Original Paper

Exogenous Hydrogen Sulfide Attenuates High Glucose-Induced Cardiotoxicity by Inhibiting NLRP3 Inflammasome Activation by Suppressing TLR4/NF- κ B Pathway in H9c2 Cells

Zena Huang^a Xiaodong Zhuang^a Chuli Xie^b Xun Hu^a Xiaobian Dong^a Yue Guo^a Shaofen Li^c Xinxue Liao^a

^aDepartment of Cardiology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou,

^bDepartment of Physiology, Zhongshan School of Medicine, SunYat-sen University, Guangzhou,

^cDepartment of Laboratory, Liwan District Chinese Medical Hospital, Guangzhou, China

Key Words

Hydrogen sulphide • Hyperglycemia • TLR4 • NLRP3 inflammasome • Cardiac cell

Abstract

Background/Aims: This study aimed to investigate whether exogenous hydrogen sulfide (H₂S) conferred cardiac protection against high glucose (HG)-induced injury by inhibiting NLRP3 inflammasome activation via a specific TLR4/NF- κ B pathway. **Methods:** H9c2 cardiac cells were exposed to 33 mM glucose for 24 h to induce HG-induced cytotoxicity. The cells were pretreated with NaHS (a donor of H₂S) before exposure to HG. Cell viability, cell apoptosis, intracellular reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and TLR4, NF- κ B, NLRP3 inflammasome, IL-1 β , IL-18 and caspase-3 expression were measured by standard methods. **Results:** H₂S attenuated HG-induced cell apoptosis, ROS expression and loss of MMP and reduced the expression of NLRP3, ASC, pro-caspase-1, caspase-1, IL-1 β , IL-18 and caspase-3. In addition, H₂S inhibited the HG-induced activation of TLR4 and NF- κ B. Furthermore, NLRP3 inflammasome activation was regulated by the TLR4 and NF- κ B pathway. **Conclusion:** The present study demonstrated for the first time that H₂S appears to suppress HG-induced cardiomyocyte inflammation and apoptosis by inhibiting the TLR4/NF- κ B pathway and its downstream NLRP3 inflammasome activation. Thus H₂S might possess potential in the treatment of diabetic cardiomyopathy.

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Published by S. Karger AG, Basel

Z. Huang and X. Zhuang made equal contributions to this article.

Professor Xinxue Liao

Department of Cardiovasology, the First Affiliated Hospital, Sun Yat-sen University, 58th, Zhongshan Road II, Guangzhou, 510080, (China)
Tel. +86-20-87332200, Fax +86-20-87332200, E-Mail liaoxinx@mail.sysu.edu.cn

Introduction

Diabetic cardiomyopathy (DCM) is the leading cause of mortality among diabetic patients [1, 2]. A pervasive feature of DCM and probably one of its major causes, is the chronic, low-level state of systemic and sterile inflammation. Previous studies from our group have reported a definite association between DCM and inflammation [3]. And hyperglycemia, as the major feature of diabetes, can cause cardiac contractile and diastolic dysfunction by activation of oxidative stress, inflammation, and apoptosis.

Toll-like receptors (TLRs) are pattern recognition receptors, playing an important role in the activation of innate and adaptive immune responses [4]. Activation of TLRs can trigger signaling cascades with release of several inflammatory cytokines [5]. In diabetic patients, there is a significant increase of TLR4 expression, as well as an increase in levels of endogenous ligands and their activated downstream signaling cascades, such as NF-κB signaling [6, 7]. And knockdown of TLR4 can attenuate NF-κB activity and expression of inflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α in diabetes [8]. So the TLR4 is supposed to play a predominant role in high glucose (HG)-induced inflammation.

The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome has emerged as an important regulator of inflammation in metabolic disorders [9]. It consists of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. NF-κB is the traditional priming signal for the transcription of the NLRP3 gene [10]. And the stimulation of NLRP3 inflammasome triggers caspase-1 activation and subsequently promotes the cleavage processing and secretion of pro-inflammatory cytokine IL-1β and IL-18 [11]. Previous studies showed that inhibition of NLRP3 significantly alleviates DCM [12]. However, whether the cardiac TLR4-NF-κB-NLRP3 pathway is involved in HG-induced cardiomyocyte injury remains unknown.

Hydrogen sulfide (H₂S) is synthesized from cysteine by cystathionine gamma lyase (CSE) and other naturally occurring enzymes. Along with nitric oxide (NO) and carbon monoxide (CO), H₂S forms part of a group of biologically active gases that are termed gasotransmitters or gasomediators. H₂S levels are decreased in diabetic patients and diabetic rats treated with streptozotocin [13]. H₂S is presented to be an important cardioprotective agent in various studies [14-17]. Guo et al. reported that H₂S could protect against doxorubicin-induced inflammation and cytotoxicity by inhibiting p38MAPK/NF-κB pathway in myocardium [18]. In diabetic models, H₂S was reported to protect cardiomyocyte from inflammation and cell death [19, 20]. Our previous study also demonstrated that exogenous H₂S protected H9c2 cardiomyocytes against HG-induced injury by inhibiting inflammatory pathway [3]. And in this study, we try to investigate whether exogenous H₂S shows cardiac protection against HG-induced injury by inhibiting NLRP3 inflammasome signaling, in particular TLR4-NF-κB pathway.

Materials and Methods

Materials

Hoechst 33258, sodium hydrosulfide (NaHS), BAY11-7082 (inhibitor of NF-κB), Rhodamine 123 (Rh123) and 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). The Cell Counter Kit-8 (CCK-8) was bought from Dojindo (Kumamoto, Kyushu, Japan). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serums (FBS) were supplied by Gibco BRL (Carlsbad, CA, USA). Western Blot Detection Kit (ECL solution) was purchased from Beyotime Biotechnology (Shanghai, China). The primary antibodies specific to TLR4, NLRP3 and ASC were supplied by Abcam Biotech (Cambridge, UK). The primary antibodies specific to caspase-1, pro-caspase-1, caspase-3, p-p65 (phosphorylated NF-κB) and t-p65 (total p65) were bought from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody was purchased from Proteintech Group (Chicago, IL, USA). HRP-conjugated secondary antibody and the enzyme linked immunosorbent assay (ELISA) kits for IL-1β and IL-18 were

purchased from Kang Chen Biotech (Shanghai, China). TAK-242 (inhibitor of TLR4) was supplied by Life Tech (Carlsbad, CA, USA). Si-RNA of NLRP3 was purchased from Ribobio Biotech (Guangzhou, China). The H9c2 embryonic rat cells were supplied by the Sun Yat-Sen University Experimental Animal Center (Guangzhou, China).

H9c2 cell culture

H9c2 cardiomyocytes were cultured in DMEM medium supplemented with 10% FBS at 37°C under an atmosphere of 5% CO₂. To explore the protective effect of H₂S on the HG-induced injury, cells were pretreated with 400 μM NaHS for 30 min prior to HG treatment, according to previous studies [18, 21-23]. To confirm the role of TLR4- NF-κB-NLRP3 pathway in HG-induced cardiac injury, 5 μM TAK-242 (inhibitor of TLR4) was co-treated with HG, while 10 μM BAY11-7082 (inhibitor of NF-κB) was pretreated for 1 h.

RNA interference

H9c2 cells were transfected at 70% confluence using Lipofectamine transfection reagent (Life Technologies, Carlsbad, USA), and siRNA against NLRP3 (Ribo Biotechnology, Shanghai, China, sense, 5'-GCUUCAGCCACAUGACUUUTT-3', and antisense, 5'-AAAGUCAUGUGGCUGAAGCTT-3') or a physiologically irrelevant negative control siRNA (sense, 5'-UUC UCC GAA CGU GUC ACG UTT-3', and antisense, 5'-ACG UGA CAC GUU CGG AGA ATT-3'). Each dried-down siRNA was dissolved in nuclease-free water to achieve a final concentration of 20 μM. Then 5 μL siRNA (20 μM) and 5 μL Lipofectamine were added to a 500 μL buffer system. The mixes were kept at room temperature for 30 min to form complexes, and equal aliquots were then added into one of the wells of a 6-well plate. The cultures were incubated at 37°C in a 5% CO₂ incubator. The medium was replaced after 12 h with DMEM that did not contain either a siRNA or the transfection reagent. Cells were collected at 12 h for analyses.

Cell viability assay

After H9c2 cells were cultured in 96-well plates and received different treatments, 10 μL CCK-8 solution was added to each well at a 1/10 dilution, followed by a further 2 h incubation in the incubator. Absorbance was measured at 450 nm with a microplate reader (Mutiskan MK3 Microplate reader, Thermo Fisher Scientific Inc, USA). The mean optical density (OD) of five wells in the indicated groups was used to calculate the percentage of cell viability according to the formula below: percentage of cell viability = OD treatment group/ OD control group × 100%. Experiments were repeated 3 times.

Assessment of apoptosis

Morphological changes of apoptosis, such as chromosomal condensation and fragmentation in the nuclei of H9c2 cells, could be observed by Hoechst 33258 staining. After different treatments, H9c2 cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 10 min. After staining with 5 mg/L Hoechst 33258 for 30 min, the cells were visualized under a fluorescent microscope (BX50-FLA; Olympus, Tokyo, Japan).

Measurement of intracellular reactive oxygen species (ROS)

After different treatments, DCFH-DA (10 μM) in FBS-free medium was added to the slides and the cells were incubated for a further 30 min at 37°C. The slides were washed three times with FBS-free DMEM medium and fluorescence was measured over the entire field of vision using a fluorescent microscope connected to an imaging system (BX50-FLA; Olympus, Tokyo, Japan). Mean fluorescence intensity (MFI) from three random fields was analyzed using Image J 1.41 software (National Institutes of Health, Bethesda, MD, USA).

Measurement of mitochondrial membrane potential (MMP)

MMP was measured using the fluorescent cell-permeable dye Rh123 that preferentially enters mitochondria based on the highly negative MMP. Depolarization of MMP results in the loss of Rh123 from the mitochondria and a decrease in intracellular fluorescence. In the present study, Rh123 (100 mg/l) was added to medium for 45 min at 37°C. Rh123 fluorescence was measured over the entire field of vision using a fluorescent microscope connected to an imaging system (BX50-FLA; Olympus Tokyo, Japan). The MFI of Rh123 from five random fields was analyzed with Image J 1.41 software, with MFI taken as an index of MMP.

Western blot assay

After the indicated treatments, H9c2 cells were harvested and lysed, and the homogenate was centrifuged at 12,000 rpm for 10 min at 4°C. The total protein in the supernatant was quantitated with a BCA protein assay kit. Total protein (30 µg from each sample) was separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The protein in the gel was transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% free-fat milk in TBS-T for 1 h at room temperature, and then incubated with primary antibodies specific to TLR4 (1:1000), p-p65 (1:1000), t-p65 (1:1000), NLRP3 (1:1000), ASC (1:500), caspase-1 (1:1000), pro-caspase-1 (1:1000), caspase-3 (1:1000) or GAPDH (1:10000) with gentle agitation at 4°C overnight and subsequently incubated with the secondary antibodies for 1.5 h at room temperature. Following three washes with TBS-T, membranes were developed using enhanced chemiluminescence and exposed to X-ray films. To quantify protein expression, the X-ray films were scanned and analyzed with ImageJ 1.41 software (National Institutes of Health, USA).

ELISA for detection of IL-1β and IL-18 in culture supernatant

After indicated treatments, levels of IL-1β and IL-18 in cell lysates were measured by ELISA according to the manufacturer's instruction. The experiments were performed at least 3 times.

Statistical analyses

All data were presented as the mean ± standard error (SE). Differences between groups were analyzed by one-way analysis of variance (ANOVA) with SPSS 13.0 (Chicago, IL, USA). $P < 0.05$ was considered to be significantly different.

Results

HG induced apoptosis and inflammation in H9c2 cells

We examined the effect of HG with different levels in cardiac cells first. CCK-8 analysis showed significant decrease of cell viability in 33 and 44 mM glucose (Fig. 1A, $p < 0.01$). Further, we detected the effect of HG with different duration, and the protein level of caspase-3, an important regulator of apoptosis, markedly elevated from 6 to 24 h (Fig. 2B, $p < 0.05 \sim p < 0.01$). Hoechst result showed increased apoptosis in HG group, presenting as nucleus DNA damage (Fig. 1C) and loss of MMP (Fig. 1D). Similarly, HG elevated the level of ROS compared with control (Fig. 1E).

Coincident with exacerbated apoptosis in HG, ELISA data revealed enhanced expression of pro-inflammatory cytokines with HG exposure. As shown in Fig. 1F–G, the level of IL-1β and IL-18 increased with a concentration-dependent manner in 22 mM to 44 mM HG ($p < 0.01$). In keeping with our previous study [19] and clinical situation, 33 mM HG treatment for 24 h was chosen as our model to mimic the hyperglycemia.

The increased expression of NLRP3 inflammasome, TLR4 and NF-κB were induced by HG

NLRP3 inflammasome consists of three subunits, NLRP3, ASC and caspase-1. Caspase-1 is transformed from pro-caspase-1. HG treatment led to a remarkable time-dependent increase in the expression of these three subunits (Fig. 2A–C, $p < 0.05 \sim p < 0.01$), as well as the amount of pro-caspase-1 (Fig. 2D, $p < 0.05$). Likewise, two important intracellular signal pathways, TLR4 and NF-κB, were both activated by HG in 12 to 24 h (Fig. 2E–F, $p < 0.05 \sim p < 0.01$).

Exogenous H₂S protected against HG-induced apoptosis and inflammation

Then we investigated the effect of H₂S on HG-induced injury. With pretreatment of NaHS for 30 min, cell viability was considerably increased (Fig. 3A, $p < 0.05$) and the expression of caspase-3 was dampened (Fig. 3B, $p < 0.05$). Similarly, H9c2 cells presented less apoptotic rate (Fig. 3C, $p < 0.01$) and decreased level of ROS (Fig. 3F, $p < 0.01$) with NaHS pretreatment, compared with HG group. Loss of MMP was also significantly ameliorated (Fig. 3D, $p < 0.05$) in HG+NaHS group.

Fig. 1. HG induced cell apoptosis and inflammation in H9c2 cardiomyocytes. H9c2 cells were treated with glucose in different concentrations, CCK-8 assay was used for cell viability detection (A). H9c2 cells were treated with 33 mM glucose for different duration, and then Western blot analysis was used for protein levels of caspase-3 (B). H9c2 cells were cultured in 33 mM glucose for 24 h, then Hoechst staining for apoptotic rate (C), fluorescence staining for MMP (D) and ROS (E) were carried out. H9c2 cells were cultured in glucose with various concentrations, and protein levels of IL-1 β (F) and IL-18 (G) were detected by ELISA. Data were presented as means $\pm$ SEM, from 3 independent experiments. * p < 0.05 vs. control, ** p < 0.01 vs. control.

Fig. 2. HG induced expression of NLRP3 inflammasome, TLR4 and NF- κ B in H9c2 cells. H9c2 cells were treated with 33 mM glucose for different duration, Western blot analyses were used for protein levels of NLRP3 (A-D), TLR4 (E) and NF- κ B (F). Data were presented as means $\pm$ SEM, from 3 independent experiments. * p < 0.05 vs. control, ** p < 0.01 vs. control.

Fig. 3. Exogenous H₂S attenuated HG-induced cell apoptosis and inflammation. H9c2 cells were cultured in 33mM glucose with or without NaHS pretreatment (400 μ M). CCK-8 assay for cell viability analysis (A). Western blot analyses for protein levels of caspase-3 (B). Hoechst staining for detection of apoptotic rate (C). Fluorescence staining for detection of MMP (D) and ROS (E). ELISA for protein levels of IL-1 β (F) and IL-18 (G). Data were presented as means $\pm$ SEM, from 3 independent experiments. ** p < 0.01 vs. control, # p < 0.05 vs. HG, ## p < 0.01 vs. HG. No significance between control and NaHS group.

Furthermore, we examined the expression of pro-inflammatory cytokines in HG+NaHS group. Pretreatment of H9c2 cells with NaHS attenuated the protein levels of IL-1 β and IL-18 (Fig. 3F-G, p < 0.01). Treatment with NaHS alone didn't show any effect on H9c2 cells (Fig. 3A-G).

NaHS alleviated HG-induced activation of NLRP3 inflammasome, TLR4 and NF- κ B

In HG + NaHS group, the expression of NLRP3 inflammasome was markedly reduced compared with HG group (Fig. 4A-D, p < 0.05 ~ p < 0.01). The increase of TLR4 and NF- κ B pathway induced by HG was also attenuated by NaHS (Fig. 4E-F, p < 0.01). Treatment with NaHS alone didn't show any effect on H9c2 cells (Fig. 4A-F).

NLRP3 inflammasome expression in HG-treated cardiac cells was suppressed by gene silencing

To explore the role of NLRP3 in HG-induced injury, we inhibited the expression of NLRP3 by NLRP3-siRNA. The protein level of NLRP3 in H9c2 cells transfected with NLRP3-siRNA was lower than the vehicle (Fig. 5A, p < 0.05). After inhibiting the expression of NLRP3, the protein levels of activated ASC and caspase-1 induced by HG simultaneously decreased compared with HG group (Fig. 5B-C, p < 0.01). NLRP3-siRNA treatment alone didn't cause alteration of the ASC and caspase-1 expression (Fig. 5B-C).

NLRP3 inflammasome was involved in apoptosis and inflammation induced by HG

Then we investigated the contribution of NLRP3 to HG-induced apoptosis. CCK-8 assay presented improved cell viability (Fig. 6A, p < 0.05) and Western blot result showed a marked

Fig. 4. Exogenous H₂S ameliorated HG-induced expression of NLRP3 inflammasome, TLR4, and NF-κB. H9c2 cells were cultured in 33mM glucose with or without NaHS pretreatment (400 μM). Western blot analyses for protein levels of NLRP3 (A-D), TLR4 (E) and NF-κB (F). Data were presented as means±SEM, from 3 independent experiments. ***p* < 0.01 vs. control, #*p* < 0.05 vs. HG, ##*p* < 0.01 vs. HG. No significance between control and NaHS group.

Fig. 5. NLRP3 gene silencing decreased the expression of ASC and caspase-1. H9c2 cells were cultured in 33mM glucose with or without pretreatment of NLRP3-siRNA. Protein levels of NLRP3 (A), ASC (B) and caspase-1 (C) were detected by Western Blot. Data were presented as means±SEM, from 3 independent experiments. **p* < 0.05 vs. control, ***p* < 0.01 vs. control, ##*p* < 0.01 vs. HG. No significance between control and NLRP3-siRNA group.

reduction of caspase-3 expression (Fig. 6B, *p* < 0.01) in H9c2 cells with NLRP3 inhibition. In keeping with this observation, we also found significant decrease in apoptotic rate (Fig. 6C,

Fig. 6. NLRP3 gene silencing ameliorated HG-induced cell apoptosis and inflammation. H9c2 cells were cultured in 33mM glucose with or without pretreatment of NLRP3-siRNA. Cell viability was detected by CCK-8 assay (A). Protein level of caspase-3 was detected by Western Blot (B). Hoechst staining for cell apoptotic rate (C). Fluorescence staining for MMP (D) and ROS (E). ELISA was used for protein levels of IL-1 β (F) and IL-18 (G). Data were presented as means $\pm$ SEM, from 3 independent experiments. ** $p < 0.01$ vs. control, ## $p < 0.05$ vs. HG, ## $p < 0.01$ vs. HG. No significance between control and NLRP3-siRNA group.

Fig. 7. TLR4 and NF- κ B induced NLRP3 inflammasome activation. Protein levels of p-p65 (A), t-p65 (A) and NLRP3 (B) in H9c2 cells with treatment were detected by Western Blot analysis. Data were presented as means $\pm$ SEM, from 3 independent experiments. ** $p < 0.01$ vs. control, ## $p < 0.01$ vs. HG.

$p < 0.01$), ROS level (Fig. 6E, $p < 0.01$) and loss of MMP (Fig. 6D, $p < 0.01$) in NLRP3-siRNA treated H9c2 cells with HG.

We also tested the expression of pro-inflammatory cytokines in HG-treated H9c2 cells with NLRP3 inhibition. As shown in Fig. 6F-G, the levels of IL-1 β and IL-18 were lower in HG+NLRP3-siRNA group than HG group ($p < 0.01$). However, H9c2 cells only treated with NLRP3-siRNA didn't present any changes (Fig. 6A-G).

TLR4 and NF- κ B mediated the HG-induced NLRP3 inflammasome activation

Above observation presented that HG activated the expression of TLR4, NF- κ B and NLRP3 inflammasome, and further we tried to figure out the association among TLR4, NF- κ B and NLRP3 inflammasome. Pretreatment of cells with TLR4 inhibitor TAK-242 inhibited

Fig. 8. Pathways involved in the protective effect of H₂S in hyperglycemia induced myocardial injury. Hyperglycemia induces activation of TLR4, causing NF- κ B facilitation. This process further activates NLRP3 inflammasome and lead to increase expression of pro-inflammatory cytokines. At last, inflammation and cell death will significantly enhance. Exogenous H₂S can suppress the activation of TLR4, and finally alleviate the inflammation and apoptosis.

the production of lipopolysaccharide-induced inflammatory mediator by binding to the intracellular domain of TLR4. The protein level of phosphorylated NF- κ B was decreased in HG+TAK-242 group than in HG group (Fig. 7A, $p < 0.01$). There was no difference in phosphorylated NF- κ B expression between TAK-242 group and control group (Fig. 7A).

Moreover, we inhibited the expression of NF- κ B by BAY11-7082, which specifically abrogated NF- κ B DNA binding. Treating with BAY11-7082, the protein level of NLRP3 decreased as compared with HG group (Fig. 7B, $p < 0.01$). The above data prompted that TLR4- NF- κ B pathway may induce activation of NLRP3 in HG-treated cardiac cells. No altered expression of NLRP3 was detected in BAY11-7082 group (Fig. 7B).

Discussion

In this study, we aimed to explore the effect and underlying mechanism of exogenous H₂S in HG-induced cardiac injury. As shown in Fig. 8, the results presented that 1) 33 mM HG caused significant exacerbated apoptosis and inflammation in H9c2 cells; 2) NLRP3, TLR4 and NF- κ B pathway were all activated in HG-treated cardiac cells; 3) 400 μ M NaHS protected against hyperglycemia-induced cardiomyocyte apoptosis and inflammation; 4) the protective effect of H₂S was associated with its ability to inhibit the activation of NLRP3 inflammasome via TLR4/ NF- κ B pathway.

H₂S, an endogenously-generated gas, has been shown to elicit cardioprotective effect in various injury models [24]. In this study, we demonstrated that exogenous H₂S protected cardiomyocytes against HG-induced inflammation and apoptosis, presenting as ameliorated apoptotic rate, dampened activation of ROS, alleviation in loss of MMP and decreased expression of pro-inflammatory cytokines. Similarly, a study *in vivo* presented that intraperitoneal or oral administration of H₂S reduced myocardial hypertrophy and reduced the degree of fibrosis [15]. Several studies have proved that H₂S could significantly reduce oxidative stress and hence improve cell viability [25, 26]. These findings indicate that H₂S is a potential therapeutic agent for the treatment of DCM.

Furthermore, we tried to investigate the mechanisms underlying the protective effect of H₂S in DCM. Actually, it is governed by multiple signaling pathways. Our previous studies found that H₂S alleviates HG-induced cardiotoxicity by inhibiting leptin signaling and mitogen-activated protein kinase [3, 27]. Meanwhile, a whole of studies demonstrated the protection of H₂S in diabetic target organs via anti-inflammation [13, 15, 24]. In the present

study, treatment of NaHS remarkably alleviated the activation of TLR4 and NF- κ B signaling, revealing that TLR4 and NF- κ B signaling may be important in H₂S protection.

TLR4 expression markedly increases in various diabetic models, paralleling with enhanced cell injury [28-31]. In diabetic mice, level of TLR4 significantly elevated [32]. Moreover, treatment with TLR4-siRNA minimized apoptosis in myocardial tissues [32]. Similarly, the present study showed a time-dependent increase of TLR4 expression in HG-treated cardiomyocytes, coincident with the exacerbated inflammation and cell death. In muscle biopsies of T2DM patients, increased TLR4 expression was found to cause activation of NF- κ B signaling with the release of pro-inflammatory cytokines [33]. As the dominant isoform of TLRs in cardiomyocytes, TLR4 can interact with cytosolic adapters. Then it induces proteasomal degradation of the inhibitors of NF- κ B (I κ Bs), enabling NF- κ B translocation into the nuclei [34]. An *in vivo* study revealed that H₂S protected against cellular injury via inhibition of NF- κ B activation [35]. In our study, blockage of TLR4 activation considerably ameliorated NF- κ B activation. Moreover, TLR4 antagonists, eritoran, and geldanamycin resulted in attenuated myocardial inflammatory responses including reduced NF- κ B nuclear translocation and decreased gene transcript of multiple pro-inflammatory cytokines [36, 37]. On above observation, we supposed an important role of TLR4 in H₂S protection against hyperglycemia-induced NF- κ B signal activation and cardiac injury.

Our data also presented that activation of NLRP3 inflammasome led to increased expression of IL-1 β and IL-18 in hyperglycemia, along with permeabilization of plasma membrane and nucleus DNA damage. Furthermore, NLRP3 silencing significantly attenuated the HG-induced activation of IL-1 β and IL-18, as well as cell death. *In vivo*, HG-induced excessive activation of NLRP3 inflammasome was observed, as well as cardiac dysfunction, fibrosis and disorganized ultrastructure [12]. NLRP3 inflammasome has been identified in a wide range of cells including cardiomyocytes [38]. NLRP3 is reported to be held inactive in cytoplasm. Once it is freed, subsequent oligomerization leads to the recruitment of procaspase-1, then promoting autocleavage and activation [39]. Active caspase-1 can finally process IL-1 β and IL-18, serving as enhancers of multiple inflammatory responses. In the diabetic state, activation of pro-inflammatory cytokines by hyperglycemia leads to the persistent inflammation in myocardium, which contributes to diabetic cardiac dysfunction [40]. The result supported our conjecture that NLRP3 inflammasome plays an important role in HG-induced inflammation and apoptosis in myocardium.

In light of the pivotal role of NLRP3 in DCM, we tried to figure out potential signal pathways involved in NLRP3 inflammasome activation. Using the inhibitors of TLR4 and NF- κ B, we found that in HG-treated cardiac cells, inhibiting TLR4 signal reduced the expression of NF- κ B, while blockage of NF- κ B downregulated NLRP3 expression. Similarly, in another study of monocytes, siRNA knockdown of TLR4 led to decreased NF- κ B activity and IL-1 β release [41]. Moreover, NF- κ B has been reported to enhance the expression of NLRP3 and IL-1 β , and NF- κ B sites in NLRP3 promoter have been identified [10, 42, 43]. And third, NF- κ B increases the amount of thioredoxin interacting protein and oxidized mitochondrial DNA, which might serve as ligands of NLRP3 [44, 45]. Based on above results, NLRP3 inflammasome activation is supposed to be a key outcome of TLR4/NF- κ B stimulation in DCM.

However, no specific NLRP3 antagonist has been identified yet, and increasing efforts are being invested as a result of successful blockade of downstream effectors IL-1 β and caspase-1 in DCM. Recent evidence reported that intravenous IgG therapy protected neurons in stroke through a mechanism involving suppression of NLRP3 inflammasome activity [46]. In the present study, Using 400 μ M NaHS, we observed a downregulation of NLRP3 expression in hyperglycemia, paralleling with ameliorated myocardial condition. These data underscores the potential benefit of exogenous H₂S in blocking NLRP3 inflammasome activation in DCM.

Nowadays, several possibilities are considered to augment the beneficial effects of H₂S in DCM: (1) stimulation of H₂S synthesizing enzymes, (2) supplying more substrate (l-cysteine) for H₂S synthesis, and (3) administration of exogenous H₂S or its donors. Statins are shown to increase H₂S level by inhibiting its metabolism [47]. Administration of SQR inhibitors can potentially inhibit H₂S breakdown [48]. An N-mercapto-based H₂S donor (NSHD)-1

can release H₂S controllably [49]. In our study, we used NaHS as a donor of exogenous H₂S supplement. In the future, more attention should be paid on the application and clinical use of H₂S.

In conclusion, we show here that exogenous H₂S can exert cardioprotection against HG/hyperglycemia induced cardiac cell inflammation and apoptosis by suppressing TLR4/ NF-κB stimulated NLRP3 inflammasome activation. Therefore, H₂S may possess the potential to be developed as a therapeutic medicine to prevent hyperglycemia-enhancing diabetic cardiomyopathy.

Acknowledgements

This study was supported by National Nature Science Foundation of China (Grant No. 81270296) and Social Developing Project of Guangdong Province in China (2014SC107).

Disclosure Statement

All the authors declared no competing interests.

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