medium; **P < 0.05, versus cells treated with CSC; #P < 0.05, versus cells treated with CSC and NaHS (n=5).
which consequently enhanced resistance to oxidative stress in the myocardium of smoking rats.

The PI3K/Akt pathway is involved in the regulation of cell survival, growth, proliferation, migration, and metabolism (Vasudevan and Garraway, 2010). Activation of PI3K results in the synthesis of PIP3 which activates the protein kinase Akt. Phosphorylated Akt regulates cellular processes by phosphorylation of a number of substrates, including PI3 kinase, Bad, caspase-9, and forkhead transcription factors (Manning and Cantley, 2007). In the present study, our findings indicated that H2S could significantly activate PI3K/Akt signaling pathway in the myocardium of smoking rats.

NRCMs transfected with PI3K-specific siRNA were used to determine whether H2S activates Nrf2 signaling via PI3K/Akt-dependent pathway. Our findings showed that inhibition of PI3K/Akt signaling could reduce Nrf2 nuclear accumulation and increase ROS generation in NRCMs treated with NaHS prior to PI3K/Akt-independent pathway. Our findings showed that inhibition of Akt and decrease the nuclear expression of Fyn, which consequently contributes to the nuclear accumulation of Nrf2.

In conclusion, our study demonstrates that H2S protects against left ventricular remodeling in smoking rats via attenuation of oxidative stress. The antioxidant effects of H2S on cardiac remodeling are possibly dependent on PI3K/Akt activation with subsequent Nrf2 nuclear translocation and increased expression of HO-1 and NQO1.

FUNDING
National Natural Science Foundation of China (No. 81400292) and the University Scientific Research Foundation of Jiangsu Province (No. 14KJB320018).

REFERENCES