Exogenous H₂S facilitating ubiquitin aggregates clearance via autophagy attenuates type 2 diabetes-induced cardiomyopathy

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Diabetic cardiomyopathy (DCM) is a serious complication of diabetes. Hydrogen sulphide (H₂S), a newly found gaseous signalling molecule, has an important role in many regulatory functions. The purpose of this study is to investigate the effects of exogenous H₂S on autophagy and its possible mechanism in DCM induced by type II diabetes (T2DCM). In this study, we found that sodium hydrosulphide (NaHS) attenuated the augment in left ventricular (LV) mass and increased LV volume, decreased reactive oxygen species (ROS) production and ameliorated H₂S production in the hearts of db/db mice. NaHS facilitated autophagosome content degradation, reduced the expression of P62 (a known substrate of autophagy) and increased the expression of microtubule-associated protein 1 light chain 3 II. It also increased the expression of autophagy-related protein 7 (ATG7) and Beclin 1 in db/db mouse hearts. NaHS increased the expression of Kelch-like ECH-associated protein 1 (Keap-1) and reduced the ubiquitylation level in the hearts of db/db mice. 1,4-Dithiothreitol, an inhibitor of disulphide bonds, increased the ubiquitylation level of Keap-1, suppressed the expression of Keap-1 and abolished the effects of NaHS on ubiquitin aggregate clearance and ROS production in H9C2 cells treated with high glucose and palmitate. Overall, we concluded that exogenous H₂S promoted ubiquitin aggregate clearance via autophagy, which might exert its antioxidative effect in db/db mouse myocardia. Moreover, exogenous H₂S increased Keap-1 expression by suppressing its ubiquitylation, which might have an important role in ubiquitin aggregate clearance via autophagy. Our findings provide new insight into the mechanisms responsible for the antioxidative effects of H₂S in the context of T2DCM.

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Diabetes mellitus has been firmly established as a major threat to human health due to its severe complications in the cardiovascular system. Diabetic cardiomyopathy (DCM) is one of the serious complications of diabetes, which greatly increases the incidence and severity of heart failure in patients with type 2 diabetes. Type 2 diabetes mellitus is defined as a protein-misfolding disease, which is characterized by misfolded and aggregated peptides and proteins. The accumulation of misfolded proteins results in a prolonged unfolded protein response, which contributes to mitochondria injury, reactive oxygen species (ROS) production and apoptosis. The prolonged unfolded protein response could also obstruct the ubiquitin–proteasome system leading to ubiquitinated proteins aggregating.

Autophagy is one of the protective factors for cell survival and is involved in eliminating damaged proteins and organelles, and autophagy has an important role in DCM. Damaged organelles could be eliminated through autophagy, which preserves their functions and suppresses mitochondrial ROS production. Meanwhile, the ubiquitin aggregates that can lead to apoptosis and ROS production are mainly cleared by autophagy.

Hydrogen sulphide (H₂S), a newly found gaseous signalling molecule, has an important role in many regulatory functions, such as vasodilatation, antioxidation and smooth muscle relaxation. In mammalian tissues, the biosynthesis of H₂S is catalysed by the pyridoxal-5-phosphate-dependent enzymes, including cystathionine-β-synthetase, cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulphurtransferase. In the cardiovascular system, H₂S synthesis is mainly catalysed by CSE. Studies also indicate that H₂S could promote disulphide formation between two Kelch-like ECH-associated protein 1 (Keap-1) molecules. Zhou et al. have demonstrated that exogenous H₂S could prevent the development of DCM by reducing ROS production through the Keap-1-nuclear respiratory factor 2 (Nrf2) signalling pathway. Meanwhile, some evidence has revealed that Keap-1 has a pivotal role in ubiquitin aggregate clearance via autophagy in HEK293T cells. Although our previous study reported that H₂S could attenuate the apoptosis of DCM induced by type 1 diabetes, the mechanism is not very clear between H₂S and DCM. Our present study reports that exogenous H₂S could protect myocardocytes by promoting autophagy in type 2 diabetes. The effect of exogenous H₂S might contribute to...
upregulating Keap-1 expression. Keap-1 could facilitate p62-mediated ubiquitin aggregate clearance via autophagy, which could be a key pathway for the protective effects of exogenous on myocardiocytes in type 2 diabetes. Therefore, we speculated that exogenous H2S was likely to increase the expression of Keap-1 by suppressing its ubiquitylation, which contributes to ubiquitin aggregate clearance via autophagy.

Results

Exogenous H2S improved cardiac diastolic function and H2S production in db/db mice. The db/db mice, leptin receptor-deficient mice, were chosen as the type 2 diabetes animal model. The blood glucose levels and serum lipids were increased, and glucose tolerance was significantly decreased in db/db mice, whereas there was no effect after sodium hydrosulphide (NaHS) injection (Supplementary Figures S1a and b). To investigate the effects of exogenous H2S on cardiac function in db/db mice, we observed the cardiac functions of mice using echocardiography. Though the ejection fraction (EF) did not change in db/db mice, the left ventricular (LV) mass was increased, and the LV volume was decreased. These alterations were ameliorated by NaHS (Figure 1a). These results suggested that db/db mice suffering from DCM could be alleviated by exogenous H2S. H2S is an important gaseous signalling molecule. Although our previous study showed that exogenous H2S improved H2S production in a type I diabetic model, here we reveal that H2S concentration can elicit changes in the hearts of db/ db mice. The H2S probe 7-azido-4-methylcoumarin (C-7Az) was used to test the H2S levels in mouse hearts. The results showed that H2S levels were decreased in the hearts of db/db mice and those levels recovered after NaHS injection (Figure 1b). We also tested the expression and activity of CSE in the hearts of db/db mice. The results showed that the expression and activity of CSE were downregulated in db/db mice, while they were elevated with the treatment of NaHS (Figures 1c and d). Furthermore, exogenous H2S protected cardiomycocytes against apoptosis in the hearts of db/db mice (Supplementary Figure S2).

H9C2 cells were treated with high glucose (HG) and palmitate (HG+Pal) to mimic cardiomycocytes in type 2 diabetes, and the results showed that the H2S levels and expression of CSE were downregulated in the HG+Pal group but were elevated with the treatment of NaHS (Supplementary Figures S3a and b). Meanwhile, the ratio of apoptotic cells was increased in the HG+Pal group, whereas it was decreased with the treatment of NaHS (Supplementary Figure S3c).

Exogenous H2S attenuated ROS production in cardiomycocytes. It has been reported that oxidative stress can promote DCM. To investigate the effect of exogenous H2S on oxidative stress, we used 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and mito-SOX staining to examine the cytosolic and mitochondrial ROS content, respectively, in mouse hearts. The results showed that the fluorescence

Figure 1  Exogenous H2S could protect cardiomycocytes and improve H2S levels in type 2 diabetes. Eight- to ten-week-old db/db mice treated with or without 100 μmol/kg NaHS by intraperitoneal injection were kept on a standard chow diet for 12 weeks. (a) The cardiac function of mice was examined by heart echocardiography. (b) The content of H2S was detected by an H2S probe in mouse myocardia (blue); scale bars: 50 μm. (c) The expression of CSE in mouse hearts was detected by western blotting. (d) The activity of CSE in mouse hearts was detected using CSE detection kits. Values are presented as the mean ± S.D. from n = 6 replicates. *P < 0.05, **P < 0.01 compared with the WT group; #P < 0.05, ##P < 0.01 compared with the db/db group.
intensity in the hearts of db/db mice was enhanced, whereas NaHS attenuated these alterations (Figures 2a and b). The expression and activity of mitochondrial catalase (Mito-CAT) and manganese-dependent superoxide dismutase (Mn-SOD) were detected to further examine the role of exogenous H2S on ROS production in the hearts of db/db mice. The results showed that the expression and activity of Mito-CAT and Mn-SOD, which were downregulated in the hearts of db/db mice, were upregulated by NaHS treatment (Figures 2c–f).

Furthermore, exogenous H2S suppressed the ROS production in H9C2 cells treated with HG and palmitate (Supplementary Figure S4), which reinforced the concept that exogenous H2S attenuated oxidative stress in cardiomyocytes.

Exogenous H2S had no significant effects on Nrf2 nuclear translocation. It has been proven that exogenous H2S attenuates oxidative stress through the Keap-1/Nrf2 pathway.19–22 Therefore, we detected the expression of Keap-1 and Nrf2. The results showed that the expression of Keap-1 was significantly upregulated by exogenous H2S (Figure 3a), whereas the expression of Nrf2 was upregulated in the hearts of both in db/db mice and NaHS injection mice (Figure 3b). However, the expression of Nrf2 in the nucleus showed no significant change (Figure 3c). These results imply that exogenous H2S had no significant effects on the Keap-1/Nrf2 pathway and that its antioxidative effects should be attributed to another mechanism.

Exogenous H2S facilitated ubiquitin aggregate clearance via autophagy by suppressing ubiquitylation of Keap-1. To find a new explanation for the antioxidative effect of H2S, the effect of Keap-1 on facilitating ubiquitin aggregate clearance was considered. It has been shown that Keap-1 has a pivotal role in eliminating ubiquitin aggregates.16 Therefore, we detected the ubiquitylation level in mouse hearts. The results showed that the ubiquitylation levels of 90–110, 70–90 and 10 kDa proteins were increased in the hearts of db/db mice and that this alteration was attenuated with the treatment of NaHS (Figure 4a). Meanwhile, the ubiquitylation levels of Keap-1, CAT and SOD were increased in the hearts of db/db mice, while they were attenuated following NaHS treatment (Figure 4b). This might be the reason for exogenous H2S upregulating Keap-1 expression and suppressing ROS production. To further investigate the effect of exogenous H2S on ubiquitin aggregate clearance, we observed the ubiquitylation level in H9C2 cells using an immunofluorescent assay. The results showed that the
ubiquitin-positive protein aggregates accumulated in the HG + Pal group, whereas NaHS promoted ubiquitin aggregate clearance (Figure 4c, red arrows). It has been reported that ubiquitin aggregates are mainly eliminated by autophagy.23 To determine whether the ubiquitylated proteins were the main source of autophagosomes content in the cells, PYR41 (3 μM), an inhibitor of ubiquitin-activating enzyme (E1), was used to suppress the ubiquitylation. The autophagosomes aggregated in the HG+Pal group and PYR41 eliminated the autophagosome aggregates (Figure 4d, red arrows). To investigate the NaHS-facilitated ubiquitinated protein aggregate clearance via autophagy, we added 3-methyladenine (3MA) under HG+Pal+NaHS conditions and detected the ubiquitinated protein level. Our results showed that the ubiquitinated protein level was augmented in the NaHS + 3MA group compared with the NaHS group and was even higher than that in the HG+Pal group (Figure 4e). These results indicate that the NaHS-facilitated ubiquitinated protein aggregate clearance occurs mainly via autophagy. The above results demonstrated that exogenous H2S could eliminate ubiquitin aggregates, which might be a new explanation for the antioxidative effect of H2S.

Exogenous H2S could promote autophagy in the hearts of db/db mice and H9C2 cells. Because ubiquitin aggregates are mainly eliminated by autophagy, and some studies have shown that autophagy is interrupted in type 2 diabetes,24,25 we examined the autophagosome content in mouse hearts using a transmission electron microscope. Autophagosomes were found in the myocardiocytes of db/db mice with or without NaHS treatment. The autophagosome contents in db/db mice were not degraded and manifested as a high-density area, whereas they were degraded following NaHS treatment, manifesting as a low-density area (Figure 5a, red arrow). The monodansylcadaverine (MDC) staining showed that autophagosomes accumulated in the hearts of db/db mice, manifesting as green patches of fluorescence (Figure 5b, red arrows), which were reduced with the treatment of NaHS, and these data reinforced the concept that exogenous H2S facilitated autophagosome content elimination. To investigate the effect of exogenous H2S on the elimination of autophagosome content, we detected two autophagosomal markers, P62 and microtubule-associated protein 1 light chain 3 II (LC3 II). The results showed that the expression of P62 and LC3 II was increased in the hearts of db/db mice, whereas exogenous H2S reduced P62 expression but increased LC3 II expression (Figures 5c and d), which was in accordance with the disruption of autophagy and lysosome bonding.26 To further investigate the effects of exogenous H2S on autophagy, we detected the expression of two upstream factors, Beclin1 and autophagy-related protein 7 (ATG7). The results demonstrated that the expression of ATG7 was decreased in the hearts of db/db mice, and the expression of both Beclin1 and ATG7 increased with the treatment of NaHS (Figures 5e and f), which indicated that exogenous H2S promoted autophagy.

To further demonstrate the effects of exogenous H2S on autophagy, we detected the autophagosomes and the expression of P62, LC3 II, ATG7 and Beclin1 in H9C2 cells. The results showed that autophagosomes accumulated in the HG+Pal group (Figure 6a, red arrows), which was attenuated...
Figure 4 Exogenous H₂S attenuated ubiquitylation levels. (a) The expression of ubiquitous protein was analysed by western blotting in mouse myocardia. (b) The ubiquitylation level of Keap-1, SOD and CAT in mice myocardium was detected by immunoprecipitation and western blotting. H9C2 cells were treated with HG (40 mM) + palmitate (Pal, 200 μM), HG+Pal+NaHS (100 μM), HG+Pal+NaHS+3MA (2 mM, an inhibitor of autophagy) and HG+Pal+PYR41 (3 μM, an inhibitor of ubiquitin-activating enzyme (E1)) for 48 h. (c) The ubiquitinated proteins in H9C2 cells were detected by an immunofluorescent assay (green); red arrows indicate ubiquitinated protein aggregates; scale bar: 100 μm. (d) The autophagosomes were detected by MDC test in H9C2 cells (green). Red arrows indicate autophagosome accumulation; scale bar: 100 μm. (e) The ubiquitinated protein level was detected by western blotting.
with NaHS treatment. In addition, in accordance with the in vivo data, the expression of P62 and LC3 II was increased in the HG+Pal group, while the expression of ATG7 and Beclin1 was decreased, and these alterations were ameliorated by exogenous H2S (Figure 6b). DL-propargylglycine (PPG), an inhibitor of CSE, was used to investigate whether endogenous H2S affects autophagosome clearance under the HG+Pal+NaHS conditions. Our results showed that NaHS reduced the content of the autophagosomes in H9C2 cells treated with HG and palmitate, while the addition of PPG had no influence (Figure 6a). However, the addition of PPG increased the expression of P62 and LC3 II simultaneously, while NaHS alone decreased the expression of the two proteins (Figure 6b). These results suggested that endogenous H2S may have a part role in autophagosomal clearance. To investigate whether Keap-1 is the target of NaHS, the Keap-1 short interfering RNA (siRNA) was used to downregulate the expression of Keap-1. The results indicated that Keap-1 siRNA reduced the expression of Beclin1, whereas the ratio of LC3 II to LC3 I was increased (Figure 6c). To investigate whether the antioxidative effect of exogenous H2S played a main role in autophagy, the inhibitor of mitochondrial ROS, Mito-TEMPO, and the inhibitor of total ROS, acetylcysteine (NAC), were applied in our study. The results indicated that Mito-TEMPO and NAC reduced the expression of Beclin1 and increased the expression of LC3 II (Figure 6d), which indicated that direct inhibition of ROS production had no significant effects on autophagosomal degradation. These data

Figure 5  Exogenous H2S could promote autophagy in the hearts of db/db mice. (a) The ultrastructure of mouse myocardia was observed using a transmission electron microscope. The red arrow indicates an autophagosome and it was amplified in the red rectangle; scale bars: 2 μm. (b) The autophagosome was detected using an MDC test in mouse myocardia (green). Red arrows indicate autophagosome accumulation. (c-f) The expression of P62, LC3 II, Beclin1 and ATG7 was detected by western blotting. Values are presented as the mean ± S.D. from n = 6 replicates. *P < 0.05, **P < 0.01 compared with the WT group; #P < 0.05, ##P < 0.01 compared with the db/db group.
suggested that the antioxidative effect of exogenous H2S attributed to its promotion of autophagy, which played a crucial role in ubiquitin aggregate clearance.

Exogenous H2S-attenuated Keap-1 ubiquitylation possibly by promoting its disulphide formation. Some studies have reported that H2S could promote disulphide formation between two Keap-1 molecules. Therefore, 1,4-dithiothreitol (DTT) was used to inhibit disulphide formation. The results showed that the effects of exogenous H2S on Keap-1 ubiquitylation and expression were blocked with DTT treatment (Figures 7a and b). The effect of NaHS on autophagosomal elimination was also blocked with DTT treatment (Figures 7c and d). To determine whether exogenous H2S could promote Keap-1 interactions with P62 and LC3 II, Keap-1 was co-precipitated with P62 and LC3 II, respectively. We found that the interaction between Keap-1 and P62 was increased with or without NaHS treatment, but exogenous H2S also increased the interaction between Keap-1 and LC3 II. DTT blocked the effect of exogenous H2S on Keap-1 (Figure 7e). Furthermore, the treatment of DTT attenuated the antioxidative effect of H2S (Figures 7f and g), which reinforced the concept that autophagy promotion might be the reason for antioxidative effect of H2S.

Discussion

The results of the current study provided new insights into the mechanisms of type 2 DCM (T2DCM) and revealed an effective protection of exogenous H2S in the model. Our results indicated that (i) exogenous H2S could attenuate ROS production and increase H2S production in db/db mouse hearts; (ii) exogenous H2S could facilitate the clearance of ubiquitin aggregates through promoting autophagy, which contributed to its antioxidative effect; and (iii) exogenous H2S could upregulate Keap-1 expression by suppressing its ubiquitylation.

DCM greatly increases the incidence and severity of heart failure in patients with diabetes. It has been reported that in patients with diabetes, increased LV mass and LV diastolic dysfunction, which are regarded as features for DCM, could be detected. In our study, we found that LV mass was increased and that LV volume was decreased in 20-week-old db/db mice. Hence, db/db mice were considered to be suffering cardiomyopathy on the twentieth week. NaHS attenuated these alterations, which might provide a new way to fight against T2DCM. H2S, as a newly found gaseous signalling molecule, has an important role in many regulatory functions. In mammalian hearts, the biosynthesis of H2S is mainly catalysed by CSE, and our previous study demonstrated that CSE was downregulated in type I diabetic hearts.
current study, we also found that H2S production was impaired in the hearts of db/db mice, which was improved by NaHS. This inferred that H2S might be an important modulator in the development of T2DCM.

Increasing evidence has shown that oxidative stress can promote the development of DCM.19 The antioxidative stress effect of H2S has an important role in the functions of H2S.30–33 It has been reported that H2S could suppress ROS production34 and increase SOD activity in cardiomyocytes.35 Our study demonstrated that exogenous H2S could suppress the production of ROS in the hearts of db/db mice. However, previous studies have demonstrated that oxidative stress can interact with autophagy, and autophagy has an important role in modulating ROS production.36–38 Autophagy is a protective factor for cell survival, which is involved in eliminating damaged proteins and organelles, and the obstruction of autophagy could result in aggregation of injured organelles and proteins, especially injured mitochondria and ubiquitinated proteins.5,39–41 Meanwhile, growing evidence has demonstrated that the development of DCM is associated with dysregulated autophagy.42,43 In addition, H2S has its regulative roles in the autophagy process during the development and progression of many diseases such as heart failure, hepatitis and Parkinson’s disease.44,45 However, the effect of H2S on autophagy in T2DCM is only slightly illuminated. Hence, our study focused on how H2S regulates autophagy in the hearts of db/db mice.

Autophagy is a well-coordinated, multi-step process regulated by autophagy-related gene products and proteins such as Beclin1 and P62.46 In our study, we found that the autophagosome contents were not degraded in the hearts of db/db mice. Moreover, the enhanced expression of LC3 II and P62, a substrate of the autophagy-lysosomal degradation pathway, also indicated that the degradation of autophagosome contents was impaired. The upstream autophagy-related proteins Beclin1 and ATG7 were also downregulated, which might ascribe to the failed degradation of the autophagosome contents. From the above results, the obstruction of autophagy might have a pivotal role in the development of T2DCM. In addition, exogenous H2S facilitated autophagosome content clearance, which seemed to improve the autophagy in T2DCM. The promotive effects of exogenous H2S on autophagy may be an important reason for decreased ROS production.

It has been reported that ubiquitin aggregate clearance mainly depends on autophagy and the disruption of autophagy results in ubiquitin aggregate accumulation in cells.47,48 We found that exogenous H2S reduced the ubiquitination level in the hearts of db/db mice. PYR41, an inhibitor of ubiquitination, abolished the autophagosomes formation, which indicated that ubiquitinated proteins were the main contents of autophagosome. However, how exogenous H2S promotion of ubiquitin aggregate clearance via autophagy still needs an explanation. Recent studies have found that Keap-1 has a crucial role in eliminating ubiquitin proteins.16 So Keap-1 may be a key factor for the promotive function of exogenous H2S on ubiquitin aggregate clearance via autophagy. We found that exogenous H2S upregulated the expression of Keap-1. It has also been reported that Keap-1 regulates the translocation of Nrf2, a negative regulator of ROS production, to the nucleus.59 However, in our study, exogenous H2S had no significant effects on the translocation of Nrf2 to the nucleus. A recent study by Ji and his colleague demonstrated that H2S suppressed diabetes-accelerated atherosclerosis via...
activation by inducing Keap-1S-sulphydration.\textsuperscript{22} The different findings between our and Ji’s studies are perhaps attributed to two different kinds of cells. Or rather, in a long-term high-sugar, high-fat and low-energy environment, the translocation of Nrf2 may be repressed and this might explain why H\textsubscript{2}S upregulates cellular antioxidants in the short-term ischaemia-reperfusion heart in a Nrf2-dependent manner\textsuperscript{50,51} but had no significant effects in our study. Meanwhile, using Mito-TEMPO and NAC alone could not reduce the expression of LC3 II, which suggested that Mito-TEMPO and NAC could not promote autophagosome clearance. Therefore, we speculated that the promotion of H\textsubscript{2}S on autophagosomal clearance was not due to its antioxidative properties and might be the mechanism for its antioxidative properties in DCM. Therefore, the protective effect of exogenous H\textsubscript{2}S on Keap-1 was focused on ubiquitin aggregate clearance via autophagy. We found that in H9C2 cells, HG and palmitate increased the interaction between Keap-1 and P62 but decreased the interaction between Keap-1 and LC3 II. This indicated that the ubiquitylation level of Keap-1 was elevated, which is tethered to P62 through molecular ubiquitin, and suppressed ubiquitin aggregate clearance via autophagy. Exogenous H\textsubscript{2}S enhanced the interaction of both Keap-1–P62 and Keap-1–LC3 II, which reinforced that exogenous H\textsubscript{2}S could facilitate ubiquitin aggregate clearance via autophagy. Meanwhile, the application of Keap-1 siRNA also repressed the effect of exogenous H\textsubscript{2}S on autophagy. From the above, the results suggested that the effect of exogenous H\textsubscript{2}S on autophagy was mainly attributed to Keap-1-mediated ubiquitin aggregate clearance.

However, it is unknown how exogenous H\textsubscript{2}S could reduce the ubiquitylation level of Keap-1, as we found. Studies have reported that H\textsubscript{2}S could promote disulphide formation between two Keap-1 molecules.\textsuperscript{22} Therefore, we detected whether exogenous H\textsubscript{2}S could suppress the ubiquitylation of Keap-1 through promoting disulphide formation of Keap-1. Therefore, DTT was used to inhibit disulphide formation. We found that DTT nearly abrogated the effect of exogenous H\textsubscript{2}S on autophagy, ROS production and Keap-1 expression. Further, DTT inhibited the interaction of Keap-1 with P62 and LC3 II, respectively. These observations suggested that exogenous H\textsubscript{2}S could facilitate ubiquitin aggregate clearance via autophagy through promoting disulphide formation of Keap-1, which might contribute to ROS scavenging. However, the details of how exogenous H\textsubscript{2}S attenuated Keap-1 ubiquitylation needs more study.

In summary, our results demonstrated that the cardiac impairment induced by type 2 diabetes might contribute to ubiquitin aggregate accumulation. Exogenous H\textsubscript{2}S could facilitate ubiquitin aggregate clearance via autophagy, which might be a new explanation for the antioxidative effect of H\textsubscript{2}S. The effect of exogenous H\textsubscript{2}S on ubiquitin aggregate clearance via autophagy might contribute to suppressing the ubiquitination of Keap-1, which upregulated the expression of Keap-1. The above evidence provides new insight into the mechanisms responsible for the antioxidative effects of H\textsubscript{2}S in the context of T2DCM (Figure 8). Upon description of the mechanism conferred by exogenous H\textsubscript{2}S protection, it is possible to provide a new avenue of therapeutic opportunities for T2DCM induced by type 2 diabetes.

Materials and Methods

Materials. H\textsubscript{2}S donor NaHS, palmitate, MDC, CSE inhibitor PPG, autophagy inhibitor 3MA, disulphide bond inhibitor DTT, mitochondrial ROS inhibitor Mito-TEMPO, ROS inhibitor NAC, E1 inhibitor PYR41 and C-7Az were purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA). Mito-TEMPO, DCFH-DA and JC-1 were purchased from Invitrogen (Grand Island, NY, USA). SOD and CAT activity assay kits were purchased from Jiancheng Institute of Bioengineering, Nanjing, China. Keap-1 siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to cleaved caspase 3 (25546-1-AP), Bax (50599-2-lg), Bcl2 (12789-1-AP), β-actin (66009-1-AP) were purchased from Proteintech (Rosemont, IL, USA).

Animals. Leptin receptor-deficient (db/db) mice (8–10 weeks old) and wild-type C57BL/6 mice were purchased from the Animal Model Institute of Nanjing (Nanjing, China). The animals were housed under diurnal lighting conditions and fed standard mouse chow and water throughout the study period. All animal experiments were

Figure 8 The protective effect of exogenous H\textsubscript{2}S on cardiomyocytes in a type 2 diabetes model. Hyperglycaemia and hyperlipidaemia induced by type 2 diabetes increase ROS production, which results in the ubiquitylation of Keap-1. As the ubiquitylation of Keap-1 was increased, the ubiquitin aggregates cannot be resolved in time. The ubiquitin aggregates accumulating in cells cause more production of ROS, which forms a vicious circle and finally induces cell death. Exogenous H\textsubscript{2}S could promote attenuated ubiquitylation of Keap-1, which has a protective effect on Keap-1. The increased expression of Keap-1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy, which suppresses the ROS production and leads to cell survival.
H2S promotes autophagy in type 2 diabetic hearts

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performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the China National Institute of Health and approved by the Animal Care Committees of Harbin Medical University, China.

Experimental groups. The animal experiment was divided into three groups. Each group included 8 mice (n=8). The wild-type C57BL/6 mice (8–10 weeks old) were kept on a standard chow diet for 12 weeks as control. The db/db mice (8–10 weeks old, blood glucose concentration ≥ 16.7 mmol/L) were divided into groups treated with vehicle or NaHS by intraperitoneal injection and kept on a standard chow diet for 12 weeks. The dose of NaHS was 100 μmol/kg used as an effective dose in previous studies.52,53

Echocardiographic analysis of cardiac function. Cardiac functions of mice were assessed using an echocardiography system (GE VIVID 7i DS, ST, CT, Fairfield, USA) after 12 weeks treated. Echocardiography was performed on self-breathing mice under anaesthesia (intraperitoneal injection of 1% pentobarbital sodium at 6 mg/kg body weight). The following LV parameters were measured, including LV mass, LV end-diastolic volume and EF.

Cell culture and treatment. Primary cultures of H9C2 rat cardiac myoblasts, purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China), were grown as monolayers at a density of 5 × 10^5 cells/cm in Dulbecco’s modified Eagle medium and incubated at 37 °C in humidified air containing 5% CO2. The medium contained 10% calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Two days after being seeded, the cultured H9C2 were randomly divided into the following seven groups: control (low glucose, 5.5 mM), HG (40 mM)+palmitate (Pal, 200 μM), HG+Pal+NaHS (100 μM), HG+Pal+NaSH-PPG (10 mM, an irreversible competitive CSE inhibitor), HG+Pal+NaSH-3MA (2 mM, an inhibitor of autophagy), HG+Pal+NaHS+DTT (20 μM, an inhibitor of disulfide bond), HG+Pal+Mito-TEMPO (2 μM, an inhibitor of mitochondrial ROS), HG+Pal+NaS-DCC (100 μM, an inhibitor of ROS) and HG+Pal+PYR41 (3 μM, an inhibitor of ubiquitin-activating enzyme (E1)). Drugs were added directly to the culture for 48 h. H9C2 cells treated with HG and palmatine classically mimic the myocardiosens in hyperglycaemia and hyperlipidaemia.54

Detection of H2S in frozen sections of mouse heart using H2S probe C-7Az. The turn-on fluorescence response of H2S in RAECs was tested C-7Az, which has been demonstrated to selectively respond to H2S. The frozen sections of mouse hearts were incubated with C-7Az to H2S in the frozen sections of mouse hearts was carried out using confocal fluorescence imaging could be used to detect H2S through the triggered fluorescence response of C-7Az.

Mitochondrial ROS and cellular ROS level analysis. Mitochondrial ROS production was measured using Mito-SOX Red mitochondrial superoxide indicator (Invitrogen). The frozen sections of mice hearts and H9C2 cells were loaded with 5 μM Mito-SOX Red at 37 °C for 15 min. Red fluorescence was measured at 583 nm following excitation at 488 nm using a Zeiss LSM 510 inverted confocal microscope (Heidenheim, Germany). Intracellular ROS levels were examined using the DCFH-DA staining method based on the conversion of non-fluorescent DCFH-DA to the highly fluorescent DCF upon intracellular oxidation by ROS. The frozen sections of mice hearts and H9C2 cells were seeded on coverslips and incubated (45 min, 37 °C, in the dark) in serum-free media containing DCFH-DA (10 μM) in the presence of control, HG and NaHS. After incubation, the conversion of DCFH-DA to the fluorescent product DCF was measured using a spectrofluorometer with excitation at 484 nm and emission at 530 nm. Background fluorescence (conversion of DCFH-DA to the highly fluorescent DCF upon intracellular oxidation by ROS) was subtracted from the fluorescence measured in the presence of ROS. The threshold of ROS production was measured using Mito-SOX Red at 37 °C for 15 min. The samples were centrifuged at 10 000 g for 3 min. The supernatants were loaded onto an SDS-PAGE gel, and the supernatant was carefully transferred to a fresh, well-labelled microfuge tube and stored at –80 °C for later use. IPs was separated by SDS-PAGE, and proteins were transferred to PVDF membrane. Samples were probed with appropriate antibodies (anti-Keap-1, anti-LC3, anti-CAT and anti-SOD) for analysis.

siRNA transfection. H9C2 cells (80% confluent) were treated according to the manufacturer’s instructions with Keap-1 siRNAs (mouse; Santa Cruz Biotechnology) for 72 h to inhibit Keap-1 expression. Transfection of H9C2 cells by siRNA was achieved using Lipofectamine 2000 (Invitrogen). In brief, Keap-1 siRNA with the transfection reagent was incubated for 20 min to form complexes, which then were added to plates containing cells and medium. The cells were incubated at 37 °C in a CO2 incubator for further analysis.

Statistical analysis. Data were presented as mean ± S.D. Data were first analysed using one-way ANOVA test. Tukey’s test was used for post hoc comparisons. The threshold of P < 0.05 was designated as statistically significant for all tests. All statistical analyses were performed using Prism 5 (GraphPad, La Jolla, CA, USA).

Conflict of Interest The authors declare no conflict of interest.

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1. Dokken B. Mechanisms of cardiovascular injury in type 2 diabetes and potential effects of dipetidyl peptidase-4 inhibition. J Cardiovasc Nurs 2016; 31: 274–283.
2. From AM, Leibson CL, Bursi F, Redfield MM, Weston SA, Jacobsen SJ et al. Diabetes in heart failure: prevalence and impact on outcome in the population. Am J Cardiol 2006; 118: 591–598.
3. Chit F, Dobson OM. Protein misfolding, functional amyloid, and human disease. Am J Rev Biochem 2006; 75: 333–366.
4. Sato A, Asano T, Isono M, Ito K. Panobinostat synergizes with bortezomib to induce endoplasmic reticulum stress and ubiquitinated protein accumulation in renal cancer cells. BMC Urol 2014; 14: 71.
5. Shang F, Taylor A. Ubiquitin-proteasome pathway and cellular responses to oxidative stress. Free Radic Biol Med 2011; 51: 5–16.
6. Meijer AJ, Cordogno P. Autophagy: a sweet process in diabetes. Cell Metab 2008; 8: 275–276.
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35. Sun WH, Liu F, Chen Y, Zhu YC. Hydrogen sulfide decreases the levels of ROS by inhibiting mitochondrial complex IV and increasing SOD activities in cardiomyocytes under ischemia/reperfusion. Biochim Biophys Acta 2012; 421: 164–169.

36. Tai H, Wang Z, Gong H, Han X, Zhou J, Wang X et al. Autophagy impairment with lysosomal and mitochondrial dysfunction is an important characteristic of oxidative stress-induced heart failure. Autophagy 2017; 13: 99–113.

37. Nakpa VP, Prakash-babu P, Vemuganti R. Crosstalk between endoplasmic reticulum stress, oxidative stress, and autophagy: potential therapeutic targets for acute CNS injuries. Mol Neurobiol 2016; 53: 332–344.

38. Xue H, Wang X, Hill K, Chen J, Lamasters J, Yang SM et al. Autophagy attenuates noise-induced hearing loss by reducing oxidative stress. Antioxid Redox Signal 2015; 22: 1308–1324.

39. Liu YB, Gao Z, Diao D, Arbab AS, Gautam SC. Pristimerin induces apoptosis in prostate cancer cells by down-regulating Bcl-2 through ROS-dependent ubiquitin-proteasomal degradation pathway. J Carcinog Mutagen 2013; suppl 6: 005.

40. Dimkovikj A, Fisher B, Hutchinson-Van R, Dobbs J, Bough Z. H2S as a physiologic vasorelaxant: from pathophysiology to treatment. J Pharmacol Exp Ther 2010; 329: 443.

41. Kan Y, Zhao J, Li J, Xia M, Tan Y, Yang ZZ et al. Disruption of calpain reduces lipotoxicity-induced cardiac injury by preventing endoplasmic reticulum stress. Biochim Biophys Acta 2016; 1862: 2023–2033.