Vascular Biology, Atherosclerosis and Endothelium Biology

Protective Effect of Hydrogen Sulfide on Balloon Injury-Induced Neointima Hyperplasia in Rat Carotid Arteries

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Endogenous hydrogen sulfide (H₂S), generated from homocysteine metabolism mainly catalyzed by cystathionine γ -lyase (CSE), possesses important functions in the cardiovascular system. In this study, we investigated the role of H₂S during the pathogenesis of neointimal formation induced by balloon injury in rats. CSE mRNA levels were reduced by 86.5% at 1 week and 64.0% at 4 weeks after balloon injury compared with the uninjured controls. CSE activity was also correspondingly reduced. Endogenous production of H₂S in the injured carotid artery was significantly inhibited at 1 week and 4 weeks after balloon injury. Treatment with NaHS (a donor of H₂S) enhanced methacholine-induced vasorelaxation of balloon-injured artery. More importantly, treatment with NaHS significantly inhibited neointima formation ($0.15 \pm 0.01 \text{ mm}^2$ versus $0.21 \pm 0.01 \text{ mm}^2$, $P < 0.001$) of the balloon-injured carotid arteries and reduced the intima/media ratio (1.05 ± 0.07 versus 1.43 ± 0.06 , $P < 0.001$). A significant decrease in vascular smooth muscle cell proliferation was demonstrated by bromodeoxyuridine incorporation at day 7 after injury. In conclusion, CSE expression and H₂S production are reduced during the development of balloon injury-induced neointimal hyperplasia, and treatment with NaHS significantly reduces neointimal lesion formation. (*Am J Pathol* 2007, 170:1406–1414; DOI: 10.2353/ajpath.2007.060939)

Atherosclerosis-associated cardiovascular disease is the leading cause of death in the developed nations and is increasing rapidly in developing countries. Atherosclerosis is a chronic, systemic disease with multiple factors involved in its initiation and progression.¹ After decades

of clinical, epidemiological, and animal studies, it has become clear that homocysteine is an independent risk factor for atherosclerosis and atherosclerosis-related cardiovascular disease.^{2–5} Animal studies have demonstrated that hyperhomocysteinemia enhances vascular neointima formation and accelerates atherosclerosis.^{6,7} It is generally known that homocysteine may induce vascular damage by promoting platelet activation, hypercoagulability and thrombosis formation, oxidative stress and activation of proinflammatory factors, endothelial dysfunction, vascular smooth muscle cell proliferation, and endoplasmic reticulum stress.^{8–15} However, the underlying mechanisms of homocysteine involved in the pathogenesis of atherosclerosis are still not fully understood. Meanwhile, clinical therapeutic targets based on the currently proposed mechanisms have not achieved as much as expected.¹⁶

Hydrogen sulfide (H₂S) has long been known as a toxic gas but has only recently been regarded as a novel gasotransmitter, possessing very important physiological and pharmacological functions in the regulation of blood pressure.¹⁷ Endogenous H₂S is generated from homocysteine metabolism, and its production is catalyzed by two key enzymes: cystathionine β -synthase and cystathionine γ -lyase (CSE).^{11,17} Moreover, CSE is the main H₂S-generating enzyme that has been identified in vascular tissues.¹⁸

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We recently demonstrated that H₂S is capable of inducing *in vitro* relaxation of the aorta of rats and lowering blood pressure.^{18,19} Zhong and colleagues²⁰ reported that reduced H₂S production as a result of CSE inhibition in rats increases blood pressure and that administration of H₂S attenuates blood pressure elevation. A recent study reported decreased CSE activity and H₂S levels in isoproterenol-induced ischemic myocardial injury and that administration of H₂S improved myocardial function, suggesting that H₂S also provides a cardiac protective effect.²¹ To date, the production and function of endogenous H₂S in the pathogenesis of atherosclerosis have not been investigated. We hypothesized that lower endogenous levels of H₂S attributable to suppression of CSE activity could be an important pathogenic factor for atherosclerosis. In this study, we demonstrated for the first time that reduced activity of CSE and production of H₂S are involved in the development of balloon injury-induced atherosclerosis in rat carotid arteries and that administration of H₂S can attenuate this neointima formation.

Materials and Methods

Chemicals

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Carotid Artery Balloon Injury

All animals used in this study were cared for in accordance with the Guidelines of the Canadian Council on Animal Care. All of the animal protocols performed were approved by the University Committee on Animal Care and Supply. Injury to the vessel wall triggering intimal hyperplasia and vascular remodeling has been used for generating animal models of atherosclerosis.²² A total of 88 male Sprague-Dawley rats (3 to 4 months old, weighing 350 to 400 g) (Charles River, Constant, QC, Canada) were used in this study. The balloon denudation injuries were performed in rat carotid artery following the procedure previously described.²² In brief, rats were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg body weight, Ketaset; Ayerst Veterinarian Laboratories, Guelph, ON, Canada) and xylazine (4.6 mg/kg body weight, Rompum; Bayer Inc., Toronto, ON, Canada). A midline incision was made in the neck to expose the left external carotid artery. A 2F Fogarty balloon embolectomy catheter (Baxter Health Care Co., Toronto, ON, Canada) was introduced into the left external carotid artery and advanced through the common carotid artery to the aortic arch. The balloon was inflated with saline (0.02 ml) until a slight resistance was felt and then was rotated while pulling it back through the common carotid artery to denude the vessel of endothelium. This procedure was repeated two more times (total of three passes) and then the catheter was removed. The external carotid was ligated, and the incision was sutured. Carotid arteries from rats receiving sham operations served as controls. The animals were euthanized with an overdose of

pentobarbital (200 mg/kg) at different time points after balloon injury, and the carotid arteries were collected for molecular, biochemical, mechanical, and histological analyses.

Experimental Design

Rats were assigned to 8 groups ($n = 12$ for groups 1 to 6 and $n = 8$ for groups 7 and 8) to determine CSE expression and activity, H₂S production, vasoreactivity, and the development of neointima formation at 1 and 4 weeks after the balloon injury. Group 1 rats received no balloon injury and served as uninjured controls. Group 2 rats underwent balloon catheter injury to the left carotid artery and were sacrificed at 1 week after balloon injury. Group 3 rats received no injury and were used as controls for week 4. Group 4 rats underwent balloon injury and were examined 4 weeks after balloon injury. Group 5 rats underwent balloon injury and received water vehicle placebo injections and were examined at 4 weeks after surgery. Group 6 rats underwent the same surgical procedure as group 5 and received H₂S in the form of sodium hydrosulfide (NaHS) (a donor of H₂S) intraperitoneally. NaHS was given at a dosage of 30 μ mol/kg body weight (10 mmol/L in saline) immediately before surgery and every day after surgery until the end of observation. Group 7 rats underwent balloon injury and received NaHS at the same dose as group 6, but an additional intraperitoneal infusion of bromodeoxyuridine (BrdU) (100 mg/kg) was given 18 hours before euthanization on day 7 after balloon injury for analysis of cellular proliferation. Group 8 rats underwent the same procedure as group 7 and received BrdU infusion but only received a water vehicle placebo injection instead of NaHS for comparison.

Quantitation of CSE mRNA Level by Real-Time Polymerase Chain Reaction (PCR)

Total RNA was collected from carotid arteries using Tri Reagent (Molecular Research Center, Cincinnati, OH). Contaminating DNA was avoided by using the DNA-free kit (Ambion, Austin, TX). Total RNA (2 μ g) was reverse-transcribed into cDNA with an AMV reverse transcriptase using random hexamer primers according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Controls containing no reverse transcriptase were used to monitor against genomic DNA contamination in each sample. Real-time PCR was performed in an iCycler iQ apparatus (Bio-Rad, Hercules, CA) associated with the iCycler optical system software (version 3.1) using SYBR Green PCR Master Mix, as previously described.²³ For quantification, the target gene was normalized by comparison to the internal standard gene β -actin. The primers of CSE (GenBank accession number AY032875) were 5'-AGCGATCACACCACAGACCAAG-3' (sense, position 432 to 453) and 5'-ATCAGCACCCAGAGCAAAGG-3' (anti-sense, position 589 to 609). Product size was determined by running PCR products on a 1.8% agarose gel. Relative mRNA quantification was calcu-

lated by using the arithmetic formula $2^{-\Delta\Delta CT}$,^{24,25} where ΔCT is the difference between the threshold cycle of a given target cDNA and an endogenous reference β -actin cDNA. Based on the calculated ΔCT value, the CSE mRNA level in the treated carotid arteries was subsequently expressed as the percentage of that in the untreated controls.

Determination of CSE Activity

CSE activity was determined using a colorimetric assay based on the determination of pyruvate formation.²⁶ Briefly, the vascular tissues were collected and homogenized in a 20 mmol/L potassium phosphate buffer (pH 7.8), and the homogenate was centrifuged at $12,000 \times g$ for 20 minutes at 4°C. Twenty μ L of supernatants were added into 170 μ L of 100 mmol/L Tris-phosphate buffer (pH 8.0) containing 2.35 mmol/L ethylenediaminetetraacetic acid, 35 μ M/L pyridoxal 5'-phosphate, and 13.7 mmol/L β -chloro-L-alanine, and the mixture was incubated at 37°C for 15 minutes. The reaction was terminated by adding 200 μ L of 6.5 mmol/L DL-propargylglycine. After 5 minutes, the resulting solution was mixed with 1.4 ml of color-producing reagent containing 0.1 mol/L piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 6.4), 0.823 mmol/L thiamine pyrophosphate, 6.857 mmol/L $MgCl_2$, 0.274 U/ml peroxidase, 0.274 mmol/L *N*-(carboxymethylamino)-4,4'-bis(dimethylamino)-diphenylamine, and 10.97 U/ml pyruvate oxidase, and incubated at 37°C for 10 minutes. The sample blank was similarly prepared, except that the sample was added to the substrate mixture. The absorbance of green dye at 727 nm was measured in a Multiskan spectrum microplate spectrophotometer. The CSE-specific activity was expressed as the absorbance at 727 nm/mg protein.

Measurement of Endogenous H_2S Production in Carotid Arteries and Plasma

Tissue H_2S production rate was measured as routinely used in our laboratory.^{18,27} In brief, carotid arteries isolated from rats were homogenized in 50 mmol/L ice-cold potassium phosphate buffer (pH 6.8). The reaction mixture contained 100 mmol/L potassium phosphate buffer, pH 7.4, 10 mmol/L L-cysteine, 2 mmol/L pyridoxal 5'-phosphate, and 10% (w/v) tissue homogenate. Cryovial test tubes (2 ml) were used as the center wells, and each contained 0.5 ml of 1% zinc acetate as a trapping solution along with a filter paper of 2×2.5 cm² to increase the air/liquid contact surface. The reaction was performed in a 25-ml Erlenmeyer flask (Pyrex; Corning, Corning, NY). The flasks containing the reaction mixture and the center wells were flushed with N_2 before being sealed with a double layer of Parafilm. The reaction was initiated by transferring the flasks from ice to a 37°C shaking water bath. After incubation at 37°C for 90 minutes, 0.5 ml of 50% trichloroacetic acid was added to stop the reaction. The flasks were sealed again and incubated at 37°C for 60 minutes to ensure complete trapping of the H_2S released from the mixture. The contents of the center wells

were transferred to test tubes containing 3.5 ml of water. Then, 0.5 ml of 20 mmol/L *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 mol/L HCl and 0.5 ml of 30 mmol/L $FeCl_3$ in 1.2 mol/L HCl were added. The absorbance of the resulting solution was determined at 670 nm with a spectrophotometer.²⁸ The H_2S concentration was calculated against the calibration curve of the standard H_2S solutions.

Serum total H_2S was measured on duplicated samples with a sulfide electrode (Lazar Research Laboratories, Los Angeles, CA) using a Fisher Accumet model 10 pH meter (Fisher Scientific, Pittsburgh, PA).²⁹ Briefly, 300 μ L of serum was mixed with 150 μ L of antioxidant buffer and 150 μ L of deionized water. After rinsing with distilled water and drying, the electrode was immersed into the sample. The electrode potential was recorded when the reading stabilized. The concentration of H_2S was calculated by comparison to the standard curve.

Tissue Contractility Study

Carotid arteries were removed and placed in cold physiological saline solution (PSS) (119 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH_2PO_4 , 1.17 mmol/L $MgSO_4 \cdot 7H_2O$, 2.5 mmol/L $CaCl_2 \cdot 2H_2O$, 25 mmol/L $NaHCO_3$, 0.03 mmol/L, and 5.5 mmol/L glucose, pH 7.4). The carotid rings were carefully dissected from the surrounding adipose tissue under a microscope (Wild M3B; Leica, Heerbrugg, Switzerland), with great care taken to protect the endothelium. The vessel segments (2.0 mm) were mounted in a four-channel wire myograph (Multi myograph model 610M; Danish MYO Technology A/S, Aarhus, Denmark) aerated with a gas mixture of 95% O_2 to 5% CO_2 at 37°C for isometric tension experiment. Tissue segments were connected to a tension transducer and an adjustable micrometer with 40 μ m of tungsten wire. The vessels were progressively stretched with an adjustable micrometer. The corresponding measured force was read, and the wall tension was calculated by dividing this force by the vessel length. Internal circumference (IC) of the segment was calculated for each level of stretch. The effective pressure was calculated by the Laplace law: when the effective pressure exceeded 100 mm Hg, the stepwise distension was stopped. IC_{100} , the internal circumference of the vessel under an effective resting transmural pressure of 100 mm Hg, was calculated [effective resting transmural pressure = wall tension/(internal circumference/ 2π)]. The vessel was set to a normal internal circumference of $IC_{0.9}$, where $IC_{0.9} = 0.9 \times IC_{100}$. Myodaq-Myodata software was used for data collection and analysis. Thirty minutes after normalization, the vessels were alerted to KPSS + 10 μ M/L noradrenaline (KPSS, PSS in which NaCl was correspondingly changed for KCl on an equimolar basis), PSS + 10 μ M/L noradrenaline, and KPSS twice, respectively, and the vessels were rinsed off with PSS for four times after each treatment. Finally, vessels were equilibrated for a further 30 minutes before beginning experimentation. The α -adrenergic agonist phenylephrine (PHE; 10^{-8} to 10^{-5} mol/L) was added cumulatively to

generate PHE dose-response curves. A concentration of PHE inducing 60 to 70% of maximal contraction response was applied to evaluate relaxation dose responses. The endothelium-dependent vasodilator methacholine (Mch; 10^{-10} to 3×10^{-5} mol/L) or H₂S (10^{-7} to 10^{-3} mol/L) was added cumulatively to generate relaxation dose responses.

Histological and Morphometric Analyses

Rats were sacrificed by intraperitoneal injection of Euthanol at various time points after balloon injury. The carotid arteries were perfusion-fixed at a constant physiological pressure of 125 mm Hg with 4% paraformaldehyde. The carotid arteries were carefully stripped of adventitia and excised between the origin at the aorta and the carotid bifurcation. The proximal segment (0.5 cm) of the denuded arteries was removed and fixed in 4% paraformaldehyde for 12 hours before being embedded in paraffin and used for morphometric analysis. The cross sections (5 μ m) of carotid artery were stained with hematoxylin and eosin and photographed. The intimal and medial cross-sectional areas of the carotid arteries were measured using an NIH Image 1.62 program (Bethesda, MD), and the intima/media ratios of the cross sections were calculated.

Immunohistological Staining

After perfusion, carotid arteries were carefully removed and denuded. The carotid artery was cut off and fixed in 4% paraformaldehyde for 12 hours, embedded in paraffin, and sectioned for immunohistostaining. Detection of DNA synthesis was performed using the BrdU incorporation method as previously described with modification.³⁰ In brief, paraffin from a histological section (5 μ m thick) was removed, and tissue sections were rehydrated with washing buffer. Endogenous peroxidase present in the tissue sections was inactivated with 0.3% hydrogen peroxide solution, followed by 0.01% proteinase K digestion at 37°C. Sections were incubated with monoclonal mouse antibody against BrdU (1/10 dilution, catalog no. 1-299-964; Roche). Primary antibodies were detected using sheep anti-mouse-Ig-alkaline phosphatase (1/10 dilution). After washing, a sufficient amount of freshly prepared color-substrate solution (nitro blue tetrazolium in 70% dimethylformamide and X-phosphate solution) (catalog no. 1-299-964; Roche) was applied to the section and incubated for 30 minutes at room temperature. Hematoxylin was used for counterstaining. Proliferation was determined by calculating the BrdU-labeling index expressed as the ratio of BrdU-positive cells to total nucleated cells. A total of 20 different fields from each artery section were randomly selected, and the numbers of BrdU-positive and -negative nuclei were counted in each field by a single blinded observer.

Apoptosis was assessed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method using a TUNEL apoptosis detection kit (catalog no. 17-141; Upstate Technology, Lake Placid, NY) following the

Figure 1. Inhibition of CSE expression and H₂S production after balloon injury. CSE expression was determined by real-time PCR and normalized to β -actin levels per unit of vascular tissue. **A** and **B**: Decreased CSE expression in balloon-injured carotid arteries at 1 week (**A**) and 4 weeks (**B**). **C** and **D**: Decreased CSE activity in carotid artery at 1 week (**C**) and 4 weeks (**D**) after balloon injury. **E** and **F**: Decreased endogenous production of H₂S from carotid artery at 1 week (**E**) and 4 weeks (**F**) after balloon injury. The results are from eight animals (* P < 0.05, ** P < 0.01 versus uninjured controls).

procedure used in our laboratory.³¹ In brief, 5- μ m sections were deparaffinized and incubated with proteinase K [1/24 (v/v) in phosphate-buffered saline] for 15 minutes at room temperature. Sections were incubated with terminal deoxynucleotidyl transferase end-labeling cocktail (a mixture of terminal deoxynucleotidyl transferase buffer, biotin-dUTP, and terminal deoxynucleotidyl transferase at a ratio of 90:5:5, respectively) for 60 minutes at 37°C. After washing and blocking, avidin-fluorescein isothiocyanate (1:10) was applied to the sections and incubated in the dark for 30 minutes at 37°C. Apoptotic cells were quantified by counting the percentage of TUNEL-positive cells against the total number of nucleated cells in 20 different fields per tissue section, and the ratio was expressed as the TUNEL index.

Statistical Analysis

All results are expressed as means $\pm$ SEM. Statistical significance was tested using a Student's *t*-test. A value of P < 0.05 was considered statistically significant.

Results

Decreased CSE Expression and Activity in Balloon-Injured Carotid Arteries

To assess the involvement of CSE in vascular remodeling, CSE expression in the carotid artery after balloon injury was determined by real-time PCR and normalized

Table 1. Plasma H₂S Levels and Their Correlation with Blood Pressure in Rats after a Bolus Intraperitoneal Injection of NaHS

	Before	1 hour	4 hours	8 hours
Blood pressure (mm Hg)	125 ± 3	102 ± 2**	108 ± 4**	124 ± 4
Plasma H ₂ S (μmol/L)	39.7 ± 6.8	69.6 ± 8.3**	48.6 ± 4.0*	38.5 ± 7.4

The plasma H₂S concentrations and blood pressure were the means of four measurements at each time point done once a week during the course of the 4-week experiments. There were no significant changes for blood pressure and H₂S during the period of observation from day 1 to week 4 except of the transient changes as shown. *n* = 8. **P* < 0.05, ***P* < 0.01, compared with those before NaHS injection.

to β-actin levels. CSE mRNA levels were significantly suppressed to 13.5 ± 2.1% (*P* < 0.01) at 1 week (Figure 1A) and 36.0 ± 6.0% (*P* < 0.05) at 4 weeks (Figure 1B) with the uninjured controls as 100%. CSE enzyme activity was reduced to 12.7 ± 1.6 OD_{727 nm}/mg protein at 1 week after balloon injury compared with uninjured controls (45.4 ± 2.6 OD_{727 nm}/mg protein, *P* < 0.01) (Figure 1C). At 4 weeks after balloon injury, the CSE enzyme activity was 18.4 ± 1.7 OD_{727 nm}/mg protein, whereas that in the uninjured controls was 42.8 ± 3.0 OD_{727 nm}/mg protein (*P* < 0.05) (Figure 1D).

Endogenous Production of H₂S in Injured Carotid Arteries

Suppression of CSE expression and enzyme activity after balloon injury significantly decreased H₂S production in the carotid artery as determined at 1 week compared with the uninjured controls (3.13 ± 0.69 nmol/g/minute versus 8.67 ± 1.43 nmol/g/minute, *P* < 0.01, *n* = 8) (Figure 1E). Endogenous production of H₂S in the carotid arteries was still significantly inhibited at 4 weeks (4.83 ± 0.94 nmol/g/minute versus 8.87 ± 1.56 nmol/g/minute, *P* < 0.05, *n* = 8) (Figure 1F) after balloon injury as compared with the uninjured controls.

Plasma H₂S Levels after Administration of NaHS

Plasma H₂S concentration was highly elevated 1 hour after intraperitoneal bolus injection of NaHS (69.6 ± 8.3 μmol/L) compared with the baseline level of 39.7 ± 6.8 μmol/L (*P* < 0.01). The elevated H₂S lasted for at least 4 hours (48.6 ± 4.0 μmol/L, *P* < 0.05) and returned to normal level 8 hours after NaHS administration (Table 1). Blood pressure was correspondingly lowered 1 hour after administration of NaHS from 125 ± 3 mm Hg to 102 ± 2 mm Hg (*P* < 0.01), and the blood pressure lowering effect lasted for at least 4 hours (108 ± 4 mm Hg, *P* < 0.01) (Table 1). The decrease in blood pressure was well correlated to plasma H₂S levels. The plasma H₂S concentrations and blood pressure were the means of four measurements at each time point determined once a week during the course of the 4-week experiments. There were no significant changes for blood pressure and H₂S during the period of observation from day 1 to week 4 except of the transient changes as shown in Table 1.

Effects of H₂S on Carotid Vasoreactivity

The contraction of the carotid artery in response to PHE increased significantly 4 weeks after balloon injury com-

pared with uninjured controls (EC₅₀ = 49.21 ± 2.66 nmol/L for injured arteries versus EC₅₀ = 167.64 ± 28.80 nmol/L for the uninjured controls) (Figure 2A). In the balloon-injured carotid artery groups, treatment with NaHS attenuated PHE-induced vasoconstriction of the

Figure 2. Effects of H₂S on the vasoactivities of carotid arteries with or without balloon injury. **A:** The vasoconstriction of the carotid in response to PHE increased significantly 4 weeks after balloon injury compared with uninjured controls (filled square, injured carotids; open square, uninjured controls). **B:** Treatment with NaHS increased methacholine-induced vasorelaxation in intact carotids compared with the untreated under precontracted condition with PHE (open square, uninjured controls; open circle, uninjured carotids with NaHS; closed square, injured without NaHS; closed circle, injured with NaHS). *n* = 6 per group. **P* < 0.05, ***P* < 0.01 versus uninjured controls (A) or injured carotids (B); #*P* < 0.05 versus injured without NaHS.

Figure 3. Therapeutic effect of exogenous H₂S on balloon injury-induced neointima formation. **A:** A representative of the uninjured carotid artery. **B:** A representative of histological change 1 week after balloon injury showing neointima formation. **C:** A representative of histological change of carotid artery 4 weeks after balloon injury, showing severe neointimal hyperplasia. **D:** A representative of histological change 4 weeks after balloon injury but receiving NaHS, showing dramatic inhibition of neointima formation compared with **C** (H&E staining). **Arrows** and **arrowheads** indicate the internal and external elastic lamina, respectively. Original magnifications, $\times 100$.

carotid compared with that of untreated rats (data not shown). Treatment with NaHS increased Mch-induced vasorelaxation of the intact carotids ($IC_{50} = 135.77 \pm 23.80$ nmol/L) as compared with untreated controls ($IC_{50} = 625.16 \pm 123.58$ nmol/L) (92.0% versus 78.6% maximal relaxation, $P < 0.01$) (Figure 2B). More importantly, treatment with NaHS enhanced Mch-induced vasorelaxation in balloon-injured carotids ($IC_{50} = 520.39 \pm 81.50$ nmol/L) as compared with untreated balloon-injured carotids ($IC_{50} = 135.27 \pm 24.43$ nmol/L) (34.0 versus 9.8% maximal relaxation, $P < 0.05$) (Figure 2B).

Inhibitory Effects of Exogenous H₂S on Balloon Injury-Induced Neointimal Formation

Development of neointimal hyperplasia after balloon injury was confirmed (Figure 3, A–C). The neointimal hyperplasia of the carotid arteries (Figure 3C) was dramatically inhibited after treatment with NaHS as compared with the untreated injured controls, determined 4 weeks after balloon injury (Figure 3D). Morphometric analysis indicated that treatment with NaHS significantly inhibited neointima formation (0.15 ± 0.01 mm², cross-sectional area) of the carotid arteries compared with untreated injured carotid arteries (0.21 ± 0.01 mm², $P < 0.001$, $n = 8$), determined 4 weeks after balloon injury (Figure 4A). There was no significant difference in the medial cross-sectional areas of the carotid arteries between the H₂S-treated and untreated groups ($P = 0.8$) (Figure 4B). However, the intima/media ratios were significantly reduced in the rats that received NaHS (1.05 ± 0.07 versus 1.43 ± 0.06 , $P < 0.001$, $n = 8$) (Figure 4C).

Anti-Proliferative and Apoptotic Effects of H₂S on Vascular Smooth Muscle Cells

Smooth muscle cell proliferation and apoptosis were determined at day 7 after arterial injury. Treatment with H₂S

induced a significant reduction in the percentage of BrdU-positive cells in the neointima compared with treatment with vehicle controls ($19.4 \pm 3.2\%$ versus $45.7 \pm 5.3\%$, $P < 0.01$, $n = 6$) (Figure 5, A–C). There was no significant difference of BrdU-labeled positive cells in media area between the two groups ($5.3 \pm 0.7\%$ versus $5.6 \pm 0.8\%$, $P > 0.05$). Using TUNEL staining, we could not detect any significant change in the rate of apoptotic cells in injured carotid arteries between the two groups in the neointima (Figure 5, D–F).

Discussion

Cystathionine β -synthase and CSE are the two key enzymes modulating homocysteine metabolism and catalyzing H₂S production. Studies have demonstrated that cystathionine β -synthase deficiency causes hyperhomocysteinemia, which in turn leads to the premature development of atherosclerosis and thrombosis.^{32–34} Based on our previous findings, CSE is expressed in vascular tissues, mainly in vascular smooth muscle cells, but not in endothelium.^{17,18} Although CSE is the main enzyme expressed in the arterial system, the role of CSE expression and H₂S production in the development of atherosclerosis have been unknown. Using an animal model of atherosclerosis induced by balloon injury, we demonstrate that CSE expression and activity are significantly reduced during the development of neointimal formation. H₂S production by carotid arteries is also dramatically decreased after balloon injury. Our findings suggest that CSE expression is down-regulated and that CSE activity is suppressed during balloon injury-induced development of atherosclerosis. Together with the increase in neointima formation, these observations indicate that the decrease in CSE activity is involved in the development of balloon injury-induced neointimal hyperplasia. On the other hand, the low level of H₂S observed during balloon injury-induced neointimal hyperplasia indicates that H₂S

Figure 4. Morphometric analysis of intima, media, and intima/media ratio of carotid arteries. **A:** Treatment with NaHS significantly inhibited neointima formation of the carotid arteries compared with untreated carotid arteries as determined 4 weeks after balloon injury. **B:** No significant change of the medial cross-sectional area of the carotids 4 weeks after balloon injury between the treated and untreated groups. **C:** The intima/media ratios were significantly reduced in the group of rats that received NaHS. $n = 8$ per group. ** $P < 0.001$ versus untreated group.

may be protective against atherosclerosis. We have previously demonstrated that the addition of the CSE inhibitor DL-propargylglycine diminishes H₂S production in mesenteric artery tissues,¹⁹ indicating the direct contribution of CSE to H₂S production in arterial tissue. Nevertheless, the functional role of H₂S in the pathogenesis of atherosclerosis has never been studied. To demonstrate whether H₂S can prevent or limit atherosclerosis, exogenous H₂S was administered to rats undergoing balloon injury to the carotid artery. Interestingly, atherosclerotic

lesion of the carotid arteries, as determined by neointimal formation and intima/media ratio, was significantly reduced in rats receiving NaHS compared with those without NaHS treatment. This finding demonstrates that administration of H₂S inhibits neointima hyperplasia.

The underlying mechanisms of the protective effect of H₂S on the development of neointimal hyperplasia were investigated in the present study. H₂S inhibits smooth muscle cell proliferation as determined by BrdU-labeled-positive nuclei induced by balloon injury. This anti-proliferative effect may explain the inhibitory effect of H₂S on balloon injury-induced neointimal formation. Earlier, Valitutti and colleagues³⁵ showed the anti-proliferative effect of H₂S on T lymphocytes. We recently demonstrated that the anti-proliferative effect of H₂S is mediated through a sustained ERK and p21Cip/WAK-1 activation.²³ It has also been shown that both endogenous and exogenous H₂S (200 to 500 μ mol/L for 12 hours) induce apoptosis of human aortic smooth muscle cells by up-regulating caspase-3 and p21(Cip/WAK-1) through activation of ERK and p38 MAPK.^{31,36} The proapoptotic effect of H₂S may play a role in inhibition of neointima formation. However, in the present study, serum H₂S was raised from 39.7 to 69.6 μ mol/L, and no apoptosis of vascular smooth muscle cells was observed using TUNEL assay. It seems that the extracellular concentrations of H₂S and difference between cultured cells *in vitro* and intact vascular tissues *in vivo* may affect the apoptotic status of vascular smooth muscle cells. Different methodologies for apoptosis could also affect the sensitivity and specificity of the detection. We think that the TUNEL assay itself is able to provide sufficient information in this study, particularly because there was no obvious apoptosis detected and apoptosis change seems unlikely to be the major contributor for attenuation of neointimal formation. However, in our future studies, we will use several assays including activated caspase-3 immunoassay to investigate the apoptotic effects of H₂S. In addition, we showed that administration of H₂S increased methacholine-induced vasorelaxation of both intact and balloon-injured carotid arteries, suggesting that H₂S can improve vasorelaxation. The H₂S-induced vasorelaxant effect is mediated mainly by stimulation of K-ATP channels in vascular smooth muscle cells but may also involve the endothelial production of nitric oxide.^{17–19} The effects on vasoconstriction and relaxation of carotid arteries seem attributable to NaHS-induced limitation of neointimal damage of vascular wall. Attenuation of neointimal hyperplasia is probably related to reduced smooth muscle cell proliferation, which is the main contributor for vasoconstriction. Reduced neointimal lesion may promote re-endothelialization and thus improve vasorelaxation.⁶

In conclusion, using the rat model of balloon injury-induced neointimal hyperplasia, we investigated the pathophysiological role of CSE/H₂S alteration in the development of neointimal formation. CSE expression and activity are reduced during the process of neointimal formation induced by balloon injury. Reduced CSE activity leads to decreased H₂S production. Treatment with H₂S attenuates the development of neointimal hyperplasia. This is the first direct demonstration showing the

Figure 5. Anti-proliferative effect of H₂S. **A** and **B**: Quantification of cellular proliferation performed at day 7 after arterial injury by BrdU incorporation in rats treated with NaHS (**A**) or vehicle (**B**). **Arrows** indicate BrdU-labeled nuclei stained as dark brown. Percentage of BrdU-labeled nuclei was calculated in **C** using the BrdU index as follows: BrdU-labeled nuclei/total nuclei $\times$ 100. $n = 6$ per group. ****** $P < 0.01$ versus controls. **D** and **E**: Quantification of apoptosis performed at day 7 after arterial injury by TUNEL in animals treated with NaHS (**D**) or vehicle (**E**). **Arrows** indicate TUNEL-positive cells. **F**: Treatment with NaHS did not increase rate of apoptosis in neointima or media. Original magnifications, $\times 100$.

importance of altered CSE/H₂S levels in the pathogenesis of atherosclerosis. Further investigation into the role of CSE/H₂S system in the development and progression of atherosclerosis may expand our understanding of the underlying mechanisms and lead to new therapies for the treatment and prevention of atherosclerosis.

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