Exogenous hydrogen sulfide protects against high glucose-induced inflammation and cytotoxicity in H9c2 cardiac cells

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Abstract. Hyperglycemia serves an important role in the pathogenesis of diabetic cardiomyopathy. The aim of the present study was to investigate whether exogenous hydrogen sulfide (H\textsubscript{2}S) protects against high glucose-induced inflammation and cytotoxicity in cardiac cells by inhibiting the p38 mitogen-activated protein kinase (MAPK)/nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) signaling pathways. Rat H9c2 myocardium cells were exposed to 33 mM glucose (high glucose, HG) for 24 h to stimulate HG-induced cytotoxicity. One group of cells was pretreated with NaHS (a donor of H\textsubscript{2}S) prior to HG exposure, and cell viability was determined using the Cell Counting Kit-8 assay. The protein expression levels of p38MAPK, the phosphorylated p65 subunit of NF-κB, iNOS, COX-2 and caspase-3 were analyzed by western blotting, and the protein expression levels of interleukin (IL)-1β and IL-6 were detected by enzyme-linked immunosorbent assay (ELISA). Pretreatment of H9c2 cells with NaHS for 30 min prior to exposure to HG significantly ameliorated the expression of p38MAPK/NF-κB and NF-κB-mediated cytotoxicity and inflammation, as evidenced by the significant increase in cell viability and decrease in iNOS, COX-2, IL-1β and IL-6 expression levels. Furthermore, treatment of cells with NaHS significantly decreased the expression of caspase-3, which suggested that NaHS attenuated HG-induced apoptosis. In conclusion, the results of the present study provided evidence to suggest that exogenous H\textsubscript{2}S protects against HG-induced cytotoxicity and inflammation in H9c2 cardiac cells. H\textsubscript{2}S may exert these cytoprotective effects via inhibition of the p38MAPK/NF-κB, COX-2 and iNOS signaling pathways.

Introduction

Over the last 30 years, the number of patients with diabetes has increased >2-fold, which renders it a major threat to human health (1). Among the complications associated with diabetes, diabetic cardiomyopathy (DCM) is characterized by structural and functional alterations in the myocardium, and is one of the leading causes of morbidity and mortality in patients with diabetes worldwide (2,3). Hyperglycemia-induced cardiac inflammation and cytotoxicity are the major pathological causes of DCM. In the hearts of leptin receptor-deficient db/db and streptozotocin (STZ)-induced diabetic mice, hyperglycemia was demonstrated to induce cytotoxicity of cardiac myocytes (4).

Recently, an increasing number of studies have focused on elucidating the signal transduction pathways associated with high glucose (HG)-induced inflammation and cytotoxicity in cardiac tissues (2,5,6). Hyperglycemia may activate the p38 mitogen-activated protein kinase (MAPK) pathway (7), which serves a critical role in the activation of nuclear factor-κB (NF-κB). NF-κB is an essential transcription factor that regulates the expression of proinflammatory genes, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin (IL)-1β and IL-6 (8). In addition, activation of NF-κB may upregulate the transcription of specific genes involved in the inflammatory response (9). iNOS mediates the nitrosylation of caspase-3, which may lead to cardiomyocyte cell death (10). COX-2 has been demonstrated to induce cardiomyocyte apoptosis in several mouse models of cardiomyopathy (11,12). Soetikno et al (13) demonstrated that curcumin prevents DCM in STZ-induced diabetic rats by inhibiting the activation of the p38MAPK/NF-κB signaling pathway. Therefore, molecules that function to inhibit p38MAPK/NF-κB, COX-2 and iNOS activation may protect against HG-induced cardiomyocyte injury.

Hydrogen sulfide (H\textsubscript{2}S) is synthesized from cysteine by cystathionine gamma lyase, as well as additional naturally occurring enzymes. Along with nitric oxide and carbon monoxide, H\textsubscript{2}S forms part of a group of biologically active gases termed gasotransmitters or gasomediators (14-16). An increasing number of previous studies have demonstrated that H\textsubscript{2}S is an important cardioprotective agent (17,18). One such
study indicated that exogenous H$_2$S exhibits cardioprotective effects by inhibiting oxidative stress and enhancing heat shock protein 90 expression levels (17). Guo et al (19) reported that H$_2$S may protect against doxorubicin-induced inflammation and cytotoxicity in cardiomyocytes by inhibiting the p38MAPK/NF-κB signaling pathway. In diabetic rats and in vitro models, H$_2$S was reported to protect cardiomyocytes from inflammation and cell death (20-22). However, the mechanisms underlying these protective effects of H$_2$S in diabetic cardiomyocytes remain unclear. Therefore, the aim of the present study was to investigate the cardioprotective effects of H$_2$S against HG-induced injury in cardiomyocytes, and to determine whether this may involve the p38MAPK/NF-κB, COX-2 and iNOS signaling pathways.

Materials and methods

Reagents. The sodium H$_2$S donor (NaHS), the selective inhibitor of NF-κB (pyridoline dithiocarbamate, PDTC), and glucose were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). The Cell Counting Kit-8 (CCK-8) assay was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Primary antibodies specific to the phosphorylated (p)-p65 as a measure of NF-κB protein expression levels, COX-2, caspase-3, and p38 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. nos. 3033, 12282, 9665 and 4511, respectively). The horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. KC-RB-035), and the enzyme linked immunosorbent assay (ELISA) kits for assessing IL-1β and IL-6 expression levels were purchased from Zhijie Kangchen Biotech Co., Inc. (Hangzhou, China). The primary antibody against iNOS (cat no. sc650) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The primary glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (cat. no. HJTW0125) was purchased from Guangzhou Jettay Biotech Co., Ltd. (Guangzhou, China). The interleukin-1 receptor antagonist (IL-1Ra) was purchased from Prospec-Tany TechnoGene, Ltd. (East Brunswick, NJ, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The radioimmunoprecipitation assay (RIPA) buffer was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Low-glucose Dulbecco's modified Eagle's medium-Ham's F12 (DMEM-F12), phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were purchased from Gibco; Thermo Fisher Scientific, Inc. H9c2 cells were obtained from the Sun Yat-sen University Experimental Animal Center (Guangzhou, China).

Cell culture and treatments. H9c2 cardiac cells were cultured in DMEM-F12 medium supplemented with 10% FBS at 37°C and 5% CO$_2$. In order to investigate the cytotoxic effects of HG, H9c2 cells were treated with 11, 22, 33 or 44 mM glucose for 24 h. To investigate the cardioprotective effects of H$_2$S on HG-induced injury, the cells were pretreated with 400 µM NaHS for 30 min prior to HG treatment. To further determine whether the anti-inflammatory effects of H$_2$S were associated with inhibition of p38MAPK/NF-κB pathway, H9c2 cells were pretreated with 3 µM SB203580 (a selective inhibitor of p38MAPK) for 60 min or 100 µM PDTC (a selective inhibitor of NF-κB) for 30 min prior to HG treatment, or were co-treated with HG plus 20 ng/ml IL-1Ra (a selective antagonist of the IL-1β receptor) for 24 h.

Cell viability assay. H9c2 cells were cultured in 96-well plates (1x10$^5$ cells/well) and were divided into the following groups: HG, HG+NaHS, HG+SB203580, HG+PDTC, HG+IL-Ra, NaHS, SB203580, PDTC or IL-1Ra before 10 µl CCK-8 solution was added to each well at a dilution of 1:10, and the cells were incubated for 2 h. The absorbance was measured at 450 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc.). The mean optical density (OD) of five wells for each treatment group was used to calculate the percentage cell viability relative to untreated control cells, according to the following formula: Cell viability (%) = (OD$_{treatment}$/OD$_{control}$) x100. The experiments were performed in triplicate.

Detection of IL-1β and IL-6 production in the culture media using ELISA. H9c2 cells were plated in 60 mm dishes at a density of 1x10$^6$ cells/well. Cells were divided into the following treatment groups: HG, HG+NaHS, HG+SB203580, HG+PDTC, HG+IL-Ra, NaHS, SB203580, PDTC or IL-1Ra. The level of IL-1β and IL-6 in the culture media was determined using ELISA, according to the manufacturer's instructions. The absorbance was read at 450 nm, and experiments were performed >5 times.

Extraction of cytoplasmic and nuclear proteins. H9c2 cells were plated in 60 mm dishes at a density of 1x10$^6$ cells/well and divided into the following treatment groups: HG, HG+NaHS, HG+SB203580, HG+PDTC, HG+IL-Ra, NaHS, SB203580, PDTC or IL-1Ra. Cytoplasmic and nuclear proteins from H9c2 cells were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagent kit, according to the manufacturer's instructions (Thermo Fisher Scientific, Inc.). Briefly, after washing three times with cold PBS, the cells were treated with cytoplasmic protein extraction buffer to isolate cytoplasmic protein fraction proteins. The nuclear proteins were subsequently extracted by adding the nuclear protein extraction buffer. Cytoplasmic and nuclear protein extracts were subject to western blot analysis.

Western blot analysis. H9c2 cells were seeded in 60-mm dishes at a density of 1x10$^6$ cells/well and divided into the following treatment groups: HG, HG+NaHS, HG+SB203580, HG+PDTC, HG+IL-Ra, NaHS, SB203580, PDTC or IL-1Ra. Cells were washed in PBS and lysed in RIPA buffer for 30 min, and the homogenate was centrifuged at 21,380 x g for 10 min at 4°C. The total protein concentration of the sample supernatant was determined using a BCA protein assay kit. Total protein (30 µg) was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred onto a polyvinylidene difluoride membrane and the membrane was blocked with 5% non-fat milk diluted in Tris-buffered saline-0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were subsequently incubated with primary antibodies specific to phosphorylated...
(p)-p65 (dilution, 1:500), iNOS (dilution, 1:1,000), COX-2 (dilution, 1:1,000), caspase-3 (dilution, 1:1,000), p-p38 (dilution, 1:1,000) or GAPDH (dilution, 1:10,000) with gentle agitation at 4°C overnight. The following day, the membranes were incubated with secondary antibodies (dilution, 1:5,000) for 1.5 h at room temperature. Following three washes with TBS-T, the membranes were developed using enhanced chemiluminescence and exposed to X-ray films. To quantify protein expression levels, the X-ray films were visualized and analyzed using ImageJ software (version, 1.41; National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. The data are presented as the mean ± standard error. Differences among treatment groups were analyzed using one-way analysis of variance with the least significant difference test. Statistical analyses were performed using the SPSS software program (version, 13.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

HG decreased cell viability in a concentration-dependent and time-dependent manner. In order to investigate the cytotoxic effects of HG, H9c2 cells were treated with 11, 22, 33 or 44 mM glucose for 24 h. As shown in Fig. 1, a significant reduction in cell viability was observed following treatment with 33 mM HG when compared with untreated controls (58.10±2.24% reduction; P<0.01). Based on these results, together with those of a previous study (21), treatment of cells with 33 mM HG for 24 h was selected to model the effects of hyperglycemia on cardiac cells in vitro.

Exogenous H₂S attenuates HG-induced cytotoxicity in H9c2 cells. As shown in Fig. 2A, exposure of H9c2 cells to HG for 24 h induced cytotoxicity, as evidenced by the significant decrease in cell viability (P<0.01). However, this effect was significantly diminished when the cells were pretreated with 400 μM NaHS for 30 min prior to HG treatment (P<0.01), which suggests that H₂S may attenuate HG-induced cytotoxicity. Similarly, pretreatment of cells with SB203580 or PDTC prior to HG treatment, significantly attenuated the cytotoxic effects of HG treatment (SB203580 and PDTC, P<0.01; Fig. 2A). These results demonstrate that the p38MAPK/NF-κB signaling pathway may be involved in HG-induced cytotoxicity. Notably, treatment of H9c2 cells with NaHS alone did not have a significant effect on cell viability (Fig. 2A).

Exogenous H₂S inhibits the p38MAPK/NF-κB signaling pathway in HG-treated H9c2 cells. The next aim of the present study was to investigate the effect of H₂S on the p38MAPK/NF-κB signaling pathway. As shown in Fig. 2B, exposure of H9c2 cells to HG for 24 h significantly enhanced the protein expression levels of p-p38MAPK when compared with untreated controls (P<0.01). In contrast, pretreatment of cells with 400 μM NaHS for 30 min prior to HG treatment was associated with a significant reduction in p-p38 expression when compared with HG-treated cells (P<0.01; Fig. 2B).

Exposure of H9c2 cells to HG for 24 h significantly increased the expression of NF-κB (P<0.01; Fig. 2C). However, pretreatment of cells with 400 μM NaHS for 30 min or 3 μM SB203580 for 60 min prior to HG treatment was associated with a significant reduction in p-p65 expression levels (NaHS, P<0.01; SB203580, P<0.01; Fig. 2C). These findings suggest that NaHS may inhibit the expression of NF-κB and the p38MAPK/NF-κB signaling pathway following exposure to HG.

Exogenous H₂S suppresses the HG-induced production of proinflammatory cytokines by inhibiting the p38MAPK/NF-κB pathway in H9c2 cells. Following exposure to HG for 24 h, the production of COX-2, iNOS, IL-1β and IL-6 (P<0.01; Fig. 3A), iNOS (P<0.01; Fig. 3B), IL-1β (P<0.01; Fig. 3C) and IL-6 (P<0.01; Fig. 3D) were significantly increased when compared with the untreated control group. However, pretreatment of cells with 400 μM NaHS for 30 min prior to HG exposure, significantly ameliorated the HG-induced increase in COX-2, iNOS, IL-1β and IL-6 expression levels (COX-2, iNOS, IL-1β and IL-6, P<0.01; Fig. 3A-D). These results suggest that H₂S may suppress the production of proinflammatory cytokines in HG-treated H9c2 cells.

Pretreatment of H9c2 cells with 100 μM PDTC for 30 min prior to HG exposure attenuated the HG-induced production of COX-2, iNOS, IL-1β and IL-6 (COX-2, iNOS, IL-1β and IL-6, P<0.01; Fig. 3A-D). In addition, H9c2 cells pretreated with 3 μM SB203580 for 60 min prior to HG exposure demonstrated a significant reduction in IL-1β and IL-6 expression (IL-1β and IL-6, P<0.01; Fig. 3C and D) when compared with HG-treated controls. This suggests that H₂S may suppress the HG-induced production of COX-2, iNOS, IL-1β and IL-6 by inhibiting the p38MAPK/NF-κB signaling pathway in H9c2 cells.

Exogenous H₂S exhibits anti-inflammatory effects by ameliorating the HG-induced increase in caspase-3 expression. The results presented so far suggest that exogenous H₂S may protect against HG-induced cytotoxicity (Fig. 2A) and reduce the production of proinflammatory cytokines (Fig. 3) in H9c2 cells. Therefore, the final aim of the present study was to investigate whether the anti-inflammatory effects of H₂S may attenuate HG-induced apoptosis. Following exposure of H9c2 cells to HG for 24 h, the expression levels of caspase-3, which is an important inducer of apoptosis (23), were significantly decreased (Fig. 3D). These results demonstrate that the p38MAPK/NF-κB signaling pathway may be involved in HG-induced cytotoxicity and apoptosis.
increased (Fig. 4). However, pretreatment of cells with 400 µM NaHS prior to HG exposure was associated with a significant reduction in caspase-3 expression levels when compared with cells treated with HG alone (P<0.01). Similarly, treatment of cells with HG plus 20 ng/ml IL-1Ra for 24 h significantly attenuated the production of caspase-3 when compared with cells exposed to HG alone (P<0.01; Fig. 4). This suggests that the IL-1 receptor may be involved in mediating the HG-induced increase in caspase-3 expression. These results indicate that exogenous H$_S$ may exhibit anti-inflammatory effects by ameliorating the HG-induced increase in caspase-3 expression, which protects against HG-induced apoptosis.

Discussion

The results of the present study suggest that exogenous H$_S$ protects cardiac cells against HG-induced inflammation and cytotoxicity, and inhibition of the p38MAPK/NF-κB, COX-2 and iNOS signaling pathways may be involved in mediating these cardioprotective effects of H$_S$ in cardiac cells in vitro. In addition, H$_S$ exhibited anti-inflammatory effects by significantly attenuating the HG-induced increase in caspase-3 expression.

Several factors have been implicated in the development of DCM, including metabolic disturbances, myocardial fibrosis, small vessel disease, autonomic dysfunction and insulin resistance (2). Metabolic disturbances, particularly hyperglycemia, initiate the development of DCM (24). Consistent with previous studies (20,25), exposure to 33 mM glucose for 24 h in the present study, was associated with cytotoxicity and the induction of inflammatory responses in H9c2 cells, as evidenced by a decrease in cell viability, increased expression of the apoptosis inducing factor-caspase 3, and an increase in the expression of multiple pro-inflammatory cytokines, including COX-2, iNOS, IL-1β and IL-6. In addition, HG-treated cardiac cells demonstrated an increase in p38MAPK and p-p65 expression, which suggests that the p38MAPK/NF-κB signaling pathway may have been involved in mediating the pro-apoptotic and cytotoxic effects of HG in H9c2 cells.

The physiological importance of H$_S$ was recognized in the last 15 years. Previous studies have demonstrated that H$_S$ influences a wide range of physiological and pathological processes, including blood vessel dilation (26-28), arterial contraction (27,29,30), neurotransmission (31), the regulation of inflammation (32,33) and cardioprotection (34,35). In patients with diabetes (36) and in rats with STZ-induced diabetes (36,37), the levels of H$_S$ in the circulation were significantly reduced. Therefore, a significant amount of attention has focused on investigating whether exogenous H$_S$ supplementation may protect cardiac cells from diabetes-induced cardiac injury. Several studies have revealed that exogenous H$_S$ protects against HG-induced cytotoxicity and inflammation in cardiac cells (20,21). A previous in vivo study demonstrated that intraperitoneal or oral administration of H$_S$ reduced myocardial hypertrophy, as well as the degree of fibrosis (18). Consistent with previous studies, the results of the present study demonstrate that exogenous H$_S$ may protect against cytotoxicity and inflammation in HG-treated H9c2 cells.

The present study investigated the mechanisms underlying the cardioprotective effects of H$_S$ against HG-induced cytotoxicity in H9c2 cells. Exogenous H$_S$ inhibited the HG-induced activation of the p38MAPK/NF-κB signaling pathway, and...
significantly attenuated the HG-induced cytotoxicity of HG-treated H9c2 cells. Consistent with these observations, H2S has been demonstrated to inhibit p38MAPK activity in various cell types, including rat aortic vascular smooth muscle cells (38), microglia (32) and neuroblastoma cells (39). In addition, H2S has been demonstrated to inhibit NF-κB activation and nuclear translocation in macrophages (40), kidney cells (41), pancreatic acinar cells (42) and heart cells (43). It is possible that activation of the p38MAPK signaling pathway may be a common mechanism involved in the pathogenesis of chronic complications associated with diabetes, as the elevation of p-p38MAPK in mouse diabetic cardiomyocytes activates the production of several cytokines (44). NF-κB is a key factor involved in inflammation, which regulates the transcription of >100 genes associated with immune and inflammatory responses (16). The functional consequence of reduced NF-κB activation in cells is a downregulation in the number of activated proinflammatory genes and cytokines, including COX-2, iNOS, IL-1β and IL-6. Zheng et al (45) observed that downregulation of the NF-κB pathway reduced the downstream expression of proinflammatory genes, including IL-1β, IL-6 and tumor necrosis factor-α, which led to attenuation of inflammation-mediated injury of the vascular wall. These data support the role of H2S as a cardioprotective agent against HG-induced inflammation.
and cytotoxicity through modulating the activity of the p38MAPK/NF-κB signaling pathway in H9c2 cells.

iNOS is an enzyme and can be induced in all cells and tissues through the action of cytokines (46). Activation of iNOS has been associated with the development of cardiovascular complications in rat models of diabetes. Bardell et al (47) demonstrated that the expression of iNOS in the mesenteric arteries is increased in rat models of diabetes.

In the present study, the expression levels of iNOS were significantly elevated in cardiac cells following exposure to HG, which were significantly attenuated by H2S treatment or following inhibition of NF-κB. A previous study, involving STZ-induced diabetic rats, demonstrated that iNOS mediated the nitrosylation of GAPDH and caspase-3, which led to cardiomyocyte death (5). Therefore, the p38MAPK/NF-κB and iNOS signaling pathways are likely to be involved in mediating the cardioprotective effects of exogenous H2S against HG-induced inflammation and cytotoxicity in H9c2 cells.

COX is an enzyme involved in the metabolism of arachidonic acid (AA), and the COX-2 isozyme is a key enzyme in the conversion of AA to prostaglandins (48). A previous study demonstrated that overexpression of COX-2 is correlated with induction of inflammatory processes (49). In the present study, the level of COX-2 expression was significantly elevated in cardiac cells following HG exposure. In addition, exposure of cardiac H9c2 cells to HG or a specific inhibitor to NF-κB, abolished the HG-induced increase in COX-2 expression. In a rat model of heart ischemia/reperfusion injury (50), inhibition of COX-2 was associated with a reduction in cellular apoptosis. Consistent with these observations, the results of the present study suggest that H2S may attenuate apoptosis in H9c2 cells following HG exposure by reducing caspase-3 expression levels. Therefore, H2S may protect cardiac cells by inhibiting COX-2 expression potentially through modulating the p38MAPK/NF-κB signaling pathway.

In the present study, pretreatment of H9c2 cells with an IL-1Ra prior to HG exposure significantly reduced caspase-3 expression. This suggests that inhibition of inflammation may protect against HG-induced apoptosis in H9c2 cells. Consistent with this observation, inhibition of the inflammatory response significantly reduced doxorubicin-induced cytotoxicity of H9c2 cells (19). In addition, p38MAPK was demonstrated to promote apoptosis in a number of in vitro models, such as pulmonary microvascular endothelial cells (51) and breast cancer cells (52), while H2S prevented apoptosis by inhibition of p38MAPK (53). Therefore, the p38MAPK/NF-κB pathway-mediated activation of the anti-inflammatory response may be associated with the cardioprotective effects of exogenous H2S treatment against HG-induced cytotoxicity in H9c2 cells.

In conclusion, the results of the present study demonstrated that exogenous H2S exhibits cardioprotective effects against HG-induced cytotoxicity and inflammation in rat cardiac cells in vitro. H2S exerted cytoprotective effects potentially via the p38MAPK/NF-κB-mediated anti-inflammatory and antiapoptotic signaling pathways. The results provide evidence to suggest that H2S may have a potential therapeutic value in hyperglycemia-induced cardiac lesions, which are predominant in DCM.

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