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Hydrogen sulfide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing GSK-3β-dependent opening of mPTP

Ling-Ling Yao,†* Xiao-Wei Huang,†* Yong-Gang Wang,‡* Yin-Xiang Cao,† Cai-Cai Zhang,† and Yi-Chun Zhu†

†Department of Physiology and Pathophysiology and Shanghai Key Laboratory of Vascular Biology, Fudan University Shanghai Medical College, and ‡Neurology Department of Shanghai Tenth People Hospital affiliated to Tongji University, Shanghai, China

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Yao LL, Huang XW, Wang YG, Cao XY, Zhang CC, Zhu YC. Hydrogen sulfide protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by preventing GSK-3β-dependent opening of mPTP. Am J Physiol Heart Circ Physiol 298: H1310–H1319, 2010. First published February 12, 2010; doi:10.1152/ajpheart.00339.2009.—Hydrogen sulfide (H2S) is an endogenously generated gaseous transmitter, which has recently been suggested to regulate cardiovascular functions. The present study aims to clarify the mechanisms underlying the cardioprotective effects of H2S. Signaling elements were examined in cardiomyocytes cultured under hypoxia/reoxygenation conditions and in a rat model of ischemia-reperfusion. In cultured cardiomyocytes, sodium hydrosulfide (NaHS; 10, 30, and 50 μmol/l) showed concentration-dependent inhibitory effects on cardiomyocyte apoptosis induced by hypoxia/reoxygenation. These effects were associated with an increase in phosphorylation of glycogen synthase kinase-3β (GSK-3β) (Ser9) and a decrease in Bax translocation, caspase-3 activation, and mitochondrial permeability transition pore (mPTP) opening. Transfection of a phosphorylation-resistant mutant of GSK-3β at Ser9 attenuated the effects of NaHS in reducing cardiomyocyte apoptosis, Bax translocation, caspase-3 activation, and mPTP opening. In a rat model of ischemia-reperfusion, NaHS administration reduced myocardial infarct size and increased the phosphorylation of GSK-3β (Ser9) at a dose of 30 μmol/kg. In conclusion, the H2S donor prevents cardiomyocyte apoptosis by inducing phosphorylation of GSK-3β (Ser9) and subsequent inhibition of mPTP opening.

cardioprotection; ischemia; gastrotransmitter

HYDROGEN SULFIDE (H2S) IS an endogenously generated gaseous transmitter, which has recently been suggested to regulate cardiovascular functions (17). Exogenous administration of H2S [in the form of sodium hydrosulfide (NaHS), a water-soluble H2S donor] has been shown to decrease blood pressure in acute (24) and chronic (20) experimental rat models. In isolated, perfused rat hearts, administration of NaHS decreases the duration and severity of arrhythmia and increases cell viability following ischemia-reperfusion (IS/RE) (6). In isolated rat cardiomyocytes exposed to severe metabolic inhibition, administration of NaHS increases cell viability and the ratio of rod-shaped cells (18). Likewise, NaHS treatment reduces myocardial infarct size in rat models of IS/RE (22) and myocardial infarction (25). In spontaneously hypertensive rats, chronic treatment with NaHS significantly decreases medial thickening of intramyocardial coronary arterioles and interstitial fibrosis (20). However, the mechanisms for the observed cardioprotective effects of H2S remain to be clarified. In our laboratory’s previous studies, the proangiogenic effect of H2S is dependent on Akt phosphorylation (7).

Glycogen synthase kinase-3β (GSK-3β) has been shown to play a pivotal role in organ development and act as an intrinsic component of the Wnt/wingless pathway. GSK-3β is regulated via phosphorylation by Akt (21). It has been shown that phosphorylation of serine at position 9 inhibits the activities of GSK-3β in neurons. Activation of the phosphatidylinositol 3-kinase/Akt pathway results in phosphorylation of GSK-3β at the serine 9 position. This event results in an inhibition of its kinase activity and provides neural protection (13). In contrast, tyrosine phosphorylation of GSK-3β at the tyrosine 216 site increases its activity (11), and this is found to be related to cell apoptosis in the PC12 cells and SH-SYSY cells (5). Interestingly, GSK-3β has recently been shown to promote the opening of the mitochondrial permeability transition pore (mPTP). mPTP opening is known to play a crucial role in lethal reperfusion injury in isolated, perfused rat hearts (12). The inhibition of GSK-3β phosphorylation results in mPTP opening (10). These findings encouraged us to investigate the role of GSK-3β and mPTP opening in H2S-induced protection against cardiomyocyte apoptosis.

MATERIALS AND METHODS

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Experimental protocols were approved by the Ethics Committee for Experimental Research, Fudan University Shanghai Medical College.

The in vivo IS/RE model and determination of infarct size and the area at risk. Male Sprague-Dawley rats (196 rats with age of 8 wk) were randomly assigned to the following groups: 1, the sham-operated control group treated with vehicle (saline solution, n = 10); 2, the IS/RE group treated with vehicle (n = 20); 3–7, the IS/RE group treated with NaHS solution at doses of 1, 10, 30, 100, and 300 μmol/kg NaHS, respectively, 10 min before IS/RE (n = 20–28 in each group) (Fig. 1A). Rats were anesthetized with pentobarbital sodium (100 mg/kg ip) and intubated and ventilated with a rodent ventilator (Chendu Instrument Factory, Si Chuan, China). A parasternal incision was made by cutting the left third and fourth ribs and intercostal muscles with surgical scissors. Myocardial ischemia was
Fig. 1. Schematic illustrations of the treatment protocols. Top: protocols for the in vivo experiments. The Sprague-Dawley rats were pretreated with different doses of sodium hydrosulfide (NaHS) or vehicle before a 30-min occlusion of the left anterior descending coronary artery, which was followed by 2-h reperfusion (IS/RE). Sham-operated rats served as control. Bottom: protocols for the in vitro experiments. Cultured ventricular myocytes were pretreated with different doses of NaHS or vehicle and then subjected to 30 min of hypoxia and 2 h of reoxygenation. In the experiments in which transfection was applied, the cells were transfected with control vector or plasmids carrying dominant-negative (DN)-Akt, glycogen synthase kinase (GSK)-β-S9A, or GSK-β-R96A 48 h before the cells were subjected to hypoxia.

inducing by passing a 6–0 silk suture beneath the left anterior descending artery at a point 1–2 mm inferior to the left auricle. The suture was tightened over a piece of PE-20 tubing (Becton, Dickinson, Sparks, MD) for 30 min and then released for 2 h. The suture was then tightened again, and rats were intravenously injected with Evans blue (Sigma-Aldrich, St. Louis, MO). The hearts were immediately excised and cut into 2-mm-thick slices parallel to the atrioventricular groove and were immerged into 10% paraformaldehyde in PBS (pH 7.4) for 2 h. The heart sections stained with Evans blue were photographed for analysis of the area at risk, which was identified as the area not stained with a blue color of Evans blue in the heart sections. The minor modification of area at risk measurement in the present study is that the heart sections stained with Evans blue were photographed for determination of area at risk before they would be stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC; Amresco, Solon, OH). Since Evans blue staining could be covered up by TTC staining, distinct borderline of area at risk can only be identified before the heart sections would be stained with TTC. The slices were then incubated with TTC in phosphate buffer (pH 7.4) at 37°C for 20 min to identify the infarct area. TTC was catalyzed by dehydrogenase enzymes to formazan, which is a red pigment and stains viable myocardium with dark red. The infarct area that does not contain dehydrogenase enzymes is not able to convert TTC into formazan and thus remains pale in color (16). The area at risk and the infarct area were quantified using a computerized planimetry program (Image pro plus, Media Cybernetics). Infarct size was expressed as a ratio of the infarct area and the area at risk. These measurements were performed in a blinded fashion. Apoptotic cardiomyocytes in the border zone of the infarct area were detected by TdT-mediated dUTP nick-end labeling (TUNEL) staining.

TUNEL of the left ventricular tissue. To determine cardiomyocyte apoptosis in a quantitative manner, six rats in each group were studied in an independent experiment with TUNEL. After IS/RE, as shown in Fig. 1, the sutures placed around the coronary artery were tightened again, and rats were intravenously injected with Evans blue. The hearts were immediately excised and cut into 2-mm-thick slices parallel to the atrioventricular groove and were immerged in 10% paraformaldehyde in PBS (pH 7.4) for 2 h. The area at risk was identified as the area not stained with the blue color of Evans blue in the heart sections. After photographing, the heart sections were cut into 4- to 5-μm-thick slides, and five slides were obtained from each tissue block. Detection of apoptotic cardiomyocytes was performed with TUNEL staining using an apoptosis detection kit (Roche, Applied Science), according to the manufacturer’s instructions. To determine the number of nuclei, the slides were incubated with Hoechst 33342 (10 μg/ml; Invitrogen) for 2 min at 37°C in dark and rinsed with PBS (pH 7.4) three times, with 5 min for each rinse. The TUNEL signals were observed with a fluorescence microscopy (Leica TCS SP2, Wetzlar). In each of the three heart section, five slides with an interval of >10 μm were examined. In each slide, the TUNEL signals (red signals) were mainly distributed around the boarder zone of the area at risk. Most of the TUNEL signals were observed in the center of the ischemic area. In contrast, the TUNEL signals were hardly observed in the nonischemic region remote from the boarder zone of the area at risk. Twelve fields were randomly selected in the boarder zone of area at risk in each slide, and the cells positive with TUNEL staining and Hoechst staining (blue signals) were counted by an observer blinded with the experimental groups (magnifications ×10). Apoptosis was determined as a ratio of the number of TUNEL-positive cells to total nuclear number in each field. Apoptosis of each animal was determined as the average of the values obtained from 180 fields.

The in vitro hypoxia/reoxygenation model. Primary cultures of neonatal cardiomyocytes were prepared according to the methods previously described (23). To establish the hypoxia/reoxygenation model, the cells were cultured in DMEM/F-12 without glucose and serum. As shown in Fig. 1, the cells were exposed to hypoxia (95% N₂ + 5% CO₂) for 30 min, followed by reoxygenation for 2 h. The cells were pretreated with different concentrations of NaHS solution for 30 min before the hypoxia/reoxygenation procedure. The control group was cultured in DMEM/F-12 with low glucose (1,000 mg/l) and 2% serum under normal air condition and treated with vehicle.

Assessment of apoptotic cell death in cultured cardiomyocytes. Apoptotic death of cardiomyocytes was determined by TUNEL and DNA ladder analysis. TUNEL was performed with a cell apoptosis detection kit (Roche, Applied Science). Briefly, after the cells were fixed and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, 50 μl TUNEL reaction mixture were added on samples, and the cell slides were incubated in humidified atmosphere for 60 min at 37°C in the dark. The slides were rinsed with PBS (pH 7.4) three times with 5 min for each rinse. To detect the nuclei, the cells were incubated with Hoechst 33342 (2 μg/ml) for 2 min at room temperature in the dark, rinsed with PBS (pH 7.4) three times with 5 min for each rinse, and observed with a fluorescence microscopy. Cell apoptosis was determined as the ratio of the number of TUNEL-positive nuclei to that of Hoechst 33342-positive nuclei from six randomly selected fields (magnification ×200). DNA ladder formation was determined by agarose gel electrophoresis. In brief, cardiomyocytes were lysed in lysis buffer and electrophoresed on 1.5% agarose gel. After staining with ethidium bromide, DNA ladder formation was visualized and photographed under ultraviolet light. The DNA ladder bands with a molecular weight smaller than 2,000 bp were quantified using the Image ProPlus software (Media Cybernetics, Bethesda, MD).

Western blot analysis. Western blot analysis was performed as described previously (23). Briefly, protein was extracted from the cultured neonatal rat cardiomyocytes. Protein samples (50 μg) were separated by 12% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Gelman-Pall, Ann Arbor, MI), and blocked with 5%
nonfat milk. After blocking, the membranes were probed with the primary antibodies, GSK-3β, phospho-GSK-3β, AKT, and phospho-AKT (Cell Signaling Technology) and Bcl-2, caspase-3, Bax, survivin, and β-actin (Santa Cruz, CA), respectively. Chemiluminescent signals were generated by the addition of the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and detected on a radiographic film. The data of the immunoblots of phosphorylated GSK-3β and AKT were represented as a ratio of the phosphorylated forms to their total forms, respectively. The immunoblots of Bcl-2, caspase-3, Bax, and survivin were all corrected to the bands of β-actin.

Plasmids transfection. The plasmids carrying GSK-3β S9A/pCS2 and GSK-3β R96A/pCS2 were generously provided by Dr. Rao Y. (Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL) (14). GSK-3β-S9A is constitutively active, and GSK-3β-R96A lacks activity for primed substrate site (8, 9, 14). The hemagglutinin-tagged dominant-negative (DN) (kinase-inactive mutant Myr-Akt-K179M) Akt is a kind gift from Dr. Jin Q. Cheng.

Fig. 2. Hydrogen sulfide (H2S) reduces infarct size in a rat model of IS/RE and decreases the number of apoptotic cells in an in vitro model of hypoxia/reoxygenation (H/R). Representative photographs (A) and bar graphs (B) show the effect of NaHS in reducing infarct size in a rat model of IS/RE. Infarct size was expressed as a ratio of the infarct area and the area at risk. The area at risk was identified as the area not stained with Evans blue, which was photographed for analysis immediately before the heart sections would be stained with TTC. The viable myocardium was stained with dark red by TTC, and the infarct area remained pale. Values are means ± SE; n = 20–28 in each group. Representative photographs (C) and bar graphs (D) showing treatment with NaHS reduced the number of apoptotic cells in the myocardium of the IS/RE, as detected with TdT-mediated dUTP nick-end labeling (TUNEL) staining. Representative photographs (E) and bar graphs (F) show the antiapoptotic effects of NaHS or H2S solution in rat cardiomyocytes cultured under H/R conditions, as detected with TUNEL staining. Apoptotic cell number (%) in D and F was represented as a ratio of the number of apoptotic cells to the total cell number. Values are means ± SE; n = 6 in each group. Bar = 400 μm in C [for higher magnifications of the areas marked in squares (arrow), bar = 40 μm]. Bar = 20 μm in E. *P < 0.05.
HYDROGEN SULFIDE PROTECTS CARDIOMYOCYTES FROM APOPTOSIS

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Material and Methods (Department of Pathology and Interdisciplinary Oncology, University of South Florida College of Medicine, H. Lee Moffitt Cancer Center, Tampa, FL). Plasmid transfection was performed as described previously (23). Cardiomyocytes were cultured in DMEM/F-12 medium containing 10% fetal bovine serum and antibiotics, and then transfected using Lipofectamine 2000 following the manufacturer’s instruction (Invitrogen). Lipofectamine 2000 was first diluted in OPTI-MEM I (Gibco BRL) and then mixed with plasmid DNA. The ratio (wt/wt) between Lipofectamine 2000 and DNA was 4:1. Following a 20-min incubation period at room temperature, the DNA-Lipofectamine 2000 mixture was added to the antibiotic-free transfection medium (DMEM containing glutamine and 10% FBS), and the culture medium was replaced with this transfection solution. Cells were then maintained in a 37°C incubator with 5% CO2 for 24 h. Then the medium was replaced with serum-free medium, and the cells were analyzed. To measure the transfection efficiency, GFP was cotransfected with GSK-3β S9A/pCS2 and GSK-3β R96A/pCS2. Twenty-four hours after incubation, cells were observed under microscope, and the transfection efficiency was GFP-positive cell to total cell number. The transfection efficiency of hemagglutinin-tagged DN Akt was measured by compared the Western blot band density in transfected groups to untransfected groups.

Assessment of mitochondrial membrane depolarization. Mitochondrial membrane depolarization was assessed by using the MitoCapture Mitochondrial Apoptosis Detection kit, according to the protocols provided by the manufacturer (BioVision, Tokyo, Japan). MitoCapture is a cationic dye that aggregates in the mitochondria and gives off a bright red fluorescence in healthy cells. In apoptotic cells, MitoCapture fails to aggregate in the mitochondria as a result of altered mitochondrial transmembrane potential and remains in the cytoplasm in its monomer form, which exhibits green fluorescence. Briefly, the cells were incubated in 1-ml incubation buffer containing 1 μl MitoCapture for 15 min at 37°C in an incubator containing 5% CO2. After staining, the fluorescent signals were measured by a TECAN multifunction microplate reader (SPG Media, London, UK). The red fluorescent signals were excited at 530 nm and detected at 630 nm, and the green fluorescence was excited at 488 nm and detected at 530 nm. Cardiomyocyte mitochondrial damage was assessed by examining mitochondrial membrane depolarization, which was indicated by the ratio of red and green signals in the micrographs.

Ca2⁺-induced mPTP opening. Isolation of mitochondria was performed using the MitoCapture Fractionation Kit (Beijingate Inst. Biotech, Peking, PR China). mPTP opening was assessed following in vitro Ca2⁺ overload, as described by Argaud et al. (2). Isolated mitochondria (6 mg proteins) were suspended in 100-μl solution buffer (70 mM sucrose, 210 mM mannitol, 0.1 mM EDTA in 50 mM Tris-HCl, pH 7.4) and were added to a test tube containing 900 μl of measuring buffer (150 mM sucrose, 50 mM KCl, 2 mM KH₂PO₄, 5 mM succinic acid to 20 mM Tris-HCl, pH 7.4). Calcium concentrations were continuously recorded with Ca²⁺-selective microelectrode (Microelectrodes, Bedford, NH) inserted into the extramitochondrial medium in conjunction with a reference electrode. The signals were processed and analyzed with the MFileb200 software (Department of Physiology and Pathophysiology, Fu Dan University Shanghai Medical College). At the end of the preincubation period, 20 μM CaCl₂ were added to the extramitochondrial medium every 30 s. As depicted in Fig. 6C, each CaCl₂ administration evoked a peak of Ca²⁺ release. Calcium was then very rapidly taken up by the mitochondria, resulting in a return of the extramitochondrial Ca²⁺ concentration to near baseline level. Repeated Ca²⁺ loading always caused a sudden increase in a massive release of Ca²⁺ from mitochondria due to mPTP opening (indicated as arrow in Fig. 6C). The cumulative amount of loaded Ca²⁺ required to trigger mPTP opening inversely represented the susceptibility of mPTP opening (2). In experiments shown in Fig. 6C, E, mitochondria were isolated from the cardiomyocytes cultured in various conditions, according to the group protocols. Whereas, in experiments shown in Fig. 6F, NaHS/vehicle was directly applied to the mitochondria isolated from the cardiomyocytes cultured in conditions without any treatment or vector transfection.

Statistical analysis. Quantitative data are presented as means ± SE. Comparison between the groups was performed by one-way ANOVA, followed by Tukey test. In all cases, a P value of <0.05 was taken to indicate statistical significance.

RESULTS

H₂S reduces infarct size in vivo and hypoxia-induced cell death in vitro. Sprague-Dawley rats were subjected to IS/RE, with or without treatment of NaHS, at doses of 1, 10, 30, 100,
and 300 μmol/kg. As shown in Fig. 2, A and B, infarct size was significantly reduced in the rats treated with NaHS at a dose of 30 μmol/kg (P < 0.05; n = 20–28 in each group). The number of apoptotic cells was significantly increased in IS/RE rats compared with that of sham-operated control. NaHS treatment (30 μmol/kg) decreased the number of apoptotic cells in the IS/RE rats (Fig. 2, C and D; P < 0.05; n = 6 in each group). As shown in Fig. 2, E and F, cultured cardiomyocytes were subjected to 30-min hypoxia (95% N₂/5% CO₂), followed by a 2-h reoxygenation (air containing 5% CO₂) period. Cardiomyocyte apoptosis was assessed by TUNEL staining. Hypoxia/reoxygenation-induced apoptosis of cardiomyocytes was significantly decreased in the presence of NaHS at concentrations of 10, 30, and 50 μmol/l and in the presence of H₂S solution at a concentration of 15 μmol/l (P < 0.05, n = 6 in each group).

Fig. 4. H₂S protects cardiomyocytes from H/R-induced apoptosis by phosphorylation of GSK-3β (Ser9). The antiapoptotic effects of H₂S were examined with or without transfection of vectors carrying GSK-3β-S9A (S9A) or GSK-3β-R96A (R96A). Representative photographs (A) and bar graphs (B) of TUNEL staining from 6 experiments show an attenuation of the antiapoptotic effects of NaHS (30 μmol/l) with or without transfection of vectors carrying GSK-3β-S9A. Transfection of vectors carrying GSK-3β-R96A also decreased apoptosis in the cardiomyocytes subjected to H/R. C: representative electrophoretic analysis of nucleosomal DNA fragmentation from 6 experiments, showing an attenuation of the antiapoptotic effects of NaHS (30 μmol/l) with or without transfection of vectors carrying GSK-3β-S9A. D: bar graphs show band density of the DNA ladder. GFP, green fluorescent protein. Values are means ± SE. Bar = 20 μm in A. *P < 0.05.
H₂S time dependently increases the phosphorylation of GSK-3β (Ser9) by inducing the phosphorylation of Akt. GSK-3β and Akt, as well as their phosphorylated forms, were examined by Western blot analysis in cardiomyocytes at several time points during the hypoxia/reoxygenation procedure. Preconditioning with NaHS caused a significant increase in the phosphorylation of both GSK-3β (Ser9) and Akt at the time points of 30, 60, and 120 min during the reoxygenation period (Fig. 3; P < 0.05). The NaHS-induced increase in the phosphorylation of GSK-3β (Ser9) and Akt was blocked by transfection with vectors carrying the DN mutant of Akt (Fig. 3; P < 0.05). Transfection efficiency for DN-AKT was 35.9%.

H₂S protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis by phosphorylation of GSK-3β at Ser9. Cardiomyocytes were transfected with the vectors carrying GSK-3β-S9A. This form is not able to be phosphorylated at the S9 site and, therefore, is constitutively active. In addition, cells were also transfected with the vectors carrying GSK-3β-R96A, which lacks activity. TUNEL staining showed that hypoxia/reoxygenation resulted in a significant increase in the number of apoptotic cardiomyocyte that could be inhibited by NaHS (30 μmol/l; Fig. 4, A and B; P < 0.05). In contrast, the antiapoptotic effect of NaHS was attenuated in cardiomyocytes transfected with the vectors carrying GSK-3β-S9A, a mutant of GSK-3β that is not able to be phosphorylated at the S9 site (P < 0.05). This observation suggests that the effect of NaHS was mediated by phosphorylation of GSK-3β (Ser9). Moreover, transfection of vectors carrying GSK-3β-R96A also decreased apoptosis in the cardiomyocytes subjected to hypoxia/reoxygenation, suggesting a role of nonphosphorylated GSK-3β (Ser9) in cardiomyocyte apoptosis (Fig. 4, A and B; P < 0.05).

Hypoxia/reoxygenation caused a significant increase in DNA ladder formation, and this effect was inhibited in the presence of NaHS (30 μmol/l), whereas the effect of NaHS was attenuated by transfection of vectors carrying GSK-3β-R96A (Fig. 4, C and D). Transfection efficiency for GSK-3β-S9A/pCS2 was ~31.7% and for GSK-3β-R96A/pCS2 was ~35.1%.

H₂S-induced inhibition of Bax translocation and caspase-3 activation is dependent on phosphorylation of GSK-3β (Ser9). Hypoxia/reoxygenation caused an increase in the translocation

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**Fig. 5.** The effects of H₂S on Bax translocation and caspase-3 activation are dependent on the phosphorylation status of GSK-3β (Ser9). The effects of NaHS (30 μmol/l) on Bax translocation, caspase-3 activation, and expression of Bcl-2 and survivin were examined by Western blot analysis in the cardiomyocytes, with or without transfection of vectors carrying GSK-3β-S9A or GSK-3β-R96A. A: representative blots showing Bax translocation, caspase-3 activation, and expression of Bcl-2 and survivin. B–G: the values were enumerated by densitometry of the bands of cytosolic and mitochondrial fraction of Bax (B), 17-kDa fraction of caspase-3 (C), 12-kDa caspase-3 (D), caspase-3 precursor (E), Bcl-2 (F), and survivin (G). Values are means ± SE; n = 5 in each group. The values were enumerated by densitometry of the bands corrected to that of β-actin. *P < 0.05.

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of Bax to mitochondria and the subsequent activation of caspase-3 in cardiomyocytes (Fig. 5, A–C; P < 0.05). Both Bax translocation and caspase-3 activation were inhibited by treatments with NaHS (30 μmol/l; P < 0.05). Importantly, the effects of NaHS preconditioning could be attenuated by transfection of vectors carrying GSK-3β-S9A (P < 0.05), suggesting that NaHS inhibited Bax translocation and caspase-3 activation via the phosphorylation of GSK-3β at the serine 9 site. Moreover, transfection of the vectors carrying GSK-3β-R96A also caused a decrease in Bax translocation and caspase-3 activation (Fig. 5, A–E; P < 0.05). For the expression of Bcl-2 and survivin, NaHS treatment showed no effect, whereas the expression of Bcl-2 and survivin was decreased in the cardiomyocytes transfected with the vectors carrying GSK-3β-S9A, either in the presence or in the absence of NaHS (P < 0.05), suggesting that the expression of the constitutive active mutant of GSK-3β downregulated the expression of Bcl-2 and survivin. In contrast, transfection of the vectors carrying GSK-3β-R96A increased the expression of Bcl-2 and survivin (Fig. 5, F and G; P < 0.05). Transfection efficiency for GSK-3β-S9A/pCS2 was ~33.5% and for GSK-3β-R96A/pCS2 was ~30.3%.

H₂S protects against mitochondrial damage and is dependent on the phosphorylation of GSK-3β (Ser9). Cardiomyocyte mitochondrial damage was assessed by examining mitochondrial membrane depolarization. The mitocapture dye accumulates in mitochondria when the mitochondrial membranes are intact, emitting a red signal (top, arrows). If the mitochondrial membranes are damaged, the mitocapture dye diffuses into the cytoplasm and emits a green signal (bottom, arrowheads). As shown in Fig. 6A, hypoxia/reoxygenation caused significant mitochondrial damage. NaHS treatment (30 μmol/l) inhibited such damage to mitochondria. The effects of NaHS were blunted by transfection of the vectors carrying GSK-3β-S9A. Transfection efficiency for GSK-3β-S9A/pCS2 was ~30.9%.

H₂S inhibits Ca²⁺-induced mPTP opening. In the control group, the cumulative amount of Ca²⁺ required to open the mPTP averaged 220 ± 20 μmol/l (Fig. 6D). Total Ca²⁺ loading required to open the mPTPs was significantly reduced in the hypoxia/reoxygenation group, averaging 95 ± 12 μmol/l, suggesting that mPTP is more likely to open under the hypoxia/reoxygenation condition (P < 0.05). In the NaHS (30 μmol/l) inhibited such damage to mitochondria. The effects of NaHS were blunted by transfection of the vectors carrying GSK-3β-S9A. Transfection efficiency for GSK-3β-S9A/pCS2 was ~30.9%.

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pretreated group, the total Ca\(^{2+}\) loading required to induce mPTP opening was significantly increased, averaging 155 ± 20 μmol/l, suggesting that NaHS inhibited the opening of mPTP (P < 0.05). Moreover, the effect of NaHS was reduced by transfection with the vectors carrying GSK-3β-S9A, suggesting that the effect of NaHS was dependent on the phosphorylation of GSK-3β at the serine 9 site (P < 0.05) (Fig. 6, C, D, and E). In an independent set of experiments, we examined the direct effect of NaHS on calcium-induced mPTP opening using mitochondrial isolated from the cardiomyocytes. The results showed that direct administration of NaHS at concentrations of 1, 10, 30, 50, 100 μmol/l to the mitochondrial suspension had no effect on calcium-induced mPTP opening (Fig. 6 F), suggesting that NaHS does not directly act on the mPTPs at these concentrations.

H\(_2\)S increases the levels of p-GSK-3β (Ser9), surviving, and caspase-3 precursor and decreases the levels of Bax in the myocardium of rats undergoing IS/RE. In the myocardium of the IS/RE rats, the phosphorylation of GSK-3β at serine 9 was significantly increased with treatment of NaHS at a dose of 30 μmol/kg (Fig. 7, A and B). The levels of survivin were increased with treatment of NaHS at doses of 30 and 100 μmol/kg (Fig. 7, A and C), while caspase-3 activation was decreased at NaHS doses of 10, 30, and 100 μmol/kg (Fig. 7, A and F). In contrast, the levels of Bax were decreased with treatment of NaHS at a dose of 30 μmol/kg (Fig. 7, A and D), whereas Bcl-2 levels decreased with treatment of NaHS at a dose of 30 μmol/kg (Fig. 7, A and E).

**DISCUSSION**

In the present study, we investigated the role of GSK-3β in mediating the antiapoptotic role of the H\(_2\)S donor NaHS in cultured cardiomyocytes of rats. We showed that phosphorylation of GSK-3β (Ser9) position was significantly increased in cultured cardiomyocytes treated with the H\(_2\)S donor. Transfection of the vectors carrying GSK-3β-S9A attenuated the antiapoptotic effects of H\(_2\)S. Juhaszova et al. (15) reports that the phosphorylation of GSK-3β at the serine 9 site promotes the inhibition of GSK-3β activity and promotes cellular protection by preventing signaling cascades associated with the induction of the mitochondrial permeability transition. In this context, our data suggest that phosphorylation of GSK-3β (Ser9) mediates the antiapoptotic effects of H\(_2\)S in cultured cardiomyocytes.

**Fig. 7.** Western blot analysis of GSK-3β, survivin, Bax expression, and caspase-3 precursor in the myocardium tissues in a rat model of IS/RE. A: representative blots showing p-GSK-3β (Ser9), GSK-3β, survivin, Bax, and caspase-3 precursor in the myocardium of IS/RE rats, with or without treatment of NaHS at doses of 10, 30, and 100 μmol/kg. B–F: the values of the bands were enumerated by densitometry for p-GSK-3β (Ser9) (B), survivin (C), Bax (D), Bcl-2 (E), and caspase-3 precursor (F). Values are means ± SE; n = 5 in each group. *P < 0.05.

**Fig. 8.** Schematic illustration of the mechanisms of H\(_2\)S-induced protection against cardiomyocyte apoptosis.
We also found that the H$_2$S inhibited the opening of mitochondrial mPTP in intact cardiomyocytes at a concentration of 30 µmol/l, and this effect was blunted by transfection of the vectors carrying GSK-3β-S9A. Halestrap et al. (12) have previously reported that the opening of the mPTP plays a pivotal role in the induction of apoptosis in cardiomyocytes. We speculate that H$_2$S-mediated inhibition of the mPTP is regulated via the phosphorylation of GSK-3β (Ser9) in intact cardiomyocytes. In contrast, the H$_2$S donor (at concentrations ranging from 1 to 100 µmol/l) was not effective at inhibiting mPTP opening in isolated mitochondria, in which intracellular signaling elements such as GSK-3β are not present. These data suggest that the inhibitory effects of H$_2$S on mPTP opening are mediated by signaling elements such as GSK-3β, which is only present in the intact cardiomyocytes (Fig. 8), and H$_2$S does not directly act on mitochondrial mPTPs at these concentrations.

Our laboratory has recently reported that Akt mediates the proangiogenic effect of H$_2$S in vascular endothelial cells (7). In the present study, Akt phosphorylation was significantly increased in response to H$_2$S treatment in cardiomyocytes. Moreover, overexpression of the DN mutant of Akt blocked H$_2$S-induced phosphorylation of GSK-3β (Ser9), suggesting that Akt was the upstream regulator of GSK-3β in cardiomyocytes (Fig. 8). In addition, H$_2$S treatments were also found to reduce Bax translocation (into the mitochondria) and caspase-3 activation, and these observations could be attenuated by blocking the phosphorylation of GSK-3β (Ser9). These data suggest that caspase-3 and Bax are the downstream effectors of GSK-3β in H$_2$S-induced cardioprotection (Fig. 8). Mitochondrial damage is a central feature of the intrinsic apoptotic pathway (4). Bax translocation to mitochondria contributes to the disruption of mitochondrial membrane potential and to the release of apoptotic proteins from the mitochondrial intermembrane space into the cytoplasm (1, 3). Our present data suggest that H$_2$S negatively regulates the apoptotic pathways by acting on the Akt/GSK-3β/Bax/caspase-3 pathway. Moreover, our data suggest that GSK-3β is the central element that mediates the protective signals of H$_2$S. Interestingly, H$_2$S was not able to directly regulate Bax and caspase-3 without inducing phosphorylation of GSK-3β (Ser9) (Fig. 8).

To further clarify whether H$_2$S also provides protection against cardiomyocyte apoptosis under in vivo conditions, the effect of H$_2$S was also examined in a rat model of cardiac IS/RE. We found that H$_2$S caused a reduction in cardiomyocyte apoptosis and myocardial infarct size in a rat model of cardiac IS/RE. In line with our results, exogenous administration of H$_2$S has been reported to reduce myocardial infarct size in rat models of IS/RE (22) and myocardial infarction (25). We further showed that H$_2$S-induced cardioprotection was associated with an increase in phosphorylation of GSK-3β (Ser9) and survivin expression, as well as a decrease in Bax expression. These data suggest that H$_2$S-induced cardioprotection in the in vivo model of IS/RE may be associated with the GSK-3β/Bax/caspase-3 pathway. These findings are in line with our in vitro model of hypoxia/reoxygenation in cultured cardiomyocytes. The exact role of GSK-3β, Bax, and caspase-3 in H$_2$S-induced cardioprotection in this rat model of IS/RE remains to be further clarified.

Our laboratory has recently demonstrated that survivin mediates the antiapoptotic effect of δ-opioid receptor stimulation in the cardiomyocytes (23). In the present study, H$_2$S treatment increased survivin levels in ischemic-reperfused rat myocardium, but not in cultured cardiomyocytes challenged with hypoxia/reoxygenation. In addition, H$_2$S-induced cardioprotection may also be mediated by different signaling pathways in neonatal cardiomyocytes and in adult myocardium. Also, we report that H$_2$S treatment did not exert any effects on Bcl-2 levels in the in vitro experiments, yet caused a decrease in cardiac Bcl-2 levels in the in vivo system. These differences require further studies. In the present study, the in vitro experiments were performed in neonatal cardiomyocytes. The gene expression pattern of neonatal cardiomyocytes is somewhat different from that of the adult cells (19). Obviously, cardiac ischemia occurs in adult hearts and is more clinically relevant. Use of neonatal cardiomyocytes is indeed a limitation of the present study. Also, the differences observed between the in vivo and in vitro effects of H$_2$S on the expression of survivin and Bcl-2 may be ascribed to the difference in the ages of the rats used in the in vivo and in vitro experiments.

It is worth noting that the doses/concentrations to provide effective protection were 30 µmol/kg and 30 µmol/l in the in vivo and in vitro experiments, respectively, while higher and lower doses/concentrations were not more effective. We found a similar dose-dependent effect in the proangiogenic effect of H$_2$S in mice at a concentration of 50 µmol/kg, while a higher dose of 200 µmol/kg is not effective (7). The mechanisms underlying this pattern of dose dependency for H$_2$S treatment is unknown.

In conclusion, H$_2$S is a novel antiapoptotic factor. The induction of the phosphorylation of GSK-3β (Ser9) by H$_2$S is a novel protective signaling pathway that functions in preventing hypoxia/reoxygenation-induced cardiomyocyte apoptosis. Akt is the upstream regulator of GSK-3β. The antiapoptotic effects of H$_2$S are also associated with an inhibition on mPTP opening, on Bax translocation, and on caspase-3 activation, whereas mPTP, Bax, and caspase-3 are the downstream effectors of GSK-3β (Fig. 8).

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES

5. Bhat RV, Stanley J, Correll MP, Fieles WE, Keith RA, Scott CW, Lee CM. Regulation and localization of tyrosine 216 phosphorylation of...


