Hydrogen sulfide induces keap1 S-sulfhydration and suppresses diabetes-accelerated atherosclerosis via Nrf2 activation

Running title: S-sulfhydration and diabetes-accelerated atherosclerosis

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Abstract:

Hydrogen sulfide (H$_2$S) has been shown to have powerful anti-oxidative and anti-inflammatory properties which can regulate multiple cardiovascular functions. However, its precise role in diabetes-accelerated atherosclerosis remains unclear. We report here that H$_2$S reduced aortic atherosclerotic plaque formation with reduction in superoxide (O$_2^-$) generation and the adhesion molecules in streptozotocin (STZ)-induced LDLr$^{-/-}$ mice but not in LDLr$^{-/-}$Nrf2$^{-/-}$ mice. In vitro, H$_2$S inhibited foam cell formation, decreased O$_2^-$ generation, as well as increased Nrf2 nuclear translocation and consequently heme oxygenase-1 (HO-1) expression up-regulation in high-glucose (HG) plus oxidized low density lipoprotein (ox-LDL)-treated primary peritoneal macrophages from wild-type but not Nrf2$^{-/-}$ mice. H$_2$S also decreased O$_2^-$ and adhesion molecules levels, increased Nrf2 nuclear translocation and HO-1 expression which were suppressed by Nrf2 knockdown in HG/ox-LDL-treated endothelial cells. H$_2$S increased S-sulfhydration of Keap1, induced Nrf2 dissociation from Keap1, enhanced Nrf2 nuclear translocation and inhibited O$_2^-$ generation which were abrogated after Keap1 mutated at Cys151, but not Cys273, in endothelial cells. Collectively, H$_2$S attenuates diabetes-accelerated atherosclerosis, which may be related to inhibition of oxidative stress via Keap1 sulfhydrylation at Cys151 to activate Nrf2 signaling. This may provide a novel therapeutic target to prevent atherosclerosis in the context of diabetes.
Introduction:

Diabetes leads to a marked increase in atherosclerosis (1). There is considerable evidence demonstrating that oxidative stress and inflammation are involved in the pathogenesis of diabetes and its complications, including atherosclerosis (2). It has been suggested that hyperglycemia-induced superoxide overproduction may be a key event in activation of pathways involved in the pathogenesis of diabetic complications (2). Approaches that limit oxidative stress may therefore translate to reduced inflammation and hence atherosclerosis.

It is well established that nuclear factor erythroid 2–related factor 2 (Nrf2) is one of the most important cellular defense mechanisms against oxidative stress. Nrf2 is broadly expressed in tissues but is only activated in response to a range of oxidative and electrophilic stimuli (3). Upon oxidative stress, Nrf2 escapes Kelch-like ECH-associated protein 1 (Keap1)-mediated repression, translocates to the nucleus, binds to antioxidant response element (ARE), and induces the expression of a battery of antioxidant proteins, one of the most important of which is heme oxygenase 1 (HO-1) (4). Nrf2 has emerged as an important target in diabetes and related complications (5,6), and low dose dh404, which is an analogue of the Nrf2 agonist bardoxolone methyl, lowers oxidative stress and protects against diabetes-associated atherosclerosis (7). These studies suggest that augmentation of antioxidant defenses
via up-regulation of the Nrf2 pathway may be novel target for the prevention and treatment of diabetic complications.

Hydrogen sulfide (H$_2$S) plays an important role in physiology and pathophysiology in several biological systems. Emerging data suggest that H$_2$S improves diabetic endothelial dysfunction (8), nephropathy (9), retinopathy (10), and cardiomyopathy (11). However, there are no published data on the potential effect of H$_2$S on accelerated atherosclerosis in diabetes.

Some recent studies indicate that H$_2$S is cytoprotective during myocardial ischemia-reperfusion injury in the setting of diabetes by alleviating oxidative stress, and the ability of H$_2$S to up-regulate cellular antioxidants in the heart in a Nrf2-dependent manner (12-14). H$_2$S may therefore play an important role in diabetes-accelerated atherosclerosis, and the effects of H$_2$S may be mediated via activation of Nrf2. In the present study, we have characterized whether and how H$_2$S targets on Nrf2 against the development of diabetes-accelerated atherosclerosis.
Research design and Methods:


LDLr<sup>−/−</sup> mice, on a C57BL/6 background, were purchased from Model Animal Research Center of Nanjing University. Nrf2<sup>−/−</sup> mice, on a C57BL/6 background, were a gift from Hongliang Li (Wuhan University), Nrf2<sup>−/−</sup> mice were crossed with LDLr<sup>−/−</sup> mice to obtain LDLr<sup>−/−</sup>Nrf2<sup>−/−</sup>. At ages 8 weeks, male mice were rendered diabetic by administering 60 mg/kg/day streptozotocin (STZ) intraperitoneally (i.p.) daily for 5 days. After STZ administration, diabetic mice were administered the H<sub>2</sub>S donor GYY4137 (133 µmol/kg/day, i.p.) or vehicle, and kept on a high fat diet for 4 weeks. The dose of GYY4137 used was based on previous publications (15). Nondiabetic LDLr<sup>−/−</sup> or LDLr<sup>−/−</sup>Nrf2<sup>−/−</sup> mice were kept on a standard chow diet for 4 weeks as control. Mice were housed (n=1/cage, n=6/group) in metabolic cages for 48h prior to metabolic analysis to acclimatise. Body weight, food intake, water intake and urinary output were determined.

All animal experiments were approved by the Committee on Animal Care of Nanjing Medical University, and were conducted according to the NIH Guidelines for the Care and Use of Laboratory Animals. All studies involving animals are reported in accordance with the ARRIVE guidelines.

2. Blood sampling.
Plasma samples were obtained from 6-h–fasted mice. Glucose was measured directly from the tail tip with a glucometer; plasma lipid levels were measured enzymatically using commercial kits (Zhong Sheng Bei Kong, Peking, China) as the manufacturer’s instructions.

3. Measurement of plasma H$_2$S

Plasma H$_2$S concentration was measured as described previously (15).

4. Analysis of Atherosclerotic Lesions

To evaluate atherosclerotic lesions, en face whole and histological sections were used to analyze. The entire aorta attached to the heart was dissected and stained with Oil Red O (ORO; Sigma Aldrich, MO, USA) (16). Lesions within the sinus were visualized after staining with ORO as well as hematoxylin and eosin (HE) and quantitated as described previously (16).

5. Cell culture and experimental conditions

Mouse peritoneal macrophages were isolated from male C57BL/6 or Nrf2$^{-/-}$ mice as described previously (17). Peritoneal cells were collected by lavage and seeded in DMEM-low glucose medium (Gibco, Grand Island, USA) with 10% FBS (Gibco).
Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords according to a previously described method (18). The endothelial cells were cultured in Endothelial Cell Medium (ECM, ScienCell, CA, USA).

EA.hy926 endothelial cells were purchased from the American Type Culture Collection (Rockville, MD, USA), and were cultured in DMEM-low glucose medium with 10% FBS.

Confluent cells (80–85%) were incubated with D-glucose (25 mM, Sigma) plus ox-LDL (50 mg/L, Yiyuan biotechnology, China), in the absence or presence of GYY4137 (50, 100 µM), sulfide-depleted GYY4137 (SDG, dissolved GYY4137 left unstoppered at room temperature for 5 days) or ZYJ1122 (a structural analogue of GYY4137 lacking sulfur) for 24 h.

6. Small interfering RNA or plasmid transfection

EA.hy926 cells were transfected with siRNA oligonucleotide against Nrf2 (sense: 5′AAGAGUAUGAGCUGGAAAAACdTdT-3′, antisense: 5′GUUUUUCCAGCUCAUACAUUCdTdT-3′, Genepharma) or negative control siRNA. HO-1 expression was silenced by HO-1 siRNA mix that was purchased from Santa Cruz. The plasmid pcDNA3-flag-Keap1 purchased from Addgene (Cambridge, MA) was termed as Keap1-WT. Single mutation at Cys-151, 273 or 288 to Ala (Haibio, Shanghai, China) was confirmed by DNA sequencing. EA.hy926 cells were
transfected with expression vectors using the Lipofectamine 3000 reagent (Invitrogen).

7. Foam Cell Formation Assay

Macrophages were fixed with 4% paraformaldehyde and stained using 0.5% ORO. Images of cells were acquired using a light microscope (Nikon, Tokyo, Japan).

8. Measurement of reactive oxygen species (ROS) formation

Superoxide production in tissue sections of upper descending thoracic aorta and cells was detected by dihydroethidium (DHE) assay as the manufacturer’s instructions. Briefly, cells or tissue were incubated with DHE for 30min. Fluorescence was measured with a Nikon TE2000 Inverted Microscope and quantified using Image-Pro Plus analysis software.

9. Immunofluorescence staining

Sections or cells were fixed and permeabilized, then blocked and incubated with antibody against Nrf2, VCAM-1, ICAM-1, CD31, α-SMA or Macrophage (Abcam, Cambridge, MA). After additional washing, sections or cells were incubated with directly conjugated fluorescent secondary antibodies, and with DAPI (Invitrogen). Fluorescence was imaged using a Nikon TE2000 Inverted Microscope. Positive cells
and total cells were quantified in 5 different sections from 6 different mice of each genotype, using Image-Pro Plus analysis software.

10. RNA Analysis

HO-1, ICAM-1, VCAM-1, thioredoxin (Trx) and glutamate cysteine ligase catalytic subunit (GCLC) mRNA expression was quantified by real-time PCR (RT-PCR) with forward and reverse primers (Supplement Table 1).

11. Isolation of Nuclear and Cytoplasmic Proteins and Western blotting

Whole-cell, cytosolic and nuclear proteins were extracted using RIPA buffer (Sigma Aldrich) or a nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific Inc.). Western Blotting was performed as described previously (19). Primary antibodies included anti-Nrf2 (Santa Cruz, MO, UAS), anti-Nrf2 (phospho S40) (Abcam), anti-HO-1 (Bioworld Technology Inc., Nanjing, China), anti-VCAM-1 (Abcam), anti-ICAM-1 (Abcam), anti-histone H3 (Bioworld), and anti-GAPDH (Abcam) antibody. Band intensities were analyzed using Image J 1.25 software.

12. Immunoprecipitation

The cells were harvested and lysed as previously described (20). Antibodies specific for Keap1 (Santa Cruz) or normal rabbit IgG were added to the supernatants followed by an incubation. Immune complexes were then precipitated with protein
A-agarose beads. Bound proteins were eluted by boiling with loading buffer and analyzed by western blotting with anti-Nrf2 antibody.

13. “Tag-Switch” method

Nrf2 and Keap1 S-sulfhydration (Keap1-SSH) was detected with “Tag-Switch” method (21). The protein of Keap1 was pulled down with immunoprecipitation, and treated with biotin-linked cyanoacetate. Samples were resuspend in Laemmli buffer, heated, and subjected to western blotting analysis using anti-biotin antibody (Santa Cruz).

Statistical analysis

Data are expressed as mean ± standard error and were analyzed by one-way ANOVA followed by Newman-Keuls Multiple Comparison Test as appropriate. All statistical analyses were performed using SPSS software, version 16.0. A value of $P<0.05$ was considered statistically significant.
Results:

1. Metabolic characteristics and plasma level of H$_2$S.

As expected, diabetic LDLr$^{-/-}$ mice fed a HFD had lower body weight, higher plasma total cholesterol, triacylglycerol, urinary output, water intake and food intake when compared with those fed standard chow, and these effects were unaffected by treatment with GYY4137 (Supplement Table2). Compared to LDLr$^{-/-}$ mice, plasma H$_2$S concentration was reduced in HFD-fed diabetic LDLr$^{-/-}$ mice which could be significantly increased by administration of GYY4137 (Supplement Table2).

2. H$_2$S decreases Atherosclerotic Lesions in diabetic LDLr$^{-/-}$ mice.

To determine the effect of H$_2$S on the formation of atherosclerotic lesions in STZ-diabetic LDLr$^{-/-}$ mice, these animals were treated with GYY4137 or vehicle for 4 weeks, and a HFD diet was used to enhance atherogenesis (Figure 1A). Initially, we measured the total aortic lesion area between the proximal ascending aorta and the bifurcation of the iliac artery by en face analysis of ORO-stained aortas. As expected, diabetic mice showed an increase in atherosclerotic plaques compared with non-diabetic control. Treatment of diabetic LDLr$^{-/-}$ mice with H$_2$S reduced lesion area (Figure 1B and Supplement Figure 1). Similar results were confirmed by HE and ORO staining in the aortic root (Figure 1C and 1D). Immunofluorescence analysis of sections from the aortic root revealed that macrophage content was increased in
diabetic LDLr<sup>−/−</sup> mice, and this effect was attenuated by H<sub>2</sub>S treatment (Figure 1E and 1F). Collectively, these data demonstrate that exogenous H<sub>2</sub>S decreases atherosclerotic lesions in diabetic LDLr<sup>−/−</sup> mice.

3. H<sub>2</sub>S reduces the level of superoxide, VCAM-1 and ICAM-1, and activates expression of Nrf2 and associated antioxidant proteins, in vessels of diabetic LDLr<sup>−/−</sup> mice.

Oxidative stress plays an important role in the pathogenesis of diabetes and its complications. To determine whether the protective role of H<sub>2</sub>S in atherosclerotic lesions might relate to reduction of ROS, we measured aortic superoxide formation by DHE assay. As expected, compared with non-diabetic LDLr<sup>−/−</sup> mice, endothelial fluorescence was increased in diabetic LDLr<sup>−/−</sup> mice. In contrast, endothelial superoxide production in diabetic LDLr<sup>−/−</sup> mice was attenuated following GYY4137 administration (Figure 2A and 2B). Several recent studies have identified Nrf2 as a critical transcription factor that regulates a battery of antioxidant genes in the face of oxidative stress (3). Recent work has also shown that H<sub>2</sub>S regulates Nrf2 in myocardial tissue (12). To examine whether Nrf2 is activated in response to H<sub>2</sub>S treatment, we investigated the intracellular localization of Nrf2. Immunofluorescence microscopy showed enhanced nuclear staining of Nrf2 in aortas of H<sub>2</sub>S-treated in comparison to untreated diabetic LDLr<sup>−/−</sup> mice (Figure 2C). To further confirm the
Nrf2 localization, we performed double staining for Nrf2 and CD31 (endothelial marker), α-SMA (smooth muscle cells marker) or macrophages marker and found Nrf2 could be clearly shown co-localized with three markers in aorta. And GYY4137 treatment increased Nrf2 nuclear translocation in aortic endothelial cells, smooth muscle cells and macrophages (Supplement Figure 2). In addition, the induction of expression of the Nrf2-related antioxidant defense enzyme HO-1 was substantially increased by H$_2$S (Figure 2D); whilst other Nrf2 target genes, such as Trx and GCLC, were unchanged (Supplement Figure 3).

Oxidative stress induces the expression of adhesion molecules such as VCAM-1 and ICAM-1, which promote the recruitment to, and accumulation of inflammatory cells within the developing atherosclerotic lesion. Therefore, the levels of VCAM-1 and ICAM-1 in aorta were determined by RT-PCR and immunofluorescence, after 4 weeks on HFD, both VCAM-1 and ICAM-1 increased in diabetic LDLr$^{-/-}$ mice, and this effect was abrogated by treatment with H$_2$S (Figure 2E-2G and Supplement Figure 4). Together, these results indicate that exogenous H$_2$S attenuates diabetes-accelerated atherosclerosis, most likely by maintaining redox balance via the Nrf2 pathway.

4. Nrf2 deficiency abolishes the protective effects of H$_2$S in STZ-induced LDLr$^{-/-}$ mice.
To further explore the pathophysiological significance of H$_2$S-induced Nrf2 activation in vivo, we mated LDLr$^{−/−}$ mice with Nrf2$^{−/−}$ mice to generate LDLr$^{−/−}$Nrf2$^{−/−}$ mice. After injection of STZ and 4 weeks of HFD, with or without concomitant GYY4137 treatment, metabolic characteristics were assessed (Supplement Table 3). Histological assessment of atherosclerotic lesions at the aortic sinus by ORO and HE staining showed a marked increase of plaques in the aortic root from LDLr$^{−/−}$Nrf2$^{−/−}$ diabetic mice fed HFD, and the aortic plaque area was now not reduced by H$_2$S treatment (Figure 3A and 3B). Moreover, the expression of superoxide, VCAM-1, and ICAM-1 were not reduced after treatment of GYY4137 in diabetic LDLr$^{−/−}$Nrf2$^{−/−}$ mice (Figure 3C-G and Supplement Figure 5). Complementary analyses of Nrf2 target gene levels in aorta revealed that the expression of HO-1 could not be augmented by H$_2$S in the presence of Nrf2 deficiency (Figure 3H). These results demonstrate that Nrf2 is necessary for the inhibitory effect of H$_2$S to be exerted on diabetes-accelerated atherosclerosis in vivo.

5. H$_2$S decreases foam cell formation and production of superoxide, and enhances HO-1 expression via activation of Nrf2, in HG plus ox-LDL treated mouse macrophages.

Accumulation of cholesterol and cholesteryl esters in macrophages and subsequent foam cell formation is a critical early event in atherogenesis. To further
investigate the molecular mechanisms underlying the effects of H$_2$S, we established a macrophage model in hyperglycaemic and hyperlipidemic conditions in vitro, which replicates some of the characteristics of macrophages in the diabetes-accelerated atherosclerotic mouse model. Mouse peritoneal macrophages from C57BL/6 were incubated with HG plus ox-LDL, with or without GYY4137 for 24 h; following which foam cell formation and ROS production were measured by ORO staining and DHE assay respectively. As expected, foam cell formation was induced in macrophages exposed to ox-LDL, and this effect was exaggerated by co-incubation with HG (data not shown). Pretreatment with GYY4137 (50 or 100 µM), but not SDG or ZYJ1122, abrogated this effect (Figure 4A and Supplement Figure 6). In addition, superoxide generation was enhanced in HG plus ox-LDL-stimulated macrophages (Figure 4B and 4C), and this too was attenuated by pretreatment with H$_2$S.

Next, to test whether Nrf2 is involved in the effects of H$_2$S on macrophage function, we investigated its intracellular localization. Immunofluorescence microscopy showed enhanced nuclear staining of Nrf2 in cells treated with H$_2$S in comparison to vehicle-treated cells, in the presence of HG plus ox-LDL (Figure 4D). Western blotting analysis of cytoplasmic and nuclear protein extracts also indicated increased nuclear accumulation of Nrf2 protein in cells treated with H$_2$S (Figure 4E and 4F), suggesting that Nrf2 is activated in response to H$_2$S exposure. Similarly, in
the presence of HG plus ox-LDL, H₂S-pretreated macrophages exhibited increased production of HO-1 (Figure 4G).

Additional experiments were performed to confirm the involvement of Nrf2 in the protective effect of H₂S, using mouse peritoneal macrophages isolated from Nrf2⁻/⁻ mice. Inhibition of foam cell formation and superoxide generation induced by HG plus ox-LDL was attenuated by H₂S treatment in Nrf2 knockout (KO) cells (Figure 5A-5C). Consistent with these results, elevation of HO-1 expression by H₂S treatment was also abolished in the Nrf2 KO group (Figure 5D). Furthermore, HO-1 knockdown by siRNA or inhibition by ZnPP (HO-1 inhibitor) also abrogated H₂S-mediated suppression of O₂⁻ generation and foam cell formation (Supplement Figure 7). These results demonstrate that Nrf2/HO-1 pathway is responsible for the inhibitory effects of H₂S on HG plus ox-LDL-induced foam cell formation and oxidative stress in macrophages.

6. H₂S decreases ROS, ICAM-1 and VCAM-1 generation, and enhances HO-1 expression via Nrf2 signaling, in HG plus ox-LDL treated endothelial cells.

It has been reported that endothelial dysfunction caused by lipotoxicity or hyperglycemia is mediated through several mechanisms including increased oxidative stress and proinflammatory responses. Therefore, we measured the effect of H₂S on oxidative stress in endothelial cells by DHE assay. Stimulation of HUVECs with HG
plus ox-LDL for 24 h caused an increase in production of superoxide, and this increase was alleviated by pretreatment with GYY4137 (50 or 100 µM) (Figure 6A and 6B) but not with SDG or ZYJ1122 (Supplement Figure 8).

To confirm whether the cytoprotective effect of H$_2$S against oxidative stress was also associated with Nrf2, we carried out immunofluorescence and western blotting for Nrf2. GYY4137 had no effect on Nrf2 phosphorylation, but can increase the Nrf2 protein expression in the nuclear in HG plus ox-LDL-stimulated endothelial cells (Figure 6C-E and Supplement Figure 9A), implying that H$_2$S may promote phosphorylation-independent Nrf2 nuclear translocation. Consistent with our in vivo study, H$_2$S reduced the expression of VCAM-1 and ICAM-1 (Figure 6F and 6G); in addition, the Nrf2 target gene HO-1 was also increased by H$_2$S pretreatment (Figure 6H and 6I). To further clarify whether H$_2$S-induced down-regulation of oxidative stress was dependent on activation of the Nrf2 pathway, EA.hy926 cells were transfected Nrf2 siRNA for 24 h before H$_2$S and HG+ox-LDL treatment. Western blotting revealed that individual transfection with Nrf2 siRNA successfully reduced Nrf2 protein expression at 24 h post-transfection, as compared to negative control siRNA-transfected (Ctl siRNA) cells (Figure 7A). Nrf2 knockdown abrogated H$_2$S-mediated suppression of ROS production induced by HG plus ox-LDL in endothelial cells (Figure 7B and 7C). Furthermore, inhibition of HO-1 expression or activity by siRNA or ZnPP abolished the protective effects of H$_2$S (Supplement
Figure 10). Together, these results indicate that the anti-oxidative and anti-inflammatory effects of H$_2$S in the presence of HG plus ox-LDL are partially mediated by Nrf2/HO-1 pathway in endothelial cells.

7. H$_2$S S-sulfhydrylated Keapl at Cys151 to regulate Nrf2 activity and reduce ROS generation in HG plus ox-LDL treated endothelial cells.

Generally, Nrf2 is retained in an unactivated state binding with Keap1 in the cytoplasm, which serves as an adaptor for the degradation of Nrf2. Nrf2 can be activated by physiological stimuli which disrupt Keap1-Nrf2 interactions leading to nuclear translocation of Nrf2 (22). To further explore the mechanisms of Nrf2 activation, we immunoprecipitated the cell lysate using an anti-Keap1 antibody and blotted for Nrf2. The results showed that GYY4137 decreased Nrf2/Keap1 interaction in HG plus ox-LDL-treated endothelial cells (Figure 8A). S-sulfhydration, the addition of one sulfhydryl to thiol side of cysteine residue and formation of persulfide group (R-S-S-H), has been identified as a novel post-translational modification by H$_2$S in eukaryotic cells. However, the covalent modification in sulfhydration is reversed by reducing agents, such as dithiothreitol (DTT) (23). We tested the S-sulfhydration of Nrf2 and found that H$_2$S donor GYY4137 or NaHS had no effect on Nrf2 S-sulfhydration (Supplement Figure 9B). We next investigated whether H$_2$S directly modified Keap1. After preincubation with GYY4137, EA.hy926 cells were
treated with HG plus ox-LDL and subjected to “Tag-Switch” assay. There was stronger S-sulfhydration of Keap1 after GYY4137 incubation (Figure 8B). To identify the S-sulfhydrated cysteine residue, Keap1 mutated at Cys151, Cys273, or Cys288 to alanine (C151A, C273A, or C288A) or wild-type (WT) was transfected into endothelial cells. H$_2$S still enhanced S-sulfhydration on Keap1 after wild-type or mutated Keap1 at Cys288 but not at Cys151 and Cys273 overexpression (Figure 8C). H$_2$S increased Nrf2 dissociation from Keap1 in HG plus ox-LDL-treated endothelial cells after Keap1-WT and Keap1-C273A but not Keap1-C151A overexpression (Figure 8D). Moreover, after Keap1 mutation at Cys151, H$_2$S failed to induce Nrf2 nuclear translocation or decrease the generation of superoxide (Figure 8E-G). These findings indicate that S-sulfhydration of Cys151 in Keap1 is critical for Nrf2 activation in HG plus ox-LDL treated endothelial cells.
**Discussion:**

A complex interaction between inflammation, lipid deposition, monocytic infiltration and endothelial dysfunction is responsible for the initiation and progression of diabetes-accelerated atherosclerosis (1). Experimental evidence for an anti-atherosclerotic effect of H$_2$S has been obtained in numerous studies in hyperlipidemic animal and cell models (15, 24-26), but the anti-atherosclerotic effect in the context of diabetes has not been previously investigated. Recent data published by our group demonstrated that treatment with H$_2$S decreased aortic atherosclerotic plaque formation and partially restored aortic endothelium-dependent relaxation in ApoE$^{-/-}$ mice fed with a HFD (15). Exogenous H$_2$S improved endothelium-dependent relaxation in isolated vascular rings incubated with high glucose, and attenuated hyperglycemia-induced DNA injury and improved cellular viability in bEnd.3 microvascular endothelial cells (8). These data suggested that exogenous H$_2$S might serve as a treatment option for diabetic-associated atherosclerosis. Indeed, in the present study, we found that H$_2$S supplementation reduces lesion area and macrophage infiltration in diabetic LDLr$^{-/-}$ mice. In agreement with these findings, we also observed that H$_2$S treatment attenuated HG+ox-LDL-induced foam cell formation. Our study provides the first evidence that H$_2$S may prevent development of diabetes-accelerated atherosclerosis, and that this does not relate to any effects on circulating blood glucose or cholesterol.
Several pathological mechanisms have been proposed for diabetic vascular complications, including diabetes-accelerated atherosclerosis, such as increased polyol pathway flux, increased advanced glycation end-product formation and activation of protein kinase C; all of these, in association with hyperglycemia-induced ROS accumulation (27). Endothelial cells and macrophages are both sources of ROS. Indeed, in our study, we have demonstrated that STZ-treated LDLr<sup>−/−</sup> mice fed a HFD showed an increase in atherosclerotic plaques compared with non-diabetic LDLr<sup>−/−</sup> mice, accompanied by increased superoxide production in aorta and this was further confirmed in HG plus ox-LDL-treated macrophages and endothelial cells. The increase in ROS promotes the recruitment and accumulation of inflammatory cells to the developing atherosclerotic lesion. H<sub>2</sub>S has also been shown to have powerful antioxidant properties. Exogenous H<sub>2</sub>S attenuates the hyperglycemia-induced enhancement of ROS formation in endothelial cells and human U937 monocytes (8,28). In line with these findings, we observed that H<sub>2</sub>S decreased superoxide generation in macrophages and endothelial cells cultured with HG plus ox-LDL. Furthermore, we showed that superoxide production in the aortas of diabetic LDLr<sup>−/−</sup> mice was reduced following H<sub>2</sub>S administration. In this study, we show for the first time that inhibition of HG+ox-LDL-generated ROS with H<sub>2</sub>S prevents the diabetes-induced increase in plaque area. Additionally, we found that H<sub>2</sub>S attenuates the increase in aortic VCAM-1 and ICAM-1 expression.
Recent studies indicate that H$_2$S may up-regulate endogenous antioxidants through an Nrf2-dependent signaling pathway (12) to combat oxidative stress. To date, the role of Nrf2 in atherosclerosis remains controversial. Myeloid Nrf2 deficiency aggravates both early and late stages of atherosclerosis in LDLr$^{-/-}$ mice (29,30). Ellagic acid improves oxidant-induced endothelial dysfunction and atherosclerosis partly via Nrf2 activation (31). In contrast to these reported protective actions, Nrf2 has also been ascribed as having potentially pro-atherogenic functions, in that ApoE$^{-/-}$Nrf2$^{-/-}$ double knockout mice exhibited reduced plaque (32). In diabetes-associated atherosclerosis, a novel analog of the Nrf2 agonist bardoxolone methyl, has been found to reduce atherosclerotic lesions as well as oxidative stress and the proinflammatory mediators ICAM-1 and VCAM-1, in STZ-induced diabetic ApoE$^{-/-}$ mice (7). Our data support previous findings as regards the protective actions of Nrf2, and suggest that H$_2$S can attenuate endothelial dysfunction, foam cell formation and atherosclerosis in the context of diabetes, at least partially via the Nrf2/HO-1 pathway.

A widely accepted model for Nrf2 nuclear accumulation describes that a modification of the Keap1 cysteines leads directly to the dissociation of the Keap1-Nrf2 complex (33). Recently, one study suggested that Keap1 can be S-sulfhydrated at Cys151 which stimulates the dissociation of Nrf2 to enable its translocation to nucleus (34). We found that Keap1 could be S-sulfhydrated at Cys151
and Cys273 simultaneously, but only the S-sulfhydration of Cys151 was involved in activation of Nrf2 which decreased the ROS generation to improve endothelial function. Kim S et al. found that thiol modification of Keap1 Cys288 is responsible for daillyl trisulfide-induced activation of Nrf2 signaling (35). However, in our study, Cys288 of Keap1 could not be S-sulfhydrated after treatment with GYY4137. This discrepancy may be attributed to different regulatory mechanisms in different cell types, the use of different H₂S treatment regiments giving rise to different kinetics of H₂S release. Nevertheless, this study demonstrates a significant role of Keap1 Cys151 S-sulfhydration in the protective effects of H₂S against diabetes-accelerated atherosclerosis.

In summary, our study provided definitive evidence that H₂S can lessen diabetes-accelerated atherosclerosis in LDLr⁻/⁻ mice and improve hyperglycemia/ox-LDL-induced injury in macrophages and endothelial cells. This protective effect of H₂S can partly be attributed to activation of Nrf2 via Keap1 S-sulfhydration at Cys151. Our findings suggest that activation of Nrf2 may be a potential novel therapeutic strategy against diabetes-associated vascular disease, and that exogenous H₂S administration in the form of an H₂S donor GYY4137 may be of therapeutic benefit in the setting of diabetes-associated atherosclerosis. Finally, our study provides new insight into the mechanisms responsible for the anti-atherosclerotic effects of H₂S in the context of diabetes.
Author Contributions.

L.X. and Y.G. researched data, contributed to discussion, and edited the manuscript. W.M., S.Z., W.W. and Y.M. researched data. G.M. contributed to discussion and proof-read the manuscript. H.F., Y.H., and Y.W. researched data. G.L. designed study and reviewed data. P.K.M., X.W., H.W., Z.Z. and Y.Y. reviewed the manuscript. A.F. contributed to discussion and re-wrote the manuscript. Z.H. reviewed data and edited the manuscript. Y.J. designed study, reviewed data and edited manuscript. All authors have approved the final version of the manuscript. Prof. Y.J. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References:


Figure Legends

Figure 1. Effects of H$_2$S on atherosclerosis in HFD fed diabetic LDLr$^{-/-}$ mice.
Diabetic LDLr$^{-/-}$ mice were fed a high-fat diet (HFD) and received daily ip injection of saline or H$_2$S donor GYY4137 (133 µmol/kg/day) for 4 weeks. A, Schema of experimental procedure. B, Lesion areas shown was quantified using Oil-Red O (ORO) staining of the thoracoabdominal aorta. C and D, Representative ORO and hematoxylin eosin (HE)-stained images and quantification of aortic sinus sections from LDLr$^{-/-}$ (n=6), STZ+HFD (n=9) and STZ+HFD+GYY4137 (n=8). Scale bars, 200 µm. E and F, Frozen sections of aortic root were stained for anti-macrophage (green) and DAPI (blue). Dotted lines indicate the boundary of lesion and aortic tunica intima. L indicates lumen. Quantitative data in the graph represent the positively stained area percentage of plaque (n=6). Scale bars, 100 µm. Data shown are mean±SEM. ***P<0.001 vs. LDLr$^{-/-}$ mice. ###P<0.001 vs. STZ+HFD mice.

Figure 2. Effects of H$_2$S on ROS formation, Nrf2, VCAM-1 and ICAM-1 expression in aortas from HFD fed diabetic LDLr$^{-/-}$ mice. A, Representative dihydroethidium (DHE) fluorescence image of aortic tissue from LDLr$^{-/-}$, STZ+HFD and STZ+HFD+GYY4137. Scale bars, 100 µm. B, Quantification of DHE fluorescence image of A, ***P<0.001 vs. LDLr$^{-/-}$ mice. ###P<0.01 vs. STZ+HFD mice, n=6. C, Representative immunostaining for Nrf2 (green) and DAPI (blue) of aorta.
Scale bars, 50 µm. D, mRNA levels of HO-1 in the aortas of LDLr^{−/−} (n=6), STZ+HFD (n=6) and STZ+HFD+GYY4137 (n=8), as determined by real-time quantitative polymerase chain reaction (RT-PCR) analysis. E and F, mRNA levels of VCAM-1 and ICAM-1 in the aortas of LDLr^{−/−} (n=6), STZ+HFD (n=6) and STZ+HFD+GYY4137 (n=6). G, Representative VCAM-1 and ICAM-1 immunostaining of aortic arch section (with arrows). Scale bars, 100 µm. *P<0.05, **P<0.01 and ***P<0.001 vs. LDLr^{−/−} mice. #P<0.05, ##P<0.01 vs. STZ+HFD mice.

ROS indicates reactive oxygen species; Nrf2, nuclear factor E2-related factor 2; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1.

Figure 3. Effects of H_{2}S on diabetes-accelerated atherosclerosis in LDLr^{−/−}Nrf2^{−/−} mice. Diabetic LDLr^{−/−}Nrf2^{−/−} mice were fed a HFD and received daily ip injection of saline or GYY4137 (133 µmol/kg/day) for 4 weeks. A and B, Representative ORO and HE-staining images and quantification of aortic sinus sections from LDLr^{−/−}Nrf2^{−/−} mice, STZ+HFD and STZ+HFD+GYY4137 (n=5). Scale bars, 200 µm. C, Representative DHE fluorescence image of aorta. Scale bars, 100 µm. D, Quantification of DHE fluorescence image of C, *P<0.05, ***P<0.001 vs. LDLr^{−/−} mice, ##P<0.01, ###P<0.001 vs. LDLr^{−/−}Nrf2^{−/−} mice, n=5-7. E, Representative VCAM-1 and ICAM-1 immunostaining of aortic arch section (with arrows). Scale bars, 100 µm. F and G, mRNA levels of VCAM-1 and ICAM-1 in the aorta of LDLr^{−/−}Nrf2^{−/−},
STZ+HFD and STZ+HFD+GYY4137 (n=5). H, mRNA levels of HO-1 in the aorta (n=5). Data shown are mean±SEM. *P<0.05, **P<0.01 vs. control. HO-1 indicates heme oxygenase-1.

Figure 4. Effects of H$_2$S on HG plus ox-LDL-treated primary peritoneal macrophages. Isolated peritoneal macrophages from C57BL/6 mice were treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (50 or 100 µM) for 24 h. A, Macrophages incubated as above and stained with ORO. Scale bars, 20 µm. B, Representative DHE stained images showing ROS generation in each condition. Scale bars, 50 µm. C, Quantification of DHE fluorescence image of B, **P<0.01 vs. untreated control. #P<0.05, ##P<0.01 vs. treatment with HG plus ox-LDL, n=5. D, Immunohistochemistry was performed on macrophages stained with antibody directed against Nrf2 (green) and DAPI (blue). Scale bars, 20 µm. E and F, Western blotting analysis and quantification of cytoplasmic and nuclear Nrf2 protein. GAPDH and histone H3 were used for normalization for cytoplasmic and nuclear proteins respectively (n=4). G, Western blotting analysis and quantification of HO-1 protein expression (n=4). Data shown are mean±SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. untreated control. ##P<0.01 vs. treatment with HG plus ox-LDL.
Figure 5. Effects of H$_2$S on HG plus ox-LDL-treated primary peritoneal macrophages from Nrf2$^{-/-}$ mice. Isolated peritoneal macrophages from C57BL/6 (wild-type, WT) and Nrf2$^{-/-}$ mice were treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) for 24 h. A, macrophages from Nrf2$^{-/-}$ mice were stained with ORO. Scale bars, 20 µm. B, Representative DHE stained images of macrophages from WT and Nrf2$^{-/-}$ mice. Scale bars, 50 µm. C, Quantification of DHE fluorescence image of B, ***$P<0.001$ vs. WT control. ###$P<0.001$ vs. WT with HG plus ox-LDL. ##&$P<0.001$ vs. Nrf2$^{-/-}$ control. n=3. D, Western blotting analysis and quantification of HO-1 protein expression in macrophages from Nrf2$^{-/-}$ mice (n=4). Data shown are mean±SEM. *$P<0.05$ vs. untreated control.

Figure 6. Effects of H$_2$S on HG plus ox-LDL-treated endothelial cells. HUVECs were treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (50 or 100 µM) for 24 h. A, Representative DHE stained images showing ROS generation. Scale bars, 100 µm. B, Quantification of DHE fluorescence image of A, **$P<0.01$ vs. untreated control. #P<0.05, ##P<0.01 vs. treatment with HG plus ox-LDL. n=5. C, Immunohistochemistry was performed on HUVECs stained with antibody directed against Nrf2 (green) and DAPI (blue). Scale bars, 20 µm. D and E, Western blotting analysis and quantification of cytoplasmic and nuclear Nrf2 protein. GAPDH and histone H3 were used for normalization for
cytoplasmic (n=5) and nuclear (n=4) proteins respectively. F and G, Western blotting analysis and quantification of VCAM-1 (n=4) and ICAM-1 (n=3) protein. H, mRNA levels of HO-1 (n=5). I, Western blotting analysis and quantification of HO-1 protein expression (n=4). Data shown are mean±SEM. *P<0.05, **P<0.01 and ***P<0.001 vs. untreated control. #P<0.05, ##P<0.01 and ###P<0.001 vs. treatment with D-glucose plus ox-LDL. HUVECs indicates human umbilical vein endothelial cells.

**Figure 7. Effects of H₂S on HG plus ox-LDL-treated Nrf2 knockdown endothelial cells.** EA.hy926 endothelial cells were transfected with control siRNA (Ctl siRNA) or Nrf2 siRNA for 24 h and then treated with D-glucose (25 mM) and ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) for 24 h. A, Western blotting analysis and quantification of Nrf2 (n=3). Data shown are mean±SEM. ***P<0.001 vs. Ctl siRNA control. B, Representative DHE staining images. Scale bars, 50 µm. C, Quantification of DHE fluorescence image of B, **P<0.01, vs. Ctl siRNA control. #P<0.05 vs. Ctl siRNA with HG plus ox-LDL. &&&P<0.001 vs. Nrf2 siRNA control, n=5.

**Figure 8. H₂S S-sulfhydrylated Keap1 at Cys151 to regulate Nrf2 transcription activity and reduce the generation of ROS in HG plus ox-LDL-treated endothelial cells.** A, EA.hy926 endothelia cells were treated with GYY4137 (100 µM) followed by D-glucose (25 mM) plus ox-LDL (50 mg/L) stimulation for 24 h, cell
lysates were immunoprecipitated with an anti-Keap1 or an anti-IgG antibody (negative control), and blotted with an anti-Nrf2 antibody (upper panel). An aliquot of total lysate was analyzed for Keap1, Nrf2 and GAPDH expression (lower panels). 

**B**, EA.hy926 endothelial cells were treated with DTT (1 mM, negative control) or D-glucose (25 mM) plus oxLDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) for 2 h. S-sulphhydration on Keap1 was detected with “Tag-Switch” method. 

**C**, After plasmid transfection of wild type Keap1(Keap1-WT) or mutated Keap1 at Cys151, Cys273, Cys288 for 24 h followed by GYY4137 (100 µM) treated for another 2 h, S-sulphhydration on Keap1 was detected with “Tag-Switch” method. 

**D**, Transfected cells were treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) for another 24 h, cell lysates immunoprecipitated with an anti-Keap1 antibody and the immunoprecipitated proteins were subjected to immunoblot analysis with anti-Nrf2 antibodies (upper panels). The total lysates was analyzed with anti-Keap1, anti-Nrf2, and anti-GAPDH antibodies (lower panels). 

**E** and **F**, Transfected cells treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) for 24h. Nuclear extracts prepared from cells were subjected to western blotting analysis for detecting the nuclear localization of Nrf2 (n=4). ROS accumulation was determined by the DHE assay. Scale bars, 50 µm. Data shown are mean±SEM. **P<0.01 vs. Keap1-WT transfected cells treatment with D-glucose plus ox-LDL.**
Quantification of DHE fluorescence image of F, **$P<0.01$ vs. untreated Keap1-WT transfected cells. ###$P<0.01$ vs. Keap1-WT transfected cells treated with D-glucose and ox-LDL. & & $P<0.01$ vs. untreated C151A transfected cells, $n=4$. 
A

B

C

ROS measured using EHE (% of control)

Control  HG+ox-LDL  HG+ox-LDL+G100

Control  HG+ox-LDL  HG+ox-LDL+G100

Ctrl siRNA  Nrf2 siRNA

Ctrl siRNA  Nrf2 siRNA

Nrf2/GAPDH (of control)

0.0  0.2  0.4  0.6  0.8

Ctrl siRNA  Nrf2 siRNA

Ctrl siRNA  Nrf2 siRNA

82x76mm (300 x 300 DPI)
Supplement Table 1. Primers for Real time qPCR.

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Data are means ±SD, n=6. *P<0.05, **P<0.01 and ***P<0.001 vs. LDLr<sup>−/−</sup> mice. #P<0.05 vs. STZ+HFD mice. TG, triacylglycerols; TC, total cholesterol; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol.
Supplement Table 3. Metabolic parameters of nondiabetic and diabetic LDLr<sup>−/−</sup>Nrf2<sup>−/−</sup> mice treated with vehicle or H<sub>2</sub>S donor GYY4137.

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Data are means ±SD, n=5-6. *P<0.05, **P<0.01 and ***P<0.001 vs. LDLr<sup>−/−</sup>Nrf2<sup>−/−</sup> mice.
Supplement Figure 1: Quantification of en face Oil Red O staining of aortas from LDLr<sup>-/-</sup>, STZ+HFD and STZ+HFD+GYY4137 mice (n=6). *P<0.05, ***P<0.001 vs. LDLr<sup>-/-</sup> mice. ##P<0.01 vs. STZ+HFD mice.
Supplement Figure 2: A, Representative immunostaining for CD31 (red), Nrf2 (green) and DAPI (blue) of aorta from LDLr<sup>−/−</sup>, STZ+HFD and STZ+HFD+GYY4137. Scale bars, 20 µm. B, Representative immunostaining for α-SMA (red), Nrf2 (green) and DAPI (blue) of aorta from LDLr<sup>−/−</sup>, STZ+HFD and STZ+HFD+GYY4137. Scale bars, 20 µm. C, Representative immunostaining for anti-Macrophage (red), Nrf2 (green) and DAPI (blue) of aortic root from STZ+HFD and STZ+HFD+GYY4137. Dotted lines indicate the boundary of lesion and aortic tunica intima. Scale bars, 50 µm.
Supplement Figure 3. GCLC and Trx gene expression in aorta from HFD fed diabetic LDLr−/− mice with or without GYY4137 treatment. RT-PCR analysis of GCLC (A) and Trx (B), GYY4137 has no effect on mRNA levels of GCLC and Trx and in the aorta of STZ+HFD+GYY4137 mice (n=6) compared with STZ+HFD mice (n=4). **P<0.01 and ***P<0.001 vs. LDLr−/− mice. GCLC indicates Glutamate-cysteine ligase catalytic subunit, Trx, Thioredoxins.
Supplement Figure 4. A, Quantitative data in the graph represent relative fluorescence intensity of aortic VCAM-1 (n=6) from LDLr<sup>−/−</sup>, STZ+HFD and STZ+HFD+GYY4137 mice. B, Quantitative data represent relative fluorescence intensity of aortic ICAM-1 (n=6). *P<0.05, ***P<0.001 vs. LDLr<sup>−/−</sup> mice. ###P<0.001 vs. STZ+HFD mice.
Supplement Figure 5: A, Quantitative data in the graph represent relative fluorescence intensity of aortic VCAM-1 (n=5) from LDLr⁻/⁻Nrf2⁻/⁻, STZ+HFD and STZ+HFD+GYY4137 mice. B, Quantitative data represent relative fluorescence intensity of aortic ICAM-1 (n=5). **P<0.01 vs. LDLr⁻/⁻Nrf2⁻/⁻ mice.
Supplement Figure 6. Isolated peritoneal macrophages from C57BL/6 were treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) or ZYJ1122 (100 µM) or SDG (sulfide-depleted GYY4137, 100 µM) for 24 h. A, Representative DHE stained images showing ROS generation in each condition. Scale bars, 100 µm. B, Quantification of DHE fluorescence image of A, ***P<0.001 vs. untreated control. ###P<0.001 vs. treatment with HG plus ox-LDL, n=3. C, Macrophages incubated as above and stained with ORO. Scale bars, 20 µm.
Supplement Figure 7. A–C, isolated peritoneal macrophages from C57BL/6 were transfected with control siRNA (Ctl siRNA) or HO-1 siRNA for 24 h and then treated with D-glucose (25 mM) and ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM, G100) for 24 h. Western blotting analysis and quantification of Nrf2 (A), DHE staining for superoxide production (B) and quantification (C) (n=3). Scale bars, 100 µm. **P<0.01, ***P<0.001 vs. Ctl siRNA control. &&&P<0.001 vs. Ctl siRNA with HG plus ox-LDL.

D and E, primary peritoneal macrophages were pretreated with HO-1 inhibitor ZnPP (10 µM) or vehicle, and then treated with D-glucose (25 mM) and ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM, G100) for 24 h. DHE staining for superoxide production (D) and quantification (E) (n=3). Scale bars, 100 µm. *P<0.05, ***P<0.001 vs. Vehicle control. ###P<0.001 vs. Vehicle with HG plus ox-LDL. &&&P<0.001 vs. ZnPP.

F and G, macrophages incubated as above and stained with ORO. Scale bars, 20 µm.
Supplement Figure 8. HUVECs were treated with D-glucose (25 mM) plus ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM) or ZYJ1122 (100 µM) or SDG (sulfide-depleted GYY4137, 100 µM) for 24 h. A, Representative DHE stained images showing ROS generation in each condition. Scale bars, 100 µm. B, Quantification of DHE fluorescence image of A, ***P<0.001 vs. untreated control. #P<0.05, ###P<0.001 vs. treatment with HG plus ox-LDL, n=3.
Supplement Figure 9. A, EA.hy926 endothelial cells were treated with GYY4137 (100 µM) followed by D-glucose (25 mM) and ox-LDL (50 mg/L) stimulation for 24 h. Western blotting analysis and quantification of p-Nrf2 protein expression (n=5). B, EA.hy926 endothelial cells were treated with DTT (1 mM, negative control) or GYY4137 (100 µM) for 2 h. Detection of protein S-sulfhydration on Nrf2 (S-sulfhydrated-Nrf2) with “Tag-Switch” method (n=5).
Supplement Figure 10. A, EA.hy926 cells were transfected with control siRNA (Ctl siRNA) or HO-1 siRNA for 24 h, western blotting analysis and quantification of Nrf2. B and C, EA.hy926 cells were transfected with control siRNA (Ctl siRNA) or HO-1 siRNA for 24 h and then treated with D-glucose (25 mM) and ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM, G100) for 24 h. DHE staining for superoxide production (B) and quantification (C) (n=3). Scale bars, 100 µm. ***P<0.001 vs. Ctl siRNA control. ##P<0.01 vs. Ctl siRNA with HG plus ox-LDL. &&&P<0.001 vs. HO-1 siRNA control. D and E, EA.hy926 cells were pretreated with HO-1 inhibitor ZnPP (10 µM) or vehicle, and then treated with D-glucose (25 mM) and ox-LDL (50 mg/L) in the presence or absence of GYY4137 (100 µM, G100) for 24 h. DHE staining for superoxide production (C) and quantification (D) (n=3). Scale bars, 100 µm. **P<0.01 vs. Vehicle control. ^P<0.05 vs. Vehicle with HG plus ox-LDL. &&&P<0.001 vs. ZnPP.