ATP-sensitive K^+ channels contribute to the protective effects of exogenous hydrogen sulfide against high glucose-induced injury in H9c2 cardiac cells

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Abstract. Hyperglycemia, as well as diabetes mellitus, has been shown to impair ATP-sensitive K^+ (K_{ATP}) channels in human vascular smooth muscle cells. Hydrogen sulfide (H_2S) is also known to be an opener of K_{ATP} channels. We previously demonstrated the cardioprotective effects exerted by H_2S against high-glucose (HG, 35 mM glucose)-induced injury in H9c2 cardiac cells. As such, we hypothesized that K_{ATP} channels play a role in the cardioprotective effects of H_2S against HG-induced injury. In this study, to examine this hypothesis, H9c2 cardiac cells were treated with HG for 24 h to establish a model of HG-induced insults. Our findings revealed that treatment of the cells with HG markedly decreased the expression level of K_{ATP} channels. However, the decreased expression of K_{ATP} channels was reversed by the treatment of the cells with 400 μM sodium hydrogen sulfide (NaHS, a donor of H_2S) for 30 min prior to exposure to HG. Additionally, the HG-induced cardiomyocyte injuries, including cytotoxicity, apoptosis, oxidative stress and mitochondrial damage, were ameliorated by treatment with NaHS or 100 μM diazoxide (a mitochondrial K_{ATP} channel opener) or 50 μM pinacidil (a non-selective K_{ATP} channel opener) for 30 min prior to exposure to HG, as indicated 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). Notably, treatment of the H9c2 cardiac cells with 100 μM 5-hydroxydecanoic acid (5-HD, a mitochondrial K_{ATP} channel blocker) or 1 mM glibenclamide (Gli, a non-selective K_{ATP} channel blocker) for 30 min prior to treatment with NaHS and exposure to HG significantly attenuated the above-mentioned cardioprotective effects exerted by NaHS. Notably, treatment of the cells with 500 μM N-acetyl-L-cysteine (NAC, a scavenger of ROS) for 60 min prior to exposure to HG markedly reduced the HG-induced inhibitory effect on the expression of K_{ATP} channels. Taken together, our results suggest that K_{ATP} channels play an important role in the cardioprotective effects of exogenous H_2S against HG-induced injury. This study also provides novel data demonstraring that there is an antagonistic interaction between ROS and K_{ATP} channels in HG-exposed H9c2 cardiac cells.

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Introduction

Hydrogen sulfide (H_2S), a well-known toxic gas with a characteristic smell of rotten eggs, has been previously described as an endogenously produced labile diffusible gasotransmitter which plays multiple roles in the cardiovascular system in general health and also in diseases (1-6). For example, in murine models of ischemia-induced heart failure, endogenous and exogenous H_2S clearly were shown to exert protective effects against left ventricular structural and functional impairment caused by ischemia-induced heart failure (7). Wang *et al* reported that H_2S attenuated ventricular dysfunction and arrested the progression of heart failure following myocardial infarction (MI) in a rat model (8). In addition, in relation to plasma H_2S levels in patients with coronary heart disease (CHD), a significant inverse correlation with the severity of CHD and changes in the

coronary artery has been noted (9). Previously, we demonstrated that exogenous H_2S protects H9c2 cardiomyocytes against chemical hypoxia- (10,11) or doxorubicin-induced (12-14) injury. The roles of H_2S in diabetes-related cardiovascular complications have attracted considerable attention, due to the following findings. First, lower circulating H_2S concentrations have been noted in animal models of diabetes (5,15,16) and patients with type 2 diabetes mellitus (DM) (5,6). Second, low blood H_2S levels may be associated with the vascular inflammation observed in diabetes since the supplementation of H_2S prevents the secretion of inflammatory factors by monocytes cultured in high-glucose (HG) medium (5). Third, exogenous H_2S protects against the development of HG-induced endothelial dysfunction (15). Fourth, exogenous H_2S alleviates myocardial ischemia/reperfusion (IR) injury in db/db mice (17). Fifth, H_2S has been shown to exert protective effects against myocardial I/R-induced damage in diabetic rats (18). Furthermore, our recent studies demonstrated that exogenous H_2S protects H9c2 cardiac cells against HG-induced injury and inflammation (19-21). Although we have reported that several factors, including antioxidant, anti-apoptotic and anti-inflammatory effects, mitochondrial protection, and the inhibition of certain intracellular signaling pathways, such as mitogen-activated protein kinase (MAPK) (19), leptin (20) and nuclear factor-κB (NF-κB) (21), contribute to the protective effects of H_2S against HG-induced cardiomyocyte injury, the mechanisms responsible for these cardioprotective effects of H_2S remain unclear. Since previous studies have indicated that H_2S activates ATP-sensitive K^+ (K_{ATP}) channels in both the heart (22,23) and vascular tissues (24) and that K_{ATP} channels are cardioprotective (23,25-29), we thus hypothesized that K_{ATP} channels are involved in the protective effects which H_2S exerts against HG-induced injury in H9c2 cardiomyocytes.

K_{ATP} channels are abundant in cardiac tissue (30). Cardiomyocytes contain K_{ATP} channels in both the sarcolemma [surface membrane (31)] and mitochondria (32,33). The opening of sarcolemmal K_{ATP} channels is associated with the shortening of cardiac action potential, and a decrease in intracellular Ca^{2+} loading and cardioprotection during ischemia (34-36). The opening of mitochondrial K_{ATP} channels contributes to the regulation of cardiac mitochondrial function (37) and cardioprotection induced by ischemic preconditioning (23,37,39). In addition, mitochondrial K_{ATP} channels ameliorate the apoptosis induced by oxidative stress in cardiac cells (26). Notably, previous research has revealed that DM is associated with the dysfunction of the cardiovascular K_{ATP} channels (40). Hyperglycemia, as well as DM, is harmful to the vasodilation mediated by K_{ATP} channels in human vascular smooth muscle cells (41-43). However, the roles of both sarcolemmal K_{ATP} channels and mitochondrial K_{ATP} channels in HG-induced cardiomyocyte injury, in particular, in relation to the cardioprotective effects of exogenous H_2S, remain unclear.

Based on our recent studies (19-21) and other previous studies (5,6,15,17,18,22-29), we hypothesized that H_2S exerts cardioprotective effects by modulating the activation of K_{ATP} channels in HG-treated cardiomyocytes. Therefore, the present study was designed to examine the following points: i) the effect of HG on the expression of cardiac K_{ATP} channels; ii) the roles of both sarcolemmal K_{ATP} channels and mitochondrial K_{ATP} channels in HG-induced cardiomyocyte injury; iii) whether exogenous H_2S protects cardiomyocytes against HG-induced injury by modulating K_{ATP} channel activity; and iv) the role of reactive oxygen species (ROS) in the inhibitory effect of HG on the expression of cardiac K_{ATP} channels.

Materials and methods

Materials and reagents. Anti-kir6.1 (R-14) antibody (sc-11224) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); anti-cleaved caspase-3 antibody (#9662) was purchased from Cell Signaling Technology, Inc. (Boston, MA, USA); anti-GAPDH antibody (10494-1-AP) was purchased from Proteintech Group, Inc. (Wuhan, China); horseradish peroxidase (HRP) conjugated secondary antibody and the BCA Protein assay kit were obtained from KangChen Biotech (Shanghai, China). Diazoxide (DZ), pinacidil (Pin), 5-hydroxydecanoic acid (5-HD) and glibenclamide (Gli) were all purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Sodium hydrosulfide (NaHS; a donor of H_2S) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and was protected from sunlight and stored at 2-4˚C. The cell counting kit-8 (CCK-8) was supplied by Dojindo Laboratories (Kumamoto, Japan). 2',7'-Dichlorofluorescein diacetate (DCFH-DA), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), Hoechst 33258 and N-acetyl-L-cysteine (NAC) were all purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) medium were obtained from Gibco-BRL (Grand Island, NY, USA). The enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech (Nanjing, China). The H9c2 cardiac cells were supplied by the Sun Yat-sen University Experimental Animal Center (Guangzhou, China).

Cell culture and treatment. The H9c2 cardiac cells, a rat cardiac myoblast cell line, were cultured in DMEM, supplemented with 10% FBS in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C. The culture medium was replaced with fresh medium every 2-3 days and expanded to new culture plates when the cells reached approximately 80% confluence.

To investigate the role of K_{ATP} channels in HG-induced cardiomyocyte injury, the H9c2 cardiac cells were treated with 100 μM DZ (a mitochondrial K_{ATP} channel opener) or 50 μM Pin (a non-selective K_{ATP} channel opener) for 30 min prior to exposure to 35 mM glucose for 24 h. To explore the protective effects of H_2S on HG-induced injury, the H9c2 cells were conditioned with 400 μM NaHS for 30 min prior to exposure to HG for 24 h. To further determine whether the protective effects of NaHS were associated with the activation of K_{ATP} channels, the cells were conditioned with 100 μM 5-HD (a mitochondrial K_{ATP} channel blocker) or 1 mM Gli (a non-selective K_{ATP} channel blocker) for 30 min prior to treatment with NaHS and 35 mM glucose for 24 h. To confirm whether there is an antagonistic interaction between ROS and K_{ATP} channels, the H9c2 cells were treated with 500 μM NAC (a scavenger of ROS) for 60 min prior to exposure to HG.

Cell viability assay. The H9c2 cells were seeded in 96-well plates at a concentration of $1x10^4$ cells/ml and incubated at 37˚C. CCK-8 assay was employed to assess the viability of the

cells. After being subjected to the above-mentioned treatments, the cells were washed with phosphate-buffered saline (PBS), and 10 μl CCK-8 solution at 10% dilution was added to each well, and the plate was then incubated for approximately 2 h in an incubator. The 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 assessment of apoptosis. Apoptotic cell death was assessed using the Hoechst 33258 staining method followed by photofluorography. The H9c2 cells were plated in 35-mm dishes at a density of $1x10^6$ cells/well. After being subjected to the indicated treatments, the cells were harvested and fixed with paraformaldehyde in 0.1 mol/l PBS (pH 7.4) for 10 min. The slides were then washed 5 times with PBS. After rinsing with PBS, the nuclear DNA was stained with 5 mg/ml Hoechst 33258 for 10 min before being rinsed briefly with PBS and then visualized under a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan). The viable H9c2 cells exhibited a uniform blue fluorescence throughout the nucleus, whereas the apoptotic cells had fragmented and condensed nuclei. The experiment was carried out 3 times.

Measurement of intracellular ROS levels. The determination of intracellular ROS levels was performed by measuring the fluorescence product formed by the oxidation of DCFH-DA, as prevoiusly described (19). Briefly, the culture medium was removed and the cells were then washed 3 times with PBS. Following the addition of fresh culture medium, the cells were incubated with DCFH-DA at a final concentration of 10 μM, for 30 min at 37˚C. The cells were then washed 5 times with PBS, and the relative amount of fluorescence product was assessed 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 for the amount of ROS. The experiment was carried out 5 times.

Measurement of mitochondrial membrane potential (MMP). As previousy described (19), MMP was assessed using a fluorescent dye, JC-1, which is a cell-permeable cationic dye that enters the mitochondria based on a highly negative MMP. The depolarization of MMP results in the loss of JC-1 from the mitochondria and a decrease in intracellular green fluorescence. The H9c2 cardiac cells were cultured on a slide with Eagle's minimal essential medium (EMEM). After being subjected to the indicated treatments, the slides were washed 3 times with PBS. The H9c2 cells were incubated with 1 mg/l JC-1 at 37˚C for 30 min in an incubator and washed 3 times with PBS. JC-1 fluorescence was then measured over the entire 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 was taken as an index of the levels of MMP. The experiment was carried out 5 times.

Western blot analysis. After being subjected to the indicated treatments, the H9c2 cardiac cells were harvested and lysed with cell lysis solution at 4˚C for 30 min. Total proteins in the cell lysates were quantified using a BCA protein assay kit. Loading buffer was added to the cytosolic extracts and, after boiling for approximately 5 min, equal amounts of supernatant from each sample were fractionated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total proteins in the gel were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for approximately 90 min at room temperature in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T) containing 5% fat-free milk] and then incubated with either anti-K_{ATP} (1:1,000 dilution), or anti-cleaved caspase-3 antibody (1:1,000 dilution) in freshly prepared TBS-T with 3% fat-free milk overnight with slow agitation at 4˚C. Following 3 washes with TBS-T, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,500 dilution; KangChen Bio-tech) in TBS-T with 3% fat-free milk for 90 min at room temperature. GAPDH was used as an internal control. The membranes were then washed 3 times with TBS-T solution for 15 min. The immunoreative 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. Each experiment was repeated 3 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 13.0 software (SPSS, Inc., Chicago, IL, USA) followed by the least significant difference (LSD) post hoc comparison test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

NaHS attenuates the HG-induced decrease in protein expression of K_{ATP} channels in H9c2 cardiac cells. In order to investigate the influence of HG (35 mM glucose) on the protein expression of K_{ATP} channels in H9c2 cardiac cells, a time-response experiment to determine the protein expression levels of K_{ATP} channels was performed. As shown in Fig. 1A and B, the cells were exposed to HG for 1, 3, 6, 9, 12 and 24 h, respectively. Following exposure to HG for 6 h, the protein expression level of K_{ATP} channels began to decrease, and the maximum decrease in expression levels was observed after the cells were exposed to HG for 12 and 24 h. Based on these results, the expression levels of K_{ATP} channels were detected at 12 h following exposure to HG in the subsequent experiments.

It is important to note that the decrease in the K_{ATP} channel levels was ameliorated by treatment with 400 μM NaHS (a donor of H_2S) for 30 min prior to exposure to HG for 12 h (Fig. 1C and D). However, the basal expression level of K_{ATP} channels was not markedly altered by treatment with 400 μM NaHS alone for 30 min. These data indicate that exogenous H_2S alleviates the decrease in the protein expression levels of K_{ATP} channels induced by HG in H9c2 cardiac cells.

K_{ATP} channels are involved in the protective effects which H_2S exerts against HG-induced cytotoxicity to H9c2 cardiac cells. Consistent with our recent studies (19-21), treatment of the cells

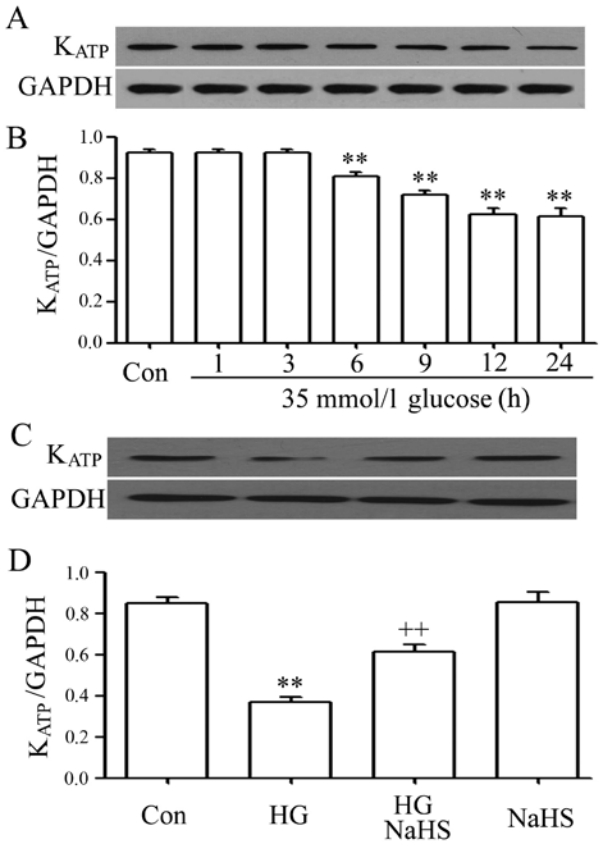

Figure 1. Sodium hydrogen sulfide (NaHS) reduces the high-glucose (HG)-induced decrease in the protein expression levels of ATP-sensitive potassium (K_{ATP}) channels in H9c2 cardiac cells. (A and C) K_{ATP} channel protein expression levels were semi-quantified by western blot analysis. (A and B) Time course of changes observed in K_{ATP} channel expression levels induced by HG (35 mM glucose) over a 24-h time period. (C and D) H9c2 cardiac cells were exposed to HG for 12 h with or without treatment with 400 μM NaHS for 30 min prior to exposure to HG. (B and D) Densitometric analysis of the K_{ATP} channel expression levels in (A and C). Data are represented as the means ± SEM (n=3). **P<0.01 vs. control (Con) group; $^{++}$P<0.01 vs. the HG-treated group.

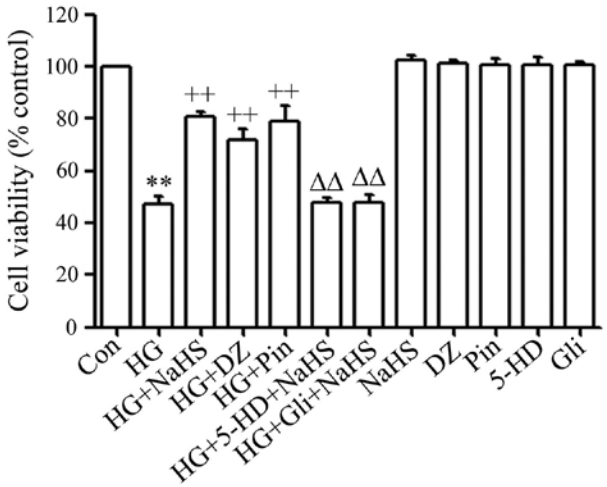

Figure 2. Effects of different treatments on the high-glucose (HG)-induced cytotoxicity in H9c2 cardiac cells. The cells were treated with 35 mM glucose for 24 h with or without pre-treatment with 400 μM sodium hydrogen sulfide (NaHS) or 100 μM diazoxide (DZ) or 50 μM pinacidil (Pin) or 100 μM 5-hydroxydecanoic acid (5-HD) or 1 mM glibenclamide (Gli) for 30 min. Cell viability was detected using the CCK-8 assay. Data are presented as the means ± SEM (n=6). **P<0.01 vs. control (Con) group; $^{++}$P<0.01 vs. the HG-treated group; $^{\Delta\Delta}$P<0.01 vs. the NaHS + HG-treated group.

with 400 μM NaHS for 30 min prior to exposure to HG for 24 h significantly inhibited HG-induced cytotoxicity, leading to an increase in cell viability (Fig. 2). To explore the role of K_{ATP} channels in HG-induced cytotoxicity, the cells were treated with 100 μM DZ (a mitochondrial K_{ATP} channel opener) or 50 μM Pin (a non-selective K_{ATP} channel opener) for 30 min prior to exposure to HG. As shown in Fig. 2, pre-treatment of the H9c2 cardiac cells with DZ or Pin considerably reduced HG-induced cytotoxicity, as evidenced by an increase in cell viability. To further investigate the role which K_{ATP} channels play in the protective effects exerted by H_2S against HG-induced cytotoxicity, the cells were treated with 100 μM 5-HD (a mitochondrial K_{ATP} channel blocker) or 1 mM Gli (a non-selective K_{ATP} channel blocker) for 30 min prior to treatment with NaHS and exposure to HG. It was demonstrated that the blockade of K_{ATP} channels with 5-HD or Gli markedly reduced the protective effects of NaHS against HG-induced cytotoxicity, resulting in a decrease in cell viability (Fig. 2). Alone, 5-HD and Gli did not significantly alter cell viability. These data suggest that K_{ATP} channels mediate the protective effects of H_2S against cytotoxicity to H9c2 cardiac cells induced by HG.

K_{ATP} channels are involved in the protective effects which H_2S exerts against HG-induced apoptosis in H9c2 cardiac cells. In agreement with our recent studies (19-21), exposure of the cells to HG for 24 h markedly increased the number of apoptotic cells (Fig. 3A, panel b), leading to an increase in the percentage of apoptotic cells (Fig. 3A, panel m). The increased number of apoptotic cells was decreased by pre-treatment with NaHS, Pin or DZ (Fig. 3A, panels c, d and e). Similarly, exposure of the cells to 35 mM glucose for 24 h markedly enhanced the expression level of cleaved caspase-3 (Fig. 3B). However, the increased expression level of cleaved caspase-3 was attenuated by treatment with 400 μM NaHS or 50 μM Pin or 100 μM DZ for 30 min prior to exposure to HG for 24 h (Fig. 3B, panels a to d). Furthermore, treatment of the H9c2 cardiac cells with 100 μM 5-HD (Fig. 3A, panel f and B, panels c and d) or 1 mM Gli (Fig. 3A, panel g and B, panels c and d) for 30 min prior to treatment with NaHS and exposure to HG blocked the above-mentioned anti-apoptotic effects of NaHS, as evidenced by the increase in the percentage of apoptotic cells (Fig. 3A, panels f and g) and the increase in cleaved caspase-3 expression (Fig. 3B, panels c and d). Alone, NaHS, Pin, DZ, 5-HD and Gli did not significantly affect the percentage of apoptotic cells or the basal expression level of cleaved caspase-3.

K_{ATP} channels are implicated in the protective effects exerted by H_2S against HG-induced oxidative stress in H9c2 cardiac cells. In agreement with our recent findings (19-21), treatment of the cells with 35 mM glucose for 24 h significantly increased the intracellular production of ROS (Fig. 4B and M). The increased ROS production was ameliorated by treatment with 400 μM NaHS for 30 min prior to exposure to HG for 24 h (Fig. 4C and M). Similarly, treatment with 50 μM Pin (Fig. 4D and M) or 100 μM DZ (Fig. 4E and M) for 30 min prior to exposure to HG for 24 h also attenuated the generation of ROS. To confirm the role played by K_{ATP} channels in

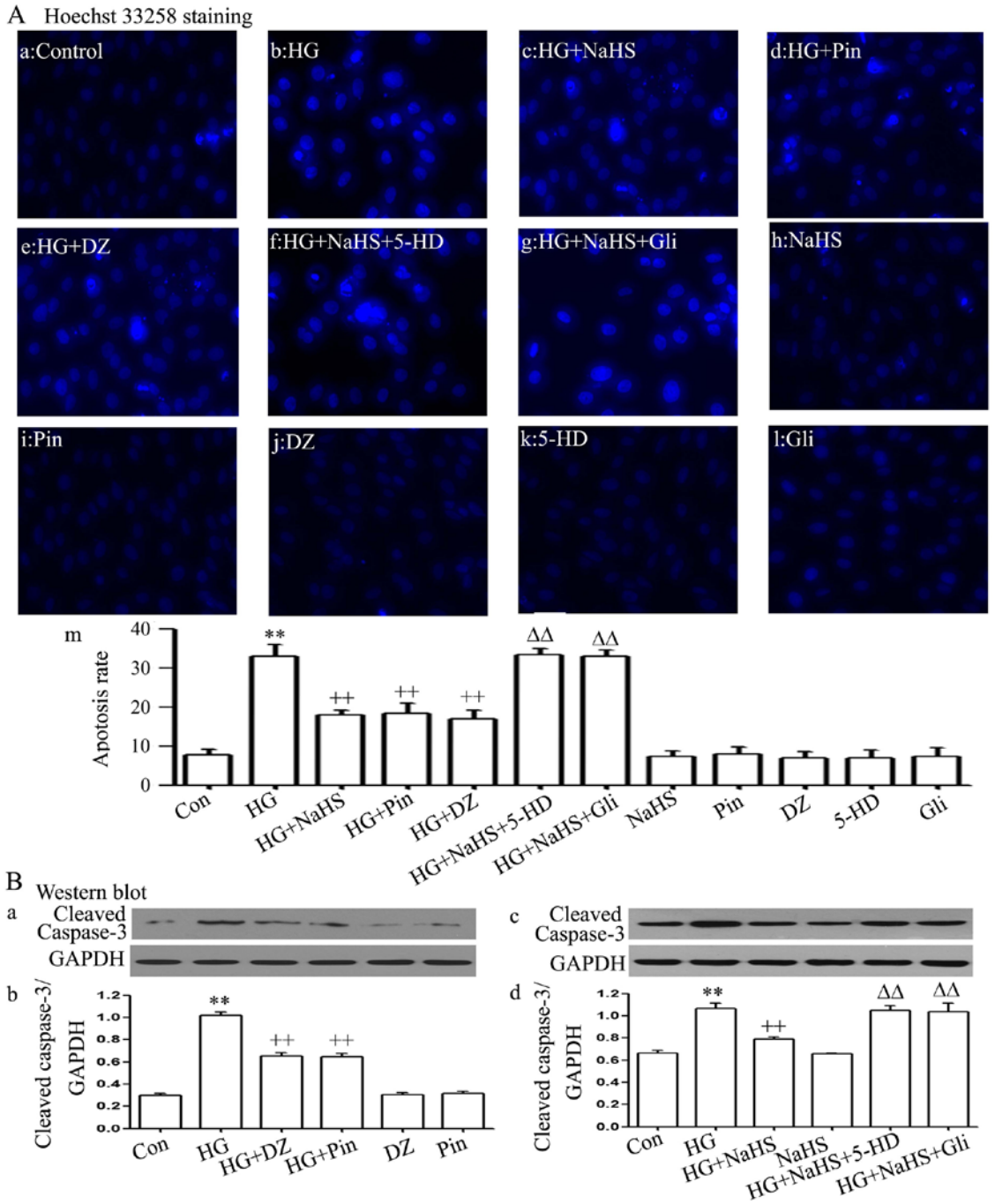

Figure 3. Role of ATP-sensitive K^+ (K_{ATP}) channels in the protective effects of H_2S against the high-glucose (HG)-induced apoptosis in H9c2 cardiac cells. (A) Hoechst 33258 nuclear stating followed by fluorescence imaging was performed to examine cell apoptosis. (B) The expression level of cleaved caspase-3 was semi-quantified by western blot analysis. (B, panels b and d) Densitometric analysis of the cleaved caspase-3 expression level in (B, panels a and c). (A, panel a) Control group. H9c2 cells were (panel b) treated with 35 mM glucose (HG) for 24 h; (panel c) treated with 400 μM sodium hydrogen sulfide (NaHS) or (panel d) 50 μM pinacidil (Pin) or (panel e) 100 μM diazoxide (DZ) for 30 min prior to exposure to HG; (panel f) treated with 100 μM 5-hydroxydecanoic acid (5-HD) or (panel g) 1 mM glibenclamide (Gli) for 30 min prior to treatment with NaHS and exposure to HG; (panel h) treated with 400 μM NaHS or (panel i) 50 μM Pin or (panel j) 100 μM DZ for 30 min followed by 24 h of culture; (panel k) treated with 100 μM 5-HD or (panel l) 1 mM Gli for 30 min followed by 24 h of culture. (m) The apoptosis rate was analyzed using a cell counter and ImageJ 1.47i software. Data are presented as the means ± SEM (n=3). **P<0.01 vs. control (Con) group; $^{++}$P<0.01 vs. the HG-treated group; $^{\Delta\Delta}$P<0.01 vs. the NaHS + HG-treated group.

the protective effects of NaHS against HG-induced oxidative stress, the cells were treated with 100 μM 5-HD or 1 mM Gli for 30 min prior to exposure to NaHS and HG. Our data indicated that pre-treatment with 5-HD (Fig. 4F and M) or Gli (Fig. 4G and M) blocked the inhibitory effects which NaHS exerted on the generation of ROS induced by HG, suggesting that the K_{ATP} channels contribute to the protective effects which H_2S exerts against the HG-induced overproduction of ROS.

K_{ATP} channels are linked to the protective effects of H_2S against HG-induced mitochondrial insults in H9c2 cardiac cells. Consistent with our recent studies (19-21), treatment of the cells

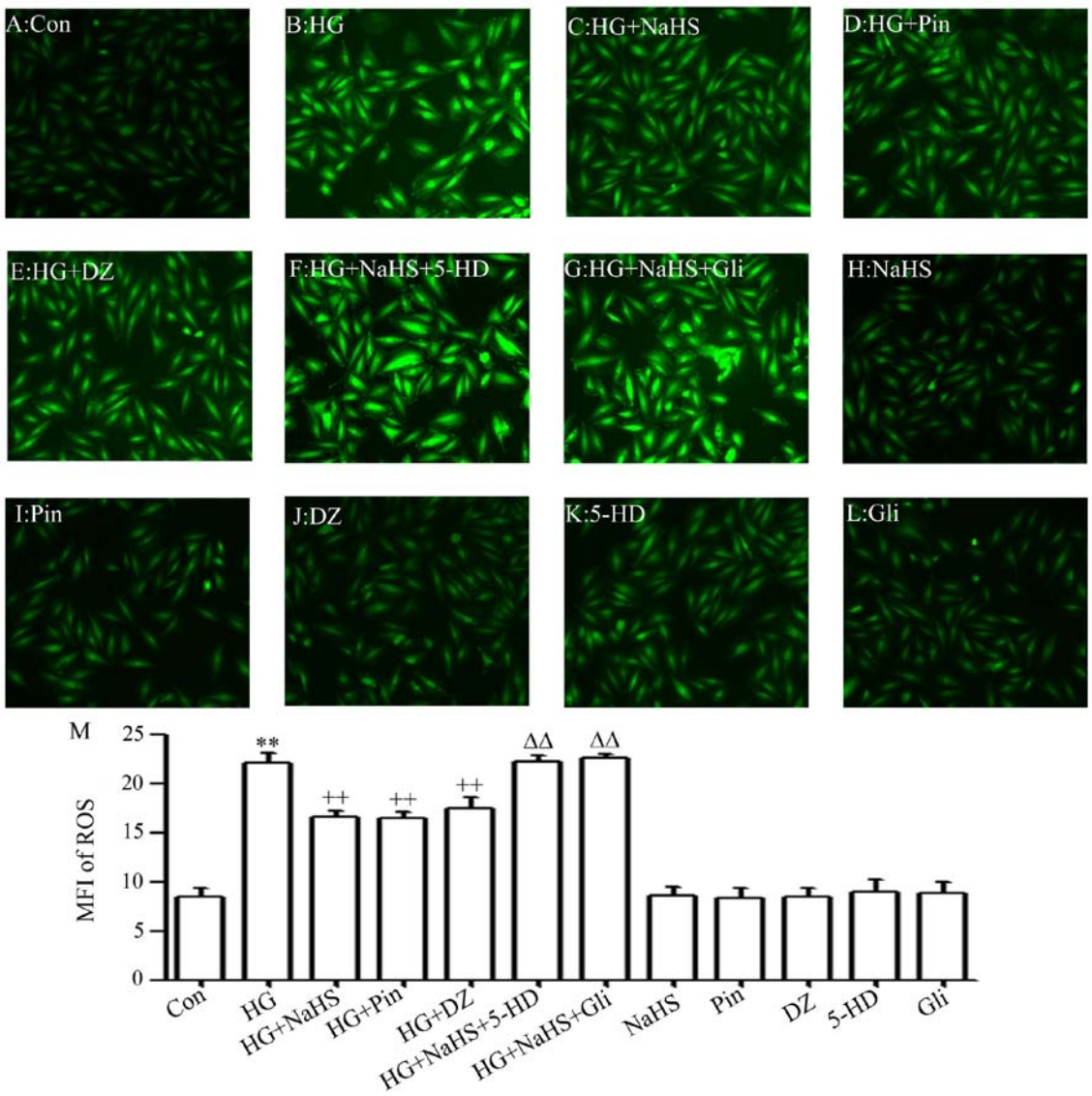

Figure 4. ATP-sensitive K^+ (K_{ATP}) channels contribute to the inhibitory effecta of H_2S on the high-glucose (HG)-induced reactive oxygen species (ROS) generation in H9c2 cardiac cells. (A-L) After the cells were subjected to the indicated treatments, intracellular ROS generation was measured by 2'7'-dichlorodihydrofluoresein diacetate (DCFH-DA) staining followed by photofluorography. (A) Control group. H9c2 cardiac cells were (B) treated with 35 mM glucose (HG) for 24 h; (C) treated with 400 μM sodium hydrogen sulfide (NaHS) or (D) 50 μM pinacidil (Pin) or (E) 100 μM diazoxide (DZ) for 30 min prior to exposure to HG; (F) treated with 100 μM 5-hydroxydecanoic acid (5-HD) or (G) 1 mM glibenclamide (Gli) for 30 min prior to treatment with NaHS and exposure to HG; (H) treated with 400 μM NaHS or (I) 50 μM Pin or (J) 100 μM DZ for 30 min followed by 24 h of culture; (K) treated with 100 μM 5-HD or (L) 1 mM Gli for 30 min followed by 24 h of culture. (M) Quantitive analysis mean fluorescence intensity (MFI) in (A-L) using ImageJ 1.47i software. Data are presented as the means ± SEM (n=5). **P<0.01 vs. control (Con) group; $^{++}$P<0.01 vs. the HG-treated group; $^{\Delta\Delta}$P<0.01 vs. the NaHS + HG-treated group.

with 35 mM glucose for 24 h markedly induced mitochondrial damage, as evidenced by the loss of MMP (Fig. 5A, panel b and 5M). In addition, we noted that the HG-induced decrease in MMP was reversed by treatment of the cells with 400 μM NaHS for 30 min prior to exposure to HG (Fig. 5A, panel c and 5M). Notably, treatment of the cells with 50 μM Pin (Fig. 5A, panel d and 5M) or 100 μM DZ (Fig. 5A, panel e and 5M) for 30 min prior to exposure to HG also blocked the HG-induced loss of MMP. Subsequently, we further explored the role of K_{ATP} channels in the protective effects of NaHS against the dissipation of MMP induced by HG. Our findings revealed that treatment with 100 μM 5-HD or 1 mM Gli for 30 min prior to treatment with NaHS and exposure to HG considerably reduced the inhibitory effects of NaHS on the HG-induced dissipation of MMP (Fig. 5A, panels f and g and 5M). These results demonstrate that K_{ATP} channels play a critical role in the protective effects of H_2S against the HG-induced mitochondrial damage.

ROS scavenger blocks the HG-induced downregulation of the expression of K_{ATP} channels in H9c2 cardiac cells. Since hyperglycemia, as well as DM, has been reported to impair K_{ATP} channels in human vascular smooth muscle cells via ROS (41-43), we investigated the role of ROS in the HG-induced decrease in K_{ATP} channel expression in the H9c2 cardiac cells. As shown in Fig. 6, treatment of the cells with 500 μM NAC (a scavenger of ROS and NAC) for 60 min prior to exposure to 35 mM glucose for 12 h blocked the inhibitory effects of HG on the expression levels of K_{ATP} channels. Alone, NAC did not

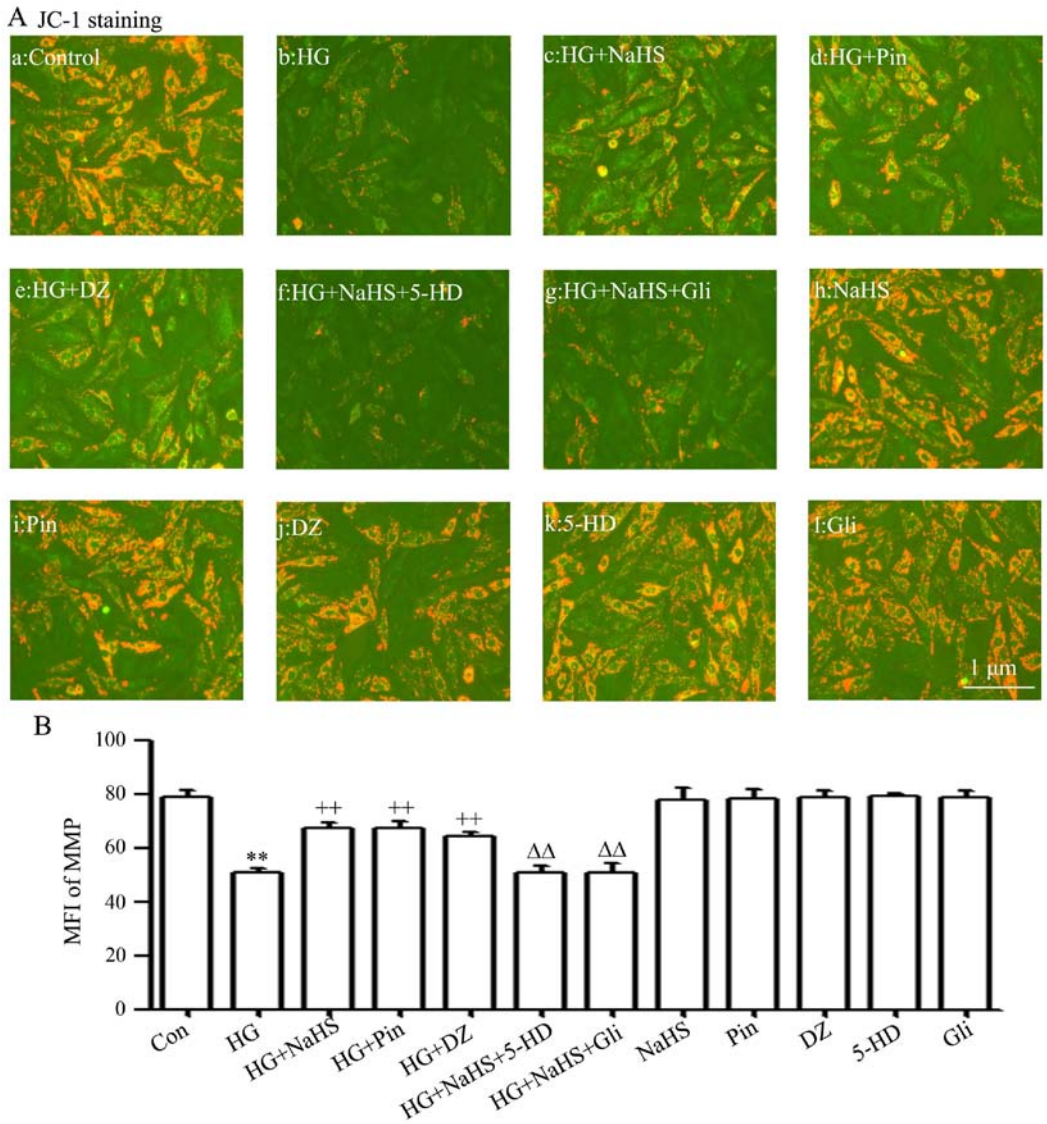

Figure 5. ATP-sensitive K^+ (K_{ATP}) channels mediate the protective effects of H_2S against the high-glucose (HG)-induced dissipation of mitochondrial membrane potential (MMP) in H9c2 cardiac cells. (A) After the cells were subjected to the indicated treatments, MMP was examined using the lipophilic cationic probe, JC-1, followed by photofluorography. (Panel a) Control group. H9c2 cardiac cells were (panel b) treated with 35 mM glucose (HG) for 24 h; (panel c) treated with 400 μM sodium hydrogen sulfide (NaHS) or (panel d) 50 μM pinacidil (Pin) or (panel e) 100 μM diazoxide (DZ) for 30 min prior to exposure to HG; (panel f) treated with 100 μM 5-hydroxydecanoic acid (5-HD) or (panel g) 1 mM glibenclamide (Gli) for 30 min prior to treatment with NaHS and exposure to HG; (panel h) treated with 400 μM NaHS or (panel i) 50 μM Pin or (panel j) 100 μM DZ for 30 min followed by 24 h of culture; (panel k) treated with 100 μM 5-HD or (panel l) 1 mM Gli for 30 min followed by 24 h of culture. (B) Quantitive analysis of the ratio of red/green fluorescence intensity, in (A) using ImageJ 1.47i software. Data are presented as the means ± SEM (n=5). **P<0.01 vs. control (Con) group; $^{++}$P<0.01 vs. the HG-treated group; $^{\Delta\Delta}$P<0.01 vs. the NaHS + HG-treated group.

significantly alter the basal expression level of K_{ATP} channels. These results suggest that ROS participates in the HG-induced downregulation of the expression of K_{ATP} channels.

Discussion

Cardiac K_{ATP} channels are key sensors and effectors of the metabolic status of cardiomyocytes, and their roles in HG-induced cardiomyocyte injury and in the cardioprotective effects of H_2S are noteworthy. The major findings of this study can be summarized as follows: i) HG markedly downregulated the expression levels of cardiac K_{ATP} channels; ii) exogenous H_2S attenuated the inhibitory effects of HG on the expression of cardiac K_{ATP} channels; iii) the K_{ATP} channel openers, DZ (a mitochondrial K_{ATP} channel opener) and Pin (a non-selective K_{ATP} channel openner), ameliorated the HG-induced cardiomyocyte injury, including cytotoxicity, apoptosis, ROS generation and the dissipation of MMP; iv) the K_{ATP} channel antagonists, namely 5-HD (a mitochondrial K_{ATP} channel antagonist) and Gli (a non-selective K_{ATP} channel antagonist), blocked the cardioprotective effects of exogenous H_2S against the HG-induced cardiomyocyte injury; v) the ROS scavenger, NAC, reduced the inhibition of cardiac K_{ATP} channel expression induced by HG. These results strongly suggest that the impairment of K_{ATP} channels is implicated in HG-induced cardiomyocyte injury and that the activation of K_{ATP} channels is linked to the protective effects which exogenous H_2S exerts against HG-induced cardiomyocyte injury.

K_{ATP} channels have the unique ability to regulate membrane excitability in response to changes in the energetic status of

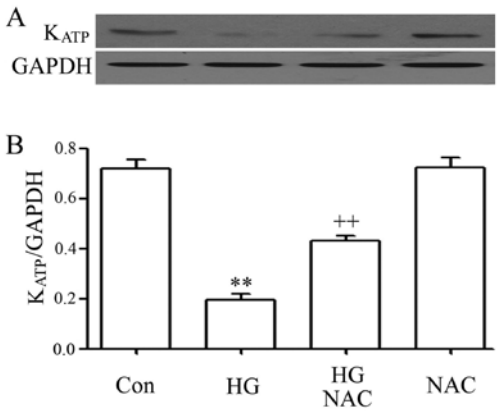

Figure 6. Reactive oxygen species (ROS) is implicated in the high glucose (HG)-induced decrease in the expression level of ATP-sensitive K^+ (K_{ATP}) channels in H9c2 cardiac cells. The cells were exposed to 35 mM glucose (HG) for 12 h with or without treatment with 500 μM NAC (a scavenger of ROS) for 60 min prior to exposure to HG. (A) K_{ATP} channel expression was semi-quantified by western blot analysis. (B) Densitometric analysis of the expression levels of K_{ATP} channel in (A). Data are represented as the means ± SEM (n=3). **P<0.01 vs. control (Con) group; $^{++}$P<0.01 vs. the HG-treated group.

cells (31,44,45). This ability serves to decrease myocardial energy consumption and vulnerability to stress (44,45). Previous research has indicated that the ability of cardiac K_{ATP} channels to affect cellular excitability and function depends on their abundance at the membrane surface (46,47). For example, an increase in cardiac sarcolemmal K_{ATP} channels enhances the speed and degree of shortening of action potentials and reduces cardiac energy consumption in response to escalating workloads (46). Several studies have indicated a correlation between dysfunction in K_{ATP} channel gating and insulin secretory disorders (40,48), such as neonatal diabetes (48). Furthermore, in human vascular smooth muscle cells, HG impairs vasorelaxation by inhibiting the activity of K_{ATP} channels (41-43). However, our knowledge of the roles of K_{ATP} channels in HG-induced cardiac cells remains incomplete.

In order to explore this issue, in the present study, we first investigated the effects of HG on the expression levels of K_{ATP} channels in H9c2 cardiac cells. Our data demonstrated that the exposure of the cells to HG markedly reduced the expression levels of K_{ATP} channels. This reduced expression level of K_{ATP} channels indirectively suggests a decrease in their presence in the HG-treated cardiac cells. Since a previous study indicated that a reduction in the number of sarcolemmal K_{ATP} channels slows cardiac action potential during shortening under hypoxia (47),. Thus, we hypothesized that the inhibition of cardiac K_{ATP} channels is a critical mechanism which underlies HG-induced cardiomyocyte injury. To confirm this hypothesis, we observed the influence of K_{ATP} channel activation on HG-induced injury. In agreement with our recent studies (19-21), the findings of this study demonstrated that treatment of the H9c2 cardiac cells with HG induced considerable injuries, including a decrease in cell viability, an increase in apoptotic cells, cleaved caspase-3 expression and ROS generation, as well as the loss of MMP. However, treatment of the H9c2 cardiac cells with DZ (a mitochondrial K_{ATP} channel opener) or Pin (a non-selective K_{ATP} channel opener) markedly attenuated the above-mentioned HG-induced injuries, as evidenced by an increase in cell viability, and a decrease in the number of apoptotic cells, decreased cleaved caspase-3 expression and ROS generation, as well as the loss of MMP. The above-mentioned results suggest that HG impairs the function of cardiac K_{ATP} channels, which contributes to HG-induced injuries. In support of our results are experimental studies which demonstrate that hyperglycemia damages the functionality of the human ether-a-go-go-related gene (HERG) K^+ channels, reduces the transient outward K^+ current, enhances the intracellular concentration of $Ca2^+$, and impairs the excitation-contraction coupling in the heart (49,50). It has also been noted that the opening of the mitochondrial K_{ATP} channels plays an important role in the regulation of cardiac mitochondrial function (37) and attenuates the oxidative stress-induced apoptosis of cardiac cells.

Of note, sulfonylurea drugs have been shown to stimulate insulin secretion by closing K_{ATP} channels (51,52). Despite the fact that their target is not completely clear, these drugs have been used to treat type 2 DM since the 1950s and they are still used today. Thus, the findings of this study drive us to explore the effects of sulfonylurea drugs on HG-induced cardiomyocyte injury in future experiments.

Another important result of this study relates to the role of the activation of K_{ATP} channels in the cardioprotective effects of exogenous H_2S against HG-induced cardiac injuries. Previously, the protective effects of H_2S on DM-related cardiovascular insults have received much attention. Exogenous H_2S attenuates I/R-induced injury in db/db mice (17) and diabetic rats (18). Our recent studies have also examined the protective effects of exogenous H_2S against HG-induced injury and inflammation in H9c2 cardiac cells (19-21). The mechanisms responsible for these cardioprotective effects of H_2S are associated with the inhibition of the MAPK (19), leptin (20) and NF-κB (21) pathways. However, the protective mechanisms of H_2S are complex, and other factors are likely involved in these cardioprotective effects of H_2S. Since the K_{ATP} channels in the heart (22,23) have been reported to be activated by H_2S, this study further investigated that roles K_{ATP} channels play in the protective effects which H_2S exerts against HG-induced cardiomyocyte injury. In agreement with our recent studies, the findings of this study demonstrated that exogenous H_2S exerted protective effects against HG-induced cardiomyocyte injuries, as indicated by an increase in cell viability, and a decrease in the number of apoptotic cells, decreased cleaved caspase-3 expression and ROS generation, as well as the loss of MMP. Notably, our results demonstrated that exogenous H_2S markedly reduced the downregulation of K_{ATP} channel expression by HG and that both 5-HD (a mitochondrial K_{ATP} channel blocker) and Gli (a non-selective K_{ATP} channel blocker) blocked the cardioprotective effects of H_2S mentioned above. These results suggest that K_{ATP} channels, in particular mitochondrial K_{ATP} channels, play a critical role in the cardioprotective effects of H_2S. Similarly, Zhao *et al* demonstrated the protective effects which H_2S exerts against arrhythmia by opening mitochondrial K_{ATP} channels (24). However, Bian *et al* (23) revealed that sarcolemmal K_{ATP} channels, but not mitochondrial K_{ATP} channels, mediate the cardioprotective effects of H_2S in isolated cardiac myocytes exposed to simulated ischemia solution. Therefore, the roles of subtypes of K_{ATP} channels involved in the cardioprotective effects of H_2S in the present study differ from the ones reported by Bian *et al* (23), although we did not

use a sarcolemmal K_{ATP} channels blocker or MCC-134 that opens sarcolemmal K_{ATP} channels and blocks minochondrial K_{ATP} channels. One possible explanation for this difference in the results may lie in the use of different experimental models.

In the present study, we also investigated the interaction between ROS and cardiac K_{ATP} channel activation. As aforementioned, both DZ and Pin ameliorated HG-induced ROS generation, suggesting that K_{ATP} channel activity had an inhibitory effect on ROS generation. A previous study reporting that K_{ATP} channel opener (DZ) reduces mitochondrial ROS production at reoxygenation (53) supports our results. On the other hand, we noted that NAC, a ROS scavenger, blocked the decrease in the expression of K_{ATP} channel protein induced by HG. Collectively, these results suggest that there is an interaction between ROS and K_{ATP} channels in HG-treated cardiac cells. The elucidation of the molecular mechanism underlying this interaction may be significant for the treatment and prevention of DM-related cardiovascular complications.

In conclusion, the present study provides novel evidence that the impairment of K_{ATP} channels is associated with HG-induced multiple cardiac injuries, including cytotoxicity, apoptosis, oxidative stress and mitochondrial damage. Therefore, the inhibition of cardiac K_{ATP} channels by insulin secretagogues should be considered to increase cardiac risk. Importantly, the present study has demonstrated that exogenous H_2S protects cardiomyocytes against HG-induced injuries by activating K_{ATP} channels. Based on the results of the present study and previous studies (5,6,15,16), we suggest that low levels of endogenous H_2S and the impairment of K_{ATP} channels are an important pathophysiological mechanism underlying huperglycemia-induced cardiovascular complications. Therefore, supplementation with H_2S and modulation of the H_2S-K_{ATP} channel pathway should be considered a potential approach to attenuating hyperglycemia-induced cardiomyocyte injury.

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