Original Article

Hydrogen sulfide attenuates high glucose-induced cardiotoxicity via enhancing autophagy activity in human AC16 cardiac cells

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Abstract: Diabetic cardiomyopathy (DCM) is a major cause of mortality and morbidity in complications of diabetes mellitus (DM) and dysregulated autophagy is proven to contribute to the physiological and pathological processes of DCM. Recent studies have shown that hydrogen sulide (H$_2$S) generation is reduced in diabetic mouse hearts and exogenous H$_2$S has cardioprotective effects in variety of cardiovascular diseases. Thus, the purpose of present study was to investigate whether exogenous H$_2$S prevents high glucose (HG)-induced cardiotoxicity through regulating autophagy in human myocardial cells (AC16 cells). We noted that treatment with different concentration of HG significantly induces cardiotoxicity, leading to decrease in the viability of AC16 cells and increase in apoptotic cells. In addition, HG treatment also increased the activity of caspase-3 and the expression of Bax (pro-apoptotic protein), and decreased the expression of Bcl-2 (anti-apoptotic protein) in AC16 cells. However, these all injuries were markedly attenuated by pre-treatment with NaHS (a donor of H$_2$S). These results suggest that exogenous H$_2$S exerts a protective role in HG-induced cardiomyocyte damages. Furthermore, HG also down-regulated the LC3 II/I ratio and suppress the expression levels of Beclin1, while the expression levels of P62 is enhanced by HG treatment, which indicates the downregulation of autophagy in HG-treated AC16 cells. Notably, pre-treatment with NaHS for 30 min markedly reverses HG-caused the inhibition of autophagy in AC16 cells. Furthermore, we found that Bafilomycin A1 (Baf, an autophagy inhibitor) attenuates the protective effects of H$_2$S on HG-induced cytotoxicity and apoptosis in AC16 cells. Taken together, these studies demonstrate that H$_2$S attenuates HG-induced cardiotoxicity through enhancing autophagy.

Keywords: Hydrogen sulfide, diabetic cardiomyopathy, high glucose, cardiocytotoxicity, autophagy

Introduction

Diabetic cardiomyopathy (DCM) is characterized by myocardial dysfunction occurring independently of coronary artery disease (CAD), valvular heart disease, or hypertension in diabetes mellitus (DM) patients [1]. Although the pathological mechanism of DCM still remains multifactorial, hyperglycaemia is considered as the main underlying pathogenic factor for myocardial damage in this condition [2]. Indeed, the cardiotoxic roles of hyperglycemia have been demonstrated in numerous cells and animal studies. Hyperglycaemia causes cardiomyocyte death, which is contributed to the production of oxidative stress, accelerated apoptosis, mitochondrial damage, hypertrophy, impaired calcium homeostasis, and fibrosis [3-6]. Vitro experiments also showed that High glucose (HG) directly induces the increases in reactive oxygen species (ROS) levels and apoptosis, while the autophagy is suppressed in glucose-induced cardiomyocyte injuries [7, 8]. However, the underlying mechanisms of DCM are still incompletely and at present, the clinical treatment cannot effectively attenuate DCM and heart failure in human treatment [9, 10]. Therefore, it is reasonable to assume the molecules mechanisms and develop a potential therapeutic strategies for DCM. Recently, the effects of dysregulated autophagy and H$_2$S on hyperglycaemia-induced cardiotoxicity in DCM have attracted considerable attention.

Autophagy is an essential intracellular catabolic pathway that the long-lived proteins and dam-
aged organelles are transferred to and degraded in the lysosomes, resulting in maintaining cellular homeostasis undergoing starvation or various other stresses [11]. General macroautophagy is referred as autophagy and tightly controlled by a variety of positive and negative regulators [12, 13]. As is known to all, under normal circumstances, the low level of autophagy is a protective mechanism of cellular stress. However, excessive autophagy can lead to cell damage. Recent researches have proved that high autophagy levels are appeared in circumstances of pressure overload, ischemia/reperfusion, heart failure, myocardial infarction, and cardiac hypertrophy [14-16], suggesting that autophagy plays a significant role in the pathogenesis of heart diseases [17]. Indeed, there are evidences pointing out that the change of autophagic response promotes the maintenance of heart functions and morphology [18]. However, the role of autophagy in DCM is more complex [19]. For example, inhibited autophagy appears adaptive feature in STZ diabetic mice (type 1 diabetic model) [20] but maladaptive feature in HFD-induced diabetes (type 2 diabetic model) [21]. Although many researches have observed the altered autophagy in HG-induced cardiotoxicity, the pathophysiologic roles of autophagy in HG-induced cardiomyocytes injures remain completely understood.

Hydrogen sulfide (H₂S) has been qualified as a new gasotransmitter along with carbon monoxide (CO) and nitric oxide (NO) with multiple physiological functions including anti-oxidant, anti-apoptosis, preservation of mitochondrial function, anti-inflammatory in physiology and pathophysiologic conditions [22]. In recent years, accumulating evidences suggest that H₂S has cardioprotective roles [23]. Exogenous H₂S has been proved to attenuate myocardial necrosis via reactive oxygen species signal pathways in streptozotocin (STZ)-treated rats [24] and rescue contractile activity by preventing cardiomyocyte apoptosis in isoproterenol-induced rats [25]. H₂S also promotes posts ischemic left ventricular function and mitochondrial respiration during myocardial ischemia-reperfusion (MI/R) damage [26]. These findings gave us rationale that H₂S might be a therapeutic strategy for diabetic-associated diseases. Recently, researches interest in the protection effects of H₂S in DCM has drawn much attention. H₂S alleviates the development of DCM through attenuation of oxidative stress, apoptosis and inflammation [27]. H₂S also attenuates HG-induced cardiotoxicity in H9c2 cells [28]. Notably, H₂S restores MI/R-impaired autophagic flux [29] and reverses high-fat-inhibited autophagy activity [30]. Hence, we speculate that the promotion of autophagy may be beneficial to the protective effect of H₂S against HG-induced cardiotoxicity in human AC16 cardiac cells.

To test this hypothesis, we used different concentrations of HG to explore and establish hyperglycemia-induced cardiotoxicity model. We then investigated the effects of bafilomycin A1 (Baf, an autophagy inhibitor) on exogenous H₂S-induced protective functions in HG-treated human AC16 cardiac cells. In this report, we, for the first time, point out that exogenous H₂S prevent HG-induced cardiotoxicity by enhancing autophagy activity.

Materials and methods

Reagents

Sodium hydrosulfide (NaHS, a donor of H₂S), Bafilomycin A1 (Baf, an autophagy inhibitor) and Hoechst 33258 staining were supplied by Sigma-Aldrich (St. Louis, MO, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Lab (Rockvile, MD, USA). Lactate dehydrogenase (LDH) and BCA Protein Assay Kit were obtained from Beyotime (Shanghai, China). Caspase-3 enzyme-linked immunosorbent assay (ELISA) Kits was bought from USCN Company (Wuhan, Hubei, China). Specific monoclonal anti-Bax and anti-Bcl-2 antibodies were purchased from Abcam (Cambridge, CB, UK). Specific monoclonal antibody to LC3 was obtained from Cell Signaling Technology Company (Beverly, MA, USA). Specific monoclonal anti-beclin-1 and anti-p62 antibodies were obtained from Epitomic Inc (Burlingame, UK). β-actin antibody was obtained from Proteintech (Danvers, MA, USA).

Cell culture and conditions

The human AC16 cardiac cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), cultured in DMEM supplemented containing 100 U/ml penicillin and streptomycin (Sigma-Aldrich, France) and

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10% FBS (Gibco). The cells were grown at 37°C in an incubator with 5% CO₂ humidified atmosphere until 60%-70% subconfluent, and then treated with normal D-glucose (5.5 mmol/L) or various concentrations of high D-glucose (HG, 33 mmol/L) in the presence or absence of NaHS (30 min) or Bafilomycin A1 (Baf, 30 min) or both for 24 h.

Cell viability assay

Human AC16 cardiac cells (under the logarithmic growth phase) were seeded in 96-well plates at density of 1×10⁴ cells/well overnight. When the culture AC16 cells reached about 70% confluence, cells were incubated with various concentrations of HG, NaHS or Baf as discussed above. After drug treatment for 24 hours, the viability of AC16 cells was accessed with CCK-8 kit according to the manufacture’s instruments and the absorbance was measured at 570 nM with a plate reader (Thermo Fisher Scientific Inc). The cell survival rate (%) was calculated as the percentage of viable cells in comparison with the control group. Each experiment was at least carried out three times, independently.

Lactate dehydrogenase (LDH) release assay

When the cell membrane is injured, lactate dehydrogenase (LDH) will transfer from the cytoplasm to the cell culture supernatant. Thus, the levels of LDH in the culture supernatant can evaluate the degree of cellular injury and cytotoxicity. After treatment of human AC16 cardiac cells with experiment reagent as described above, the LDH contents were determined by LDH release assay kit according to the manufacturer’s instructions. The LDH activity was detected at 490 nm and opposite to the cell activity. Each independent experiment was repeated for three times.

Cell morphology assay with Hoechst 33258 staining

Cells in logarithmic growth phase were seeded at density of 1×10⁵ cells/well in 12-well plates. After treatment with experiment reagent as described above, the AC16 cells were washed with 4°C PBS for three times and then fixed with 4% paraformaldehyde for 10 min. After washed with PBS for three times again and stained with Hoechst 33258 (5 μg/ml) for 10 min at room temperature in a dark, the morphology of apoptotic cells was observed by fluorescence microscopy (BX50-FLA, Olympus, Tokyo, Japan). Apoptotic cells were unevenly stained, with strong blue fluorescence, while the morphology of the normal nuclei was complete and showed uniform blue fluorescence.

Measurement of caspase-3 activity

After treatment of human AC16 cardiac cells with experiment reagent as described above, the activity of caspase-3 was determined by caspase-3 enzyme-linked immunosorbent assay (ELISA) Kits according to the manufacture's instruments. The caspase activity was measured at 490 nm on an ELISA plate (Molecular Devices, USA). Each independent experiment was repeated for three times.

Western blot analysis

After treatment of human AC16 cardiac cells with experiment reagent for 24 h, cells were rinsed for three times with ice-cold PBS and lysed in RIPA buffer (Beyotime). After centrifugation at 12,000×g for 10 min at 4°C, the proteins were quantified using BCA assay. Equal amounts of protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked at room temperature for 2 h in 5% non-fat milk (Tris-buffered saline (TBST) containing 0.1% Tween-20 and 5% milk) and then incubated with primary antibodies against Bax, Bcl-2, LC3, Beclin-1, p62 and β-actin at 1:1000 dilutions in TBST containing 3% milk at 4°C overnight, respectively. After washed with TBST for three times, the membranes were incubated with appropriate HRP-conjugated secondary antibodies (Proteintech, USA) at 1:5,000 dilutions for 2 h at room temperature. Following washed with TBST, the membranes were processed for an enhanced chemi-luminescence (ECL) kit (Amersham Biosciences, UK) and exposed to X-ray film. The expression levels of protein were quantified by Image J software. Each experiment was repeated for three times, independently.

Statistical analysis

All data were presented as the means ± standard error of the mean (SEM). The difference
between groups was determined by one-way analysis of variance (ANOVA) using SPSS17.0 software (Chicago, IL, USA), and followed by the LSD test. *P < 0.05, **P < 0.01, versus control group.

**Results**

**High glucose reduces the viability of human AC16 cardiac cells**

To address the effect of high glucose (HG) with different concentrations (11, 22, 33, and 44 mM) on human AC16 cells, we first measured the viability and LDH releases under HG treatment. As shown in Figure 1, the CCK-8 result shown that treatment of AC16 cells with HG at 33 and 44 mM concentrations for 24 h significantly decreases the cell viability, while low concentrations of glucose (11 and 22 mM) have no effect on the cell viability (Figure 1A). Likely, HG concentration-dependently increased the levels of LDH in culture supernatant (Figure 1B). These results suggested HG-induced cytotoxicity in human AC16 cardiac cells.

**High glucose induces apoptosis in human AC16 cardiac cells**

In addition, Hoechst 33258 staining result shown that 33 and 44 mM of HG obviously exhibit the phenomenon of nuclear cracking and condensation in human AC16 cells comparing with control group (Figure 2A). The activation of caspase-3 and the change of Bax/Bcl-2 ration play an important role in the process of apoptosis [31]. We found that HG at concentrations 22, 33 and 44 mM markedly increase the activity of caspase-3 (Figure 2B). In addition, western blot results shown that treatment with HG significantly causes the up-regulation of Bax protein (Figure 2C) and down-regulation of Bcl-2 protein in human AC16 cardiac cells (Figure 2D). Combining with the above all results suggested that HG induces cardiomyocyte apoptosis.

**High glucose inhibits autophagy in human AC16 cardiac cells**

To investigate whether HG could alter autophagic activity in cardiomyocytes, we test the effects of HG at different concentrations on the markers of autophagy (LC3-II/I ration, the levels of Beclin-1 and P62) by western blot assay in human AC16 cardiac cells. As shown in Figure 3, compared with normal control, treatment with HG for 24 h concentration-dependent decreased the expression levels of beclin-1 (Figure 3A) and ratio of LC3-II/I (Figure 3B) in human AC16 cardiac cells, while the level of p62 protein was obviously up-regulated by HG treatment (Figure 3C). In summary, these results suggested that HG reduces autophagy activity in human AC16 cells.

NaHS, a donor of H$_2$S, mitigates high glucose-caused cardiomyocytes damages in human AC16 cardiac cells. Emerging literatures have confirmed that H$_2$S has myocardial protection...
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Therefore, we attempted to further explore whether H2S can inhibit the HG-induced cardiomyocytes injury. To validate this hypothesis, we pre-treated of human AC16 cells with NaHS for 30 min prior to HG (33 mM) for 24 h and measured the cell viability and apoptosis. CCK-8 assay result shown that pre-treatment with NaHS (200 and 400 µM) for 30 min weakened the HG-induced the down-regulation of cell viability (Figure 4A). In addition, NaHS (200 and 400 µM) improved the phenomenon of nuclear condensation and cracking induced by HG (33 mM) in human AC16 cardiac cells (Figure 4B). NaHS also reversed HG-induced the up-regulation of caspase-3 activity (Figure 4C) and Bax protein expression (Figure 4D) as well as the down-regulation of Bcl-2 protein expression (Figure 4E) in a concentration-dependent manner. These results indicated the cardioprotection effects of exogenous H2S on HG-induced cardiotoxicity.

NaHS reverses high glucose-exhibited the inhibition of autophagy in human AC16 cardiac cells

Emerging evidences have shown that H2S is a potent regulator of autophagic flux and plays an
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important role in myocardial diseases by regulating autophagy [29, 33]. Thus, we suspect that H\textsubscript{2}S may have an effect on the inhibition of autophagy induced by HG. As shown by western bolt assay in Figure 5, the decreases in the levels of beclin-1 protein (Figure 5A) and ratio of LC3-II/I (Figure 5B) induced by HG (33 mM) were significantly reversed by pre-treatment with NaHS (200 and 400 µM) for 30 min, respectively. NaHS also reduced the increase in the expression of p62 protein induced by HG (33 mM) in a dose-independence manner in human AC16 cells (Figure 5C). The above results suggested that H\textsubscript{2}S protects human AC16 cardiac cells against HG-induced toxicity and the protection mechanism may be related to regulate the autophagy activity.

**Inhibition of autophagy attenuates the protective effects of NaHS on HG-induced cardiotoxicity in human AC16 cardiac cells**

To further assess autophagy to the protective effects of H\textsubscript{2}S, AC16 cells were pre-treated with Bafilomycin A1 (Baf, an inhibitor of autophagy, 50 nM) for 30 min. As shown in Figure 6A, NaHS-caused recovery of AC16 cells activity was obviously reversed by pre-treated with Baf. Additionally, Hoechst 33258 staining result shown that NaHS-induced improvement of cell morphology throughout HG treatment was also abrogated by pre-treatment with Baf (Figure 6B). Simultaneously, the mitigation effect of NaSH on the increased in the activity of caspase-3 (Figure 6C) and expression of Bax protein (Figure 6D), and the decreased in the expression of Bcl-2 protein (Figure 6E) were also abolished by Baf. These results show that enhancement of autophagy mediates the protective functions of H\textsubscript{2}S against HG-induced cardiotoxicity.

**Discussion**

Diabetes mellitus (DM) is a serious disease that threatens human health and the prevention and treatment of diabetes and its complications (such as diabetic cardiomyopathy,
DCM) have drawn a lot of attentions in the world. Although it is well-known that hydrogen sulfide (H\textsubscript{2}S) has a protective effect in heart failure among diabetic patients, the underlying protective mechanisms of H\textsubscript{2}S in diabetic cardiomyopathy (DCM) are not yet fully understood. Hyperglycemia is an important feature in the pathology of diabetic cardiomyopathy. Hence, in present study, the high glucose (HG, 33 mM)-caused human AC16 cardiac cells injury was represented as the cell model of DCM and used to investigate the protective effects of H\textsubscript{2}S against HG-induced damages and its underlying protective mechanisms. We found that pretreatment with NaHS attenuated HG-induced cytotoxicity and apoptosis in human AC16 cardiac cells. Furthermore, the inhibition of autophagy induced by HG was also reversed by NaHS. Notably, Bafilomycin A1 (Baf, an autophagy inhibitor) attenuated NaHS-mediated cardioprotection effects against HG-induced toxicity. These results suggest that the beneficial
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functions of H₂S in HG-treated human AC16 cardiac cells were mediated by upregulating autophagy activity.

Increasing evidences have shown that H₂S has the potential capacity to protect heart against arrhythmia, myocardial infarction, fibrosis, ischemia-reperfusion injury, hypertrophy, and heart failure [34]. Recent evidences exhibit that exogenous H₂S also attenuates HG-induced cardiotoxicity in H9c2 cardiac cells [28, 35]. Consistent with these studies, in present study, we found that the exposure of AC16 cells to HG for 48 h significantly induced cytotoxicity and apoptosis, as evidenced by decreases in cell viability and LDH activity, as well as the increase in apoptotic cells, while these injuries were obviously reversed by pretreatment with NaHS (a donor of H₂S). Caspase-3 is called executor of cell apoptosis which is necessary killer proteinase in cell apoptosis process [31]. Bax and Bcl-2 proteins are the most important opposite regulatory factor in the process of apoptosis. Bax promotes apoptosis, and conversely, Bcl-2 inhibits apoptosis. A number of studies suggest that intracellular ratio of Bax/Bcl-2 reflects the induction of apoptotic cell death after HG treatment [36, 37]. The research by Meng G et al shows that GYY4137 (a slow-releasing H₂S donor) can reduce the caspase-3 activity and the expression of Bcl-2 but increase the expression of Bcl-2 in ischemia and reperfusion-treated myocardium [38]. Similarly, we found that the exposure of AC16 cells to HG increases the activity of caspase-3 and the expression of Bax protein, as well as reduce the expression of Bcl-2 protein, which were attenuated by NaHS. Caspase and Bcl-2 protein family play an important regulating effect in the mitochondrial apoptosis pathway [39]. The triggering of apoptosis promotes the transfer of Bcl-2 protein family to mitochondria and disrupts the mitochondrial membrane, resulting in the release of cytochrome C, which activates caspase protein family and finally triggers cell death and apoptosis. Wang Y et al has shown that H₂S alleviates hyperhomocysteinemia-induced myocardial damage through protecting cadiac mito-
Hence, combined with the above research, we supposed that HG induced cardiotoxicity, at least in part, through mitochondrial-dependent apoptotic pathway and H$_2$S may attenuate HG-induced injuries through cardiac mitochondrial protection. The further researches are needed to determine the exact role of mitochondrion in HG-induced apoptosis or the protective effects of H$_2$S using inhibitors and agonists.

Accumulating evidences have suggested that autophagy, another form of programmed death, plays a vital role in the pathophysiology of DCM [17]. Autophagy is a lysosome-mediated catabolic processes and initiated with the genera-
tion of Beclin1 to form double-membrane structure, which with the help of LC3-II (microtubule-associated protein 1 light chain 3 II) [41]. The ratio of LC3-II/LC3-I (two forms of LC3) is positively correlated with the extent of autophagosome formation [42]. In this study, we found inhibition of autophagy induced by HG treatment, as evidenced by the decreased in the expression of Beclin1 and the ration of LC3-II/LC3-I in AC16 cells, consistent with the resulted proved by Dong C et al [43]. P62 is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy lysosome. The expression of p62 in the cells is negatively correlated with the autophagy activity or autophagy flux. In the current study, we found HG treatment markedly increases the expression of p62 protein in AC16 cell, suggesting that HG may cause the down-regulation of autophagy activity via destroying the function of the lysosomal in cardiac myocytes. Further study using lysosomal inhibitors or agonists to verify this hypothesis is essential, which will provide a new perspective for the treatment of DCM.

Importantly, in current study, we manifest the autophagy contributes to the underling protective mechanism of H₂S in HG-treated human AC16 cardiac cells. H₂S can activate autophagy or restore autophagic flux to protect against myocardial ischemia and reperfusion-induced myocardial damage [29]. The current also proved that NaHS pretreatment restores the level of autophagy activity under HG treatment, as evidenced by the increases in the expression of Beclin1 and the ratio of LC3-II/LC3-I, as well as the decrease in the expression of p62. Notably, the protection effects of H₂S against HG-exhibited cardiotoxicity were significantly reversed by Bafilomycin A1 (Baf, a lysosomal protease inhibitor). These results suggest that H₂S protects AC16 cells against HG-induced damage through enhancing the autophagy activity. Recent evidences suggest that enhancing autophagy can reduce hepatic/R injury, which is related to its anti-apoptotic and anti-inflammatory activity [44]. The relationship of mitochondria, apoptosis and autophagy are complex and whether this relationship contribute to the protection roles of H₂S in DCM is further needed to investigate.

In summary, the study has provided a novel evidence that H₂S prevent cardiomyocyte injuries induced by HG treatment by enhancing autophagy activity in human AC16 cells. This study provides an important role of autophagy in the protection effects of H₂S against DCM and highlights the therapeutic potential of H₂S to prevent diabetic cardiovascular complications.

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Disclosure of conflict of interest

None.

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