Hydrogen sulfide lowers hyperhomocysteinemia dependent on cystathionine γ lyase S-sulfhydration in ApoE-knockout atherosclerotic mice

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Funding information
CAMS Innovation Fund for Medical Sciences, Grant/Award Number: CIFMS, 2016-12M-1-006; National Natural Science Foundation of China, Grant/Award Numbers: 81600677, 81870318, 81670379 and 81470552; State Key Laboratory of Cardiovascular Disease, Grant/Award Number: 2018fk-02

Background and Purpose: Hydrogen sulfide donors can block the cardiovascular injury of hyperhomocysteinemia. H2S also lowers serum homocysteine in rats with mild hyperhomocysteinemia, but the pharmacological mechanism is unknown. The present study investigated the mechanism(s) involved.

Experimental Approach: ApoE-knockout mice were fed a Paigen diet and L-methionine in drinking water for 16 weeks to create a mouse model of atherosclerosis with hyperhomocysteinemia. H2S donors (NaHS and GYY4137) were administered by intraperitoneal injection. We also assayed the H2S produced (by methylene blue assay and mito-HS [H2S fluorescence probe]), cystathionine γ lyase (CSE) mRNA and protein expression, and CSE sulfhydration and nitrosylation and its activity.

Key Results: H2S donor treatment significantly lowered atherosclerotic plaque area, macrophage infiltration, and serum homocysteine level in the mouse model of atherosclerosis with co-existing hyperhomocysteinemia. mRNA and protein levels of CSE, a key enzyme catalyzing homocysteine trans-sulfuration, were down-regulated with hyperhomocysteinemia, and CSE catalytic activity was inhibited. All these effects were reversed with H2S donor treatment. Hyperhomocysteinemia induced CSE nitrosylation, whereas H2S sulfhydrated CSE at the same cysteine residues. Nitrosylated CSE decreased and sulfhydrated CSE increased its catalytic and binding activities towards L-homocysteine. Mutation of C252, C255, C307,

Abbreviations: 3-MST, 3-mercaptoppyruvate sulfurtransferase; CBS, cystathionine β synthase; CSE, cystathionine γ lyase; ER, endoplasmic reticulum; GSNO, S-nitrosoglutathione; HHcy, hyperhomocysteinemia; LDL-c, LDL cholesterol
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and C310 residues in CSE abolished CSE nitrosylation or sulfhydration and prevented its binding to L-homocysteine.

**Conclusions and Implications:** Sulfhydration or nitrosylation of CSE represents a yin/yang regulation of catalysis or binding to L-homocysteine. H$_2$S donor treatment enhanced CSE sulfhydration, thus lowering serum L-homocysteine, which contributed in part to the anti-atherosclerosis effects in ApoE-knockout mice with hyperhomocysteinemia.

### 1 | INTRODUCTION

Hyperhomocysteinemia (HHcy) usually results from a deficiency of the enzymes or the cofactors involved in the metabolism of homocysteine, such as cystathionine β synthase (CBS) and 5,10-methylenetetrahydrofolate reductase. It can also be caused by mutation of methionine, excessive methionine uptake, certain diseases (e.g., hypothyroidism), and as a side effects of some drugs (metformin, methotrexate, and nicotinic acid; Kim, Kim, Roh, & Kwon, 2018). This condition is a widely accepted independent risk factor for atherosclerosis. Elevated levels of homocysteine induce chronic inflammation, oxidative stress, and endoplasmic reticulum (ER) stress and mediate cell proliferation and migration. The result is endothelial dysfunction (Lai & Kan, 2015), increased thrombosis (Thambyrajah & Townend, 2000), accelerated vascular smooth muscle cell proliferation and migration (Luo et al., 2012), and impaired cholesterol transport in monocytes and macrophages (Ide, Keller, & Weiss, 2006; Lentz, 2005), all of which promote the pathogenesis of atherosclerosis.

**Hydrogen sulfide**, a novel gasotransmitter, is a product of the metabolism of homocysteine by a trans-sulfuration pathway catalyzed by CBS and cystathionine y lyase (CSE; Kabil & Banerjee, 2014). Treatment with H$_2$S inhibits HHcy-induced oxidative stress in vascular smooth muscle cells (Chang et al., 2008; Yan et al., 2006), ER stress in cardiomyocytes (Chang et al., 2008; Wei et al., 2010), cardiac remodeling by microRNA 133a (Kesherwani, Nandi, Sharawat, Shahshahan, & Mishra, 2015), plasma lipid peroxidation (Olas & Kontek, 2015), and platelet activation (d’Emmanuele di Villa Bianca et al., 2013). Thus, H$_2$S could be a novel inhibitor of the cardiovascular injuries induced by HHcy. Moreover, treatment with H$_2$S donor compounds lowered total blood levels of homocysteine (from 36 to 18 μM) in a rat model of mild-HHcy (Chang et al., 2008). However, the molecular mechanism(s) underlying these effects is still unknown.

The enzyme CSE can directly metabolise homocysteine to generate cystathionine, lanthionine, or α-ketobutyrate and concomitantly release H$_2$S (Chiku et al., 2009). We hypothesized that CSE activity contributed to the lowering of homocysteine levels by H$_2$S, in models of HHcy. Because HHcy was first correlated with atherosclerosis and the molecular mechanism of this injury is clear, we selected atherosclerosis as the animal model. In this mouse model of atherosclerosis with HHcy, we treated mice with two H$_2$S donors—NaH$_5$ (a rapid release donor) and GY4137 (a slow release donor)—and measured serum homocysteine, plaque changes, and expression of CSE protein.

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**What is already known**

- Treatment with H$_2$S donor compounds lowers serum total homocysteine in a rat model.

**What this study adds**

- H$_2$S sulfhydrated cystathionine y lyase and enhanced its catalytic activity to decrease homocysteine levels.
- Treatment with H$_2$S donor compounds decreased atherosclerosis in a mouse model with hyperhomocysteinemia.

**What is the clinical significance**

- H$_2$S donors may be a novel drug choice in cardiovascular diseases with hyperhomocysteinemia.

In vitro, we focused on possible modifications to CSE protein and subsequent changes to its binding and catalytic activities towards homocysteine, to test our hypothesis.

### 2 | METHODS

#### 2.1 | Mouse model of atherosclerosis

All animal care and experimental procedures complied with the Animal Management Rule of the Ministry of Health, People’s Republic of China (document No. 55, 2001) and the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, updated 2011), and were approved by the Laboratory Animal Ethics Committee of Peking University. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath & Lilley, 2015) and with the recommendations made by the British Journal of Pharmacology. A total of 45 male ApoE−/− mice (8 weeks old, 20–25 g, SPF grade, three mice per cage) were supplied by the Animal Center, Peking University Health Science Center. The atherosclerosis–HHcy model was created by giving mice a Paigen diet (D12109C, Research diets, USA) and drinking water containing L-methionine (1 g·kg$^{-1}$) in 12-hr light/12-hr dark cycle for 16 weeks. Mice were randomly
assigned to three treatment groups (n = 15 each): control (receiving 0.9% NaCl), NaHS (5.6 mg·kg\(^{-1}\)) or GYY4137 (3.6 mg·kg\(^{-1}\)), as H\(_2\)S donors. All treatments were given for 16 weeks, by intraperitoneal injection (100 μl), twice a day. The doses shown for NaHS and GYY4137 are the final daily dose administered.

2.2 | Histology

The area of atherosclerotic lesions in the aortic roots was quantified on cross sections of mouse aortas as described by Zhao et al. (2015). In brief, mice were anaesthetized by isoflurane inhalation and then sodium pentobarbital (50 mg·kg\(^{-1}\), i.p.). Blood was collected from the angular artery. Mice were killed by cervical dislocation and then perfused with 30-ml PBS, then with 4% paraformaldehyde via the left ventricle. Serial sections (10 μm thick) of frozen heart tissue were obtained by cutting from the apex of the heart towards the origin of the aorta. Sections were mounted from the point where all three aortic valve cusps became clearly visible. Every fifth section was used for Oil-red O (ORO) staining and counterstained with haematoxylin, so that each ORO-stained slide represented tissue separated by a 50-μm distance. Atherosclerotic lesion area was measured by using Image J and reported as mean area of Oli Red O staining per section in the first five sections for each mouse. Lesion area of the aorta with en face staining was quantified by using Image J (ImageJ, RRID:SCR_003070).

After being blocked, aortic root cross sections were incubated with the primary antibody anti-galectin-3 antibody (Mac-2, a marker of activated macrophages, ab2785, Abcam) or anti-CSE antibody (ab189916, Abcam), washed, and then incubated with HRP-conjugated secondary antibody (ab7061 or ab7083 respectively) for 1 hr at room temperature; the brown colour was developed by using DAB, and images were acquired. Mouse IgG replaced the primary antibody and then corresponding secondary antibody as a negative control.

2.3 | Serum homocysteine and lipid profile measurement

Serum lipid profile including levels of total cholesterol, LDL cholesterol (LDL-c), and HDL cholesterol were analysed by using an automatic chemistry analyser (IDEXX Catalyst One, USA). Serum total homocysteine level was determined by enzymic assay (DZ568A, Diazyme, San Diego, CA, USA).

2.4 | Cell culture

293-HEK (CLS Cat# 300192/p777_HEK293, RRID:CVCL_0045) and HepG2 cell (CLS Cat# 300198/p2277_Hep-G2, RRID:CVCL_0027) lines were purchased from the National Infrastructure of Cell Line Resource (China) and cultured in DMEM containing 10% FBS and 100 U·ml\(^{-1}\) penicillin–streptomycin at 37°C in a 5% CO\(_2\) atmosphere.

2.5 | Cellular H\(_2\)S product assay

HepG2 cells were treated with different concentrations of L-homocysteine, NaHS (100 μM), S-nitrosglutathione (GSNO, 500 μM), DTT (1 mM), and ascorbate (1 mM) for 12 hr and then washed three times with PBS. Cells were scraped into and homogenized in potassium phosphate buffer (50 mM, pH = 6.8). A reaction mixture containing 100-μl cellular lysate, 2-mM pyridoxal 5’-phosphate, (P3675, Sigma), 1-mM L-cysteine, or L-homocysteine in 1-ml potassium phosphate buffer (100 mM, pH = 7.4) was added to Erlenmeyer flasks. Central wells contained 0.5 ml of 1% zinc acetate as trapping solution. Reactions were performed in a 37°C shaking water bath for 90 min; then 1 ml of 20% trichloroacetic acid was added to stop the reaction. The flasks were sealed again and incubated in a shaking water bath for 1 hr at 37°C to ensure complete trapping of H\(_2\)S. The H\(_2\)S production was assayed by the methylene blue method and measured at 670 nm by spectrophotometry. The H\(_2\)S production was expressed as pmol·min\(^{-1}\)·mg\(^{-1}\) protein.

To measure intracellular H\(_2\)S levels, HepG2 cells were treated with different drugs for 2 hr and then washed three times with PBS. Fresh culture medium (detection of CSE activity) or Krebs Henseleit buffer solution containing L-homocysteine (1 mM; detection of CSE metabolism of L-homocysteine) and 10-μM mito-HS (H\(_2\)S fluorescence probe) was added and cells were continuously cultured for 1 hr in a dark box in a CO\(_2\) incubator. Fluorescence images were acquired by confocal microscopy (Wu, Liang, & Tang, 2016).

2.6 | Biotin switch assay for protein sulphydration and nitrosylation

The assay was performed as described by Mustafa et al., (2009). Briefly, cells were homogenized in HEN buffer (composition: 100 mM HEPES (pH 7.8), 1 mM EDTA, 0.1 mM Neocuproine). Homogenates were centrifuged at 14,000 g (4°C) for 15 min. The supernatant was collected and blocked with HEN buffer (containing 2.5% SDS and 20-mM methyl methanethiosulfonate) at 50°C for 20 min. Methyl methanethiosulfonate was removed by precipitating proteins with acetone at −20°C for 20 min. After acetone removal, protein was resuspended in HEN buffer containing 1% SDS, with 1-mM sodium ascorbate for subsequent nitrosylation or without sodium ascorbate for subsequent sulphydration, and 4-mM biotin-HPDP was added. Samples were then incubated for 4 hr at room temperature. Biotinylated protein was pulled down by streptavidin magnet beads and eluted by SDS-PAGE loading buffer for analysis by western blot.

2.7 | Plasmid transfection of CSE mutation

The human CSE (NM_001902.5)-GFP fusion protein plasmid (pENTER-eGFP-CSE-FLAG-6 × His) and mutation 1 (C252S + C255S, M1), mutation 2 (C307S + C310S, M2), and mutation 3 (C252S + C255S + C307S + C310S, M3) plasmids were constructed
by Shandong Vigene Biosciences. Plasmids were transfected into 293-HEK cells for biotin switch assay and microscale thermophoresis assay.

### 2.8 Western blot analysis

Total protein from cells or tissues was quantified by BCA assay, denatured by boiling, separated by SDS-PAGE, and transferred to nitrocellulose membranes (1620113, Bio-Rad, USA), which were blocked and then incubated with anti-CSE or anti-CBS (LifeSpan Cat# LS-C6843-500, RRID:AB_796261) antibody (1:1,000, with TBST) at 4°C overnight, washed and then incubated with HRP-conjugated secondary antibody (ab7083, donkey anti-rabbit IgG) for 1 hr at room temperature. After washing, ECL (1705062, Bio-Rad) was used to acquire images. The same membranes were stripped with stripping buffer (2% SDS, 62.5-mM Tris–HCl, pH = 6.8, 0.008% β-mercaptoethanol) at 50°C for 20 min and then washed five times with TBST. After re-blocking with TBST (containing 3% milk), incubating with anti-GAPDH (ab125247, 1:10,000), washing with TBST, and then incubating with HRP-conjugated secondary antibody (ab7061, donkey anti-mouse IgG), images were acquired by chemiluminescence assay. The density of bands was analysed by using Image J (Liao et al., 2015).

### 2.9 Quantitative real-time PCR

Total RNA was extracted from aorta and liver tissues or HepG2 cells and reverse transcribed for quantitative RT-PCR analysis with SYBR green I dye in the ABI7500 Quantitative PCR System. The expression of CSE, CBS, and 3-mercaptopuruvate sulfurtransferase (3-MST) was normalized to that of GAPDH and analysed by the 2^−ΔΔCt method. The primers are in Table S1.

### 2.10 Microscale thermophoresis assay for measuring CSE binding activity to L-homocysteine

Prepared wild-type or mutant human eGFP-CSE fusion protein plasmids (M1, M2, and M3) were transfected into 293-HEK cells for 24 hr. The cells were then treated with different drugs (NaHS, GYY4137, DTT, GSNO, ascorbate, and DL-propargylglycine) for 4 hr and then washed three times with PBS. Cells were homogenized with binding buffer (20-mM Tris–HCl, pH 6.8, 130-mM NaCl, 2-mM EDTA, 1% NP40, and 10% glyceride). Homogenates were centrifuged at 14,000 g for 15 min at 4°C and supernatants collected. The supernatant, containing the eGFP-CSE fusion protein, was diluted to provide eGFP fluorescence density from 600 to 1,000. This concentration of fusion protein was then divided into 12 tubes (50 μl/tube). A range of L-homocysteine solutions (125–0.061mM) were prepared by two-fold dilution and 50 μl of each dilution was added to the CSE-GFP fusion protein in the 12 tubes After mixing the samples were siphoned into capillary tubes, and binding activity (as changes in fluorescence) was measured, using the Monolith NT.115 instrument (NanoTemper Technologies; Wienken, Baaske, Rothbauer, Braun, & Duhr, 2010).

### 2.11 Data and statistical analysis

The data and statistical analysis comply with the recommendations of the British Journal of Pharmacology on experimental design and analysis in pharmacology (Curtis et al., 2018). All data collection and analysis were performed in a blinded manner. The experiments were not blinded as the H2S donors had to be dissolved, each day, in different vehicles, NaHS in saline and GYY4137 in DMSO. There were 15 mice in each experimental group, aortic tissues for different experiments, so n<15; 4 mice were used to provide blood samples for preliminary experiments, the final number of blood samples were 11. Data are presented as mean ± SD; n indicates the numbers of independent experiments. All statistical analysis were performed by Graph prism 7.0, RRID:SCR_002798. Differences between the three experimental groups (HHcy, HHcy + NaHS, and HHcy + GYY4137) were analysed by one-way ANOVA, followed by the Student–Newman–Keuls test. Differences in cellular experiments were also analysed by one-way ANOVA. Differences in protein expression (HHcy and HHcy + NaHS, or HHcy and HHcy + GYY4137) were analysed by a two-tailed t-test. Statistical significance was set at P < .05.

### 2.12 Materials

DMEM, 1,640 culture medium, and TRizol reagent were from Invitrogen (Carlsbad, CA). L-homocysteine (69453), DL-homocysteine (44925), L-methionine (M9625), DL-propargylglycine (P7888), DTT (43815), NaHS (161527), GYY4137 (SML0100) and L-ascorbic acid (795437) were from Sigma-Aldrich (St. Louis, MO).

### 2.13 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

### 3 RESULTS

#### 3.1 Treatment with H2S donor compounds reduced atherosclerosis association with HHcy

To confirm the effects of H2S on atherosclerosis in the presence of HHcy, we used ApoE-knockout mice given a Paigen diet and L-methionine in drinking water (1 g·kg⁻¹) for 16 weeks to generate the mouse model of atherosclerosis with HHcy. H2S donors (NaHS and GYY4137) were administered by intraperitoneal injection, with normal saline injection as a control. The treatments were given as two injections per day for 16 weeks. As assessed by aortic en face Oil Red O staining, treatment with H2S donor compounds significantly decreased the atherosclerotic plaque areas (Figure 1a). Aortic root...
plaque size was also lowered by H2S (Figure 1b). Immunohistochemical staining for Mac-2 confirmed that treatment with H2S donors reduced macrophage infiltration (Figure 1c), as with mouse IgG as a negative control (Figure S1). Treatment with H2S donor compounds also lowered serum levels of cholesterol (Figure 1d) and LDL-c (Figure 1e) but slightly enhanced that of HDL cholesterol (Figure 1f). These data confirmed that H2S can block the atherogenic effects of HHcy.

In the present model, L-methionine in drinking water induced a moderate HHcy level (74 ± 12 μM), and the two H2S donor treatments lowered the serum homocysteine level by 25% (55 ± 11 μM, for NaHS) and 30% (51 ± 9 μM, for GYY4137) respectively (Figure 1g). This finding was similar to those from a rat model of mild HHcy (Chang et al., 2008).

3.2 | HHcy impaired the CSE/H2S system, which was reversed by H2S donor treatment

To determine the involvement of CSE activity in the lowering of homocysteine levels by H2S donors, we first measured CSE protein expression in aortic root plaque (rabbit IgG as a negative control). Treatment with NaHS or GYY4137 increased the expression of CSE protein in aortic root plaques, compared with that in untreated ApoE-knockout mice with HHcy (Figure 2a). Consistent with these data, the mRNA levels of aortic CSE (Figure 2b) and 3-MST (Figure 2c), but not those for CBS, were up-regulated by treatment with H2S donor compounds (Figure 2d).

Liver is a major organ for the metabolism of homocysteine by trans-sulfuration, which contributes to lowering serum homocysteine
We therefore measured changes in the CSE/H₂S system in mouse liver. H₂S level was elevated in liver after treatment with NaHS or GYY4137, compared with HHcy alone (Figure 2e). Accordingly, levels of CSE protein (Figure 2f) and mRNA (Figure 2g) but not CBS and 3'-MST were up-regulated after H₂S donor treatment (Figure 2f,h). Thus, the in vivo data indicated that H₂S treatment up-regulated the endogenous CSE/H₂S system.

As with previous findings of the effects of HHcy on the CSE/H₂S system in macrophages (Li et al., 2015), we also found that DL-homocysteine, concentration dependently reduced H₂S production in HepG2 cells (Figure 3a). With an H₂S-fluorescence probe used to track the generation of H₂S in live cells, both DL-homocysteine and L-homocysteine (100 µM) lowered endogenous H₂S generation, compared with the NaHS treatment, as a positive control (Figure 3b). Consistent with the lower H₂S production with HHcy, levels of CSE mRNA (Figure 3c) and protein (Figure 3d) were concentration dependently down-regulated. These data suggest that the presence of HHcy impaired the CSE/H₂S system, which may exacerbate the HHcy injury because of the dysfunction in the trans-sulfuration metabolic pathway for homocysteine. In this HHcy (100 µM)-burdened cell model, H₂S donor treatment (NaHS and GYY4137) up-regulated the levels of CSE mRNA (Figure 3e) and protein (Figure 3f). This cellular evidence also shows that treatment with H₂S donor compounds restored the CSE/H₂S system impaired by HHcy.
3.3 HHcy increased nitrosylation of CSE, whereas H₂S promoted sulfhydration of CSE

Protein nitrosylation or sulfhydration at the same cysteine residue could induce opposing biological actions, and sulfhydration of CSE had already been identified (Mustafa et al., 2009). Whether nitrosylation or sulfhydration of CSE contributed to changes in its enzyme activity was not known. We treated HepG2 cells with L-homocysteine (12.5 to 200 μM) and found that high concentration of homocysteine greatly reduced sulfhydration of CSE but that its nitrosylation was enhanced at low concentrations (Figure 4a). In our mouse model, treatment with H₂S donor compounds also similarly enhanced sulfhydration of liver CSE and lowered its levels of nitrosylation (Figure 4b).

3.4 The yin/yang regulation of CSE enzyme activity by sulfhydration and nitrosylation

Next, we investigated the effects of changing levels of sulfhydration or nitrosylation on CSE enzyme activity metabolising homocysteine to produce H₂S. First, we treated HepG2 cells with NaHS (1 mM), GSNO (500 μM), DTT (1 mM) or ascorbate (1 mM) for 2 hr to change the sulfhydration or nitrosylation of endogenous CSE and then assayed CSE activity towards L-homocysteine, both in the live cells and in vitro. Incubation of the cells with NaHS increased the sulfhydration of endogenous CSE (Figure 5a) and then enhanced intracellular H₂S generation from L-homocysteine (Figure 5b). Incubation with DTT decreased CSE sulfhydration (Figure 5a) and decreased H₂S generation.
(Figure 5b). Exposure of cells to GSNO induced nitrosylation of CSE (Figure 5a) and reduced intracellular $\text{H}_2\text{S}$ generation, whereas ascorbate enhanced it (Figure 5b). To confirm this finding, we treated HepG2 cells with each of these agents for 12 hr and then measured CSE activity in cell extracts in vitro. Consistent with the results from live cells, $\text{H}_2\text{S}$ generation from L-homocysteine in vitro was increased by NaHS but decreased by DTT and GSNO (Figure 5b).

To confirm the effect of sulfhydration or nitrosylation of CSE on its activity, we prepared a CSE-GFP fusion plasmid and assessed the binding of CSE to L-homocysteine by microscale thermophoresis. Under physiological condition, different concentrations of L-homocysteine bound to CSE induced dynamic fluorescence density changes indicating the binding activity. Because of the limitation of the method it is not to achieve saturation binding and thus not possible to determine the usual enzymic parameters such as $K_D$ and $V_{\text{max}}$. In this assay we used DL-propargylglycine as a positive control. Sulfhydrated CSE (as after NaHS or GYY4137 treatment) increased and nitrosylated CSE (GSNO treatment) decreased its binding to L-homocysteine, which was confirmed by DTT (to remove sulfhydration) or ascorbate (to remove nitrosylation; Figure 6a).

To identify the modification sites of CSE, we constructed the human CSE (NM_001902.5)-eGFP fusion plasmid and mutated M1 (C252S + C255S), M2 (C307S + C310S), and M3 (C252S + C255S + C307S + C310S)-eGFP fusion plasmids. The biotin switch assay was used to assess sulfhydration or nitrosylation of CSE. The M1 or M2 mutation significantly decreased the levels of CSE sulfhydration or nitrosylation, and M3 mutation abolished these modifications (Figure 6b). M1, M2, and M3 mutations reduced the binding activity of both sulfhydrated CSE (Figure 6c) and nitrosylated CSE (Figure 6d). Of note, the M3 mutation almost abolished binding of CSE to L-homocysteine (Figure 6c,d). Consistent with the binding results, the $\text{H}_2\text{S}$ production catalysed by mutated CSE was also decreased (Figure 6e). These data suggest that the cysteine residues at position 252, 255, 307, and 310 in CSE are sites at which the enzyme was sulfhydrated or nitrosylated, and that these residues might be essential for the binding of CSE to L-homocysteine. Sulfhydrated CSE enhanced but nitrosylated CSE inhibited binding to L-homocysteine, which indicated a yin/yang regulation of its catalytic activity.

4 | DISCUSSION

Homocysteine is an independent risk factor for cardiovascular diseases such as atherosclerosis and stroke. Folic acid or vitamin B12 or B6 administration can increase the re-methylation of homocysteine and thus reduce its levels, but many clinical trials have reported that lowering homocysteine did not reduce the risk of myocardial
infarction, stroke, or death (Marti-Carvajal, Sola, & Lathyris, 2015; Marti-Carvajal, Sola, Lathyris, & Dayer, 2017; Marti-Carvajal, Sola, Lathyris, Karakitsiou, & Simancas-Racines, 2013; Marti-Carvajal, Sola, Lathyris, & Salanti, 2009). H2S is a metabolic product of homocysteine by the trans-sulfuration pathway, catalyzed by CBS and CSE. H2S also had protective effects on cardiovascular injury induced by homocysteine (Chang et al., 2008; d’Emmanuele di Villa Bianca et al., 2013; Kesherwani et al., 2015; Sen et al., 2012; Wei et al., 2010; Yan et al., 2006). As well, treatment with H2S donor compounds lowered serum homocysteine in a rat model of mild HHcy (Chang et al., 2008). Thus, H2S donors may be a novel therapeutic choice for HHcy. The key scientific question is how H2S lowers homocysteine. CSE is a key enzyme in the homocysteine trans-sulfuration metabolic pathway and can directly metabolise homocysteine to release H2S (Chiku et al., 2009). In the present study, we found that sulfhydrated CSE increased, but nitrosylation at the same cysteine sites decreased, its activity towards homocysteine. Treatment with H2S donor compounds enhanced sulfhydration of CSE, reduced homocysteine level and then reduced atherosclerosis in the HHcy mouse model (Figure 7).
High levels of homocysteine can induce oxidative stress, inflammation, ER stress, and epigenetic changes, cause endothelial dysfunction (Lai & Kan, 2015) and vascular smooth muscle cell dysplasia (Luo et al., 2012), and accelerate thrombosis (Dionisio, Jardin, Salido, & Rosado, 2010) and foam cell formation (Chernyavskiy, Veeranki, Sen, & Tyagi, 2016), thus promoting atherogenesis. Although some studies have shown that folic acid supplementation reduced atherosclerotic plaque size in ApoE−knockout mice (Cui et al., 2017) or LDL receptor−knockout mice (Pan et al., 2018), it did not reduce early atherosclerosis with HHcy in humans (Cacciapuoti, 2013). H2S donors per se can decrease atherosclerosis. They can also inhibit platelet activation induced by HHcy (Wang et al., 2017); reduce ROS levels, then decrease cytotoxicity of homocysteine to vascular smooth muscle cells (Olas & Kontek, 2015; Yan et al., 2006); directly scavenge hydrogen peroxide and superoxide; and inhibit ER stress of the aorta and heart in animals with HHcy (Chang et al., 2008; Wei et al., 2010). All of these data indicate that H2S antagonizes the cardiovascular damage induced by HHcy, which might contribute to atherogenesis.

Importantly, our study provides evidence, in an animal model, that treatment with H2S donor compounds decreased atherosclerotic plaque size and macrophage infiltration in plaques of ApoE−knockout mice with HHcy.

In our earlier work, we found that H2S donor treatment lowered serum homocysteine level in rats with mild HHcy (Chang et al., 2008). In the present study, treatment with two H2S donors also lowered total serum homocysteine level in a mouse model of moderate HHcy, which confirmed the effects of H2S. Nevertheless, the molecular mechanism was not clear. In the HHcy condition, hepatocyte CSE—a key metabolic enzyme of homocysteine—was downregulated, consistent with an effect on macrophages, which may be due to increased DNA methylation of the CSE promoter by upregulating DNA methyltransferase (Li et al., 2015) and then decreasing CSE transcription (Figure 3). H2S promoted the expression of the DNA demethylases ten−eleven translocation 1 (Tet1) and Tet2 (Peng et al., 2017; Yang et al., 2015), which may cause demethylation of the CSE promoter induced by HHcy, and then up-regulate CSE.
FIGURE 7  Schematic diagram of the present findings. CSE is a key enzyme of the trans-sulfuration pathway of homocysteine (Hcy) metabolism, and its activity contributes to H2S generation from homocysteine. Increased serum homocysteine can down-regulate CSE expression and nitrosylate CSE (CSE-SNO) and thus reduce CSE catalytic activity and H2S production, which inhibits the homocysteine trans-sulfuration pathway and exacerbates homocysteine accumulation (HHcy) to promote atherosclerosis. H2S donor treatment up-regulated CSE protein levels and its sulfhydration (CSE-SSH) and enhanced its binding and catalytic activities, thus lowering serum homocysteine, and decreasing atherosclerotic plaques mRNA and protein levels (Figure 3). These findings highlight that homocysteine and the CSE–H2S system create an interactive antagonistic feedback loop.

Protein post-translational modification is essential for regulating its function. Some reports indicated that HHcy activates GSNO reductase to reduce Akt nitrosylation (Li et al., 2018), which promotes atherosclerosis (Chen et al., 2014; Chen et al., 2015), but these findings differ from our study. In these studies, the homocysteine concentrations were high (0.5–2 mM), so the protein-nitrosylation effect may be due to an overoxidative-redox response or cellular toxicity. Our homocysteine concentrations (12.5 to 200 μM, a physiological concentration for HHcy in humans) dependently enhanced CSE nitrosylation at C252, C255, C307, and C310 sites in hepatocytes (a major location for homocysteine metabolism). Nitrosylation of CSE lowered its binding and its catalytic activity towards homocysteine, maintained HHcy level, and lowered H2S production. Thus, HHcy impaired the CSE–H2S system and then inhibited its trans-sulfuration metabolic pathway, causing a vicious circle to maintain HHcy.

Sulfhydration is another post-translational modification of the cysteine residues in proteins. Nitrosylation or sulfhydration at the same site in proteins has opposing effects on function. Nitrosylation of GAPDH inhibits, but sulfhydration enhances, its activity (Mustafa et al., 2009). Knockout of CSE (Mustafa et al., 2009), or inhibition of CSE activity (Zheng et al., 2017) lowered protein sulfhydration, which suggests that CSE might be a key enzyme for sulfhydration. In our experimental conditions, H2S donor treatment increased CSE protein expression and its sulfhydration but decreased its nitrosylation. Sulfhydrated CSE can increase binding and metabolism of L-homocysteine. Thus, post-translational modification with nitrosylation or sulfhydration represents a yin/yang regulation of CSE activity. HHcy-induced nitrosylation of CSE and inhibited its trans-sulfuration activity. Treatment with H2S donor compounds increased sulfhydration of CSE, restoring its activity and, consequently, lowering serum levels of homocysteine.

Current clinical treatment is to use vitamin B12 and B6 or folic acid to lower serum homocysteine level. Although these treatments are safe and are effective in lowering homocysteine, the risk of cardiovascular events is not reduced. Our study has highlighted that H2S donor compounds are novel homocysteine-lowering agents and also reduce atherosclerosis in the presence of HHcy. Many studies also support the antagonist effects of H2S on injuries to the cardiovascular system, kidneys and the nervous system. Thus, H2S donor compounds might be a new target for drug design for the treatment of HHcy.

ACKNOWLEDGEMENTS
This work was supported by the National Natural Science Foundation of China (Grants 81470552, 81670379, 81870318, and 81600677), CAMS Innovation Fund for Medical Sciences (CIFMS, Grant 2016-12M-1-006), and State Key Laboratory of Cardiovascular Disease (Grant 2018fk-02).

AUTHOR CONTRIBUTIONS
B.G. designed and supervised this project. J.F. and F.Z. performed most of the experiments. S.L., C.C., S.J., and J.Z. performed some experiments. Q.C. and J.Y. interpreted and analysed the data. B.G., J.C., and G.X. wrote the paper. All authors read and approved the final manuscript.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJR guidelines for Design & Analysis, Immunoblotting and Immunocytochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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How to cite this article: Fan J, Zheng F, Li S, et al. Hydrogen sulfide lowers hyperhomocysteinemia dependent on cystathionine γ lyase S-sulfhydration in ApoE-knockout atherosclerotic mice. *Br J Pharmacol*. 2019;176:3180–3192. https://doi.org/10.1111/bph.14719