# Exogenous H₂S protects H9c2 cardiac cells against high glucose-induced injury and inflammation by inhibiting the activation of the NF-κB and IL-1β pathways

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**Abstract.** Hyperglycemia has been reported to activate the nuclear factor-κB (NF-κB) pathway. We have previously demonstrated that exogenous hydrogen sulfide (H₂S) protects cardiomyocytes against high glucose (HG)-induced injury by inhibiting the activity of p38 mitogen-activated protein kinase (MAPK), which can activate the NF-κB pathway and induce interleukin (IL)-1β production. In the present study, we aimed to investigate the hypothesis that exogenous H₂S protects cardiomyocytes against HG-induced injury and inflammation through the inhibition of the NF-κB/IL-1β pathway. H9c2 cardiac cells were treated with 35 mM glucose (HG) for 24 h to establish a model of HG-induced damage. Our results demonstrated that treatment of the cells with 400 $\mu$M sodium hydrogen sulfide (NaHS, a donor of H₂S) or 100 $\mu$M pyrrolidine dithiocarbamate (PDTC, an inhibitor of NF-κB) for 30 min prior to exposure to HG markedly attenuated the HG-induced increase in the expression levels of the phosphorylated (p)-NF-κB p65 subunit. Notably, pre-treatment of the H9c2 cardiac cells with NaHS or PDTC significantly suppressed the HG-induced injury, including cytotoxicity, apoptosis, oxidative stress and mitochondrial insults, as evidenced by an increase in cell viability, as well as a decrease in the number of apoptotic cells, the expression of cleaved caspase-3, the generation of reactive oxygen species (ROS) and the dissipation of mitochondrial membrane potential (MMP). In addition, pre-treatment of the cells with NaHS or PDTC ameliorated the HG-induced inflammatory response, leading to a decrease in the levels of IL-1β, IL-6 and tumor necrosis factor-α (TNF-α). Importantly, co-treatment of the H9c2 cells with 20 ng/ml IL-1 receptor antagonist (IL-1Ra) and HG markedly reduced the HG-induced increase in p-NF-κB p65 expression, cytotoxicity, the number of apoptotic cells, as well as the production of TNF-α. In conclusion, the present study presents novel mechanistic evidence that exogenous H₂S protects H9c2 cardiac cells against HG-induced inflammation and injury, including cytotoxicity, apoptosis, overproduction of ROS and the dissipation of MMP, by inhibiting the NF-κB/IL-1β pathway. We also provide new data indicating that the positive interaction between the NF-κB pathway and IL-1β is critical in HG-induced injury and inflammation in H9c2 cardiac cells.

## Introduction

Hyperglycemia is not only one of the main clinical characteristics of diabetes mellitus (DM), but is also a risk factor for the development of cardiovascular complications associated with chronic diabetes, such as diabetic cardiomyopathy (1,2). Multiple factors have been reported to contribute to hyperglycemia-induced cardiac damage, such as oxidative stress (3-6), mitochondrial dysfunction (5-8), apoptosis (5-9) and activation of several signaling molecules, including mitogen-activated protein kinase (MAPK) (3,5,10,11), leptin (6,12) and p53 (7,9). Another signaling molecule involved in hyperglycemia-induced cardiomyocyte damage may be nuclear factor-κB (NF-κB). NF-κB is a dimeric transcription factor that regulates numerous genes associated with stress responses, including oxidative stress, apoptosis and inflammation. The NF-κB family is composed of 5 subunits, Rel A (p65), Rel B, c-Rel, NF-κB1 (p50) and NF-κB2 (p52), each of which may form hemo- or heterodi-

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mers. Among these, the nuclear translocation of the p65 subunit is a key step in the activation of NF-κB (13). NF-κB is known to be activated by p38 MAPK (a member of the MAPK family) in cardiomyocytes (14-16). Accumulating evidence indicates that NF-κB plays a significant role in cardiac damage induced by various stimuli (14-16). Guo *et al* demonstrated the involvement of the NF-κB pathway in doxorubicin-induced cardiac cytotoxicity, resulting in a decrease in cell viability (14,15). The NF-κB pathway has also been implicated in G-protein-coupled receptor angonist-elicited cardiomyocyte hypertrophy (16). Recently, the roles of NF-κB in hyperglycemia-induced cardiac insults and inflammation have attracted attention. As shown in a previous study, in mice with streptozotocin-induced DM, a marked increase in cardiac hypertrophy, fibrosis and inflammation was observed with a corresponding upregulation of reactive oxygen species (ROS) generation, interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, cellular adhesion molecules and phosphorylated (p)-p38 MAPK expression, and NF-κB activation (17), suggesting that the NF-κB pathway participates in hyperglycemia-induced myocardial damage and inflammation. However, the authors did not observe the effects of the inhibition of NF-κB activation on cardiac injury and inflammation induced by hyperglycemia. Thus, it is important to further clarify the role of the inhibition of NF-κB activation in the protective effects against hyperglycemia-induced cardiac insults and inflammation.

In a recent study, we demonstrated that exogenous hydrogen sulfide (H₂S) protects H9c2 cardiac cells against high glucose (HG)-induced injury, including cytotoxicity, apoptosis, oxidative stress and mitochondrial damage, by inhibiting the activation of the p38 MAPK, extracellular signal-regulated protein kinase 1/2 (ERK1/2) (5) and leptin pathways (18). H₂S, previously regarded as a poisonous gas, has been recognized as the third endogenous gasotransmitter signaling molecule alongside nitric oxide (NO) and carbon monoxide (19,20). H₂S exerts its cytoprotective effects in various models of cardiac injury. In isoproterenol-treated rat hearts, treatment with H₂S has been shown to attenuate myocardial necrosis and rescue contractile activity (21). In an *in vivo* model of mice subjected to ischemia/reperfusion (I/R), pre-treatment with Na₂S markedly reduced the extent of myocardial injury, oxidative stress and apoptosis (22). Recently, it was also demonstrated that exogenous H₂S protects H9c2 cardiac cells against chemical hypoxia-induced injury by inhibiting oxidative stress and enhancing heat shock protein 90 (HSP90) expression (23,24). Of note, since lower circulating H₂S levels have been measured in animal models of diabetes (25-27) and patients with type 2 DM (26,28), the role of H₂S in hyperglycemia-induced cardiac insults has attracted considerable attention. Peake *et al* reported that the exogenous administration of H₂S (Na₂S) ameliorates myocardial I/R injury in db/db mice (4). Furthermore, H₂S has been shown to exert protective effects against myocardial I/R-induced injury in diabetic rats (29). Of note, although H₂S has been shown to exert cytoprotective and anti-inflammatory effects by inhibiting the NF-κB pathway in doxorubicin-treated cardiac cells (15) or cobalt chloride (CoCl₂, a hypoxia mimetic agent)-treated human skin keratinocytes (HaCaT cells) (30), whether exogenous H₂S protects cardiomyocytes against HG-induced injury and inflammation by inhibiting NF-κB activation remains unclear. To investigate this hypothesis, in the current study, H9c2 cardiac cells were exposed to 35 mM glucose (HG) to establish a model of HG-induced cardiac injury and inflammation. We then examined the following: i) the effects of exogenous H₂S on the HG-induced increase in NF-κB activation; ii) the roles of NF-κB activation in HG-induced cardiac injury and inflammation; iii) the roles of the inhibition of NF-κB activation in the protective effects of exogenous H₂S against HG-induced injury and inflammation; and iv) the interaction between NF-κB and IL-1β in H9c2 cardiac cells.

## Materials and methods

*Materials.* Sodium hydrogen sulfide (NaHS), was obtained from Sigma Chemical Co., (St. Louis, MO, USA) protected from sunlight and stored at 2-4°C. Pyrrolidine dithiocarbamate (PDTC), 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbocyanine iodide (JC-1) and Hoechst 33258 were purchased from Sigma-Aldrich (Sigma Chemicals Co.) and stored at -20°C. IL-1 receptor antagonist (IL-1Ra) was purchased from ProSpec (Ness-Ziona, Israel). The enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, IL-6 and TNF-α were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The Cell Counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum (FBS) and DMEM were obtained from Gibco-BRL (Grand Island, NY, USA). Anti-p-NF-κB p65 antibody, anti-total (t)-NF-κB p65 antibody and anti-cleaved caspase-3 antibody were procured from Cell Signaling Technology, Inc. (Boston, MA, USA); horseradish peroxidase (HRP)-conjugated secondary antibody and the BCA protein assay kit were obtained from KangChen Bio-tech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from Nanjing KeyGen Biotech Co. Ltd., (Nanjing, China). Lysis buffer was purchased from the Beyotime Institute of Biotechnology (Shanghai, China) and Urea sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was purchased from Hangzhou Fude Chemical Co., Ltd. (Hangzhou, China).

*Cell culture and treatment.* The H9c2 cardiac cells, a rat cardiac myoblast cell line, were supplied by the Sun Yat-sen University Experimental Animal Center (Guangzhou, Guangdong, China). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37°C under an atmosphere of 5% CO₂ and 95% air. For the treatment procedure, the cells were cultured in DMEM (5.5 mM glucose) medium for 12 h pior to the administration of 35 mM glucose (final concentration) for 24 h. The glucose concentration of the control group was 5.5 mM. To explore the protective effects of H₂S against HG (35 mM glucose)-induced injury, the cells were treated with 400 μM NaHS (a well-known H₂S donor) for 30 min prior to exposure to 35 mM glucose for 24 h. To further determine whether the protective effects of H₂S are associated with the inhibition of the acvitation of the NF-κB/IL-1 pathway, the H9c2 cells were treated with 100 μM PDTC (a specific inhibitor of NF-κB) prior to exposure to 35 mM glucose for 24 h or co-treated with 20 ng/ml IL-1Ra (an antagonist of IL-1) and 35 mM glucose for 24 h.

*Cell viability assay.* The H9c2 cells were seeded in 96-well plates at a concentration of $1 \times 10^4$ cells/ml and incubated at 37°C. CCK-8 assay was employed to assess the viability of the H9c2 cardiac cells. After the indicated treatments, 10 $\mu$l CCK-8 solution at a 1/10 dilution was added to each well and then the plate was incubated for 2 h in an incubator. Absorbance at 450 nm was assayed using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) of 3 wells in the indicated groups were used to calculate the percentage of cell viability according to the following formula: cell viability (%) = ($OD_{treatment\ group}/OD_{control\ group}$) x100%. The experiment was repeated 5 times.

*Hoechst 33258 nuclear staining for the determination of apoptosis.* Apoptotic cell death was observed by Hoechst 33258 staining followed by photofluorography. In brief, the H9c2 cells were plated in 35-mm dishes at a density of $1 \times 10^6$ cells/well. After the indicated treatments, the cells were fixed with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) for 10 min. The slides were then washed 5 times with PBS. After staining with 5 mg/ml Hoechst 33258 for 15 min, the H9c2 cells were washed 5 times with PBS, and the cells were then visualized under a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan). Viable H9c2 cells displayed a uniform blue fluorescence throughout the nucleus and a normal nuclear size. However, apoptotic H9c2 cells showed condensed, distorted or fractured nuclei. The experiment was carried out 3 times.

*Examination of intracellular ROS generation.* Intracellular ROS generation was determined by the oxidative conversion of the cell-permeable oxidation of DCFH-DA to fluorescent DCF. The H9c2 cells were cultured on a slide with DMEM. After the different treatments, the slides were washed 3 times with PBS. DCFH-DA solution (10 $\mu$M) in serum-free medium was added to the slides, and the cells were then incubated at 37°C for a further 30 min in an incubator. The slides were washed 5 times with PBS, and DCF fluorescence was measured over the entire field of vision by using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The mean fluorescence intensity (MFI) from 5 random fields was measured using ImageJ 1.47i software and the MFI was used as an index of the amount of ROS. The experiment was carried out 5 times.

*Measurement of mitochondrial membrane potential (MMP).* MMP was assessed using a fluorescent dye, JC-1, a cell-permeable cationic dye that preferentially enters the mitochondria based on the highly negative MMP. The depolarization of MMP results in the loss of MMP from the mitochondria and a decrease in red/green fluorescence. The cells were cultured on a slide with DMEM. After the indicated treatments, the slides were washed 3 times with PBS. The cells were incubated with 1 mg/l JC-1 at 37°C for 30 min in an incubator, washed briefly with PBS 3 times and air dried again. Fluorescence was then measured over the whole field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA, Olympus). The MFI of JC-1 from 5 random fields was analyzed using ImageJ 1.47i software, and the MFI was taken as an index of the levels of MMP. The experiment was carried out 5 times.

*Western blot analysis.* After the indicated treatments, the H9c2 cells were harvested and lysed with cell lysis solution at 4°C for 30 min. Total protein was quantified using the BCA protein assay kit. Loading buffer was added to cytosolic extracts, followed by boiling for 5 min, and the same amounts of supernatant from each sample were fractionated by 10% SDS-PAGE, and then the total proteins were transferred into polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% fat-free milk for 60 min in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T)] at room temperature, and incubated with either anti-t-NF-κB p65 (1:1,000 dilution), anti-p-NF-κB p65 (1:1,000 dilution), or anti-cleaved caspase-3 (1:1,000 dilution) in freshly prepared TBS-T with 3% free-fat milk overnight with gentle agitation at 4°C. The membranes were washed for 15 min with TBS-T and incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,500 dilution; KangChen Bio-tech, Inc.) in TBS-T with 3% fat-free milk for 1.5 h at room temperature. The membranes were then washed 3 times with TBS-T for 15 min. The immunoreactive signals were visualized by ECL detection. In order to quantify protein expression, the X-ray films were scanned and analyzed using ImageJ 1.47i software. The experiment was carried out 3 times.

*ELISA for the detection of IL-1β, IL-6 and TNF-α in the culture supernatant.* The H9c2 cells were plated in 96-well plates. After the indicated treatments, the levels of IL-1β, IL-6 and TNF-α in the culture medium were determined by ELISA according to the manufacturer's instructions (Wuhan Boster Biological Technology, Ltd.). The experiment was performed at least 5 times.

*Statistical analysis.* All data are presented as the means ± SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA), followed by the least significant difference (LSD) post hoc comparison test. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

## Results

*NaHS and NF-κB inhibitor ameliorate the HG-induced increase in the expression of p-NF-κB p65 in H9c2 cardiac cells.* To explore the effects of HG (35 mM glucose) on the activation of the NF-κB pathway in H9c2 cells, a time-response experiment to determine the expression level of NF-κB was performed. As shown in Fig. 1A-C, after the cells were exposured to HG for 30, 60, 90, 120, 150, 180 and 210 min, the expression levels of the p-NF-κB p65 subunit were significantly enhanced, reaching a peak at 60 and 90 min. However, the expression levels of the t-NF-κB p65 subunit remained unaltered following exposure of the cells to HG for the indicated periods of time. Based on these data, the p-NF-κB expression level was determined at 90 min following exposure to HG in the following experiments.

Of note, the increased expression of p-NF-κB p65 was reduced by treatment of the cells with 400 $\mu$M NaHS (a donor of H₂S) for 30 min prior to exposure to HG for 90 min (Fig. 1D-F). Similarly, treatment of the cells with 100 $\mu$M PDTC, an inhibitor
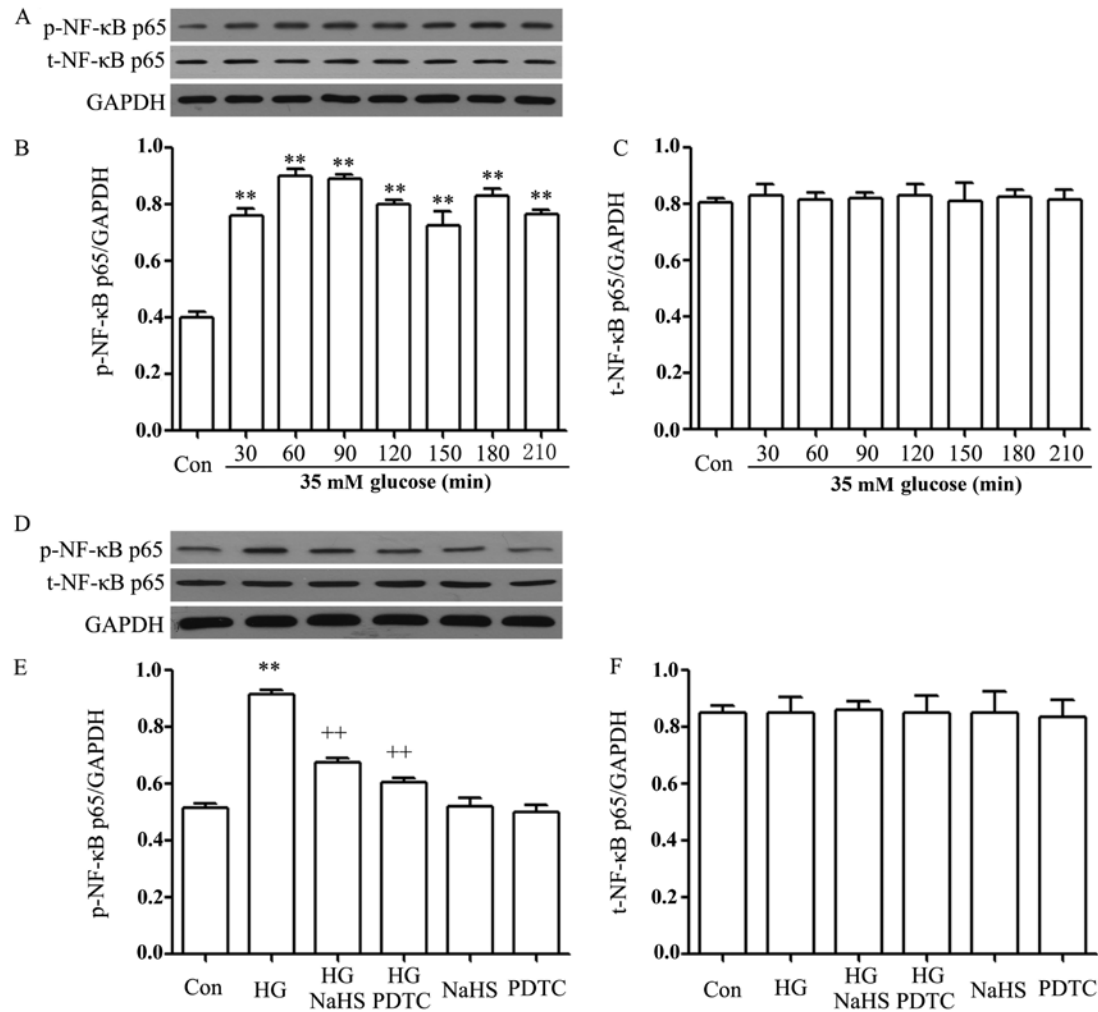
Figure 1. Sodium hydrogen sulfide (NaHS) and pyrrolidine dithiocarbamate (PDTC) alleviate the high glucose (HG)-induced increase in the expression of phosphorylated (p)-nuclear factor-κB (NF-κB) p65 subunit in H9c2 cardiac cells. (A and D) NF-κB expression levels were semi-quantified by western blot analysis. (A-C) Time course of changes observed in the NF-κB expression levels induced by HG (35 mM glucose) over a 210-min time period. (D-F) H9c2 cardiac cells were exposed to HG for 90 min with or without treatment with 400 $\mu$M NaHS or 100 $\mu$M PDTC (an inhibitor of NF-κB) for 30 min prior to exposure to HG. (B,C,E and F) Densitometric analysis of the p-NF-κB p65 and total (t)-NF-κB p65 expression levels in (A) and (D). Data are presented as the means ± SEM (n=3). **$p<0.01$ vs. the control (Con) group; ++$p<0.01$ vs. the HG-treated group.
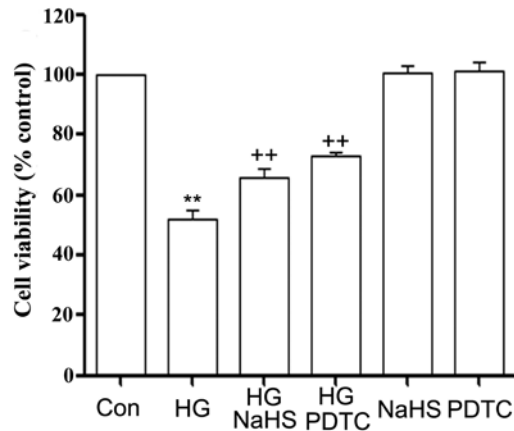


Figure 2. Exogenous hydrogen sulfide ($H_2S$) and nuclear factor-κB (NF-κB) inhibitor protect H9c2 cardiac cells against high glucose (HG)-induced cytotoxicity. The cells were treated with 35 mM glucose for 24 h with or without pre-treatment with 400 $\mu$M sodium hydrogen sulfide (NaHS) or 100 $\mu$M pyrrolidine dithiocarbamate (PDTC) for 30 min. Cell viability was examined using the Cell Counting kit-8 (CCK-8) assay. Data are shown as the means ± SEM (n=3). **$p<0.01$ vs. the control (Con) group; ++$p<0.01$ vs. the HG-treated group.

of NF-κB, for 30 min prior to exposure to HG also attenuated the increased expression of p-NF-κB p65 (Fig. 1D and E). The basal expression levels of p-NF-κB p65 (control) were not altered by the separate treatment with 400 $\mu$M NaHS or 100 $\mu$M PDTC (Fig. 1D and F).

*Role of inhibition of NF-κB activation in the protective effects of $H_2S$ against HG-induced cytotoxicity in H9c2 cardiac cells.* In agreement with our previous studies (5,18), treatment of the cells with 400 $\mu$M NaHS for 30 min prior to exposure to HG for 24 h markedly decreased HG-induced cytotoxicity, as indicated by an increase in cell viability (Fig. 2). Since we demonstrated that the expression level of p-NF-κB p65 was increased by HG treatment (Fig. 1), we then wished to confirm the role of NF-κB activation in the HG-induced cytotoxicity. As shown in Fig. 2, treatment of the H9c2 cardiac cells with 100 $\mu$M PDTC (an inhibitor of NF-κB) for 30 min prior to exposure to 35 mM glucose markedly blocked the HG-induced cytotoxicity, leading to an increase
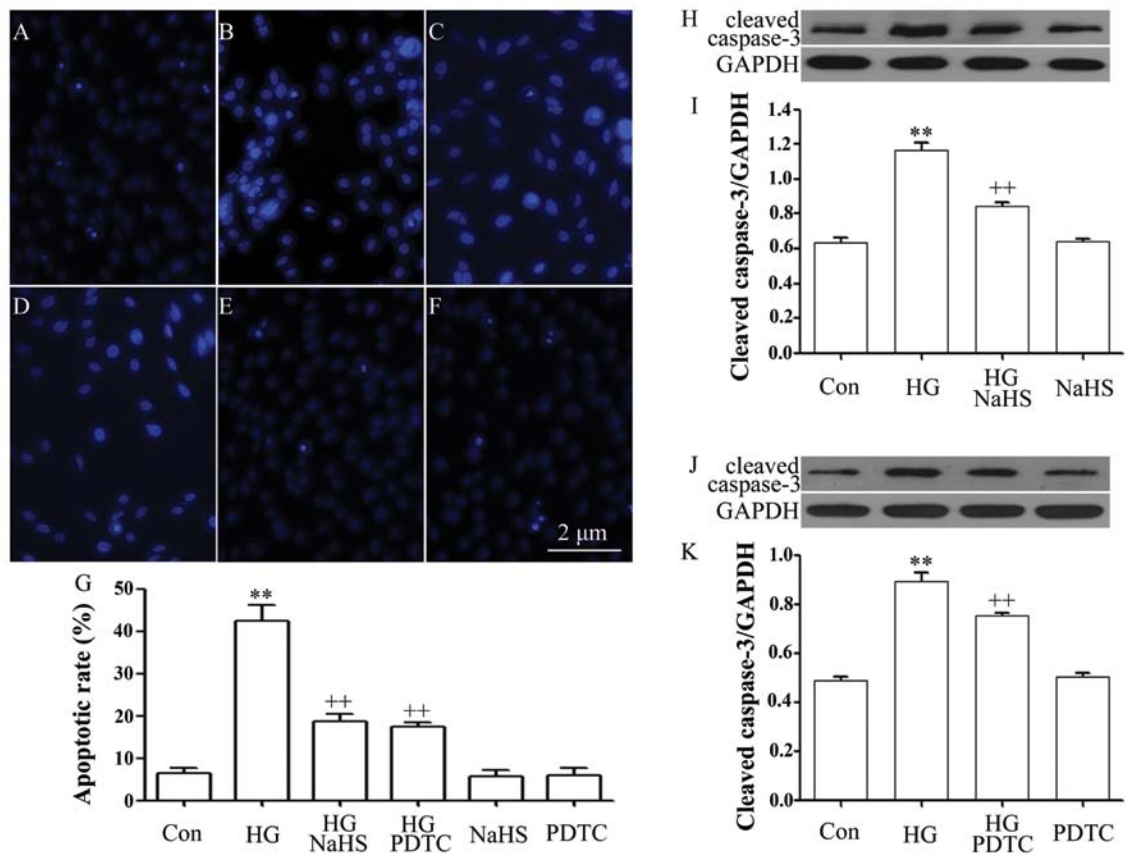
Figure 3. Exogenous hydrogen sulfide ($H_2S$) and nuclear factor-κB (NF-κB) inhibitor attenuate high glucose (HG)-induced apoptosis in H9c2 cells. (A-F) Hoechst 33258 nuclear staining followed by fluorescence imaging was used to detect cell apoptosis. (A) Control group. H9c2 cells were (B) treated with 35 mM glucose [high glucose (HG)] for 24 h; (C) treated with 400 μM sodium hydrogen sulfide (NaHS) or (D) 100 μM pyrrolidine dithiocarbamate (PDTC) for 30 min prior to exposure to HG; (E) treated with 400 μM NaHS for 30 min followed by 24 h of culture; (F) treated with 100 μM PDTC for 30 min followed by 24 h of culture. (G) The apoptotic rate was analyzed using a cell counter and ImageJ 1.47i software. (H and J) The expression level of cleaved caspase-3 was semi-quantified by western blot analysis. (I and K) Densitometric analysis of the cleaved caspase-3 expression levels in (H) and (J). Data are shown as the means ± SEM (n=3). **p<0.01 vs. the control (Con) group; ++p<0.01 vs. the HG-treated group.

in cell viability, indicating the involvement of NF-κB activation in HG-induced cytotoxicity.

*Role of inhibition of NF-κB activation in the protective effects of $H_2S$ against HG-induced apoptosis in H9c2 cells.* As illustrated in Fig. 3, exposure of the cells to 35 mM glucose for 24 h induced significant apoptosis, as evidenced by an increase in the number of apoptotic cells (Fig. 3B and G) and in the expression of cleaved caspase-3 (Fig. 3H and J). However, the increased number of apoptotic cells and the cleaved caspase-3 expression level were markedly diminished by treatment with 400 μM NaHS for 30 min prior to exposure to HG (Fig. 3C and G-I). Similarly, treatment of the cells with 100 μM PDTC for 30 min prior to exposure to HG also considerably blocked HG-induced apoptosis, leading to a decrease in the number of apoptotic cells and the expression level of cleaved caspase-3 (Fig. 3D, G, J and K). Alone, NaHS or PDTC did not significantly alter the percentage of apoptotic cells and the basal expression level of cleaved caspase-3. These results reveal that the inhibition of the activation of NF-κB plays a role in the protective effects of exogenous $H_2S$ against HG-induced apoptosis.

*Role of inhibition of NF-κB activation in the inhibitory effects of $H_2S$ on the HG-induced generation of ROS in H9c2 cardiac cells.* Consistent with our previous studies (5,6,10,18), exposure of the cells to 35 mM glucose for 24 h markedly increased the intracellular generation of ROS (Fig. 4B). The elevated ROS generation was reduced by treatment of the cells with 400 μM NaHS for 30 min prior to exposure to HG (Fig. 4C). To determine whether the activation of NF-κB plays a role in HG-induced oxidative stress, the H9c2 cells were treated with 100 μM PDTC for 30 min prior to exposure to HG. It was shown that pre-treatment with PDTC markedly reduced the increased intracellular ROS generation induced by HG (Fig. 4D), indicating the contribution of the activation of the NF-κB pathway to HG-induced oxidative stress in H9c2 cells.

*Role of the inhibition of NF-κB activation in the protective effects of $H_2S$ against the HG-induced dissipation of MMP in H9c2 cardiac cells.* It has been reported that HG induces mitochondrial damage in cardiomyocytes (5,6,10,18). As illustrated in Fig. 5B and G, exposure of the cells to 35 mM glucose for 24 h induced the marked dissipation of MMP. The dissipation
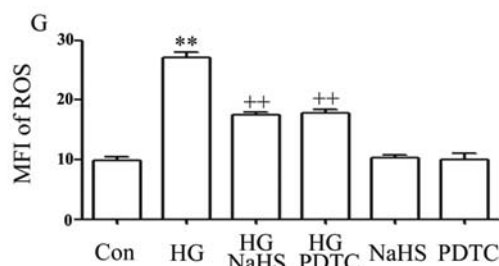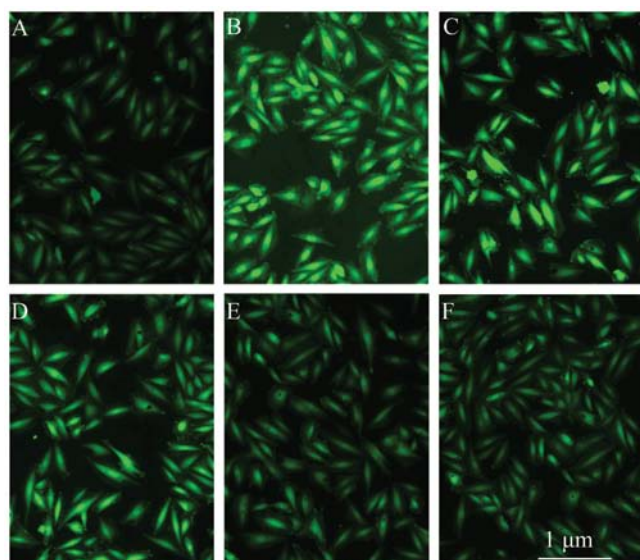
Figure 4. Exogenous hydrogen sulfide (H₂S) and nuclear factor-κB (NF-κB) inhibitor attenuate the high glucose (HG)-induced overproduction of reactive oxygen species (ROS ) in H9c2 cells. (A-F) After the cells were treated with the indicated treatments, intracellular ROS generation was detected by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining followed by photofluorography. (A) Control group. H9c2 cells were (B) treated with 35 mM glucose [high glucose (HG)] for 24 h; (C) treated with 400 $\mu$M sodium hydrogen sulfide (NaHS) or (D) 100 $\mu$M pyrrolidine dithiocarbamate (PDTC) for 30 min prior to exposure to HG; (E) treated with 400 $\mu$M NaHS for 30 min followed by 24 h of culture; (F) treated with 100 $\mu$M PDTC for 30 min followed by 24 h of culture. (G) Quantitative analysis of mean fluorescence intensity (MFI) of DCF in (A-F) using ImageJ 1.47i software. Data are shown as the means ± SEM (n=3). **p<0.01 vs. the control (Con) group; ++p<0.01 vs. the HG-treated group.
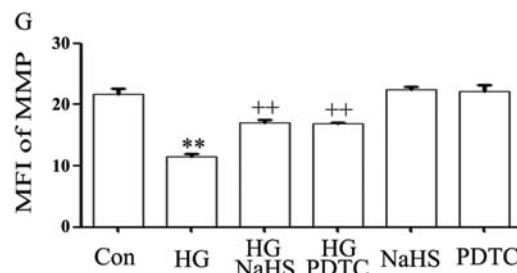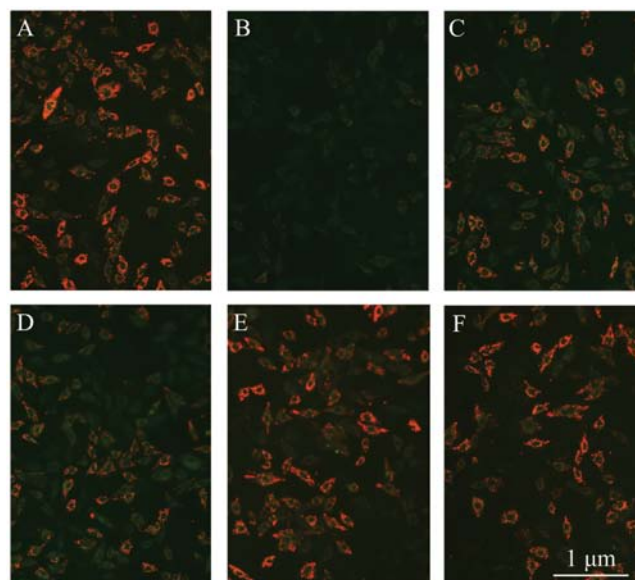


Figure 5. Exogenous hydrogen sulfide (H₂S) and nuclear factor-κB (NF-κB) inhibitor attenuate the high glucose (HG)-induced dissipation of mitochondrial membrane potential (MMP) in H9c2 cardiac cells. (A-F) After the cells were treated with the indicated treatments, MMP was detected by the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol-carbo-cyanine iodide (JC-1) staining followed by photofluorography. (A) Control group. H9c2 cells were (B) treated with 35 mM glucose [high glucose (HG)] for 24 h; (C) treated with 400 $\mu$M sodium hydrogen sulfide (NaHS) or (D) 100 $\mu$M pyrrolidine dithiocarbamate (PDTC) for 30 min prior to exposure to HG; (E) treated with 400 $\mu$M NaHS for 30 min followed by 24 h of culture; (F) treated with 100 $\mu$M PDTC for 30 min followed by 24 h of culture. (G) Quantitative analysis of the ratio of red/green fluorescence intensity in (A-F) by using ImageJ 1.47i software. Data are shown as the means ± SEM (n=3). **p<0.01 vs. the control (Con) group; ++p<0.01 vs. the HG-treated group.

of MMP was blocked by pre-treatment with 400 $\mu$M NaHS for 30 min prior to exposure to HG (Fig. 5C and G). Additionally, treatment of the H9c2 cells with 100 $\mu$M PDTC for 30 min prior to exposure to HG for 24 h also markedly reduced the dissipation of MMP, suggesting that the activation of NF-κB participates in the HG-induced loss of MMP in H9c2 cells.

*NaHS and NF-κB inhibitor reduce the HG-induced production of pro-inflammatory cytokines in H9c2 cardiac cells.* As indicated in Fig. 6, after the cells were treated with 35 mM glucose (HG) for 24 h, the levels of IL-1β (Fig. 6A), IL-6 (Fig. 6B) and TNF-α (Fig. 6C) were markedly enhanced, compared with the control group (p<0.01). However, the increased levels of IL-1β, IL-6 and TNF-α induced by HG were markedly attenuated by treatment with 400 $\mu$M NaHS for 30 min prior to exposure to HG for 24 h, revealing the inhibitory effects of exogenous H₂S on the production of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α

induced by HG. Similarly, treatment of the cells with 100 $\mu$M PDTC for 30 min prior to exposure to HG also alleviated the increased production of IL-1β, IL-6 and TNF-α (Fig. 6A-C), suggesting that the activation of NF-κB contributes to the HG-induced inflammatory response in H9c2 cardiac cells.

*IL-1Ra suppresses the HG-induced upregulation of p-NF-κB p65 expression in H9c2 cardiac cells.* Since the above results (Fig. 1) revealed that exposure to HG upregulated the expression of the p-NF-κB p65 subunit and that the activation of NF-κB was involved in the production of IL-1β induced by HG (Fig. 6), and a recent study reported that IL-1Ra reduces hyperglycemia and tissue inflammation in type 2 diabetic GK rats (31), we then wished to explore whether IL-1β contributes to the activation of NF-κB by HG. As shown in Fig. 7, exposure of the H9c2 cells to 35 mM glucose for 90 min significantly enhanced the expression level of p-NF-κB p65. However, co-treatment of the cells with
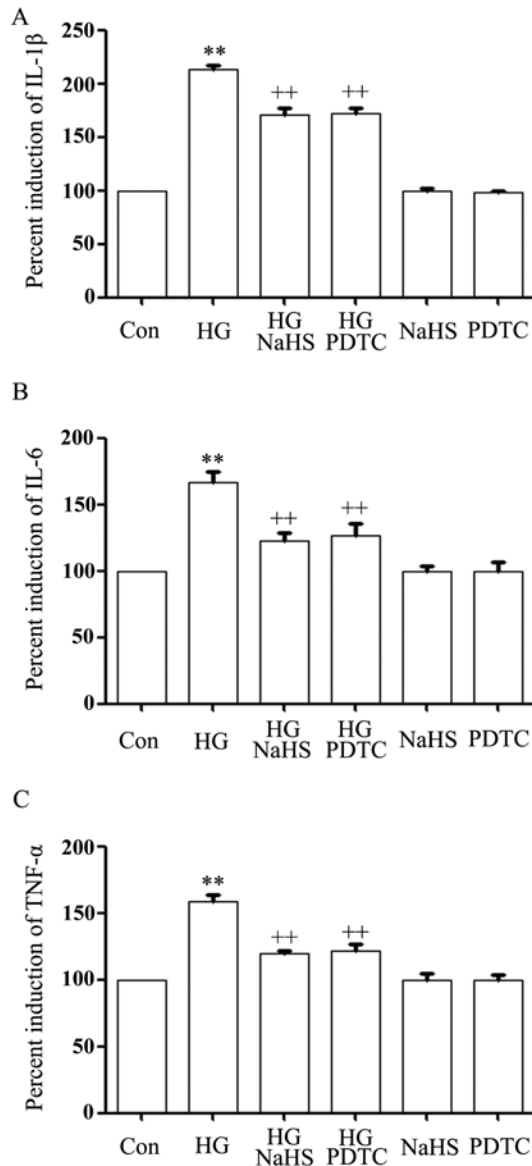
Figure 6. Exogenous hydrogen sulfide (H$_2$S) and nuclear factor-κB (NF-κB) inhibitor attenuate the high glucose (HG)-induced production of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) in H9c2 cells. The cells were treated with 35 mM glucose (HG) for 24 h with or without treatment with either 400 μM sodium hydrogen sulfide (NaHS) or 100 μM pyrrolidine dithiocarbamate (PDTC) for 30 min prior to exposure to HG for 24 h. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels of (A) IL-1β, (B) IL-6 and (C) TNF-α in cell supernatants. Data are shown as the means ± SEM (n=5). **p<0.01 vs. the control (Con) group; ++p<0.01 vs. the HG-treated group.



Figure 7. IL-1 receptor antagonist (IL-1Ra) attenuates the high glucose (HG)-induced increase in phosphorylated (p)-nuclear factor-κB (NF-κB) p65 expression in H9c2 cells. (A) After the cells were treated with the indicated treatments, NF-κB expression levels were semi-quantified by western blot analysis. (B and C) Densitometric analysis of the phosphorylated (p)-NF-κB and total (t)-NF-κB p65 levels in (A). Data are presented as the means ± SEM (n=5). **p<0.01 vs. the control (Con) group; ++p<0.01 vs. the HG-treated group.

20 ng/ml IL-1Ra and 35 mM glucose for 90 min significantly attenuated the HG-induced increase in p-NF-κB p65 expression (Fig. 7A and B). Alone, IL-1Ra did not affect the basal expression of p-NF-κB in the H9c2 cells (Fig. 7A and C).

*IL-1Ra attenuates HG-induced cytotoxicity, apoptosis and TNF-α production in H9c2 cardiac cells.* As shown in Fig. 8, co-treatment of the cells with 20 ng/ml IL-1Ra and 35 mM glucose for 24 h markedly attenuated the HG-induced cytotoxicity, apoptosis and TNF-α production, as evidenced by an increase in cell viability (Fig. 8A), as well as by a decrease in the number of apoptotic cells (Fig. 8B, panels b and e) and
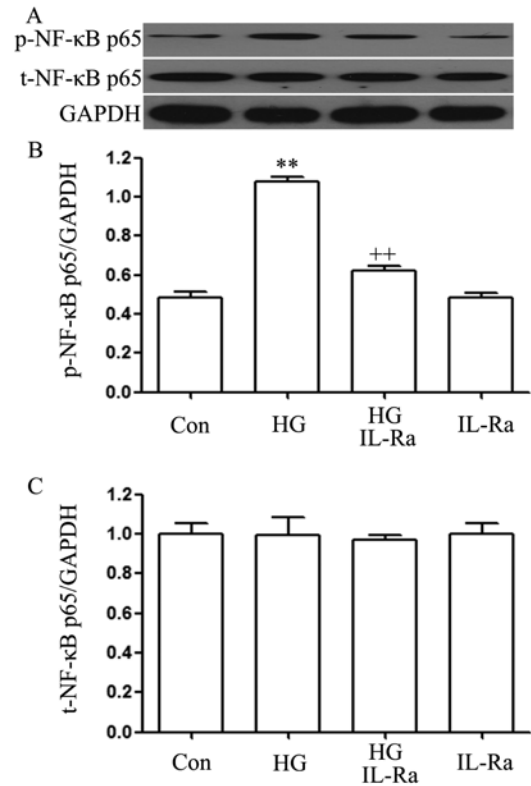
TNF-α production (Fig. 8C). Alone, IL-1Ra did not affect cell viability, the number of apoptotic cells and the basal production of TNF-α in the H9c2 cells (Fig. 8A-C).

## Discussion

To the best of our knowledge, the present study demonstrates for the first time that exogenous H$_2$S protects H9c2 cardiac cells against HG-induced injury and inflammation by inhibiting the activation of NF-κB and IL-1β and that the positive interaction between NF-κB and IL-1β contributes to the HG-induced injury and inflammation. Several lines of evidence support our conclusion: i) exogenous H$_2$S attenuated the HG-induced increase in p-NF-κB p65 expression, and IL-1β production; ii) exogenous H$_2$S inhibited HG-induced cardiomyocyte injury and inflammation, as evidenced by an increase in cell viability, and a decrease in the number of apoptotic cells and cleaved caspase-3 expression, as well as a decrease in ROS production and the dissipation of MMP and the production of IL-1β, IL-6 and TNF-α; iii) similar to the inhibitory effects of exogenous H$_2$S, pre-treatment of the H9c2 cells with PDTC (an inhibitor of NF-κB) also ameliorated the HG-induced injury and inflammation; and iv) co-treatment of the H9c2 cells with IL-Ra (an inhibitor of IL-1β receptor) and HG alleviated the cytotoxicity, apoptosis and upregulation of p-NF-κB expression induced by HG.
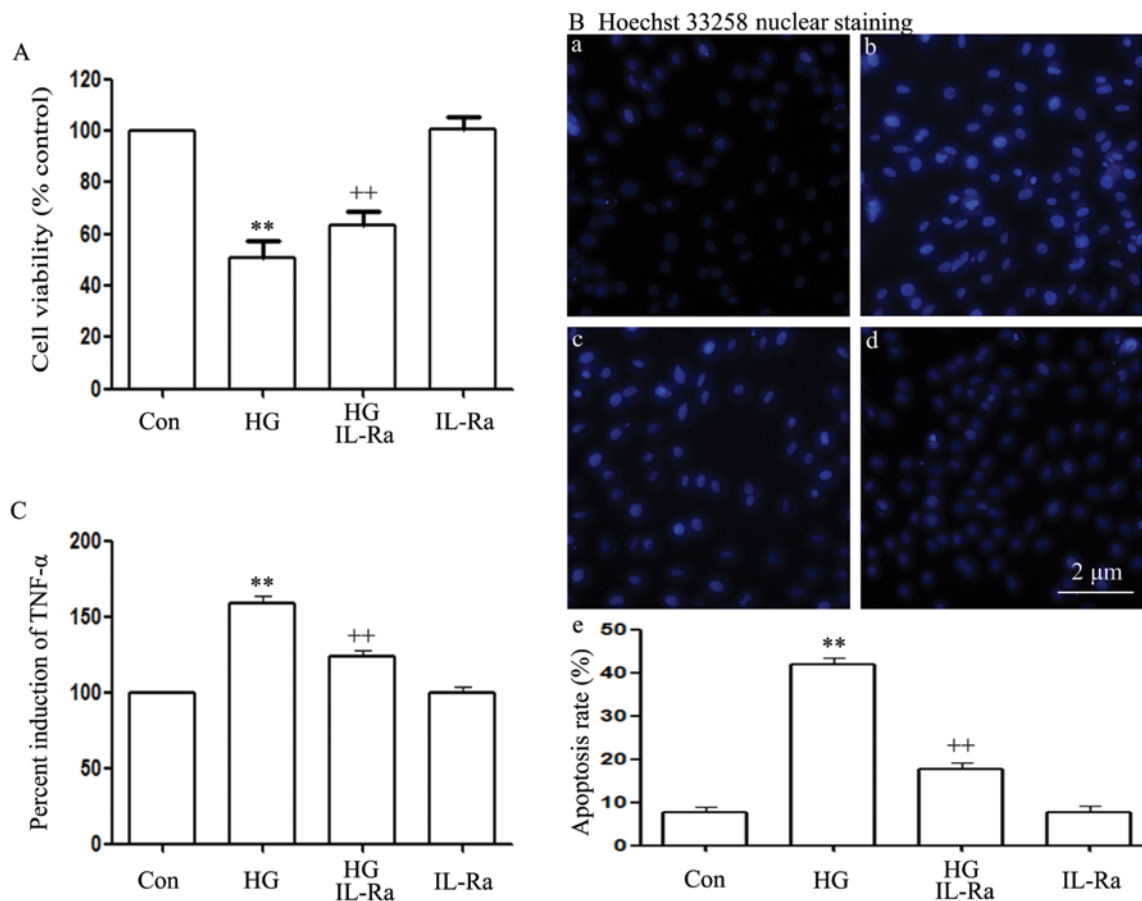
Figure 8. IL-1 receptor antagonist (IL-1Ra) attenuates high glucose (HG)-induced cytotoxicity, apoptosis and tumor necrosis factor-α (TNF-α) production in H9c2 cardiac cells. The cells were co-treated with 20 ng/ml IL-1Ra and 35 mM glucose for 24 h. (A) Cell viability was examined using the Cell Counting kit-8 (CCK-8) assay. (B, panels a-d) Hoechst 33258 nuclear staining followed by fluorescence imaging was used to detect cell apoptosis. (B, panel a) Control group. H9c2 cells were (B, panel b) treated with 35 mM glucose (HG) for 24 h; (B, panel c) co-treated with 20 ng/ml IL-1Ra and 35 mM glucose for 24 h; (B, panel d) treated with 20 ng/ml IL-1Ra for 24 h; (B, panel e) the apoptotic rate was analyzed using a cell counter and ImageJ 1.47i software. (C) The cells were treated with 35 mM glucose (HG) for 24 h in the absence or presence of co-treatment with 20 ng/ml IL-1Ra for 24 h. Enzyme-linked immunosorbent assay (ELISA) was performed to test the levels of TNF-α in cell supernatants. Data are presented as the means ± SEM (n=3). $^{**}$p<0.01 vs. the control (Con) group; $^{++}$p<0.01 vs. the HG-treated group.

NF-κB is a key transcription factor that regulates inflammatory processes (32). Previous studies have demonstrated that NF-κB is involved in cellular damage and inflammation induced by various stimuli, including chemical hypoxia (30), doxorubicin (14,15) and lipopolysaccharide (LPS) (33). NF-κB may participate in some of the downstream effects of NADH oxide on cardiac hypertrophy and it regulates the expression of inflammatory genes, such as TNF-α and IL-6 (34,35). Recently, the pathophysiological roles of the NF-κB pathway in diatetic complications, such as diabetic cardiomyopathy, have attracted considerable attention (17,31,36). In diabetic rats, NF-κB is activated (17,31,36). However, the roles of the NF-κB pathway in HG-induced cardiac injury and inflammation remain poorly understood. In order to explore this, in this study, we first observed the effects of HG on the activation of NF-κB in H9c2 cardiac cells. In agreement with the results of previous studies (17,31,36), it was shown that exposure to HG significantly induced an increase in p-NF-κB expression, suggesting the activation of the NF-κB pathway by HG. Secondly, we investigated the roles of the NF-κB activation in HG-induced injury. Our results demonstrated that pre-treatment of the H9c2 cardiac cells with PDTC (an

inhibitor of NF-κB) markedly suppressed HG-induced multiple injury, including cytotoxicity, apoptosis, oxidative stress and mitochondrial damage, as characterized by an increase in cell viability, as well as a decrease in the number of apoptotic cells, cleaved caspase-3 expression, ROS generation and the dissipation of MMP. These findings suggest that NF-κB activation is involved in HG-induced multiple damage in H9c2 cells. Since our (30), as well as other previous studies (37) have shown that ROS generation induces the activation of NF-κB in other cell types, the combination of the results of this study with those of other studies (30,38) suggests that there may be a crosstalk between the ROS and NF-κB pathway in HG-treated H9c2 cells. To confirm this hypothesis, further studies are required. Moreover, since data from diabetic patients (39,40) and diabetic animals (17,31,36) have demonstrated that hyperglycemia is associated with tissue inflammation, in this study, we examined the effects of NF-κB activation on pro-inflammatory factors. Similar to the results of previous studies (17,31,36,39,40), this study demonstrated that exposure to HG induced significant cardiac inflammatory responses, as indicated by the increased production of IL-1β, IL-6 and TNF-α. However, the increased production of IL-1β, IL-6 and TNF-α was reduced by PDTC,

suggesting the involvement of NF-κB activation in the HG-induced production of pro-inflammatory factors. These data provide clear evidence that the activation of the NF-κB pathway contributes to HG-induced injury and inflammation in H9c2 cardiac cells, which confirms and adds to the results of previous studies demonstrating the roles of NF-κB activation in hyperglycemia-induced cardiac damage and inflammation (41).

Another major finding of this study relates to a positive interaction between NF-κB and IL-1β which is critical for the induction of cardiomyocyte injury and inflammation induced by HG. This consideration is based on the results of a previous study demonstrating an interaction between NF-κB and IL-1β in aged murine lungs in response to endotoxic stress (42). IL-1 has been shown to play critical roles in the pathogenesis of heart disease (31,43). IL-1 binds to the transmembrane IL-1 type receptor (IL-1R) and initiates a downstream signal. Its natural antagonist, IL-1Ra, competitively interacts with the same receptor to prevent IL-1 signaling (44). Ehses *et al* demonstrated that IL-Ra reduces hyperglycemia and tissue inflammation in type 2 diabetic rats (31). Similarly, we observed the inhibitory effects of IL-1 Ra on the HG-induced cardiac cytotoxicity, apoptosis and inflammation. In addition, this study confirmed the contribution of NF-κB activation to the HG-induced cytotoxicity, apoptosis, oxidative stress, loss of MMP and inflammation. Therefore, we speculate that there may be an interaction between NF-κB and IL-1β in the induction of injury and inflammation in HG-exposed H9c2 cardiac cells. Significantly, our data demonstrate that PDTC inhibits the HG-induced production of IL-1β and that IL-1Ra attenuates the increased expression of p-NF-κB p65 subunit expression, which clearly confirms our hypothesis. These results provide novel mechanistic evidence for the roles of both NF-κB and IL-1β in the HG-induced cardiac injury and inflammation.

Another important finding of the present study relates to the roles of the inhibition of the NF-κB and IL-1β pathways in the cardioprotective effects of exogenous $H_2S$ against HG-induced multiple cardiac injury and inflammation. Accumulating evidence indicates that $H_2S$ exerts cardioprotective effects (4,5,15,18,21-24). $H_2S$ therapy offers protection against several models of myocardial injury in the setting of type 1 (25,29,45) or type 2 (4) diabetes by attenuating apoptosis and oxidative stress. Recently, we demonstrated that exogenous $H_2S$ alleviates cytotoxicity, apoptosis, oxidative stress and the loss of MMP by inhibiting the p38 MAPK, ERK1/2 and leptin pathways in HG-treated H9c2 cardiac cells (5,18). Since we have reported that exogenous $H_2S$ suppresses the p38 MAPK/NF-κB-mediated inflammatory response and cytotoxicity in doxorubicin-treated H9c2 cardiac cells (15), this study further explored whether $H_2S$ protects the cells against HG-induced injury and inflammation by inhibiting the NF-κB and IL-1β pathways. Our findings demonstrated that exogenous $H_2S$ markedly reduced NF-κB activation and IL-1β production. In addition, similar to the inhibitory effects of both PDTC and IL-1Ra as indicated above, pre-treatment with $H_2S$ markedly attenuated the HG-induced cardiac injury and inflammation. These results suggest that the inhibition of the NF-κB and IL-1β pathways may be a critical mechanism responsible for the protective effects of exogenous $H_2S$ against HG-induced cardiac injury and inflammation. Similarily, several recent studies have indicated that exogenous $H_2S$ reduces the chemical hypoxia-induced (30) or doxorubicin-induced (15) cell injury and inflammation through the inhibition of NF-κB and IL-1β. These studies (15,30) support our results.

In conclusion, the present study provides novel evidence that a positive interaction between the NF-κB and IL-1β pathways contributes to the HG-induced cardiac injury and inflammation and that exogenous $H_2S$ protects H9c2 cardiac cells against HG-induced injury and inflammation through the inhibition of the NF-κB and IL-1β pathways. These findings may aid in the development of novel therapeutic approaches for the prevention and treatment of hyperglycemia-related injury and inflammation.

## Acknowledgements

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