Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats

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Geng B, Cui Y, Zhao J, Yu F, Zhu Y, Xu G, Zhang Z, Tang C, Du J. Hydrogen sulfide downregulates the aortic L-arginine/nitric oxide pathway in rats. Am J Physiol Regul Integr Comp Physiol 293: R1608–R1618, 2007. First published July 18, 2007; doi:10.1152/ajpregu.00207.2006.—The aim of the present study was to investigate the effect of hydrogen sulfide (H2S) signaling by nitric oxide (NO) in isolated rat aortas and cultured human umbilical vein endothelial cells (HUVECs). Both administration of H2S and NaHS, as well as endogenous H2S, reduced NO formation, endothelial nitric oxide synthase (eNOS) activity, eNOS transcript abundance, and L-arginine (L-Arg) transport (all P < 0.01). The kinetics analysis of eNOS activity and L-Arg transport showed that H2S reduced Vmax values (all P < 0.01) without modifying Km parameters. Use of selective NOS inhibitors verified that eNOS [vs. inducible NOS (iNOS) and neuronal NOS (nNOS)] was the specific target of H2S. Nitric oxide; nitric oxide synthase; L-arginine transport

HYDROGEN SULFIDE (H2S) IS a well-known pungent gas, and its toxicology has been extensively studied (40). Recently, H2S has been found to be endogenously generated in various mammalian tissues by 2 pyridoxal-5′-phosphate-dependent enzymes, such as cystathionine β-synthase (CBS), and cystathionine γ-lyase (CSE) (6), with L-cysteine used as a main substrate. The gas may be a functional regulator in the nervous and cardiovascular systems (21). In the cardiovascular system, CSE may be a key enzyme by which H2S is generated (50). H2S causes a dose-dependent relaxation of the isolated rat aorta (16, 50) and mesenteric arterioles (4), induces transient hypotension in anesthetized rats in vivo (50), reduces proliferation of vascular smooth muscle cells (VSMCs) (8, 46), and exerts a negative inotropic action of the heart in vivo and in vitro (13). Significantly, there is evidence linking the endogenous CSE/H2S pathway to the pathogenesis of arterial hypertension (45, 52), pulmonary hypertension induced by hypoxia (5), septic and endotoxin shock (18), hemorrhagic shock (27), ischemic cardiac disease (2, 11), ischemic brain damage (33), and hyperdynamic circulation in cirrhosis (10). Growing evidence suggests that in addition to nitric oxide (NO) and carbon monoxide (CO) (12), endogenous H2S may be a novel cardiovascular gasotransmitter. As an endogenous ligand, H2S directly opens the KATP channel in VSMCs and relaxes vascular smooth muscles in an endothelium-independent manner (50), which is different from the effect of NO and CO.

Interestingly, the administration of sodium nitroprusside (SNP), an NO donor, potentiates the vasorelaxation effects induced by H2S (16) and upregulates H2S production in rat vascular tissues in a concentration-dependent manner (48). In contrast, pretreatment with H2S attenuates the vasorelaxant effect induced by SNP in aortic rings (49). In rats with septic and endotoxin shock, the plasma level of H2S is positively correlated with that of NO (18). In the brain, H2S acts as an endogenous peroxynitrite scavenger (42). All of these results suggest that NO and H2S signaling are interrelated. Of note, the expression of the H2S-generating enzyme CSE had been observed in VSMCs (50), but not in the vascular endothelium, suggesting that H2S is generated in vascular tissues mainly from VSMCs. Whether and how endogenous H2S from VSMCs affect the endothelial NO production pathway is unknown. In the present study, we examined the effects of H2S on NO production, endothelial NO synthase (eNOS) activity, and expression, and L-Arginine (L-Arg, substrate of NOS) transport in isolated rat aortas and cultured human umbilical vein endothelial cells (HUVECs) to explore the regulatory role of H2S in the vascular L-Arg/NOS/NO pathway.

MATERIALS AND METHODS

Animals. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (documentation no. 55, 2001, Ministry of Health of P.R. China) and the Guide for the Care and Use of the Laboratory Animals of the First Hospital, Peking University. Male Sprague-Dawley (SD) rats (250–300 g) were provided by the Animal Department, Health Science Center of Peking University. All animals were maintained on normal rat chow with unrestricted water and on a 12:12-h light-dark cycle.

Materials. HUVECs (926 cell line) were purchased from the American Type Culture Collection (Manassas, VA). Tris-base, Dowex AG 50
w-x8 (Na⁺), NADPH, tetrahydrobipterin (BH₄), calmodulin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), l-Arg, l-carnitine, spermidine, pinacinic, glibenclamide, N-ethylmaleimide (NEM), DL-propargylglycine (PAG), l-cysteine, pyridoxal-5'-phosphate, and sodium hydrosulfide (NaHS) were purchased from Sigma (St. Louis, MO). l-[³H]Arg (1.5 TBq/mmol) was from NEN Life Science Products (Boston, MA); and eNOS antibody (SC-654), P-eNOS(Ser1177)-R antibody (SC-12971), iNOS antibody (SC-7271), nNOS antibody (SC-648), phospho-Akt1/2/3 (Ser473) antibody (SC-7985R), Akt antibody (SC-8312), and β-tubulin antibody (SC-5274) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

H₂S-saturated solution (0.09 mol/l at room temperature) was made by bubbling with pure H₂S gas (offered by Beijing XianHeYu, China) and stored at ~70°C.

The specific primers used for sample loading calibration were synthesized by SBS (Beijing, China) and were as follows: eNOS-S, 5′-GTGGTGTCCGGCGGATGTG-3′; eNOS-A, 5′-AAAGACAGGGGCAAGCGGTG-3′; cationic amino acid transporter-1 (CAT-1)-S, 5′-TGAGAGACGGGATGAGG-3′; CAT-1-A, 5′-TAGAGAAACGCCTCCTAGTG-3′; GAPDH-S, 5′-GAGATTGGCCTGATTGGG-3′; and GAPDH-A, 5′-GGAAGATGGTGATGGGATT3′. Other chemicals and reagents were of analytical grade.

Organ culture of aortas in vitro. At the selected time, recipient rats were anesthetized with pentobarbital sodium (30 mg/kg ip) and then decapitated quickly. The entire thoraco-abdominal aorta was quickly cleaned of adherent adipose tissues and reagents were of analytical grade.

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Transport kinetics of \textit{l}-Arg into endothelial cells was measured by the method of Leoncini et al. (25), with minor modifications. Briefly, kinetic experiments were performed in cells incubated for 15 min with KH buffer containing \textit{l}-Arg (10 to 320 \textmu mol/l) and 2 \textmu Ci/ml \textit{l}-[\textsuperscript{3}H]Arg. Transport was terminated by removing the media and washing the cells three times with ice-cold 10 mmol/l unlabeled \textit{l}-Arg in PBS. Cells were lysed with 0.5% Triton X-100 in 0.5 mol/l NaOH, and radioactive activity [\textsuperscript{3}H]-Arg taken by cells was assayed by liquid scintillation counting.

**RT-PCR assay of gene expression of eNOS and CAT-1 in HUVECs.**

The mRNA levels of eNOS and CAT-1 were measured by RT-PCR, as described previously by our laboratory (19). Total RNA from cells was extracted with Trizol reagent (Appleygen Technologies, Beijing, China). RT-PCR was performed in a total volume of 25 \textmu l. After denaturing at 95°C for 5 min, we ran PCR at 94°C for 30 s, 61°C for 30 s, and 72°C for 40 s for 30 cycles. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. The optical density of the bands of eNOS cDNA (553 bp) and CAT-1 cDNA (250 bp) was measured by use of the Gel Documentation System (Bio-Rad, Hercules, CA). The PCR products were amplified again with the human GAPDH primers at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 20 cycles, and the optical density of the GAPDH band (205 bp) was considered as the relative amount of eNOS or CAT-1 gene expression.

Western blot analysis. Protein isolation and Western blot analysis were carried out as previously described (3). Briefly, a cellular lysate was prepared from the HUVECs and separated by SDS-PAGE (on 7.5% gels). The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) by electroblotting (Bio-Rad). Proteins were detected with polyclonal anti-eNOS, anti-P-eNOS, anti-iNOS, anti-nNOS, anti-Akt, and anti-P-Akt antibodies and a monoclonal anti-tubulin antibody. After incubation with the appropriate secondary antibodies conjugated to horseradish peroxidase, the membrane was washed, and color was developed by the use of an appropriate secondary antibodies conjugated to horseradish peroxidase. The activity of both agents was largely blocked by pretreatment with glibenclamide (10 \textmu mol/l).

**RESULTS**

\textit{H}2\textit{S} inhibited NO generation of aortic tissues. After \textit{l}-Arg administration to cultured aortic tissues, the level of nitrate plus nitrite, both stable products of NO, continuously accumulated in the medium up to 6 h (Fig. 1A). After administration of \textit{H}2\textit{S} (50 \textmu mol/l) and NaHS (50 \textmu mol/l), the NO products in the medium were decreased, by 63% and 47% (\textit{P} < 0.01) after 2 h, 48% and 30% (\textit{P} < 0.01) after 4 h, and 32% and 19% (\textit{P} < 0.05) after 6 h, respectively, compared with that in controls (Fig. 1A). According to the Fig. 1A results, we selected 2 h as a time of best inhibitory effect and observed the effects of various concentrations (from 1 to 1,000 \textmu mol/l) of \textit{H}2\textit{S} on NO release at this time. Administration of both \textit{H}2\textit{S} and NaHS significantly reduced NO production in a concentration-dependent manner after a 2-h incubation (Fig. 1B). The IC\textsubscript{50} value was 19.5 \textmu mol/l for \textit{H}2\textit{S} [95% confidence interval (CI), 7.06–53.09 \textmu mol/l] and 15.9 \textmu mol/l for NaHS (95% CI, 7.99–31.59 \textmu mol/l). Although the IC\textsubscript{50} of \textit{H}2\textit{S} was a little higher than that of NaHS, the inhibitory effect of \textit{H}2\textit{S} was stronger than that of NaHS (Fig. 1C). The results showed that exogenous \textit{H}2\textit{S} inhibits vascular NO production in vitro.

We felt it important to explore the effect of endogenously generated \textit{H}2\textit{S}. Administration of \textit{l}-cysteine (\textit{l}-Cys, the substrate of CSE) and pyridoxal-5’-phosphate (PLP, a cofactor of CSE) increased \textit{H}2\textit{S} release from incubated aortic tissues with \textit{H}2\textit{S} concentration in the culture medium reaching 37.5 ± 2.7 \textmu mol/l (Fig. 1D); baseline \textit{H}2\textit{S} concentration in the culture medium was undetectable. Plasma \textit{H}2\textit{S} concentration was 45.6 ± 10.59 \textmu mol/l, according to values reported by Zhao et al. (50). At the same time, \textit{l}-Cys and PLP treatment reduced NO production by 43% compared with that in controls (\textit{P} < 0.01, Fig. 1E). The effect of \textit{l}-Cys and PLP treatment on NO production was blocked by preincubation with an inhibitor of CSE, DL-propargylglycine (PAG) (Fig. 1E), which prevented endogenous \textit{H}2\textit{S} formation (the \textit{H}2\textit{S} concentration in culture medium could not be detected by sensitive sulfur electrode, as shown in Fig. 1D).

The \textit{K}_{\text{ATP}} channel is an important molecular target of \textit{H}2\textit{S} in cardiovascular tissues (4, 47, 50). To assess the role of \textit{K}_{\text{ATP}} channel in \textit{H}2\textit{S}-induced decrease of NO production, the \textit{K}_{\text{ATP}} channel opener pinacidil and \textit{K}_{\text{ATP}} channel inhibitor glibenclamide were used. Pinacidil reduced NO production by 34% and \textit{H}2\textit{S} (100 \textmu mol/l) level by 30% (\textit{P} < 0.01) (Fig. 1F). The activity of both agents was largely blocked by pretreatment with glibenclamide (10 \textmu mol/l). These results suggest that the \textit{K}_{\text{ATP}} channel is involved in inhibition of \textit{H}2\textit{S}-induced NO generation.

\textit{H}2\textit{S} downregulated aortic eNOS activity and changed its kinetics. eNOS is the principle form of NO synthase in normal arterial endothelium. On incubation, aortic tissue slices with \textit{H}2\textit{S} (50 \textmu mol/l) and NaHS (50 \textmu mol/l), reduced eNOS activity by 56% and 47% (\textit{P} < 0.01) after 2 h, 39% and 27% (\textit{P} < 0.01) after 4 h, and 12% and 11% (\textit{P} > 0.05, Fig. 2A) after 6 h, respectively, compared with that in controls. In addition, \textit{H}2\textit{S} and NaHS (from 1 to 1,000 \textmu mol/l) markedly decreased aortic eNOS activity in a concentration-dependent manner after 2 h incubation (Fig. 2B). The IC\textsubscript{50} values of \textit{H}2\textit{S} and NaHS for eNOS activity were 11.49 \textmu mol/l (95% CI, 9.53–19.87 \textmu mol/l) and 31.97 \textmu mol/l (95% CI, 11.93–85.70 \textmu mol/l), respectively. \textit{H}2\textit{S} was stronger in inhibiting eNOS activity than NaHS (Fig. 2C). The results are similar to those with NO generation, which suggests that the \textit{H}2\textit{S} reduced vascular NO generation, at least in part, by inhibiting eNOS activity.

The kinetics of eNOS regulation by \textit{H}2\textit{S} were studied in HUVEC cultures. Incubation of HUVEC with NaHS (50 \textmu mol/l, 2 h) inhibited the maximal activity of eNOS (\textit{V}_{\text{max}}, 75.1 ± 2.9 vs. control 113.0 ± 4.0 \textmu mol·min\textsuperscript{-1}·mg protein\textsuperscript{−1}, \textit{P} < 0.01) without modifying the \textit{K}_{m} value for \textit{l}-Arg (33.6 ± 4.4 vs. control 39.3 ± 3.0 \textmu mol/l, \textit{P} > 0.05, Fig. 2D), thus significantly decreasing the enzyme activity efficiency (ratio of \textit{V}_{\text{max}} to \textit{K}_{m}: 2.24 ± 0.66 vs. 2.88 ± 1.33, \textit{P} < 0.01). We investigated the effect on aortic eNOS activity of endogenously generated \textit{H}2\textit{S} by adding \textit{l}-Cys and PLP to HUVECs. eNOS activity was reduced by 48% (\textit{P} < 0.01; Fig. 2E), an effect that
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was attenuated by pretreatment with PAG (a CSE inhibitor) (48). L-canavanine, a selective iNOS inhibitor (26), and spermidine, an nNOS inhibitor, alone or together, did not affect the inhibition effects of H2S (100 μmol/l) on NOS activity (Fig. 2F), which indicates that H2S specifically regulates eNOS activity. Like H2S, pinacidil inhibited eNOS activity, which was not blocked by the iNOS or nNOS inhibitors used. However, glibenclamide blocked inhibition of eNOS activity by H2S and pinacidil. Collectively, these results suggest that a KATP channel is involved in the impact of H2S on eNOS activity.

Both Ca2+ -dependent and -independent pathways are involved in the regulation of eNOS activity. There is evidence that H2S modulates Ca2+ influx in cardiovascular cells (1), suggesting that H2S may inhibit eNOS activity through a Ca2+ -dependent pathway. Whether a Ca2+ -independent pathway is involved in the inhibition of eNOS activity by H2S is unknown. We detected phosphorylation of Ser1177, which activates eNOS by a Ca2+ -independent pathway (17), in eNOS protein. Incubation with H2S (500 μmol/l) for 2 h inhibited the phosphorylation of eNOS Ser1177 without changing eNOS expression (Fig. 2G; P < 0.01). Pinacidil also slightly inhibited eNOS phosphorylation. Pretreatment with glibenclamide (10 μmol/l) partially blocked the effects of H2S and pinacidil on eNOS phosphorylation (Fig. 2G). The results confirmed the role of a KATP channel in the regulation of H2S in eNOS activity.

Akt (also called protein kinase B) mediates eNOS phosphorylation of Ser1177 in vitro and in vivo and plays a role in both calcium-dependent and -independent eNOS activation (15). H2S treatment for 30 min significantly reduced Akt phosphorylation without changing total Akt protein expression (Fig. 2H). Pinacidil also reduced Akt phosphorylation, and glibenclamide blocked the effects of H2S and pinacidil. This suggests that Akt signaling is involved in the regulation of H2S to eNOS phosphorylation, which partially modulates by opening KATP channels.

H2S inhibited l-[3H]Arg uptake by aortic tissues and changed l-[3H]l-Arg transport kinetics in HUVECs. To determine the impact of H2S on transcellular transport of l-Arg, we measured l-[3H]l-Arg uptake in aortic tissues. H2S (50 μmol/l) and NaHS (50 μmol/l) decreased l-[3H]Arg uptake by 58% and 50% (P < 0.01) after 2 h, 39% and 30% (P < 0.01) after 4 h, and 12% and 8% (P > 0.05) after 6 h, respectively (Fig. 3A). In addition, incubation with H2S and NaHS for 2 h inhibited the l-[3H]Arg uptake in a concentration-dependent manner (1 to 1,000 μmol/l; Fig. 3B). The IC50 values were 84.08 μmol/l (95% CI: 27.28–259.20 μmol/l) and 39.15 μmol/l (95% CI: 10.46–146.50 μmol/l) for H2S and NaHS.
Fig. 2. H₂S downregulates eNOS activity without changing iNOS activity. A: alterations in aortic eNOS and iNOS activities induced by H₂S and NaHS (50 μmol/l) after 2, 4, and 6 h of incubation. B: effect of different concentrations of H₂S and NaHS (1–1,000 μmol/l) on aortic eNOS and iNOS activities after 2-h treatment. C: inhibitory effect of H₂S and NaHS on eNOS activity. Control as 100%. D: eNOS enzyme activity assay at various concentrations of L-arginine (L-Arg; 0.1–3.2 mmol/l) in human umbilical vein endothelial cells (HUVECs) with or without treatment with physiological concentrations of H₂S (50 μmol/l). E: effect on eNOS and iNOS activities of endogenous H₂S generated by treatment with L-Cys plus PLP. F: alteration of nNOS and iNOS activity after treatment with H₂S (100 μmol/l) and pinacidil (10 μmol/l), and the effects of the use of the selective iNOS inhibitor-L canavanine (2.5 mmol/l), the nNOS-selective inhibitor spermidine (0.5 mmol/l), and the nonselective K_ATP channel inhibitor glibenclamide (10 μmol/l) downregulate eNOS phosphorylation at Ser1177 but do not change eNOS protein expression after 2-h incubation; glibenclamide (Gli) blocked the inhibitory effects in part (n = 3). H: H₂S (500 μmol/l) and pinacidil (10 μmol/l) reduced phosphorylation of Akt, and glibenclamide blocked the inhibitory effects. All values are means ± SD. *P < 0.05 H₂S vs. control; †P < 0.05 NaHS vs. control; ‡P < 0.05 vs. control; *P < 0.01 vs. control; #P < 0.05 vs. H₂S treatment; &P < 0.05 vs. pinacidil. $P < 0.01 vs. control iNOS activity (n = 5).
we investigated the kinetics of L-Arg transport in HUVECs. 

H2S vs. 73.0 min, P administered or endogenously generated, inhibits L-Arg transport (17.5 ± 1.2 vs. control 20.6 ± 1.1 pmol/10^6 cells/min, P < 0.01). Without modifying the K_m value (84.4 ± 14.8 vs. 73.0 ± 10.4 pmol/l, P > 0.05) and thus reduced significantly the transport efficiency (V_max/K_m: 0.21 ± 0.08 vs. 0.28 ± 0.11, P < 0.01) (Fig. 3D).

In addition, administration of L-Cys and PLP inhibited L-Arg uptake, which was blocked by PAG pretreatment, as shown in Fig. 3E. The results suggest that H_2S, whether exogenously administered or endogenously generated, inhibits L-Arg transport.

HUVECs transport arginine through two Na^+-independent systems. System y+ is sensitive to N-ethylmaleimide (NEM) and relates to the expression of CAT1 and CAT2B. System y+L relates to the expression of y+LAT2, y+LAT1, and 4F2hc (35). To identify which type of transporter system acts as a main target of H_2S, we investigated L-Arg uptake with or without NEM (0.5 mmol/l, pretreatment 20 min). NEM reduced L-Arg uptake by 32% compared with control (Fig. 3F, P < 0.01). NEM pretreatment plus H_2S (100 μmol/l) did not change the L-Arg influx compared with the effect of NEM alone (Fig. 3F; P > 0.05). The K_ATP channel opener pinacidil slightly reduced L-Arg uptake (12% decrease compared with control) as did H_2S, but NEM intensified the inhibitory effects of pinacidil on L-Arg uptake compared with NEM treatment alone (Fig. 3F, P < 0.01). Simultaneously, glibenclamide blocked, in part, the inhibitory effects of both H_2S and pinacidil.

H_2S down-regulated CAT-1 and eNOS gene transcript level in HUVECs. To determine whether H_2S modulates the transcript level of CAT-1, mRNA expression of the y+ transporter inhibitor NEM, the alteration of L-Arg uptake by H_2S and pinacidil. All values are means ± SD. *P < 0.05 H_2S vs. control; **P < 0.05 NaHS vs. control; ***P < 0.05; **P < 0.01 vs. control, and #P < 0.05 vs. H_2S treatment. (n = 5).

H_2S inhibited eNOS but did not change iNOS and nNOS protein expression in HUVECs. To confirm whether H_2S modulates eNOS, eNOS protein expression was assayed by Western blot analysis in HUVECs after 6-h H_2S incubation. H_2S (100, 500, and 1,000 μmol/l) significantly inhibited eNOS protein expression but not in a concentration-dependent manner, and the inhibitory effect was partially blocked by glibenclamide preincubation (10 μmol/l, Fig. 5A). Fig. 5B confirmed the above phenomena and showed pinacidil-induced inhibition of eNOS protein expression and block of this by glibenclamide.

Induction of iNOS protein expression by H_2S was not observed (Fig. 5C). nNOS is another constitutive NOS that is
expressed in endothelial cells. Neither H$_2$S nor pinacidil altered nNOS expression (Fig. 5D).

NaHS inhibited $l$-Arg/NOS/NO pathway in rats in vivo. To demonstrate the in vivo effects of H$_2$S on the vascular $l$-Arg/NOS/NO pathway, we analyzed plasma levels of nitrite and nitrate after intraperitoneal administration of 14 μmol/kg NaHS in rats. At 2 h after NaHS injection, the plasma content of nitrite plus nitrate was 21% (24.44 ± 0.80 vs. 31.00 ± 2.66 μmol/l less than in controls, $P < 0.01$, Fig. 6A). Aortic eNOS activity was reduced by 42% (23.06 ± 5.08 vs. 39.50 ± 8.67 pmol·min$^{-1}$·mg protein$^{-1}$, $P < 0.01$, Fig. 6B), and $l$-Arg uptake decreased by 30% (88.35 ± 14.31 vs. 125.40 ± 18.96 pmol·min$^{-1}$·mg protein$^{-1}$, $P < 0.01$, Fig. 6C). iNOS activity was not induced by H$_2$S injection in the in vivo experiments (Fig. 6B).

DISCUSSION

Recently, the novel endogenous gas H$_2$S has been recognized as another cardiovascular gasotransmitter that exerts important cardiovascular effects similar to those of NO and CO. However, H$_2$S, as opposed to NO and CO, is specifically released from VSMCs but not endothelium, opens the K$_{ATP}$ channel, induces cellular membrane hyperpolarization, and causes vessel dilation in an autocrine/paracrine manner (40, 41, 50), which differs from the effect of NO and CO. Some studies have reported that NO enhanced endogenous H$_2$S production (48) and its vascular dilation effect (16), whereas H$_2$S inhibited vascular relaxation induced by NO (49). In the present study, we found that endogenous H$_2$S downregulated the vascular $l$-Arg/NOS/NO pathway. This was mediated in part through the K$_{ATP}$ channel. Taken together, these results suggest a delicate crosstalk between endothelial and VSMCs by a complex interaction of the gasotransmitters NO and H$_2$S (Fig. 7).

The physiological serum concentration of H$_2$S in Sprague-Dawley (SD) rats has been reported to be 45.6+/-14.2 μmol/l (50). In the present study, an exogenously administered physiological concentration of H$_2$S or its donor, NaHS, inhibited NO release from cultured aortic tissues. The inhibitory effect of H$_2$S weakened with incubation time but remained detectable for 6 h or more, consistent with anticipated changes of H$_2$S concentration in the medium (as a gasotransmitter, H$_2$S could be metabolized in the cell quickly) (44). According to the concentration-dependent inhibitory effects of H$_2$S, the IC$_{50}$ value of H$_2$S was calculated as approximately one-half of the physiological serum concentration. Endogenous H$_2$S generation from aortic tissues is 3.6 nmol·min$^{-1}$·g wet tissue$^{-1}$ (48). SNP (from 10 to 1,000 nmol/l) increased aortic H$_2$S production in a concentration-dependent manner; 1 μmol/l SNP can increase H$_2$S by 2 nmol·min$^{-1}$·g wet tissue$^{-1}$ (48). One possibility is a negative feedback regulation in which accumulated NO enhances H$_2$S release from VSMCs during the short term and, over accumulative H$_2$S, inhibits NO formation, achieving dynamic balance in the ultimate.

H$_2$S endogenously generated by $l$-Cys plus PLP reduced NO production by 43%. Pretreatment with PAG, a CSE inhibitor (51) and pinacidil, a nonselective K$_{ATP}$ channel opener, decreased aortic NO production, similar to the effect of H$_2$S. Glibenclamide, a nonselective inhibitor, blocked the inhibitory effects of H$_2$S and pinacidil. Collectively, these data suggest that endogenously generated H$_2$S downregulates vascular NO production, and opening of the K$_{ATP}$ channel could be one of the possible mechanisms.

NO is generated from the conversion of $l$-Arg to $l$-citrulline by the enzymatic action of NADPH-dependent NO synthases (NOS). NOS exists in 3 distinct isofoms, a constitutive neuronal NOS (NOS I or nNOS), an inducible NOS (NOS II or iNOS), and a constitutive endothelial NOS (NOS III or eNOS) (1). In the vaculature, NO is produced mainly in the endothelium by constitutively expressed eNOS. The present results suggest that exogenous H$_2$S or its donor NaHS reduced eNOS activity within 4 h in a concentration (from 1 to 1,000 μmol/l)-dependent manner. The IC$_{50}$ values for H$_2$S (11.49 μmol/l) were ~26% of the physiological serum H$_2$S concentration, and lower than that of H$_2$S-inhibited NO production (19.5 μmol/l). The kinetic study showed that H$_2$S did not modify the enzyme affinity to $l$-Arg (represented as $K_m$ value) but decreased the maximum value of catalytic velocity ($V_{max}$), indicating that H$_2$S reduces eNOS catalytic efficiency. H$_2$S endogenously generated by administration of $l$-Cys plus PLP also inhibited eNOS activity.

To verify the specificity of H$_2$S-mediated inhibition of eNOS activity, we investigated the effect of single or combined administration of the selective iNOS inhibitor $l$-canavanine and nNOS inhibitor spermidine. These treatments did not change the inhibitory effect of H$_2$S on constitutive NO activity. Although iNOS activity could be induced by stimuli such as cytokines, reactive oxygen species, or some drugs (1), we detected very low iNOS activity in normal cultured aortic tissues, and neither H$_2$S nor NaHS incubation affected iNOS activity during the entire incubation period. Additionally, H$_2$S inhibits eNOS transcript level and protein expression. These
findings suggest that eNOS is an important regulatory target of H2S-affected NO generation. L-Arg transport is another key step to limiting NO generation besides NOS catalysis (8). In the present study, exogenous administration of both H2S and NaHS significantly reduced L-[3H]Arg transporter activity within 4 h and returned to control levels after 6 h. Inhibition was concentration dependent in the 1 to 1,000 μmol/l range, and the inhibitory effect of H2S (from 12.4% to 55.3%) was higher than that of NaHS (from 3.6% to 38.8%). The IC50 values were 84 μmol/l for H2S, and approximated 182% of physiological serum concentrations.

Intracellular levels of L-Arg are not generally considered rate-limiting for eNOS catalysis (51). Consumption of intracellular L-Arg initiates and stimulates its uptake by cells (30), and at this time, NO generation is dependent on L-Arg uptake. These may explain why the IC50 of H2S for L-Arg uptake was higher than that for NO generation. Kinetic analysis of the L-Arg transport showed that H2S inhibited the Vmax of L-[3H]Arg uptake but did not influence its Km value. Furthermore, administration of L-Cys plus PLP increased endogenous H2S production and reduced L-Arg transport. L-Arg enters mammalian cells through several membrane-bound cationic amino acid transporters: systems y+, b0, +, and y+L (8). Previous studies have demonstrated that Arg transport in the vascular endothelium is mainly attributed to system y+, and to some extent to system y+L (35). NEM, a system y+ selective inhibitor, reduced L-Arg influx per se. After pretreatment with NEM, H2S did not change L-Arg influx, suggesting that the NEM-sensitive system y+ transport activity contributes to the regulation of L-Arg uptake by H2S. In addition, the present study found that H2S also decreased the CAT1 (encoding the system y+ transporter) transcript level by 34%. All of the above results suggest that endogenous H2S inhibits L-Arg transport under physiological conditions and that the system y+ transporter is the main pathway regulated by H2S.

The effect of injection of NaHS on the L-Arg/NOS/NO pathway was also examined in vivo in rats. According to our
previous work, injection of NaHS (14 \( \mu \text{mol/kg} \)) after 2 h increased plasma H\(_2\)S concentration by 67% over baseline (52). In the present study, the plasma level of nitrate plus nitrite was decreased by 21%, and aortic eNOS activity by 42%, and L-Arg by 30% 2 h after injection of NaHS. These in vivo results were similar to those of the in vitro experiments and support the notion that H\(_2\)S downregulates the vascular L-Arg/NOS/NO pathway (i.e., inhibited L-Arg uptake, eNOS activity, and NO generation). Venous injection of H\(_2\)S can directly induce vasodilation and cause transient hypotension (16, 50), and we have previously reported that intraperitoneal bolus injection of NaHS reduces arterial blood pressure. Increasing plasma H\(_2\)S level from 39 to 79 \( \mu \text{mol/l} \) reduced arterial blood pressure by 22 mmHg (from 134 to 112 mmHg); but plasma H\(_2\)S concentration increased from 79 to 123 \( \mu \text{mol/l} \), while arterial blood pressure fell by 6 mmHg (from 112 to 106 mmHg) (52). The present in vivo studies that show inhibition of the L-Arg/NOS/NO pathway by H\(_2\)S may explain, in part, this phenomenon.

Our findings support the concept that eNOS is an important target molecule of H\(_2\)S regulation. L-Arg transport from plasma into cells is regulated by H\(_2\)S, including the interaction of eNOS with caveolin-1, heat shock protein 90 (Hsp90), or membrane phospholipids, and enzyme translocation and phosphorylation (1). Ser1177 appears to be the most important site of eNOS phosphorylation and is affected by most, if not all, of the diverse stimuli that promote eNOS activation. Phosphorylation of eNOS-Ser1177 increases eNOS sensitivity to Ca\(^{2+}\)/calmodulin binding and leads to eNOS activation (28). Our study found that H\(_2\)S downregulated eNOS phosphorylation at Ser1177. This result implies that H\(_2\)S inhibits eNOS activity partly through inhibition eNOS phosphorylation at Ser1177. Akt-mediated-phosphorylation of eNOS-Ser1177 is a crucial step of eNOS activation induced by estrogen, insulin, VEGF, statins, and shear stress (15). H\(_2\)S reduced Akt phosphorylation without influencing total Akt protein, suggesting that the Akt pathway may contribute to regulation of H\(_2\)S by eNOS-Ser1177 phosphorylation. H\(_2\)S is an endogenous opener of the K\(_{\text{ATP}}\) channel in many cell types, e.g., (39, 47, 50). The K\(_{\text{ATP}}\) channel inhibitor glibenclamide blocked the effects of H\(_2\)S on eNOS activity (Fig. 2, G and H), eNOS phosphorylation, and Akt phosphorylation. Glibenclamide also blocked inhibition of eNOS phosphorylation by the nonselective K\(_{\text{ATP}}\) channel opener pinacidil. These data imply that H\(_2\)S may open K\(_{\text{ATP}}\) channels, followed by reduced Akt phosphorylation and inhibition of eNOS phosphorylation, leading to downregulation of eNOS activity.

Our study found that H\(_2\)S also decreased eNOS transcript abundance. Numerous physiological and pathophysiological stimuli have been identified to modulate eNOS expression. Reactive oxygen species, as a signal molecule, play an important role in eNOS transcription and posttranslational regulation. Effects of H\(_2\)O\(_2\) on eNOS transcription and posttranslational modification were reported in bovine and human endothelial cells. Chronic oxidative stress caused by excessive H\(_2\)O\(_2\) production in vivo evokes a compensatory response involving increased eNOS transcript (36). Our previous work showed that H\(_2\)S reduced oxidative radical release and scavenged H\(_2\)O\(_2\) directly (11). H\(_2\)S may reduce the H\(_2\)O\(_2\) signal in eNOS transcription and posttranslation and thereby reduce eNOS transcription. Fig. 5D revealed that K\(_{\text{ATP}}\) channel opening by H\(_2\)S and pinacidil inhibited eNOS protein expression, effects that were blocked by glibenclamide. These data suggest that K\(_{\text{ATP}}\) channels are involved in the inhibition of eNOS protein expression by H\(_2\)S. However, the precise mechanisms of inhibition of eNOS protein expression by H\(_2\)S need to be further investigated.

L-Arg transporter could be an additional target molecule of H\(_2\)S regulation. L-Arg transport from plasma into cells is

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**Fig. 6.** H\(_2\)S inhibited NO production in vivo. Six rats were intraperitoneally injected with NaHS (14 \( \mu \text{mol/kg} \)) for 2 h, and 6 controls with normal saline. A: nitrate plus nitrite plasma concentration after 2 h. B: aortic eNOS and iNOS activities are shown. C: L-[\(^3\)H]-Arg uptake in aortic tissues. **P < 0.01 vs. control.

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**Fig. 7.** Summary of the interaction between H\(_2\)S and NO in artery.
mediated by several different classes of CATs. System y^+ activity, selective for cationic amino acids only, is encoded by 4 genes (CAT1, CAT2, CAT3, and CAT4) representing different isoforms (35). Systems y^+ (CAT-1) and y^-L (heavy chain subunit-4F2hc) have been detected in the endothelium (9). Transport of cationic amino acids via CAT-1 is voltage dependent with alterations of membrane potential affecting both V_{max} and K_{m} values for influx (22). L-Arg transport is sensitive to the membrane potential; it is stimulated by drugs that cause membrane hyperpolarization and inhibited by those that cause membrane depolarization (51). H_{2}S opens the K_{ATP} channel and induces membrane hyperpolarization (50), which may contribute to L-Arg transport activity, and furthermore, it results in an accumulation of the intracellular pool of L-Arg available for eNOS. However, opening K_{ATP} channels alone is not enough to explain the mechanisms of H_{2}S-mediated L-Arg influx. Administration of pinacidil reduced L-Arg influx, as did H_{2}S, but NEM could not block the inhibitory effects of pinacidil. Glibenclamide blocked the effect of pinacidil to a lesser extent than H_{2}S, suggesting that the mechanisms involved in the H_{2}S regulation of L-Arg influx may be more complex.

Administration of pinacidil-reduced L-Arg influx as did H_{2}S, but NEM could not block the effects of pinacidil. Glibenclamide blocked almost completely the inhibition effects of pinacidil but only slightly blocked the effects of H_{2}S, which suggests that the mechanisms involved in the H_{2}S regulation of L-Arg influx are complex. Opening K_{ATP} channels alone is not enough to explain the mechanisms of H_{2}S-mediated L-Arg influx.

Oh et al. (29) found that H_{2}S could inhibit NO production in LPS-stimulated macrophages through a mechanism that involves heme oxygenase-1 (HO-1)/CO. H_{2}S increased pulmonary arterial HO-1 gene transcription and protein expression (32). CO, derived from VSMCs and endothelium, has vasodilatory and antiproliferative effects and acts as a competitive antagonist for NO-mediated sGC activation or displaces internal stores of NO. As a heme ligand, CO potentially inhibits NOS activity and reduces NO production (34, 38).

Taken together, these results suggest that the gasotransmitter family, including NO, CO, and H_{2}S, derived from vascular tissues in a paracrine/autocrine manner, interacts in the regulation of vascular homeostasis. The interaction of these gasotransmitters in distinct locations of the blood vessels could maintain a dynamic balance and form a regulatory “network” (41). Imbalances of the network regulation may contribute to pathogenesis of cardiovascular diseases. Research into the network regulation of gasotransmitters should reveal a novel prospect for understanding the mechanism of cardiovascular diseases and a novel target for prevention and treatment.

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HS DOWNREGULATES L-ARGININE/NO PATHWAY


