Role of Hydrogen Sulfide in the Development of Atherosclerotic Lesions in Apolipoprotein E Knockout Mice

Yanfei Wang, Xia Zhao, Hongfang Jin, Hongling Wei, Wei Li, Dingfang Bu, Xiuying Tang, Yali Ren, Chaoshu Tang, Junbao Du

Objective—We explored the effect of hydrogen sulfide (H₂S) on atherosclerotic progression, particularly on intracellular adhesion molecule-1 (ICAM-1) in apolipoprotein-E knockout (apoE⁻/⁻) mice and human umbilical vein endothelial cells (HUVECs).

Methods and Results—ApoE⁻/⁻ mice were treated with sodium hydrosulfide (NaHS) or DL-propargyglycine (PPG); HUVECs were pretreated with NaHS. Compared with control mice, apoE⁻/⁻ mice showed decreased plasma H₂S level and aortic H₂S production but increased plasma ICAM-1 and aortic ICAM-1 protein and mRNA. Compared with apoE⁻/⁻ mice, apoE⁻/⁻ + NaHS mice showed increased plasma H₂S level, but decreased size of atherosclerotic plaque and plasma and aortic ICAM-1 levels, whereas apoE⁻/⁻ + PPG mice showed decreased plasma H₂S level but enlarged plaque size and increased plasma and aortic ICAM-1 levels. NaHS suppressed ICAM-1 expression in tumor necrosis factor (TNF)-α–treated HUVECs. NaHS inhibited IκB degradation and NF-κB nuclear translocation in HUVECs treated with TNF-α.

Conclusions—The vascular CSE/H₂S pathway was disturbed in apoE⁻/⁻ mice. H₂S exerted an antiatherogenic effect and inhibited ICAM-1 expression in apoE⁻/⁻ mice. H₂S inhibited ICAM-1 expression in TNF-α-induced HUVECs via the NF-κB pathway. (Arterioscler Thromb Vasc Biol. 2009;29:173-179.)

Key Words: hydrogen sulfide ■ intercellular adhesion molecule-1 ■ atherosclerosis ■ apolipoprotein E knockout mice ■ human umbilical vein endothelial cells

Atherosclerosis is an important underlying pathology of cardiovascular diseases, the leading cause of morbidity and mortality in many countries. Over the past 50 years, numerous studies attempting to explain the complex events leading to atherosclerosis have been undertaken. Nitric oxide and carbon monoxide, which are small gaseous transmitters, freely permeable to membrane, endogenously and enzymatically generated, and have specific functions, are recommended as gasotransmitters. They have been closely implicated in endothelial dysfunction and vascular remodeling in atherosclerotic arteries; researches into the two gasotransmitters have improved the understanding of atherogenesis. However, the mechanisms of atherosclerosis have not been fully elucidated.

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Hydrogen sulfide (H₂S) has long been known as a toxic gas, but only recently has it been regarded as a novel gasotransmitter. Endogenous H₂S is generated from L-cysteine catalyzed by cystathionine-γ-lyase (CSE) in the cardiovascular system. It possesses important physiological and pathophysiologic functions, and exerts regulatory effects on the pathogenesis of various cardiovascular diseases such as hypertension, pulmonary hypertension, shock, and myocardial injury. A significant decrease in plasma H₂S was observed in children with hyperlipidemia and in patients with coronary heart disease, and decreased plasma H₂S level was correlated with the severity of coronary heart disease and coronary artery lesions. Furthermore, CSE expression and H₂S production were reduced during the development of balloon injury-induced neointimal hyperplasia, and treatment with an H₂S donor significantly reduced formation of neointimal lesions. However, whether there is a disturbance of the vascular CSE/H₂S pathway in the pathogenesis of atherosclerosis and whether H₂S affects the process of atherosclerosis is unclear.

Inflammation may contribute to atherosclerosis by various mechanisms depending on disease stage. All phases of atherosclerosis involve recruitment of inflammatory cells from the circulation and their migration across the endothelium...
lum. This process is predominantly mediated by cellular adhesion molecules, which are expressed on the vascular endothelium and on circulating leukocytes in response to several inflammatory stimuli. The circulating form of a membrane-bound inter cellular adhesion molecule-1 (ICAM-1) has been proposed as a marker of vascular inflammation in atherosclerosis; it has been shown to predict cardiovascular risk and future cardiovascular disease. ICAM-1, a member of the immunoglobulin superfamily, induces firm adhesion of inflammatory cells at the vascular surface. ICAM-1 is minimally expressed in normal endothelium; however, at the early stage of atherosclerosis, it is usually significantly upregulated and has been consistently observed in atherosclerotic plaques. Reduced or deficient expression of ICAM-1 provides direct protection from the formation of atherosclerotic lesions in mice, probably by slowing the recruitment of inflammatory cells from the blood. 

H₂S produces antiinflammatory effects in neurocytes. H₂S donors were shown to inhibit aspirin-induced leukocyte adherence to the endothelium of rat mesenteric venules. But an association between H₂S and ICAM-1 in the progression of atherosclerosis has not been studied, and little is known about the regulatory effect of H₂S on ICAM-1 expression in the pathogenesis of atherosclerosis. Possible effects of H₂S on the production of vascular ICAM-1 in apolipoprotein-E knockout (apoE⁻/⁻) mice; and the possible mechanisms underlying the effect of H₂S on ICAM-1 in human umbilical vein endothelial cells (HUVECs).

**Materials and Methods**

**Animals**
Six-week-old male C57BL/6 mice and homozygous apoE⁻/⁻ mice obtained from Jackson Laboratory (Bar Harbor, Me) were raised in the Experimental Animal Center of Peking University. Mice were obtained from Jackson Laboratory (Bar Harbor, Me) were raised in the Experimental Animal Center of Peking University. Mice were randomly divided into 4 groups (n=8 each): control, apoE⁻/⁻, apoE⁻/⁻ + sodium hydrosulfide (NaHS), and apoE⁻/⁻ + DL-propargylylglycine (PPG) and observed for 10 weeks. Please see supplemental materials (available online at http://atvb.ahajournals.org).

**Assessment of Aortic Atherosclerotic Lesions**
Please see supplemental materials.

**Measurement of Plasma Lipids**
Please see supplemental materials.

**Measurement of H₂S Content in Plasma and H₂S Production in Tissue**
H₂S was measured using a sulfide electrode. Please see supplemental materials.

**Measurement of ICAM-1 Content in Plasma by Quantitative Sandwich ELISA**
Please see supplemental materials.

**Measurement of ICAM-1 and 1κBα Protein Expression by Western Blotting**
Please see supplemental materials.

**Determination of CSE and ICAM-1 mRNA Expression by Quantitative RT-PCR**
Please see supplemental materials.

**Immunohistochemistry for α-Actin, CSE and ICAM-1 in the Aortic Root**
Please see supplemental materials.

**Culture of HUVECs and Rat Aortic Smooth Muscle Cells (ASMCs)**
Please see supplemental materials.

**Localization of NF-κB p65 by Western Blotting and Immunofluorescence**
Please see supplemental materials.

**Differentiation of THP-1 Cells and Formation of Foam Cells**
Please see supplemental materials.

**Statistical analysis**
Please see supplemental materials.

**Results**

**Changes in Body Weight and Levels of Plasma Lipids in Mice**
Body weights and plasma lipids were measured in all mice at the end of the experiment. There were no significant differences in the body weights of mice among groups. Compared with control mice, apoE⁻/⁻ mice showed increased plasma level of total cholesterol (TC; 9.20±2.26 versus 2.06±0.17 mmol/L), triglyceride (TG; 1.07±0.46 versus 0.62±0.19 mmol/L), and low-density lipoprotein-cholesterol (LDL-C; 1.26±0.29 versus 0.17±0.02 mmol/L) but a decreased level of high-density lipoprotein-cholesterol (HDL-C; 0.78±0.42 versus 1.36±0.14 mmol/L; all P<0.01; please see Supplemental Table I). Plasma levels of lipids did not significantly differ between apoE⁻/⁻ mice with or without treatment.

**Changes in Vascular CSE/H₂S Pathway During Atherosclerosis**
Compared with control mice, apoE⁻/⁻ mice showed significantly lower H₂S level in plasma (44.64±4.52 versus 57.69±7.03 μmol/L) and aortic production of H₂S (1.98±0.30 versus 2.46±0.82 nmol/min·mg protein) in apoE⁻/⁻ mice (please see Supplemental Figure II). In apoE⁻/⁻ + NaHS mice, plasma H₂S was significantly increased (52.21±7.24 μmol/L, P<0.05) and aortic CSE mRNA significantly decreased (0.55±0.22, P<0.05), as compared with apoE⁻/⁻ mice. In apoE⁻/⁻ + PPG mice, plasma H₂S (36.39±5.87 μmol/L) and production rates of H₂S in the aorta (0.89±0.47 versus 1.98±0.82 nmol/min·mg protein), liver (0.10±0.08 versus 0.68±0.46 nmol/min·mg protein), and kidney (0.03±0.02 versus 0.17±0.08 nmol/min·mg protein) showed significantly lower levels than in apoE⁻/⁻ mice. These results suggest that H₂S is a critical factor in the regulation of atherosclerosis.
Whereas in PPG-treated apoE−/− mice, it was significantly diminished or enlarged in NaHS- or PPG-treated apoE−/− mice, respectively, CSE expression as seen on immunohistochemistry was decreased or increased accordingly (please see supplemental Figure II).

**Effect of H2S on the Size of Aortic Atherosclerotic Plaque**

Oil-red O staining of atherosclerotic plaques showed red and was not seen in control mice, but was widely observed in apoE−/− mice (139317 ± 30363 μm²). Compared with the size of plaque in apoE−/− mice, it was significantly diminished (85928 ± 1923 μm², P < 0.05) in apoE−/− + NaHS mice, but increased (312702 ± 41065 μm², P < 0.01) in apoE−/− + PPG mice (Figure 1A and 1B).

**Effect of H2S on Circulating ICAM-1 Level and Aortic ICAM-1 Expression in Mice**

Compared with control mice, apoE−/− mice showed a significantly decreased level of ICAM-1 in plasma (0.37 ± 0.02 versus 0.21 ± 0.07 μg/L, P < 0.01) and higher levels of aortic ICAM-1 protein (2.07 ± 1.04 versus 1.08 ± 0.44, P < 0.05) and mRNA (10.75 ± 4.86 versus 1.59 ± 0.88, P < 0.01; Figure 2A through 2C). Compared with apoE−/− mice, apoE−/− + NaHS mice showed a significantly decreased level of ICAM-1 in plasma (0.28 ± 0.05 μg/L, P < 0.01) and decreased aortic levels of protein.) were significantly decreased (all P < 0.05), whereas aortic CSE mRNA was significantly increased (8.06 ± 3.60, P < 0.01) as compared with apoE−/− mice (please see supplemental Figure IA through IE). With the size of the plaque diminished or enlarged in NaHS- or PPG-treated apoE−/− mice, respectively, CSE expression as seen on immunohistochemistry was decreased or increased accordingly (please see supplemental Figure II).

**Effect of H2S on ICAM-1 mRNA in ASMCs**

NaHS treatment (50 μmol/L, 100 μmol/L, and 500 μmol/L) dose-dependently decreased the expression of CSE mRNA in ASMCs, whereas PPG treatment (10 mmol/L) significantly increased the expression of CSE mRNA in ASMCs (please see supplemental Figure IF through IG).

**Effect of NaHS on Smooth Muscle Cell Expression in Aortic Atherosclerotic Plaque**

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**Effect of H2S on Smooth Muscle Cell Expression in Aortic Atherosclerotic Plaque**

SMC expression in the atherosclerotic plaque was identified by immunohistochemical staining of α-actin. The latter was expressed mainly in the aortic media layer in control mice, but in the plaque and aortic media in apoE−/− mice. In NaHS-treated apoE−/− mice, α-actin expression was seen only on the surface of the plaque and in the aortic media, whereas in PPG-treated apoE−/− mice, α-actin was widely expressed in the plaque (please see supplemental Figure II).

**Effect of H2S on Aortic Ultrastructure**

Control mice showed thin and flat endothelial cells, a clear intercellular tight junction, a thick internal elastic membrane, and fusiform-shaped medium SMCs in the aorta (please see supplemental Figure III). In apoE−/− mice, endothelial cells were pillar-shaped, with swollen mitochondria and expanding endoplasmic reticulum; some endothelial cells were disrupted and fell off; the subendothelial tissue was thickened; and much cell debris and lipid droplets, even SMCs, were seen in the thickened subendothelial tissue. The internal elastic membrane was uneven. Medium SMCs grew vertically with many organelles in the cytoplasm, and some even broke through the internal elastic membrane. In one segment of aorta in apoE−/− + NaHS mice showed an ultrastructure similar to that of control mice, except that some endothelial cells were slightly larger and part of the endoplasmic reticulum was expanded slightly; and in another segment of aorta, there was vascular degeneration in the endothelial cells with swollen mitochondria, the subendothelial tissue was thickened with cell debris, the internal elastic membrane was even, and medium SMCs grew vertically. ApoE−/− + PPG mice showed more severe ultrastructural disorder of aortic tissue; endothelial cells were completely disrupted and had fallen off in certain areas, with exposed subendothelial tissue comprising much cell debris, lipid droplets, and shrunken nuclei.

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**Effect of H2S on Circulating ICAM-1 Level and Aortic ICAM-1 Expression in Mice**

Compared with control mice, apoE−/− mice showed a higher level of ICAM-1 in plasma (0.37 ± 0.02 versus 0.21 ± 0.07 μg/L, P < 0.01) and higher levels of aortic ICAM-1 protein (2.07 ± 1.04 versus 1.08 ± 0.44, P < 0.05) and mRNA (10.75 ± 4.86 versus 1.59 ± 0.88, P < 0.01; Figure 2A through 2C). Compared with apoE−/− mice, apoE−/− + NaHS mice showed a significantly decreased level of ICAM-1 in plasma (0.28 ± 0.05 μg/L, P < 0.01) and decreased aortic levels of
ICAM-1 protein (0.94±0.64, P<0.01) and mRNA (4.18±1.84, P<0.05), but apoE−/−+PPG mice showed a significantly increased level of ICAM-1 in plasma (0.43±0.05 μg/L, P<0.05) and increased aortic levels of ICAM-1 protein (2.99±0.97, P<0.05) and mRNA (19.09±10.15, P<0.01).

Immunohistochemistry revealed sparse expression of ICAM-1 protein in the endothelium of control mice, but overexpression in the atherosclerotic plaque of apoE−/− mice (please see supplemental Figure II). In apoE−/−+NaHS mice, ICAM-1 was expressed in the endothelium (the surface of atherosclerotic plaque), but in apoE−/−+PPG mice, it was widely expressed in the atherosclerotic plaque and slightly around SMCs in the media.

**Effect of H2S on ICAM-1 Protein Expression in TNF-α-Stimulated HUVECs**

To explore the effect of H2S on ICAM-1 expression, HUVECs were pretreated with NaHS (1 μmol/L, 10 μmol/L and 100 μmol/L) for 6 hours and then stimulated with 10 ng/mL TNF-α for 6 hours in the continuous presence of NaHS. ICAM-1 protein expression was much higher in TNF-α-treated HUVECs than in HUVECs without any treatment (P<0.01). NaHS treatment at 1 μmol/L, 10 μmol/L and 100 μmol/L significantly decreased ICAM-1 protein expressions in TNF-α-stimulated HUVECs (Figure 3A).

**Effect of H2S on IκBα Degradation in TNF-α-Stimulated HUVECs**

IκBα protein expressions in HUVECs treated with TNF-α+NaHS were increased by 0%, 24.1%, 22.2%, 52.2%, and 26.8%, compared with those in HUVECs stimulated with TNF-α for 0 minutes, 5 minutes, 15 minutes, 30 minutes, and 60 minutes, respectively (Figure 3B). NaHS significantly suppressed degradation of IκBα when TNF-α treatment lasted for 30 minutes.

**Effect of H2S on NF-κB Nuclear Translocation in TNF-α-Stimulated HUVECs**

HUVECs were pretreated with 100 μmol/L NaHS for 12 hours and then stimulated with 10 ng/mL TNF-α for 30 minutes to explore the effect of H2S on NF-κB nuclear translocation. Nuclear NF-κB p65 protein was increased and NF-κB nuclear translocation was promoted in HUVECs stimulated with TNF-α, whereas NaHS treatment significantly suppressed NF-κB nuclear translocation in TNF-α-stimulated HUVECs (Figure 3C and 3D).

**Effect of H2S on Formation of Foam Cells in THP-1-Derived Macrophages**

Oxidized-LDL (100 mg/L) induced almost all THP-1-derived macrophages to form foam cells, whereas NaHS (50 μmol/L, 100 μmol/L and 500 μmol/L) dose-dependently suppressed the number of foam cells induced by oxidized-LDL (please see supplemental Figure IVA and IVB).

**Discussion**

In the present study, apoE−/− mice used to characterize the role of endogenous H2S in atherosclerosis showed elevated plasma levels of TC, TG, and LDL-C, and a reduced level of HDL-C, which was in accordance with previous studies.27–28 Atherosclerosis develops and progresses spontaneously in apoE−/− mice, so the effect of manipulations on atherosclerotic progression can be investigated.27–29 In apoE−/− mice, H2S level in plasma and H2S production rate in atherosclerotic aortic tissues were significantly decreased, whereas there were no changes in the production rates of H2S in the liver and kidney. This suggested that the decrease in plasma H2S was probably attributable to the reduction of H2S production rate in the aorta, however mRNA expression of CSE in the aorta was significantly increased, which indicates that the CSE/H2S pathway is probably involved in atherosclerotic progression. The decreased level of H2S in plasma and decreased H2S production rate in the aorta may generate a positive feedback, resulting in a compensatory increase in CSE gene expression. CSE protein was located mainly in the media layer of the aorta, as demonstrated by immunohistochemistry in control mice, because CSE is expressed solely by vascular SMCs in the cardiovascular system.6–7 It was expressed in the plaque and media of the aorta in apoE−/− mice, possibly because of SMC migration.12 SMCs were identified in the atherosclerotic plaque of apoE−/− mice by
α-actin immunohistochemistry, which was in accordance with previous studies.30–31

We treated apoE−/− mice with NaHS or PPG to explore the role of endogenous H2S in atherosclerosis pathogenesis. NaHS was used as a H2S donor for the following reasons: (1) NaHS dissociates into Na+ and HS− in solution, then the latter associates with H+ to produce H2S. It does not matter whether H2S solution is prepared by bubbling H2S gas or by dissolving NaHS. In physiological saline, about one-third of the H2S exists in the undissociated form (H2S) and the remaining two-thirds as HS− in equilibrium with H2S; (2) NaHS enables definition of the concentrations of H2S in solution more accurately and reproducibly than bubbling H2S gas; (3) the influence of ≤1 mmol/L sodium ion in the physiological experiments is negligible in plasma containing 140 mmol/L sodium ion; and (4) the concentrations of NaHS used did not change the pH of the medium.32 PPG was used as an inhibitor of H2S production because it is a potent, active site-directed, irreversible inhibitor of CSE.33

NaHS treatment significantly elevated the H2S level in plasma and reduced the CSE mRNA level in the aorta of apoE−/− mice, whereas PPG significantly reduced the H2S level in plasma and elevated the CSE mRNA level in the aorta. This suggests the possibility of a positive feedback between circulating H2S level and CSE gene expression during atherosclerosis. To further investigate the above results, we examined in vitro expression of CSE mRNA. NaHS treatment (50 μmol/L, 100 μmol/L, and 500 μmol/L) dose-dependently decreased the CSE mRNA level, whereas PPG treatment (10 mmol/L) significantly increased the CSE mRNA level in ASMCs, which was in accordance with what we observed in vivo.
The elevation of plasma H$_2$S in apoE$^{-/-} +$NaHS mice resulted from the production of H$_2$S from NaHS. The reduction of plasma H$_2$S in apoE$^{-/-} +$PPG mice was probably attributable to the inhibition of CSE in the aorta, liver, and kidney, the organs where most H$_2$S is produced.

Neither NaHS nor PPG treatment altered the body weight or plasma levels of lipids in apoE$^{-/-}$ mice. The size of the atherosclerotic plaque in the aortic root was diminished significantly and the ultrastructural disorder of the aorta was relieved by NaHS treatment, but became much more severe by PPG treatment. This suggests that H$_2$S may have a protective effect against atherosclerosis.

The inflammatory process mediated by cellular adhesion molecules is involved in all phases of atherosclerosis. ICAM-1 plays an important role in immune and inflammatory responses, including atherosclerosis, because of its critical function in mediating adhesion of inflammatory cells to the endothelium, as well as transmigration of leukocytes. The level of ICAM-1 in plasma and expression of ICAM-1 in the atherosclerotic aorta of apoE$^{-/-}$ mice were upregulated, which was also found in human atherosclerotic plaques.

Previous studies indicated that H$_2$S attenuated ICAM-1 upregulation induced by nonsteroidal antiinflammatory drugs. Whether H$_2$S exerts a regulatory effect on ICAM-1 expression in the development of atherosclerosis is unknown. We therefore investigated the effect of H$_2$S on secretion and expression of ICAM-1 in the atherosclerotic aorta of apoE$^{-/-}$ mice by treatment with NaHS or PPG. Treatment with NaHS diminished the size of the atherosclerotic plaque in the aortic root; significantly decreased plasma ICAM-1 content and aortic ICAM-1 protein and mRNA levels; and the expression of ICAM-1 protein in the endothelium for the recruitment of circulating cells into the vessel wall was sparsely distributed on the surface of the atherosclerotic plaque. However, treatment with PPG (inhibitor of endogenous generation of H$_2$S) enlarged the size of the atherosclerotic plaque, and significantly increased plasma ICAM-1 level and aortic ICAM-1 protein and mRNA levels. ICAM-1 protein was generously expressed in the atherosclerotic plaque and slightly around SMCs in the media layer. Expression of adhesion molecules on SMCs might facilitate the accumulation of transmigrated leukocytes within the vascular wall.

Expression of ICAM-1 in H$_2$S-treated HUVECs showed IκB degradation and NF-κB nuclear translocation, whereas NaHS pretreatment significantly suppressed IκB degradation and NF-κB nuclear translocation, therefore inhibited NF-κB activation.

Adhesion molecules play a pivotal part in atherogenesis, and there were other elements leading to atherosclerosis, such as the migration and proliferation of SMCs and macrophages, the formation of foam cells, etc. The above results indicate that H$_2$S inhibited ICAM-1 expression probably via suppression of NF-κB activation. The inflammatory process mediated by cellular adhesion molecules is considered an early marker for atherosclerosis involves other mechanisms merits further studies.

Conclusions

The present study directly demonstrates for the first time the involvement of the CSE/H$_2$S pathway and its regulatory role in the development and progression of atherosclerosis and the possible underlying mechanisms, including the inhibitory effect of H$_2$S on adhesion molecules such as ICAM-1 involving the NF-κB pathway in vivo and in vitro. The effect of H$_2$S on ICAM-1 suggested that the gasotransmitter H$_2$S was a fast regulatory signal from SMCs to endothelial cells of the blood vessels for that H$_2$S is mainly from vascular SMCs and ICAM-1 is formed in endothelial cells. The discovery of the role of CSE/H$_2$S pathway in atherosclerosis and adhesion molecules may expand our understanding of the underlying mechanisms of atherosclerosis, and the biological effects and pathophysiologic functions of H$_2$S. This may lead to a potential novel therapy for atherosclerosis.

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Disclosures

None.

References


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The Role of Hydrogen Sulfide in the Development of Atherosclerotic Lesions in Apolipoprotein E-Knockout Mice

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Materials and Methods

Materials

Sodium hydrosulfide (NaHS), DL-propargylglycine (PPG), phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), oil-red O, hematoxylin and pyridoxal 5’-phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trizol reagent, Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640 medium and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). Oligo (dT)15 primer and M-MLV reverse transcriptase were purchased from Promega (Madison, WI, USA). All other chemicals were of analytical grade and purchased from Beijing Chemical Reagents (Beijing, China).

Animals

Six-week-old male C57BL/6 mice and homozygous apoE−/− mice in a C57BL/6 background were obtained from Jackson Laboratory (Maine, NE, USA) and raised in the Experimental Animal Center of Peking University. Mice were randomly divided into four groups (n=8 each) depending on treatment: control, apoE+/+, apoE+/− + NaHS (H2S donor; 56 μmol/kg body weight/day) and apoE+/− + PPG (CSE inhibitor; 37.5 mg/kg body weight/day). NaHS (freshly prepared each day) and PPG were dissolved in physiologic saline and injected intraperitoneally into mice. Untreated mice were injected daily with saline [1]. Mice had free access to normal food and water and were observed for 10 weeks. Procedures were in accordance with the guidelines for animal research of our institute.

Tissue processing

After 10 weeks of treatment, animals were killed by exsanguination under anesthesia with use of pentobarbital sodium (40 mg/kg body weight, i.p.). The heart and arterial tree were
perfused with phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4). The heart with attached aortic arch was fixed in 4% formalin, embedded in ornithine carbamoyltransferase (OCT), and serially sectioned at 6 μm using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) for morphometric analysis. Two millimeters of the descending aorta from the arch just behind the left subclavian artery was fixed in 3% glutaraldehyde and post-fixed in 1% phosphate-buffered osmium tetroxide, then embedded with epon 812, and cut into ultra-thin sections (60–90 nm) using a Leica UCT ultramicrotome. Sections were stained with uranyl acetate and lead citrate, and examined on a JEM-1230 transmission electron microscope (JEOL Limited, Tokyo, Japan). The remaining descending aorta from the arch down to the bifurcation was excised, freed of peri-adventitial fat in situ, frozen in liquid nitrogen, and stored at –80°C until use.

**Assessment of aortic atherosclerotic lesions**

For quantification of atherosclerotic lesions, 10–12 sections taken from every four consecutive sections clearly showing tricuspid valves were stained with oil-red O and counterstained with hematoxylin [2]. Images were captured and lesion areas were quantified with Leica Qwin imaging software (Leica Microsystems). Lesion size from a particular location was calculated from the average of the areas quantified from the 10–12 sections.

**Measurement of plasma lipids**

Mice were fasted for at least 8 h at the end of the experiment, and blood was collected into heparin-coated tubes. Plasma was separated and plasma levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) were determined by a Hitachi 7180 Autoanalyzer (Hitachi,
Measurement of H$_2$S content in plasma

Plasma H$_2$S was measured using a sulfide electrode (PXS-270, Shanghai, China) [3]. Briefly, 0.3 mL of plasma was mixed with 0.3 mL of antioxidant buffer. After being rinsed with distilled water and dried, the electrode was immersed into the sample. Electrode potential was recorded when the reading stabilized. H$_2$S concentration was calculated according to the standard curve.

Measurement of H$_2$S production in tissue

H$_2$S production rate in tissue was measured as described previously with minor modification [1]. Briefly, tissues were homogenized in 50 mmol/L of ice-cold potassium phosphate buffer (pH 6.8). Reaction mixture contained 10 mmol/L L-cysteine, 2 mmol/L pyridoxal 5’-phosphate, 100 mmol/L potassium phosphate buffer (pH 7.4) and 10% (W/V) homogenates. Center wells each contained 0.5 mL of 1 mol/L sodium hydroxide. After incubation at 37°C for 90 min, 0.5 mL of 50% trichloroacetic acid was added to the mixture to terminate the reaction. Flasks were incubated for an additional 60 min at 37°C to ensure complete trapping of H$_2$S. Contents of the center wells were transferred to test tubes and mixed with 0.5 mL of antioxidant buffer. H$_2$S content in the solution was measured in an identical way to measurement in plasma. Measurement was done in duplicate for each sample. H$_2$S production rate was expressed in nmol/(min·mg protein).

Measurement of ICAM-1 content in plasma

Plasma concentration of soluble ICAM-1 was measured in duplicate by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, USA) according
to manufacturer’s instructions.

**Measurement of ICAM-1 and IκBα protein expression by Western blotting**

Total protein in aortic tissues or HUVECs was extracted in ice-cold protein lysis buffer containing 50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetra-acetic acid, 1% NP-40, 0.25% sodium deoxycholate, 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), and protease and phosphatase inhibitors. Protein samples (20 µg) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membrane. Membrane was incubated overnight with primary antibodies against ICAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (Lab Version Corporation, Fremont, CA, USA) or IκBα (Cell Signaling Technology, Danvers, MA, USA) at 4°C, and then incubated in horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactions were visualized by electrochemical luminescence (ECL) and exposed to Kodak X-ray film (Eastman Kodak Company, Rochester, NY, USA). β-actin was used for normalization.

**Determination of CSE and ICAM-1 mRNA expression by quantitative real-time polymerase chain reaction (RT-PCR)**

Total RNA was extracted using Trizol reagent and transcribed into cDNA using oligo (dT)15 primer and M-MLV reverse transcriptase. Quantitative RT-PCR was done on an ABI PRISM 7300 instrument (Applied Biosystems, Foster, CA, USA). Samples and standards were determined in duplicate. β-actin was used for normalization. TaqMan probes were modified by 5’-FAM and 3’-TAMRA. Specific sequences of primers and TaqMan probes are listed in supplemental Table I.
Immunohistochemistry for α-actin, CSE and ICAM-1 in the aortic root

Primary antibodies specific for α-actin (Assay Designs Incorporated, Michigan, USA), CSE (Abnova Corporation, Taipei City, Taiwan) and ICAM-1 (Santa Cruz Biotechnology) were used for immunohistochemical study. Briefly, slides of 6-μm cross-section were incubated overnight with α-actin, CSE or ICAM-1 primary antibodies at 4°C. After the slides were incubated with appropriate biotinylated secondary antibodies and HRP streptavidin, diaminobenzidine (DAB) was added for color development and hematoxylin for counterstaining. Brown granules in atherosclerotic plaque under microscopy were defined as positive signals.

Culture of HUVECs

HUVECs were obtained as cryopreserved cultures (Cascade Biologics, OR, USA) and grown in endothelial cell growth medium (medium 200; Cascade Biologics) supplemented with 2% FBS, 1 μg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL human fibroblast growth factor and 10 μg/mL heparin. Cells were used between passages 3 and 5. Culture purity was verified by staining with monoclonal antibody against human factor VIII-related antigen. HUVECs were pretreated with NaHS (1 μmol/L, 10 μmol/L and 100 μmol/L) for 6 h and stimulated with 10 ng/mL tumor necrosis factor-α (TNFα) for a further 6 h in the continuous presence of NaHS. ICAM-1 protein expression was determined by Western blotting (as described above). In other experiments, HUVECs were pretreated with 100 μmol/L NaHS for 12 h and stimulated with 10 ng/mL TNFα for the indicated times, and IκBα protein expression was determined by Western blotting.

Localization of NFκB p65 by Western blotting and immunofluorescence
For determination of NFκB localization, HUVECs were pretreated with 100 μmol/L NaHS for 12 h and stimulated with 10 ng/mL TNFα for 30 min. Western blotting was carried out with nuclear protein fractions using anti-human NFκB p65 primary antibody (1:1000) (Cell Signaling Technology, Danvers, MA, USA). Nuclear protein of HUVECs was extracted according to manufacturer’s instructions using the Nuclear and Cytoplasmic Extraction Kit (Kangchen Bio-tech, Shanghai, China). To measure NFκB expression in situ, confluent HUVECs (controls or 12-h NaHS-treated cells) on slides were exposed to TNFα (10 ng/mL) for 30 min. They were fixed in 95% ethanol for 30 min at 37°C, washed with PBS, blocked with 5% bovine serum albumin (BSA) in PBS for 30 min at 37°C, then reacted with rabbit anti-human NFκB p65 antibody (1:25 dilution in PBS) overnight at 4°C. After washing, slides were incubated for 1 h at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and propidium iodide (fluorescent DNA dye), then viewed on a confocal fluorescent microscopy. Expression of NFκB p65 showed green fluorescence, and red represented nuclei. NFκB nuclear translocation occurred in the cells in which green and red overlapped.

Cell culture of rat aortic smooth muscle cells (ASMCs)

Male Sprague–Dawley rats (150–180 g) were killed by anesthetic overdose (pentobarbital sodium; i.p.). The thoracic aorta was stripped of adventitia and removed to culture media in a sterile manner. Rat ASMCs were isolated and cultured according to the method of Jin et al [4]. Briefly, cells were maintained in DMEM containing 5 mmol/L glucose, 10% FBS, and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) in a CO2 incubator at 37°C. Subcultures of ASMCs from passage 4 to 6 were used in experiments. ASMCs were starved
with serum-free DMEM for 24 h before experiments. Cells were then treated with NaHS (50 μmol/L, 100 μmol/L and 500 μmol/L) or PPG (1 mmol/L, 5 mmol/L and 10 mmol/L) for 12 h. Total RNA was extracted for determination of CSE mRNA expression as described above.

**Cell culture of THP-1 monocytes**

THP-1 (promonocytic cell line) was obtained from the American Type Culture Collection (ATCC number TIB-202). Cells were grown in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 U/mL streptomycin. They were maintained at 37°C in a humidified atmosphere of 5% CO₂.

**Differentiation of THP-1 cells and formation of foam cells**

THP-1 cells were induced to differentiate into macrophages by incubation with PMA (200 nmol/L) in 12-well plates at a density of 0.5–1 × 10⁶ cells/well for 48 h. Oxidized-LDL (100 mg/L) was added to the macrophages for 24 h to form foam cells. The latter were identified by oil-red O staining. Formation of foam cells was expressed as the ratio of the number of foam cells and total cells.

**Statistical analysis**

Data analysis involved use of SPSS v11.5 for Windows (SPSS Incorporated, Chicago, IL, USA). Results are expressed as mean ± standard deviation (SD). Comparisons among groups involved one-way ANOVA followed by the LSD test, and the two sample t-test was used for comparison between two groups. $P<0.05$ was considered statistically significant.
References


<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene number</th>
<th>Product size</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>TaqMan Probe</th>
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<td>mouse β-actin</td>
<td>NM_007393.3</td>
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<td>5’-AAGGCCAACCGTGAAAAGATG-3’</td>
<td>5’-CACAGCCTGGATGGCTACGT-3’</td>
<td>5’-TTTGAGACCTTTCAACACCCCAGCCA-3’</td>
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<td>126 bp</td>
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<td>5’-CTGAGCTTTCAGCAATCTGACCCCATG-3’</td>
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CSE: cystathionine-γ-lyase, ICAM-1: intercellular adhesion molecule-1
### Supplemental Table II. Changes in body weight and plasma lipids in mice of each group (Mean±SD)

<table>
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<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>TC (mmol/L)</th>
<th>TG (mmol/L)</th>
<th>HDL-C (mmol/L)</th>
<th>LDL-C (mmol/L)</th>
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<tr>
<td>Control</td>
<td>26.7±2.2</td>
<td>2.06±0.17</td>
<td>0.62±0.19</td>
<td>1.36±0.14</td>
<td>0.17±0.02</td>
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<tr>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>27.5±2.4</td>
<td>9.20±2.26**</td>
<td>1.07±0.46**</td>
<td>0.78±0.42**</td>
<td>1.26±0.29**</td>
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<tr>
<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;+NaHS</td>
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<td>9.13±5.49</td>
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<td>ApoE&lt;sup&gt;-/-&lt;/sup&gt;+PPG</td>
<td>26.6±2.2</td>
<td>10.30±4.23</td>
<td>0.84±0.22</td>
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TC: total cholesterol, TG: triglyceride, HDL-C: high-density lipoprotein-cholesterol, LDL-C: low-density lipoprotein-cholesterol

Compared with control mice: **P<0.01
Supplemental Figure Legends:

Supplemental Figure I. Changes in CSE/H$_2$S pathway (mean ± SD). H$_2$S level in plasma of mice (A). Expression of CSE mRNA in the aorta of mice (B). H$_2$S production rates in the aorta (C), liver (D) and kidney (E) of mice. Expressions of CSE mRNA in cultured rat ASMCs treated with NaHS (F) and PPG (G).

* $P<0.05$; ** $P<0.01$ compared with control mice

# $P<0.05$; ## $P<0.01$ compared with apoE$^{-/-}$ mice

† $P<0.05$; †† $P<0.01$ compared with rat ASMCs without treatment
Supplemental Figure II. Immunohistochemistry of CSE, ICAM-1 and α-actin expressions in the aortic root of mice in each group (DAB, 200×). Brown granules were considered to be positive signals.
Supplemental Figure III. Ultrastructure of the aortic atherosclerotic plaque of mice in each group by electron microscopy.

EC: endothelial cell, SMC: smooth muscle cell, IEM: internal elastic membrane
Supplemental Figure IV. Effect of NaHS on formation of foam cells in THP-1-derived macrophages. (A) Oil-red O-stained foam cells (400×). Foam cells were stained red by oil-red O. (B) Ratio of the numbers of foam cells and total cells. **P<0.01: compared with vehicle treatment.