Decreased Endogenous Production of Hydrogen Sulfide Accelerates Atherosclerosis

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Background—Cystathionine γ-lyase (CSE) produces hydrogen sulfide (H₂S) in the cardiovascular system. The deficiency of CSE in mice leads to a decreased endogenous H₂S level, an age-dependent increase in blood pressure, and impaired endothelium-dependent vasorelaxation. To date, there is no direct evidence for a causative role of altered metabolism of endogenous H₂S in atherosclerosis development.

Methods and Results—Six-week-old CSE gene knockout and wild-type mice were fed with either a control chow or atherogenic paigen-type diet for 12 weeks. Plasma lipid profile and homocysteine levels, blood pressure, oxidative stress, atherosclerotic lesion size in the aortic roots, cell proliferation, and adhesion molecule expression were then analyzed. CSE-knockout mice fed with atherogenic diet developed early fatty streak lesions in the aortic root, elevated plasma levels of cholesterol and low-density lipoprotein cholesterol, hyperhomocysteinemia, increased lesional oxidative stress and adhesion molecule expression, and enhanced aortic intimal proliferation. Treatment of CSE-knockout mice with NaHS, but not N-acetylcysteine or ezetimibe, inhibited the accelerated atherosclerosis development. Double knockout of CSE and apolipoprotein E gene expression in mice exacerbated atherosclerosis development more than that in the mice with only apolipoprotein E or CSE knockout.

Conclusions—Endogenously synthesized H₂S protects vascular tissues from atherogenic damage by reducing vessel intimal proliferation and inhibiting adhesion molecule expression. Decreased endogenous H₂S production predisposes the animals to vascular remodeling and early development of atherosclerosis. The CSE/H₂S pathway is an important therapeutic target for protection against atherosclerosis.

Key words: apolipoprotein E  ■  atherosclerosis  ■  cystathionine γ-lyase  ■  hydrogen sulfide  ■  oxidative stress

Atherosclerosis is characterized with plaque formation in large and medium-sized blood vessels. The stiffened and narrowed blood vessels limit blood circulation and increase plaque thrombogenicity, which threatens the functionality of vital organs such as the heart and brain. The development of atherosclerosis is a chronic pathological process. Vascular remodeling and inflammation, endothelial dysfunction, smooth muscle cell (SMC) proliferation and migration, and accumulation of cholesterol-rich lipoproteins in blood vessel walls are early events of atherogenesis, resulting in the recruitment of circulating monocytes, their adhesion to endothelium via adhesion molecules, and their differentiation into macrophages. The subendothelial accumulation of cholesterol-laden macrophages is morphologically recognized as foam cells. In humans, these fatty streaks can progress to more advanced lesions characterized by a lipid-rich necrotic core and a fibrous cap consisting of SMCs and collagen.

Lesion rupture can result from the decreased viability of SMCs that is necessary for collagen production and for the structural integrity of the fibrous cap following the release of matrix metalloproteinases from apoptotic macrophages.

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Hydrogen sulfide (H₂S), a member of the gasotransmitter family, plays a number of important physiological roles within the body, including protection against cardiovascular disease. Cystathionine γ-lyase (CSE) endogenously produces H₂S in the cardiovascular system, and the deficiency of CSE in mice leads to decreased endogenous H₂S level, age-dependent increase in blood pressure, impaired endothelium-dependent vasorelaxation, and accumulation of homocysteine in the blood. Administration of NaHS (a H₂S donor) protects rat aortic SMCs from the cytotoxicity caused...
by hyperhomocysteine or reactive oxygen species (ROS), which are considered independent atherogenic risk factors.

The correlation of H$_2$S metabolism with atherosclerosis development has been suggested. We previously reported that CSE expression and H$_2$S production were decreased during the development of neointimal hyperplasia in rat carotid artery, and that treatment with exogenous H$_2$S significantly reduced neointimal lesion formation.

Furthermore, treatment of apolipoprotein E knockout (apoE-KO) mice with exogenous H$_2$S decreased, whereas inhibition of CSE with 3,3'-dipropargylglycine increased atherosclerotic lesion size in these animals. However, these previous studies are inconclusive regarding a pathogenic role of endogenous H$_2$S or the treatment benefits of exogenous H$_2$S donors. The release of H$_2$S by H$_2$S donors is time dependent, and the in vivo levels of H$_2$S after the application of H$_2$S donors are difficult to control. The use of CSE inhibitors is cautioned because these inhibitors apparently target the pyridoxal phosphate binding sites and may not be entirely specific for CSE.

To date, there is no direct evidence for a causative role of the altered metabolism of endogenous H$_2$S in atherosclerosis. We hypothesized that the physiological level of endogenous H$_2$S limits the development of atherosclerosis, and the down-regulation of the CSE/H$_2$S system predisposes vascular tissues to remodeling and the early development of atherosclerosis. These hypotheses were proved in this study by using CSE gene-deficient (CSE-KO) mice as an ideal animal model for investigation into the role of endogenous H$_2$S in the pathogenesis of atherosclerosis. We also produced CSE/apoE double knockout (DKO) mice which offered an additional approach to examine the effects of endogenous H$_2$S on atherosclerosis development even in the absence of dietary manipulation.

### Methods

#### Animals and Diet

All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care Committee of Lakehead University, Ontario, Canada. In-house–bred male CSE-KO mice (C57BL/6J29),

background) were housed in a controlled environment with access to food and water ad libitum on a 12-hour light/dark cycle. Mice were initially fed with a standard rodent chow diet (Rodent RQ 22–5, Zelnger Bros Inc, PA) until 6 weeks of age and then switched to either a high-fat atherogenic (paigen-type) diet (TD.0208, Harlan Tekald, Madison, WI) or control diet (TD.05230, Harlan Tekald, Madison, WI). In all high-fat atherogenic diet feeding experiments, control mice were fed with TD.05230 diet that contains the same ingredients as a high-fat atherogenic diet, with the exception of fat content. The excess fat in a high-fat diet was replaced with carbohydrates in the control diet. Both diets were matched for energy content, and mice were allowed free access to food and water for 12 weeks. During the 12 weeks of experimental dietary feeding, all mice were supplemented with dl-cysteine in the drinking water (1 mg/mL) according to Mani et al. H$_2$S supplement experiments were performed by intraperitoneal injection of NaHS (39 µmol/kg body weight) with phosphate-buffered saline (PBS) injection in other mice as the control. In the antihypertensive drug–treated group, mice were treated with hydralazine in drinking water at 250 mg/L for 12 weeks. In 1 study, atherogenic diet–fed wild-type (WT) and CSE-KO mice were treated with N-acetylcysteine (NAC) in drinking water at 1 mg/mL concentration. In another study, atherogenic diet–fed WT and CSE-KO mice were treated with ezetimibe (Jai-Radhe, Ahmedabad, India), a lipid-lowering drug, during the last 3 weeks of the 12-week feeding period at 80 mg/kg diet.

CSE/apoE DKO mice were developed by breeding CSE-KO mice with apoE-KO mice and DKO mice were selected by genotyping polymerase chain reaction. Genotyping for the apoE locus was done according to the protocol provided by the Jackson Laboratory (Bar Harbor, ME) with the use of the forward primer oLMR0180 5'-GGCTACGGAGGAGCCGAGCC-3', together with the reverse primer oLMR0181 5'-TGGTACATTTGAGCTGTCG-3' and oLMR0182 5'-GCCGCCGAGACTGTAC-3' where oLMR0180 and oLMR0181 amplify a 155-bp fragment of the WT locus, and oLMR0180 and oLMR0182 amplify a 245-bp fragment from the targeted allele. CSE-KO genotyping was conducted as described previously. DKO mouse genotype was further confirmed by Western blot analysis by using commercially available antibodies against either CSE (Abnova, Walnut, CA) or apoE (Santa Cruz Biotechnology). Age-matched male WT, CSE-KO, apoE-KO, and DKO mice were selected and fed with a standard chow diet (Rodent RQ 22–5) for 24 weeks. Although standard rodent chow diet contains approximately equivalent nutritive value with the control diet, standard rodent chow diet was used only in the DKO experiment from weaning to the end of the experiment.

#### Blood Pressure and Lipid Profile Measurements

Systolic blood pressure was measured by using a standard tail-cuff noninvasive measurement system as described previously. Mice receiving different feeding treatments were fasted for 12 to 14 hours before blood sample collection. Plasma was separated by centrifugation and stored at −80°C; and lipid profiles, including total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride concentrations, were determined by standard enzymatic colorimetric techniques (Roche Modular-P800, Roche Diagnostics, Laval, Quebec) according to the manufacturer’s procedures. Plasma homocysteine was measured as described before.

#### Assessment of Atherosclerotic Lesions

Quantitation of atherosclerotic lesions in the aortic root was done as described previously. In brief, the heart and common arteries removed from mice were fixed with 4% paraformaldehyde and embedded in paraffin. Serial sections were cut at 4-µm thickness through the aortic root and stained with hematoxylin and eosin. The consecutive sections were used for immunohistochemistry. For each section, images were captured and the surface area of the lesions was measured by using the ImagePro image analysis software (Media Cybernetics Inc, Rockville, MD). Detection of cell proliferation was performed by using the bromodeoxyuridine incorporation method with modifications.

#### Ultrasound Biomicroscopy

Ultrasound biomicroscopy (Vevo 2100, Visualsonics, Toronto, Canada) with a transducer frequency of 40 MHz was used for vascular imaging in anesthetized mice. In brief, mice were anesthetized with 2.5% isoflurane initially and then with 1.5% isoflurane during the entire procedure to keep the heart rate between 400 and 500 beats/min. The aorta, carotid artery, and aortic arch were imaged in the 2-dimensional short- and long-axis view, and pulse-wave Doppler measurements were conducted to analysis flow velocities. Heart rate, posterior wall thickness, end-diastolic and end-systolic internal dimensions of the aorta and carotid artery were also measured from the motion-mode image. The Vechostrain measurements were performed by using the software Vevo 2100 (version 1.1.1, Visualsonics, Toronto, Canada).

#### Culture of SMCs and Detection of ICAM-1 Expression

SMCs from the mesenteric artery of WT and CSE-KO mice were isolated and identified as described previously. SMCs were treated with 100 µmol/L NaHS for 24 hours. The expression levels of intracellular adhesion molecule-1 (ICAM-1) (Santa Cruz Biotechnology)
and nuclear factor kappa B (NF-κB) (Cell Signaling Technology, Boston, MA) were detected with Western blotting.²⁴

**Oxidative Stress Measurements**

Total glutathione (GSH) (Cayman Chemical, Ann Arbor, MI), ROS level (Cell-Bioslab, San Diego, CA), total thiols levels (Rel Assay Diagnostics, Gaziantep, Türkiye), superoxide dismutase (SOD) (Cayman Chemical) activity, and malondialdehyde formation (Cayman Chemical) were determined in plasma and SMCs with the use of commercial assay kits. The activities of glutathione peroxidase (GPx) and glutathione reductase (GR) were measured as described previously.²⁴

**Statistical Analysis**

Statistical Analyses were performed with OriginPro 9.0 (OriginLab Corporation, MA) and SPSS 21.0 software (SPSS Inc, IL). All the data sets were tested for normality of distribution using the Shapiro-Wilk test and presented as either mean±standard error of the mean or median and range as appropriate. Comparison between 2 groups was performed by using the Student t test or Mann-Whitney U test (for nonparametric) as appropriate. Comparisons among 3 or more groups were performed by using 2-way analysis of variance (Tukey post hoc tests). The categorical data were analyzed with the Fisher exact test. Statistical significance level was set at P<0.05.

**Results**

**Body Weight and Plasma Lipid Profile**

During the 12 weeks of diet treatments, WT mice fed with the control or atherogenic diet increased their body weight significantly, whereas CSE-KO mice with the same diet treatments did not show any significant change in body weight (Figure 1A and IB in the online-only Data Supplement). Plasma total cholesterol and HDL-cholesterol levels were similar in the control diet-fed CSE-KO and WT mice, whereas LDL-cholesterol was higher in CSE-KO than in WT mice (Figure 1A through 1C). With the atherogenic diet feeding, plasma cholesterol levels showed a 2-fold increase in CSE-KO mice but no change in WT mice (Figure 1A). In comparison with WT mice, atherogenic diet-fed CSE-KO mice had significantly elevated plasma LDL-cholesterol (Figure 1B), HDL-cholesterol (Figure 1C), and total cholesterol/HDL ratio (Figure 1D). The ratio of total cholesterol versus HDL in CSE-KO mice was, in fact, lower than that in WT mice on the same atherogenic diet (Figure 1E). In contrast to the changes in cholesterol, plasma triglyceride levels were significantly lower in CSE-KO mice fed with the control or atherogenic diet than in WT mice (Figure 1F).

**Atherosclerotic Lesion Formation**

Small fatty streaks composed of foam cells or individual macrophage-like cells were observed in the aortic root from CSE-KO mice fed with the atherogenic diet, but not in WT mice (Figure 2A, a through d). The accumulation of macrophages and foam cells in the aortic atherosclerotic lesion area were identified by immunohistochemistry with the use of anti-Mac-3 antibody in CSE-KO mice (Figure 2A, e and f). Control diet-fed WT and CSE-KO mice were devoid of advanced atherosclerotic lesions (Figure 2A, g and h). Quantitative assessment revealed a significant increase in the atherosclerotic lesion area in CSE-KO mice fed with the atherogenic diet in comparison with WT mice on the same diet (Figure 2B). Noninvasive real-time ultrasound biomicroscopy on living animals discovered atherosclerotic plaques in the carotid artery, aortic arch, and abdominal aorta of atherogenic diet–fed CSE-KO mice, whereas no plaques were observed in atherogenic and control diet–fed WT mice or in control diet–fed CSE-KO mice (Figure 2C and Figure II in the online-only Data Supplement). Color Doppler blood flow analysis further confirmed the lesion development in atherogenic diet–fed CSE-KO mice in which plaque formation obstructed and reduced blood flow (online-only Data Supplement Video I).

To investigate whether the lack of endogenous H₂S affects cell proliferation, the animals were intraperitoneally injected with bromodeoxyuridine to determine the percentage of cell proliferation in the aortic root (Figure III in the online-only Data Supplement). 4′,6-Diamidino-2-phenylindole (1 μg/mL) was used as a counterstain to calculate the percentage of proliferating cells. CSE-KO mice fed with the atherogenic diet had a significantly higher percentage of bromodeoxyuridine-positive cells in the intima of the aortic root (5.4±1.3%) than that from WT mice (P<0.05).

**Hypertension Is Not the Cause of Atherosclerosis Development in CSE-KO Mice**

Both control diet and atherogenic diet–fed CSE-KO mice showed hypertension in comparison with WT mice groups (Figure 3A). To elucidate the role of hypertension in atherosclerosis development of CSE-KO mice fed with atherogenic diet, WT and CSE-KO mice were treated with hydralazine in drinking water for 12 weeks (250 mg/L). This treatment lowered blood pressure to normotensive level in CSE-KO mice, but it did not significantly change the blood pressure of WT mice (Figure 3B). After 12 weeks of atherogenic diet feeding and hypertensive drug treatment, WT and CSE-KO mice were analyzed for atherosclerosis development by using routine aortic root histology. Blood pressure lowered to normoten-sive levels in CSE-KO mice did not ameliorate atherosclerosis development (Figure 3C).

**Antiatherogenic Effect of H₂S**

To test whether the development of early atherosclerotic lesions in CSE-KO mice is attributable to the lack of H₂S production, WT and CSE-KO mice were treated with NaHS, an exogenous H₂S donor, for 12 weeks. NaHS treatment significantly decreased atherosclerotic lesions and improved the plasma lipid profile in atherogenic diet–fed CSE-KO mice in comparison with PBS-injected atherogenic diet–fed CSE-KO mice (Figure 4). Severe hyperhomocysteinemia was found in CSE-KO mice, but not in WT mice, fed with either the control or atherogenic diet (Figure IVA in the online-only Data Supplement). We observed no significant differences in plasma homocysteine levels between PBS- and NaHS-injected WT and CSE-KO mice (Figure IVB in the online-only Data Supplement). Similarly, total thiol content of plasma was significantly decreased in atherogenic diet–fed CSE-KO mice in comparison with WT mice on the same diet, whereas CSE-KO and WT mice fed with the control diet did not show any significant difference (Figure IVC in the online-only Data Supplement). NaHS treatment did not significantly change plasma total thiol levels of atherogenic diet–fed WT
and CSE-KO mice groups in comparison with PBS treatment (Figure IVD in the online-only Data Supplement). Ezetimibe treatment significantly decreased total cholesterol, LDL-cholesterol, and HDL-cholesterol in CSE-KO mice (Figure V through VC in the online-only Data Supplement), but it did not significantly improve atherosclerotic lesion size in CSE-KO mice (Figure VE in the online-only Data Supplement).

CSE Deficiency Accelerated Atherosclerosis Development in ApoE-KO Mice

We crossed CSE-KO mice with apoE-KO mice to develop DKO mice (Figure VI in the online-only Data Supplement). DKO mice were not different in general health and body weight than apoE-KO mice. Age-matched WT, CSE-KO, apoE-KO, and DKO mice were fed with standard rodent chow diet for 24 weeks, and plasma lipid profile and atherosclerotic lesion size were analyzed. Despite similar plasma levels of total cholesterol, LDL-cholesterol, and triglyceride with apoE-KO mice, the aortic root lesion area was significantly greater in DKO mice (Figure 5). In contrast to apoE-KO mice, DKO mice showed significantly increased plasma HDL-cholesterol levels (Figure 5C). DKO mice treated with NaHS for 12 weeks exhibited significantly decreased atherosclerotic lesion without altering plasma lipid profile (Figure 5A, 5B, and 5D), with the exception that the HDL-cholesterol level was decreased (Figure 5C).

Increased Oxidative Stress and Adhesion Molecules Expression

CSE-KO and WT mice fed with the control diet did not show any significant difference in their blood GSH, plasma SOD, and malondialdehyde levels, whereas atherogenic diet–fed CSE-KO mice showed significantly lower levels of blood GSH (Figure 6A) and plasma SOD (Figure 6B) and higher levels of malondialdehyde (Figure 6C) than those in WT mice. CSE-KO mice fed with control or atherogenic diet showed significantly decreased liver GSH levels than WT mice, whereas heart GSH levels only decreased in atherogenic diet–fed CSE-KO mice and no significant difference was found on control diet–fed CSE-KO and WT mice (Figure VII in the online-only Data Supplement). Furthermore, GPx and GR activities were reduced in vascular tissues and SMCs from CSE-KO mice in comparison with those from WT mice (Figure 6D and 6E). We also found intensive immunoreactivities to nitrotyrosine in atherogenic diet–fed CSE-KO mice aortic root, further confirming increased oxidative stress (Figure VIII in the online-only Data Supplement).
To determine the interaction of H\textsubscript{2}S and ROS, an in vitro experiment was performed by using vascular SMCs from both CSE-KO and WT mice in the presence or absence of NaHS treatment. ROS levels of CSE-KO SMCs were significantly higher than those of WT SMCs, which were reversed by NaHS treatment (Figure 6F).

Although no change was detected in plasma homocysteine levels, the intraperitoneal injection of NaHS significantly decreased oxidative stress in atherogenic diet–fed CSE-KO mice in comparison with control PBS injection. NaHS treatment significantly increased liver GSH (Figure IXA in the online-only Data Supplement) and plasma SOD levels (Figure IXB in the online-only Data Supplement), and decreased plasma malondialdehyde levels in CSE-KO mice (Figure IXC in the online-only Data Supplement). In addition, WT and CSE-KO mice fed with atherogenic diet were treated with NAC. NAC treatment did not decrease plasma total cholesterol and LDL-cholesterol levels in CSE-KO mice (Figure 7A and 7B), but it significantly decreased oxidative stress in CSE-KO mice, which was confirmed by increased liver GSH, plasma SOD activity, and total thiol levels, and by decreased plasma malondialdehyde levels, as well (Figure 7C through 7F). On the other hand, NAC treatment did not reduce the atherosclerotic lesions developed in CSE-KO mice (Figure 7G).

To elucidate the involvement of adhesion molecules in the development of atherosclerosis, we quantitatively analyzed the expression of aortic ICAM-1. As shown in Figure 8A, the transcriptional expression of ICAM-1 in aortae of CSE-KO mice fed with the control or atherogenic diet was significantly increased in comparison with that of WT mice. NaHS treatment, but not PBS control, reversed the increased aortic ICAM-1 expression in CSE-KO mice fed with atherogenic diet (Figure IXD in the online-only Data Supplement).

SMCs isolated from CSE-KO mice also showed increased ICAM-1 protein expression in comparison with WT SMCs (Figure 8B). In addition, NF-κB expression was significantly increased in the nuclear fraction of CSE-KO SMCs in comparison with WT SMCs (Figure 8C). NF-κB is a well-known inducer for ICAM-1 expression. Increased ICAM-1 expression

![Figure 2. Atherosclerotic lesion development in CSE-KO mice. A, Representative histological sections of aortic roots from CSE-KO and WT mice on atherogenic diet for 12 weeks (H&E stain). Areas in the rectangles of Aa and Ab are enlarged in Ac and Ad. Foam cells forming fatty streaks are observed in CSE-KO mice (Ac), but not in WT mice (Ad). Positive immunostaining for the macrophages is observed in the aorta from CSE-KO mice (Ae) but only faintly in WT mice (Af). Control diet–fed WT and CSE-KO mice did not develop any lesion (Ag and Ah). Scale bars, 200 μm. *P<0.05 vs WT mice, n=5 to 9 for each group. B, Quantitative comparisons of the mean areas of aortic atherosclerotic lesions between WT and CSE-KO mice with the use of Mac-3 staining. C, Ultrasound scanning of the left common carotid artery. CSE-KO mice fed with the atherogenic diet showed plaque (arrow) in the left common carotid artery (Cd), whereas WT mice fed with the control or atherogenic diet and CSE-KO mice fed with the control diet did not develop any plaque (Ca through Cc). Areas in the rectangles (Ca through Cd) are enlarged in (Ce through Ch), respectively. n=7 for each group. CSE indicates cystathionine γ-lyase; H&E, hematoxylin and eosin; KO, knockout; and WT, wild type.](http://circ.ahajournals.org/)

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in CSE-KO mice could stem from increased NF-κB activation. Treatment of cultured SMCs with NaHS (100 µmol/L) for 24 hours significantly decreased ICAM-1 protein expression in CSE-KO SMCs, but not in WT SMCs (Figure 8D).

**Discussion**

It has been reported previously that atherosclerosis development in apoE-KO mice coincided with reduced blood H2S level.17 However, whether the impaired CSE/H2S pathway is the cause or consequence, or a simple parallel event of atherosclerosis development, was not clear.17,25 Our previous studies reported that endogenous H2S inhibits vascular SMC proliferation and CSE deficiency increases SMC proliferation.23 In the present study, we showed that the expression of CSE and a physiological level of endogenous H2S play important roles in maintaining the structural and functional integrity of vascular tissues. CSE-KO mice on the atherogenic diet, but not the control diet, developed moderate fatty streak lesions, consisting predominantly of macrophages and proliferating vascular cells in the aortic root. In addition to the aorta, other major blood vessels of atherogenic diet-fed CSE-KO mice developed atherosclerosis, as observed by noninvasive real-time ultrasound biomicroscopy imaging. Obstructed and reduced blood flow at the plaque area further confirms the lesion development in atherogenic diet–fed CSE-KO mice. Deficiency in the CSE/H2S pathway leads to increased aortic intimal proliferation and increased aortic adhesion molecule expression, which may be one of the underlying pathogenic mechanisms for the vascular remodeling and the early development of atherosclerosis in atherogenic diet–fed CSE-KO mice.

Analysis of cholesterol, HDL, LDL, and triglyceride levels in the plasma revealed considerable differences between CSE-KO and WT mice. This is the first documented evidence for the direct impact of CSE deficiency on lipid metabolism in mice fed with control or atherogenic diets. CSE-KO mice on the atherogenic diet showed increased plasma total cholesterol and LDL- and HDL-cholesterol levels. Hypercholesterolemia, increased LDL, and decreased HDL are known risk factors for the development of atherosclerosis.26,27 Insufficient control of plasma lipid metabolism and increased oxidative stress in CSE-KO mice might also contribute subsequently to the development of atherosclerosis.

The involvement of hypertension in atherosclerosis development has not been clear and the available reports have been conflicting.28,29 Antihypertensive treatment of apoE/
endothelial nitric oxide synthase DKO mice abolished the deleterious effects of endothelial nitric oxide synthase deficiency on atherosclerosis development. In contrast, Chen et al reported that control of hypertension in apoE/endothelial nitric oxide synthase DKO mice during a 16-week period did not prevent atherosclerotic lesion formation. CSE-KO mice have a hypertension phenotype. In our study, the treatment of atherogenic diet–fed CSE-KO mice with the antihypertensive compound hydralazine lowered blood pressure but failed to slow accelerated atherosclerosis development. These findings suggest that hypertension does not contribute to atherosclerosis development in CSE-KO mice.

The correlation of the development of early atherosclerotic lesions in CSE-KO mice with the lack of endogenous H$_2$S production was substantiated by NaHS treatment experiments. NaHS treatment of atherogenic diet–fed CSE-KO mice, with PBS injection as control, significantly decreased atherosclerotic lesions and improved the plasma lipid profile. Wang et al reported that neither NaHS nor dl-propargylglycine treatment altered the plasma lipid profile of apoE-KO mice. This is not surprising given that the abnormal expression of CSE and endogenous production of H$_2$S have not been reported in apoE-KO mice and that dl-propargylglycine cannot completely block CSE activity in living animals in addition to its nonspecific effect on other enzymes. It is known that low LDL-cholesterol levels limit atherosclerosis development. Could the protection of CSE-KO mice from atherosclerosis offered by NaHS treatment be related to decreased plasma total-cholesterol and LDL-cholesterol levels? To this end, we treated atherogenic diet–fed CSE-KO mice with ezetimibe. Ezetimibe is a cholesterol absorption inhibitor, and its action takes place in the jejunal enterocyte brush border of the small intestine. Ezetimibe treatment normalized the plasma lipid profile of CSE-KO mice but did not improve or delay atherosclerosis development in these animals. These data indicate that the altered cholesterol and LDL-cholesterol homeostasis does not play a critical pathogenic role in the accelerated
atherosclerosis development in CSE-KO mice. In addition to CSE, cystathionine-β synthase is another important enzyme in the reverse transsulfuration pathway. Previously, Namekata et al.\textsuperscript{32} reported abnormal lipid metabolism with increased LDL and decreased HDL-cholesterol levels in cystathionine-β synthase knockout mice even at the young age of 2 to 3 weeks. However, there is no report to date that altered lipid metabolism alone in this mouse strain caused atherosclerosis development.

Hyperhomocysteinemia represents a major risk factor of cardiovascular disease.\textsuperscript{33,34} Endogenous H\textsubscript{2}S impacts homocysteine metabolism.\textsuperscript{35} Plasma homocysteine levels of CSE-KO mice fed with the control or atherogenic diet were 18 times higher than those of age-matched WT mice, which is in line with our previous study.\textsuperscript{20} Zhou et al.\textsuperscript{35} reported that diet-induced hyperhomocysteinemia does not independently cause atherosclerosis in C57BL/6J mice. In our study, no significant change was observed in plasma hyperhomocysteinemia levels after NaHS treatment of CSE-KO mice. Furthermore, CSE-KO mice fed with control diet developed hyperhomocysteinemia without developing atherosclerosis. Together, these data suggest that atherosclerotic lesion development in CSE-KO mice is unlikely to be attributable to hyperhomocysteinemia.

To confirm the role of H\textsubscript{2}S in the development of atherosclerosis in the absence of dietary manipulation, atherosclerotic lesion development was assessed in CSE/apoE DKO mice fed with a regular chow diet for 24 weeks. With this diet regime, CSE-KO and WT mice did not, but apoE-KO and DKO mice did develop atherosclerosis. Although apoE-KO and DKO mice have similar plasma cholesterol and LDL-cholesterol levels, DKO mice showed accelerated atherosclerosis in the aortic root more than apoE-KO mice did. NaHS treatment reduced atherosclerotic lesion of DKO mice to the comparable level of apoE-KO mice without any influence on plasma lipid levels. These results further support the notion that atherosclerosis development in CSE-KO mice is independent of plasma lipid homeostasis.

Increased oxidative stress also contributes to the development of atherosclerosis.\textsuperscript{36} GSH is cytoprotective against oxidative damage by neutralizing lipid peroxidase and
hydrogen peroxide.\textsuperscript{37,38} Oxidative stress promotes the oxidation of LDL.\textsuperscript{39} CSE-KO mice fed with the atherogenic diet showed lower levels of malondialdehyde and vascular nitrotyrosine, reflecting increased oxidative stress. We previously reported that GSH levels in vascular tissues (mesentery arteries and aorta) and isolated vascular SMCs of CSE-KO mice were significantly lower than in WT mice.\textsuperscript{14} GPx and GR activities in both vascular tissues and isolated vascular SMCs of CSE-KO mice are also decreased. GPx and GR are actively involved in the reduction and recycling of glutathione disulfide. Hydrogen peroxide generated from superoxide by SOD was further reduced to H$_2$O by GPx. GSH oxidized to glutathione disulfide during the GPx reaction was regenerated by GR, whereas NADPH was consumed as a cosubstrate.\textsuperscript{40} As a result, decreased GPx and GR activities would lead to increased oxidative stress and reduced GSH biosynthesis from glutathione disulfide. Although previous results showed no significant difference in endothelial nitric oxide synthase expression and nitric oxide levels between WT and CSE-KO mice,\textsuperscript{14,41} the nitric oxide signaling pathway in CSE-KO mice may have been impaired because of the lowered thiols level and elevated ROS level. Considering the potential reaction between H$_2$S and S-nitrosothiols to form thionitrous acid,\textsuperscript{42} H$_2$S deficiency and nitric oxide deficiency could have been intertwined in CSE-KO mice.\textsuperscript{43}

NaHS treatment of atherogenic diet–fed CSE-KO mice results in decreased oxidative stress, which was verified by increased liver GSH and plasma SOD levels and decreased plasma malondialdehyde levels. In addition, NaHS treatment significantly decreased ROS levels in cultured SMCs from CSE-KO mice. These results further support the role of H$_2$S as a strong antioxidant. On the other hand, NAC treatment of atherogenic diet–fed CSE-KO mice significantly decreased oxidative stress but had no significant impact on plasma lipid profile and atherosclerotic lesion development.

Adhesion molecules play key roles in the development of atherosclerosis and plaque instability.\textsuperscript{44} Their expression is markedly upregulated in the vascular lesion area and plasma.\textsuperscript{44} Both endothelial cells and vascular SMCs overexpress adhesion molecules in atherosclerosis.\textsuperscript{45} A casual relationship between adhesion molecules and atherosclerosis has been explored
with transgenic mouse models. Bourdillon et al reported that increased expression of ICAM-1 leads to the formation and progression of atherosclerotic lesions. Our present study shows a 30-fold upregulation of ICAM-1 expression in the aorta of CSE-KO mice fed with the atherogenic diet. Although the control diet–fed CSE-KO mice did not have early atherosclerosis development or abnormal lipid metabolism, these mice showed a 3-fold increase in ICAM-1 mRNA level in comparison with that of WT mice with the same diet. It appears that endogenous H2S inhibits the expression of vascular ICAM-1 and this inhibitory effect becomes even more important when the animals are predisposed to atherogenic stimulations. This notion is supported by the studies on atherogenic diet–fed CSE-KO mice aortic tissue and cultured CSE-KO SMCs, of which NaHS treatment decreased the expression level of ICAM-1. We further demonstrated that the increased expression of ICAM-1 in the absence of endogenous H2S may be associated with the increased NF-κB activation.

In conclusion, direct evidence for a protective role of endogenous H2S against the development of atherosclerosis has been obtained for the first time. Endogenously produced H2S protects vascular tissues from atherogenic damage by reducing vascular intimal proliferation, inhibiting adhesion molecule expression and oxidative stress, and maintaining normal lipid metabolism. Deficiency in CSE expression and lower endogenous H2S level should be considered as risk factors and biomarkers for atherosclerosis development, alone or compounding with other atherosclerotic risk factors, such as apoE deficiency. Hence, the CSE/H2S pathway represents an important therapeutic target for the prevention of and intervention in the early stage of atherosclerosis.
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Disclosures

None.

References

Atherosclerosis is a proliferative vascular disease that threatens the functionality of vital organs such as the heart and brain. Hydrogen sulfide (H₂S) is a gasotransmitter that regulates many physiological and pathophysiological processes, including vascular proliferative diseases. H₂S is endogenously generated by cystathionine γ-lyase in vascular tissues, and the deficiency of cystathionine γ-lyase in mice leads to a decreased endogenous H₂S level, an age-dependent increase in blood pressure, and impaired endothelium-dependent vasorelaxation. The discoveries from this study show that the deficiency in cystathionine γ-lyase expression predisposes the experimental mice to accelerated early development of atherosclerosis, characterized with early fatty streak lesions in the aortic root, elevated plasma levels of cholesterol and low-density lipoprotein, and increased lesion oxidative stress and adhesion molecule expression, and enhanced aortic intimal proliferation. Atherosclerosis development becomes faster and more severe when both the cystathionine γ-lyase gene and apolipoprotein E gene are knockout. Rescue of these atherosclerotic mice with H₂S donor, but not with antihypertensive or β-lactamase gene and proliferation. Atherosclerosis development becomes faster and more severe when both the cystathionine γ-lyase expression predisposes the experimental mice to accelerated early development of atherosclerosis, characterized with early fatty streak lesions in the aortic root, elevated plasma levels of cholesterol and low-density lipoprotein, and the experimental mice to accelerated early development of atherosclerosis, characterized with early fatty streak lesions in the aortic root, elevated plasma levels of cholesterol and low-density lipoprotein, and increased lesion oxidative stress and adhesion molecule expression, and enhanced aortic intimal proliferation. Atherosclerosis development becomes faster and more severe when both the cystathionine γ-lyase gene and apolipoprotein E gene are knockout. Rescue of these atherosclerotic mice with H₂S donor, but not with antihypertensive or antioxidant or lipid-lowering drugs per se, prevented atherosclerosis development. This study identifies the deficiency in endogenous H₂S production as a risk factor for atherosclerosis, alone or compounding with other atherosclerotic risk factors, such as abnormal lipid metabolism. Consequently, endogenous level of H₂S would find its application as a biomarker for early diagnosis of atherosclerosis and its prognosis. Armed with the cellular and molecular mechanisms for the effects of H₂S on atherosclerosis development, our clinical intervention and prevention of atherosclerosis can also be more effective and better focused.
Decreased Endogenous Production of Hydrogen Sulfide Accelerates Atherosclerosis
Sarathi Mani, Hongzhu Li, Ashley Untereiner, Lingyun Wu, Guangdong Yang, Richard C. Austin, Jeffrey G. Dickhout, Sárka Lhoták, Qing H. Meng and Rui Wang

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Supplemental Material

Decreased endogenous production of hydrogen sulfide accelerates atherosclerosis

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Supplementary Methods

Assessment of atherosclerotic lesion

Tissue preparation. After 12 weeks on atherogenic or control diet, mice were anesthetised and euthanized. Mouse hearts were perfused with 10 ml of phosphate buffered saline (PBS) and 10 ml of 4% paraformaldehyde. The heart and common arteries were removed, washed with PBS, and fixed with 4% paraformaldehyde for at least 24 hours. Hearts with the attached ascending aorta (1-2 mm) were cut open along the line connecting the tips of the atria. The upper part of the heart was processed and embedded in paraffin with the cut side down. Serial sections were cut at 4 µm thickness through the aortic root. The section at the confluence of the aortic valve leaflets and three more sections spaced at 80 microns apart along the aortic root were stained with hematoxylin and eosin (H&E). The consecutive sections were used for immunohistochemistry.¹²

Immunohistochemistry. After deparaffinizing and blocking the endogenous peroxidase with 0.5% hydrogen peroxide in methanol, the antigen retrieval using rice steamer was performed. Sections were blocked with 5% normal serum of the species in which the secondary antibody was raised, for 10 minutes, and incubated with rat anti-Mac-3 antibody (BD Biosciences, Mississauga, Ontario) diluted in the blocking serum for 1 hour followed by biotinylated secondary antibody (Vector Laboratories, Burlington, Ontario) diluted 1/500 in 0.05 M/L Tris buffer, pH 7.5, for 30 minutes. Sections were incubated in streptavidin-peroxidase (Zymed laboratories, San-Francisco, CA) diluted 1/20 in Tris buffer for 10 minutes followed by development in the peroxidase substrate Nova Red (Vector Laboratories, Burlington, Ontario)
according to manufacturer’s instruction. After counterstaining with hematoxylin, sections were dehydrated and mounted in Permount (Fisher Scientific, Ottawa, Ontario).

Lesion measurement. To identify small fatty streaks or individual macrophage-like cells as macrophages, Mac-3 immunohistochemistry was performed on consecutive sections. The area of red-stained cells within the aortic wall was measured using the ImagePro image analysis software (Media Cybernetics Inc, Rockville, USA).

Detection of proliferating cells in histological sections

Detection of cell proliferation was performed using bromodeoxyuridine (BrdU) incorporation method with modified procedure. After the mice were intraperitoneally injected with BrdU at 100 mg/kg body weight for 16 h, heart tissue from mice was dissected, cleaned and fixed by immersion in 4% paraformaldehyde for 18 h. The samples were then incubated in 30% sucrose at 4°C for 3 days. After embedding in optimal cutting temperature compound (Calbiochem), 10 µm thick sections of aortic-root were cut using a cryostat and mounted on poly-L-lysine-coated slides. The sections were incubated with anti-BrdU-FLUOS monoclonal mouse antibody against BrdU conjugated with fluorescein (Roche, Mississauga, ON) for 45 min at 37°C in a humidity chamber. DAPI (1 µg/ml) was used as a counter-stain. After washing, the sections were observed under a fluorescence microscopy (Olympus). Proliferation was determined by calculating the BrdU-labelling index expressed as the ratio of BrdU-positive cells to total nucleated cells. At least two arbitrarily chosen fields per tissue cross section and four sections per tissue sample were examined.

Real-time PCR analysis
Total RNA from aortic tissues were isolated with TRIzol Reagents according to the manufacturer protocol (Invitrogen, Burlington, Ontario). The first strand cDNA was reverse-transcribed using M-MuLV Reverse-Transcriptase (New England Biolabs, Pickering, Ontario) and real-time PCR was performed in an iCycler iQ5 associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix (Bio-Rad, Mississauga, ON). Briefly, all PCR reactions were performed in a volume of 20 μl and the cycling was conducted at 95°C for 90 s followed by 40 cycles of 95°C for 30 s, annealing at 55°C for 30 s and at 72°C for 30 s. The primers of intracellular adhesion molecule-1 (ICAM-1, Gene Bank Accession Number NM_010493) were 5’-AAATGACCTGCAGACGGAAG-3’ and 5’-TCCTCCTGAGCCTTCT GTAA-3’ with a product of 286 bp. The targeted mRNA levels, normalized with the level of β-actin, were calculated from standard curves of total RNA (Ambion, Austin, Tx) utilizing the iCycler optical system software.

Western blotting analysis

Tissues or cells were harvested and lysed. Equal amounts of proteins were boiled and separated with SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane, as described previously. Membranes were blocked with Tris-buffered saline containing 5% non-fat milk at room temperature for 1h, then incubated overnight at 4°C with primary antibody. The primary antibody dilutions were 1:1,000 for ICAM-1, NFkB, and Lamin; and 1:10,000 for β-actin. The membrane was then washed three times with 1xphosphate-buffered saline-Tween 20 (PBST) buffer and incubated in PBST solution with horseradish peroxidase-conjugated secondary antibody (diluted 1:5,000) for 1 h at room temperature on a shaker. Finally, the
membrane was washed with PBST solution for 3 times. The immunoreactions were visualized with enhanced chemiluminescent and exposed to x-ray film (Kodak, Rochester, NY).

Oxidative stress measurements

The activities of glutathione peroxidise (GPx) and glutathione reductase (GR) were measured according as described previously. The measurement of GPx was coupled to the reduction of GSSG formed in the peroxidase reaction. The activity of GR was determined by monitoring the decrease in absorbance at 340 nm based on the oxidation of NADPH which is coupled to the reduction of one GSSG back to two GSH. The enzyme activity for both GPx and GR was expressed as nmole NADPH oxidized/min/mg protein. Protein concentrations were determined by bicinchoninic acid procedure using bovine serum albumin standards.

Reactive oxygen species (ROS) in plasma and SMCs were measured by DCFH assay (Cell Biolabs, San Diego, CA) according to the manufacturer protocol. For in vitro cell culture experiment, the cells were loaded with a membrane-permeable and non-fluorescent probe DCFH-DA (5 μM) for 2 h at 37°C in PBS, protected from light. Thereafter, the cells were washed 3 times with PBS to remove the excess probe, followed by the treatment with or without NaHS (50 μM) for 12 h. Oxidized DCF was quantified by monitoring DCF fluorescence intensity with excitation at 485 nm and emission at 527 nm with a Fluoroskan Ascent plate reader (Thermo Labsystem, Nepean, Ontario) using Ascent software and expressed as arbitrary units.
Supplemental Figure 1

A.

Change in body weight (g)

Weeks

B.

Body weight (g)

Initial  Final

WT-C  KO-C
WT-A  KO-A
Supplemental Figure 2

A

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B

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Supplemental Figure 3

WT  CSE-KO

BrdU

DAPI

BrdU-positive cells (%)

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*
Supplemental Figure 6

A

PCR genotyping for CSE gene

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PCR genotyping for apoE gene

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B

WT  CSE-KO  apoE  DKO

CSE

apoE

β-Actin
Supplemental Figure 7

A

Liver GSH levels (mmol/mg tissue)

Control | Atherogenic

B

Heart GSH levels (mmol/mg tissue)

Control | Atherogenic

WT | CSE-KO
Supplemental Figure Legends

Supplemental Figure 1. Body weight changes of CSE-KO mice. (A) Body weight changes during the feeding periods with different diets. WT mice fed with control (WT-C) and atherogenic diet (WT-A) showed significantly increased body weight, whereas CSE-KO mice fed with control (KO-C) or atherogenic diet (KO-A) did not gain body weight. n=20 for each group. (B) Body weight changes before (Initial) and after (Final) the 12-week feeding regimes with control or atherogenic diets. n=20 for each group.

Supplemental Figure 2. Ultrasound scanning of aortic arch (A) and abdominal aorta (B). Only CSE-KO mice fed with atherogenic diet developed plaque. Plaques are indicated by arrows. n=7 for each group.

Supplemental Figure 3. Aortic intimal cell proliferation in CSE-KO mice fed with atherogenic diet. Quantitation of cell proliferation in aortic root with BrdU incorporation. Percentage of BrdU-positive cells was calculated as BrdU-labeled nuclei/total nuclei stained with DAPI × 100. M, media. I, intima. Scale bar=20 µm and n=5 for each group. * p<0.05 vs WT mice.

Supplemental Figure 4. Plasma homocysteine (A and B) and total thiol (C and D) levels of WT and CSE-KO mice with different feeding conditions. n=6 for each group, *p<0.05 vs WT mice.

Supplemental Figure 5. Correlation of cholesterol homeostasis with atherosclerosis development of CSE-KO mice. Plasma levels of total cholesterol (A), LDL-cholesterol (B), HDL-cholesterol (C), and triglycerides (D) of WT and CSE-KO mice with or without ezetimibe
treatment. (E) Effect of ezetimibe treatment on atherosclerotic lesion development of atherogenic diet-fed WT and CSE-KO mice. Areas in the rectangles of upper panels (a, b, e and f) were enlarged in the corresponding lower panels (c, d, g and h). Scale bars = 200 microns. n= 5-8 for each group. * p< 0.05 vs WT mice; #p< 0.05 vs CSE-KO without ezetimibe treatment.

Supplemental Figure 6. Genotyping of CSE/apoE double knockout (DKO) mice. (A)
Genotyping results of 4 representative mice. PCR genotyping for CSE gene: R1 together with the two forward primers F1 and N1 where F1 and R1 amplify a 473 bp fragment of the wild-type locus, and N1 and R1 amplify a 428 bp fragment from the targeted allele. PCR genotyping for apoE gene: oIMR0180 together with the two reverse primers oIMR0181 and oIMR0182 where oIMR0180 and oIMR0181 amplify a 155 bp fragment of the wild-type locus, and oIMR0180 and oIMR0182 amplify a 245 bp fragment from the targeted allele. Lane 1 is from a DKO mouse (CSE<sup>-/-</sup>/apoE<sup>-/-</sup>); Lane 2 is from a CSE<sup>-/-</sup>/apoE<sup>-/+</sup> mouse; Lane 3 is from a CSE<sup>-/+</sup>/apoE<sup>-/+</sup> mice and Lane 4 is from CSE<sup>-/-</sup>/apoE<sup>-/+</sup> mice. (B) Western blot analysis of the representative WT, CSE-KO, apoE-KO, and CSE/apoE DKO mice.

Supplemental Figure 7. Tissue glutathione levels. Total glutathione levels in liver (A) and heart (B) of WT and CSE-KO mice fed with control or atherogenic diet. n=6 for each group, * p< 0.05 vs. WT.

Supplemental Figure 8. Immunohistochemical staining for nitrotyrosine in aortic root of WT and CSE-KO mice fed with atherogenic diet. Areas in the rectangles of (a) and (b) are enlarged
in (c) and (d), respectively. CSE-KO mice fed with atherogenic diet, but not WT mice, showed strong staining for nitrotyrosine (foam cell - FC). Scale bars = 200 microns.

Supplemental Figure 9. NaHS reverses oxidative stress and ICAM-1 expression in atherogenic diet-fed CSE-KO mice. Changes in liver GSH (A), plasma SOD (B), plasma malondialdehyde (C), and aorta ICAM-1 expression (D) were detected in atherogenic diet-fed CSE-KO mice, but not in WT mice. n=6 for each group, *p<0.05 vs WT mice; #p<0.05 vs PBS-treated CSE-KO mice.
Supplemental References


Supplemental Video Legend

Supplemental video 1. Reduced blood flow through the plaqued vessels with Ultrasound scanning. Representative recordings from atherogenic diet-fed WT and CSE-KO mice. n=7 for each group.