

Hydrogen Sulfide Is an Endogenous Inhibitor of Phosphodiesterase Activity

Mariarosaria Bucci, Andreas Papapetropoulos, Valentina Vellecco, Zongmin Zhou, Anastasia Pyriochou, Charis Roussos, Fiorentina Roviezzo, Vincenzo Brancaleone, Giuseppe Cirino

Objective—Recent studies have demonstrated that hydrogen sulfide (H₂S) is produced within the vessel wall from L-cysteine regulating several aspects of vascular homeostasis. H₂S generated from cystathione γ -lyase (CSE) contributes to vascular tone; however, the molecular mechanisms underlying the vasorelaxing effects of H₂S are still under investigation.

Methods and Results—Using isolated aortic rings, we observed that addition of L-cysteine led to a concentration-dependent relaxation that was prevented by the CSE inhibitors DL-propargylglycine (PAG) and β -cyano-L-alanine (BCA). Moreover, incubation with PAG or BCA resulted in a rightward shift in sodium nitroprusside- and isoproterenol-induced relaxation. Aortic tissues exposed to PAG or BCA contained lower levels of cGMP, exposure of cells to exogenous H₂S or overexpression of CSE raised cGMP concentration. RNA silencing of CSE expression reduced intracellular cGMP levels confirming a positive role for endogenous H₂S on cGMP accumulation. The ability of H₂S to enhance cGMP levels was greatly reduced by the nonselective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine. Finally, addition of H₂S to a cell-free system inhibited both cGMP and cAMP breakdown.

Conclusion—These findings provide direct evidence that H₂S acts as an endogenous inhibitor of phosphodiesterase activity and reinforce the notion that this gasotransmitter could be therapeutically exploited. (*Arterioscler Thromb Vasc Biol.* 2010;30:1998-2004.)

Key Words: endothelium ■ hypertension ■ signal transduction ■ vascular muscle ■ vasodilation ■ cystathione γ -lyase ■ hydrogen sulfide ■ cAMP ■ cGMP ■ phosphodiesterase

Nitric oxide (NO) is believed to account for most of the endothelium-derived relaxing factor activity released within the vessel wall, at least in some vessels.¹ On muscarinic stimulation, NO is produced following the conversion of L-arginine to NO by endothelial nitric oxide synthase (eNOS).² NO diffuses from the endothelium to the underlying smooth muscle cell layer, where it stimulates soluble guanylate cyclase to produce cGMP. cGMP in turn activates protein kinase G (PKG), which initiates a cascade of events leading to relaxation.^{2,3} Hydrogen sulfide (H₂S) is emerging as a new gaseous signaling molecule in the cardiovascular system.^{4,5} Vascular endothelial cells express cystathionine γ -lyase (CSE) and produce measurable amounts of this gasotransmitter.⁶ Recent evidence suggests that H₂S exhibits endothelium-derived relaxing factor activity.⁶ In addition, it has been shown that muscarinic stimulation leads to CSE activation in the endothelium, triggering the conversion of L-cysteine to H₂S and that CSE, like eNOS, is a calcium/calmodulin-dependent enzyme. Therefore, within the vascular wall, these

2 pathways coexist and serve a similar function. The relative amounts of NO versus H₂S likely depend on the vascular bed studied⁷ or on the state of the tissue, eg, healthy versus diseased.^{5,8}

Mice with targeted disruption of the CSE locus (CSE null mice) exhibit hypertension, similarly to what is observed in eNOS-null mice.⁶ Moreover, resistance arteries isolated from CSE knockout mice display a marked inhibition of methacholine-induced relaxation. These observations, along with data in the literature showing similar effects for the 2 gasotransmitters, strongly suggest that NO and H₂S either cooperate dynamically to maintain vessel homeostasis or serve as each other's backup system under pathological conditions.^{9,10}

Although the mechanism of action of NO-induced vasorelaxation is well understood, that of H₂S remains less clear. Glibenclamide attenuates the vasorelaxing action of H₂S, but a residual dilatory response is observed,^{9,11} suggesting that although KATP channel activation is important for H₂S-

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From the Department of Experimental Pharmacology, Faculty of Pharmacy, University of Naples–Federico II, Naples, Italy (M.B., V.V., F.R., V.B., G.C.); the Laboratory for Molecular Pharmacology, Department of Pharmacy, University of Patras, Greece (A. Papapetropoulos, A. Pyriochou), and the “G. P. Livanos” Laboratory, Department of Critical Care and Pulmonary Services, University of Athens, Greece (Z.Z., C.R.).

Dr Bucci and Dr Papapetropoulos contributed equally to this work.

Correspondence to Giuseppe Cirino, PhD, FBPharmacolS, Department of Experimental Pharmacology, Faculty of Pharmacy, University of Naples–Federico II, Naples, 80131 Italy. E-mail cirino@unina.it

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induced vasodilation, additional mediators contributing to tone reduction by H₂S must exist. Therefore, the aim of the present study was to investigate the contribution of cyclic nucleotides in H₂S-stimulated relaxation in vascular tissue.

Methods

Isolated Aortic Rings Experimental Protocol

Male Wistar rats 8 to 10 weeks of age were euthanized, and thoracic aortas were rapidly dissected and cleaned from fat and connective tissue. Rings 2 to 3 mm in length were cut and placed in organ baths (2.5 mL) filled with oxygenated (95% O₂ to 5% CO₂) Krebs solution at 37°C, mounted to isometric force transducers and connected to a Graphtec linear recorder. After ≈60 minutes of equilibration period, rings were challenged with phenylephrine (PE) (1 μmol/L) until the responses were reproducible. To verify the integrity of the endothelium, an acetylcholine (ACh) cumulative concentration-response curve (10 nmol/L to 30 μmol/L) was performed on PE-precontracted rings. Tissues that relaxed less than 80% were discarded. In a separate set of experiments, rings were precontracted with PE (1 μmol/L), once a plateau was reached, L-cysteine (1 μmol/L to 10 mmol/L), ACh (10 nmol/L to 30 μmol/L), and sodium nitroprusside (SNP) (1 nmol/L to 3 μmol/L) cumulative concentration-response curves were performed. To inhibit CSE, aortic rings were exposed to DL-propargylglycine (PAG) and β-cyano-L-alanine (BCA) (1, 3, and 10 mmol/L) for 15 and 60 minutes, respectively, and then cumulative-concentration response curves to different vasorelaxing agents were performed.

H₂S Measurements

H₂S determination was performed according to Stipanuk and Beck¹² with some modifications. Aortic rings were homogenized in a lysis buffer (potassium phosphate buffer [100 mmol/L, pH=7.4] sodium orthovanadate [10 mmol/L], and protease inhibitor). The homogenates were added in a reaction mixture (total volume 500 μL) containing pyridoxal-5'-phosphate (2 mmol/L, 20 μL), L-cysteine (10 mmol/L, 20 μL), and saline (30 μL). After 30 minutes, ZnAc (1%, 250 μL) was added to trap H₂S, followed by trichloroacetic acid (TCA) (10%, 250 μL). Subsequently, N,N-Dihethyl-p-phenylenediamine sulphate (DPD) (20 mmol/L, 133 μL) in 7.2 mol/L HCl and FeCl₃ (30 mmol/L, 133 μL) in 1.2 mol/L HCl were added. Absorbance was measured at 650 nm after 20 minutes. The H₂S concentration of each sample was calculated against a calibration curve of sodium hydrosulfide (NaHS) (3.12 to 250 μmol/L), and results were expressed as nmol of H₂S/mg of protein.

cGMP Determination

Aortic rings were placed in Krebs solution at 37°C and incubated with vehicle, PAG, or BCA (10 mmol/L). Rings were stimulated with SNP (1 μmol/L); after 15 minutes, tissues were snap frozen and homogenized in 8 volumes of buffer containing 5% trichloroacetic acid per gram of tissue. cGMP was then extracted and measured using a commercially available enzyme immunoassay kit (Assay Designs, Ann Arbor, Mich) following the manufacturer's instructions. For experiments in cultured cells, the cells were incubated for 5 minutes (unless otherwise indicated) with NaHS. In some experiments, cells were pretreated with 3-isobutyl-1-methylxanthine (IBMX). To overexpress CSE in smooth muscle cells, gene transfer was accomplished using an adenovirus at 10 multiplicities of infection (a green fluorescent protein-expressing adenovirus was used as a control). After 48 hours, cells were washed and incubated for 5 minutes with a solution containing L-cysteine (1 mmol/L) in the presence or absence of PAG pretreatment (10 mmol/L, 30 minutes). To extract cGMP, cells were lysed in 0.1 N HCl, and cGMP content was measured in the extracts using a commercial kit.

Cell Culture and Transfections

Human umbilical vein endothelial cells were grown in M199 medium supplemented with 15% fetal bovine serum, endothelial cell

growth supplement, heparin, and antibiotics. Rat aortic smooth muscle cells were isolated from 12- to 14-week-old male Wistar rats and cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. For transfections, COS-7 cells were used. The cells were plated in 24-well plates at a density of 5×10⁴ cells per well and allowed to grow overnight. Cells were then transfected with appropriate plasmids using ExGen 500 in vitro transfection reagent according to the manufacturer's instructions. cGMP levels or expression of proteins was measured 48 hours after transfection. For transfections of human umbilical vein endothelial cells with the control or CSE short interfering RNA (siRNA), the following protocol was used. Cells were plated in a 24-well plate and allowed to reach 75% confluence. The medium was then replaced, and the transfection reagent (2 μL · 5 nmol⁻¹ · L⁻¹ siRNA) was prepared in serum-free medium and added to the cells. Plates were then vigorously agitated to disperse siRNA evenly. Twenty-four hours posttransfection, cells were used for cGMP measurements or were lysed to determine protein levels.

Phosphodiesterase Activity Assay

The ability of NaHS to modulate phosphodiesterase (PDE) activity was evaluated by using a colorimetric cyclic nucleotide PDE assay kit. Briefly, the rationale of this assay relies on the cleavage of cGMP or cAMP by a cyclic nucleotide PDE. The 5'-GMP and 5'-AMP released were further cleaved into the nucleosides and phosphate. The phosphate generated is quantified using a colorimetric reaction using Biomol Green reagent in a modified Malachite Green assay. A nonspecific cyclic nucleotide PDE inhibitor, IBMX was included as a positive control for inhibitor screening. Different concentrations of NaHS (3, 10, 30 nmol/L) were tested and compared with IBMX (40 μmol/L); the procedure was performed according to manufacturer instructions.

Statistical Analysis

All data are expressed as mean±SEM. Results were analyzed by 1- or 2-way ANOVA, followed by a post hoc test for multiple comparisons. A value of *P*<0.05 was considered significant.

Results

Inhibition of CSE Attenuates Endothelium-Dependent and Endothelium-Independent Relaxations

Incubation of rat aortic rings with L-cysteine resulted in a concentration-dependent relaxation (Figure 1A and 1B). The maximal relaxation observed was 40%, occurring at 3 mmol/L L-cysteine. Treatment of aortic tissue with either one of the CSE inhibitors BCA or PAG inhibited the production of H₂S in tissue homogenates (Figure 1C). The lack of complete inhibition of the colorimetric signal by the CSE inhibitors is likely due to the fact that this widely used assay also detects additional molecules carrying sulfhydryl groups or the presence of alternative pathways that generate H₂S in vascular tissues. To determine whether endogenously produced H₂S contributes to the ACh-induced vasodilatory response, rings were incubated with increasing amounts of BCA or PAG. In these experiments, we found that CSE inhibition reduced endothelium-dependent relaxations (Figure 2A and 2B).

To determine whether endogenous H₂S alters endothelium-independent relaxation elicited by an NO donor (SNP), aortic rings were exposed to PAG or BCA before stimulation with SNP. Such pretreatment resulted in a rightward shift of the SNP relaxation curve and increased the EC₅₀ of SNP (log EC₅₀=−8.43±0.089, −7.41±0.062, and −7.71±0.07 for vehicle, PAG, and BCA, respectively; Figure 2C and 2D). It

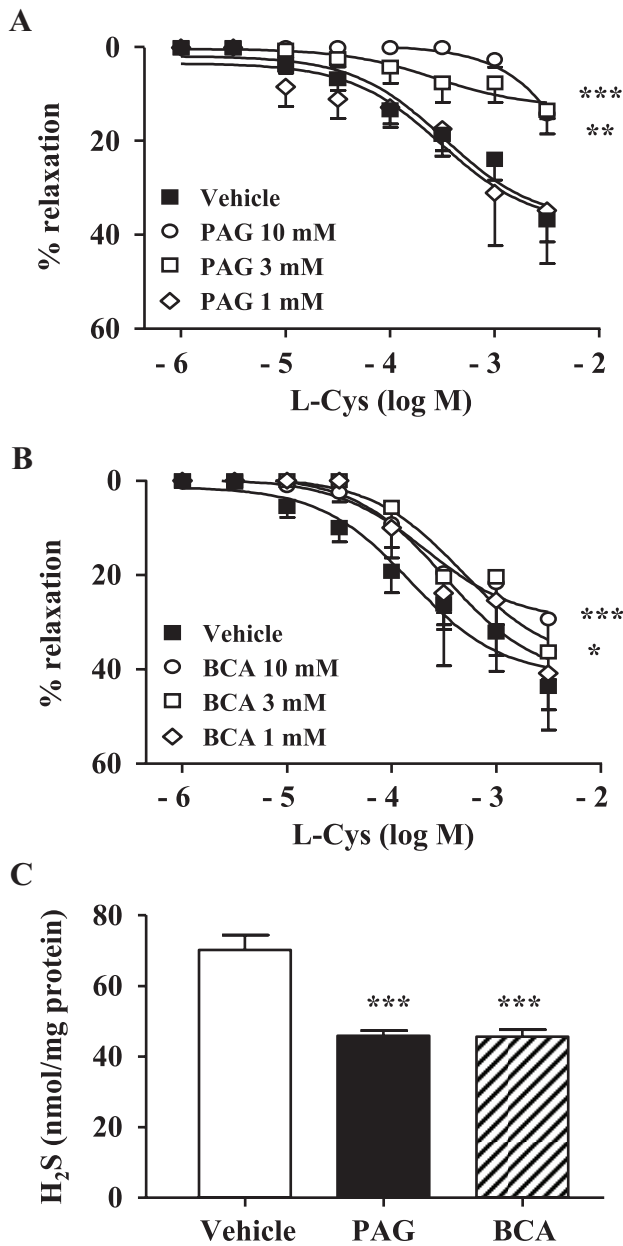


Figure 1. CSE inhibition reduces H₂S biosynthesis and modulates vascular activity in rat aorta. Aortic rings were incubated with either PAG (A) (1, 3 and 10 mmol/L) or BCA (B) (1, 3, and 10 mmol/L) for 15 and 60 minutes, respectively. Then, aortic rings were contracted with PE (1 μmol/L); once the plateau was reached, cumulative-concentration response curves to L-cysteine (L-cys) were performed. For A and B, n=6, *P<0.05, **P<0.01, ***P<0.001 from vehicle. C, Aortic homogenates were incubated with PAG or BCA (10 mmol/L) for 15 minutes; L-cys and pyridoxal 5'-phosphate were then added, and samples were transferred to 37°C to measure H₂S formation. n=4, ***P<0.001 from vehicle.

should be noted that the 2 CSE inhibitors had no effect on eNOS activity and did not quench the NO produced by directly reacting with it (supplemental Figure I, available online at <http://atvb.ahajournals.org>), suggesting that the observed effect was due to inhibition of H₂S production rather than an interference of BCA or PAG with the eNOS/NO pathway.

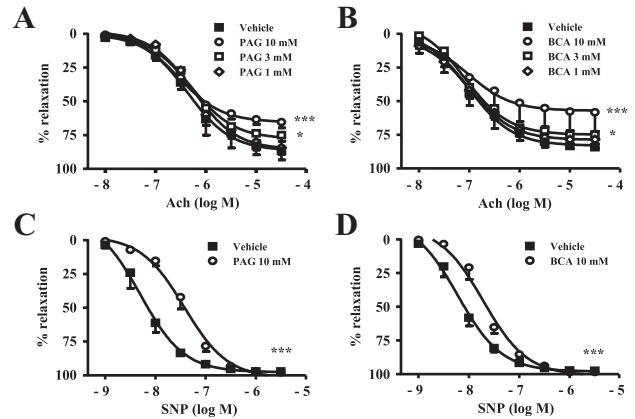


Figure 2. CSE inhibition reduces both endothelium-dependent and endothelium-independent vasodilation in rat aorta. Aortic rings were incubated with the indicated concentration of PAG (A and C) or BCA (B and D) for 15 and 60 minutes, respectively. Thereafter, aortic rings were contracted with PE (1 μmol/L), and once a plateau was reached, cumulative-concentration response curves to Ach (A and B) or SNP (C and D) were performed. n=6, *P<0.05, ***P<0.001 from vehicle.

H₂S Regulates cGMP Levels

To study the mechanism through which H₂S modulates the response to SNP, we determined the effects of H₂S on cGMP levels. We found that blockade of CSE activity resulted in a significant reduction of SNP-induced cGMP accumulation in aortic tissue (Figure 3A). In addition, incubation of cultured rat aortic smooth muscle cells with NaHS, an H₂S donor, led to a concentration-dependent increase in cGMP levels (Figure 3B) that reached a plateau at 50 μM NaHS. The NaHS-induced rise in cGMP was evident as early as 1 minute with cGMP levels, reached a maximum at 3 minutes, and remained elevated for at least 10 minutes (Figure 3C).

To confirm our observations that H₂S has the ability to enhance cGMP levels, we infected smooth muscle cells with an adenovirus carrying the CSE cDNA. CSE overexpression elevated intracellular cGMP in a PAG-sensitive manner (Figure 4A). To confirm that endogenous H₂S can raise intracellular cGMP levels, we used a siRNA approach to knock down CSE expression. Indeed, similarly to what had been observed with the pharmacological CSE inhibitors in tissue homogenates, reduction in CSE levels in cells attenuated cGMP levels (Figure 4B).

H₂S Inhibits PDE Activity

To investigate whether the increase in cGMP observed after exposure to H₂S was due to inhibition of its breakdown, we used the nonselective PDE inhibitor IBMX. We observed that H₂S caused an increase in cGMP levels that was of similar magnitude to IBMX (Figure 5A). Moreover, in cells pre-treated with IBMX, the ability of NaHS to enhance cGMP levels was greatly attenuated, suggesting that H₂S and IBMX share a common target.

We next used a heterologous expression system to determine whether H₂S can inhibit PDE5, a cGMP-specific PDE that is widely distributed in the cardiovascular system. Stimulation of COS-7 with exogenous H₂S (NaHS) increased cGMP levels, probably because of the inhibition of PDEs

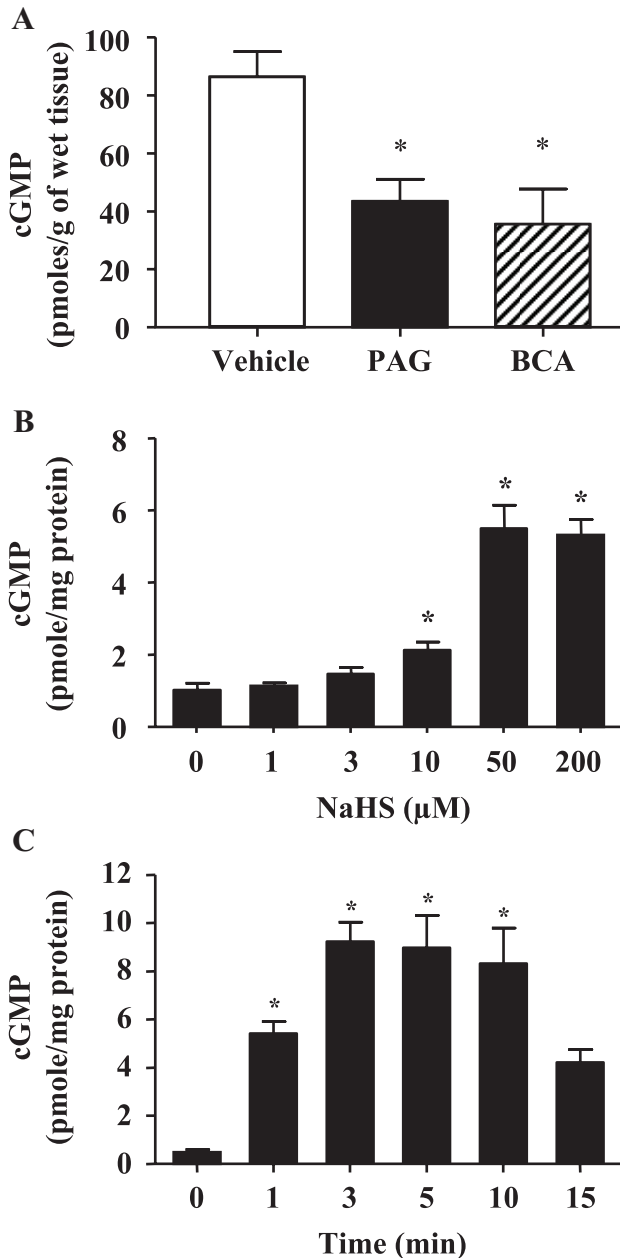


Figure 3. H₂S enhances cGMP levels. A, Rat aortas were incubated with PAG or BCA (10 mmol/L) for 15 or 60 minutes, respectively; tissues were then stimulated with 1 μmol/L SNP for 15 minutes. At the end of the incubation period, cGMP was extracted and measured by enzyme immunoassay. n=3; **P*<0.05 from vehicle. B, Rat aortic smooth muscle cells were exposed to the indicated concentration of NaHS for 15 minutes. n=4; **P*<0.05 from control. C, Smooth muscle cells were stimulated with NaHS (50 μM) for 1 to 15 minutes; cGMP was then extracted and measured by EIA. n=4; **P*<0.05 from control.

expressed endogenously by COS-7 cells. The effect of NaHS calculated as percentage increase in cGMP levels was significantly enhanced in COS-7 cells overexpressing PDE5A (236±26%) compared with that of β-galactosidase-expressing cells (168±23%) (Figure 5B and supplemental Figure II). This latter result suggests that H₂S inhibits PDE5A activity, confirming our hypothesis that H₂S acts as a PDE inhibitor.

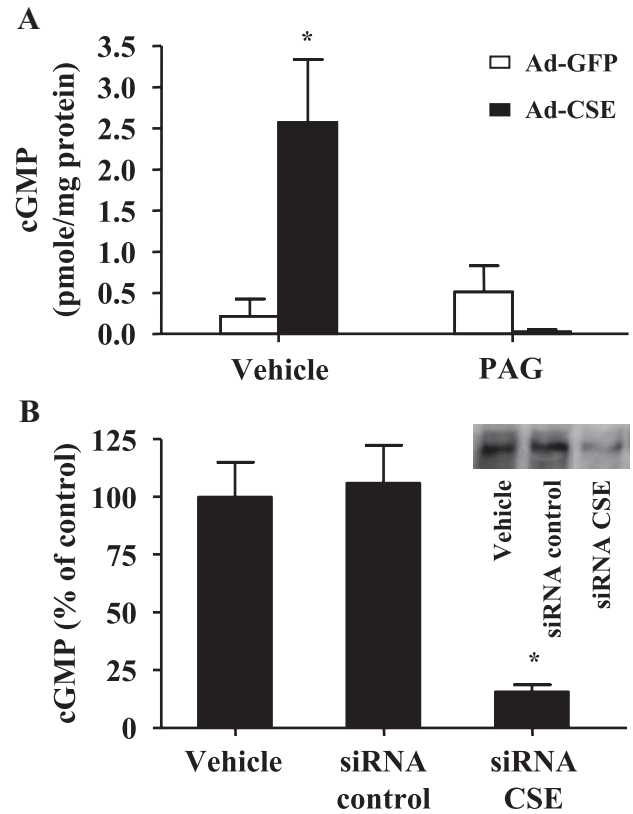


Figure 4. Overexpression of CSE increases intracellular cGMP, whereas knockdown of CSE reduces it. A, Rat aortic smooth muscle cells were infected with a green fluorescent protein (GFP)- or CSE-expressing adenovirus (10 multicopies of infection). After 48 hours, cells were incubated for 30 minutes with 10 mmol/L PAG, and cGMP was extracted and measured with enzyme immunoassay; n=4; **P*<0.05 from control adenovirus (Ad)-GFP. B, Human umbilical vein endothelial cells were incubated with an siRNA against the H₂S-producing enzyme CSE. Such treatment significantly reduced the expression of CSE (inset); after 24 hours, cGMP was extracted with HCl and measured. n=4; **P*<0.05 from control siRNA.

To directly test whether H₂S inhibits PDE activity, we used a cell-free assay. The assay uses a mixture of different semipurified PDE isoforms and allows the evaluation of PDE activity by measuring the breakdown products of cGMP and cAMP. Initially, we ruled out that NaHS interferes with any of the assay reagents; we then proceeded to demonstrate that nanomolar amounts of NaHS (3, 10, 30 nmol/L) inhibited cGMP-PDE activity in a concentration-dependent manner (Figure 5C and Supplemental Table I). In particular, 10 to 30 nmol/L NaHS significantly, and comparably to IBMX, inhibited PDE activity, causing a reduction in the breakdown product 5'-GMP (Figure 5C). Similar results were observed for cAMP catabolism PDEs (Figure 6A). To evaluate whether inhibition of endogenously produced H₂S interferes with vasodilatory responses that are cAMP-mediated, rings were incubated with BCA or PAG before being exposed to isoproterenol (Figure 6B). In these experiments, we found that CSE inhibition shifted the isoproterenol-induced relaxation curves to the right.

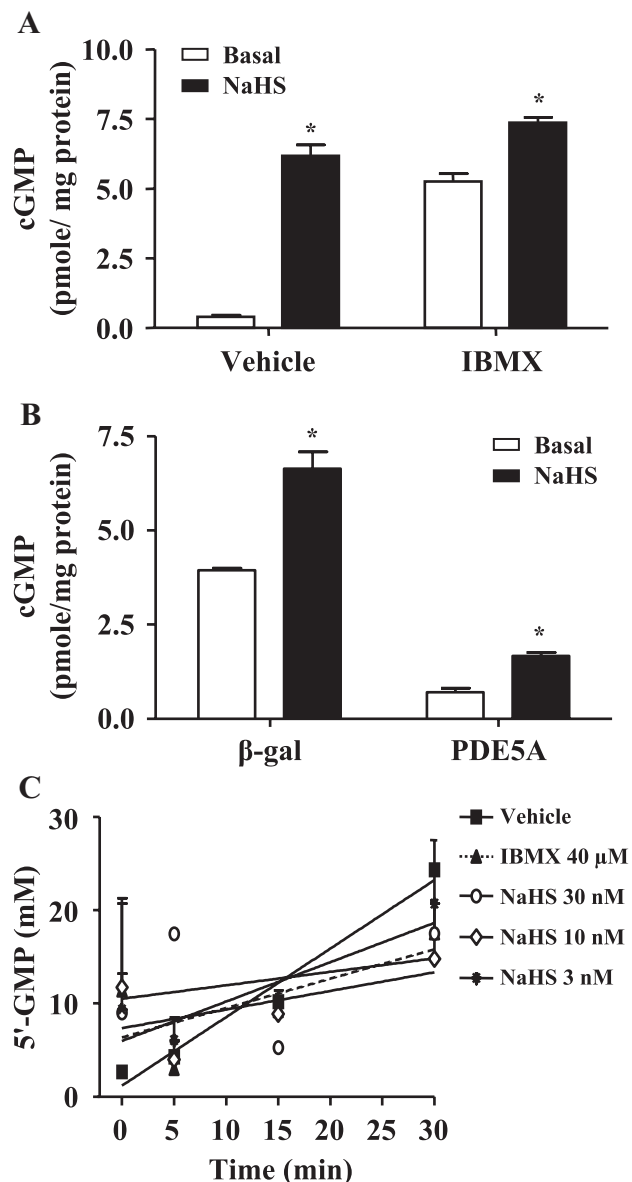


Figure 5. H₂S inhibits cGMP-degrading PDE activity. A, Smooth muscle cells were pretreated with vehicle or IBMX (5 mmol/L) for 15 minutes and then stimulated with NaHS (50 μ mol/L) for an additional 5 minutes. cGMP was then extracted and measured by enzyme immunoassay. $n=4$; * $P<0.05$ from basal. B, COS-7 cells were transfected with β -galactosidase (β -gal) or PDE5. After 48 hours, cells were exposed to vehicle or 50 μ mol/L NaHS for 5 minutes before cGMP extraction. $n=4$; * $P<0.05$ from control; $P<0.05$ from β -galactosidase. C, cGMP-degrading PDE activity was evaluated in the presence of the indicated concentration of NaHS or IBMX, using a commercially available cyclic nucleotide PDE kit.

Discussion

The main findings of the present study are that (1) L-cysteine promotes vasodilation through activation of CSE and H₂S production; (2) both endothelium-dependent and endothelium-independent relaxations in the rat aorta are attenuated on inhibition of endogenously produced H₂S; (3) exposure of smooth muscle cells to H₂S increases their intracellular cGMP content, whereas inhibition of cell-derived H₂S reduces cGMP levels; and (4) H₂S inhibits PDE activity and modulates cyclic nucleotide levels.

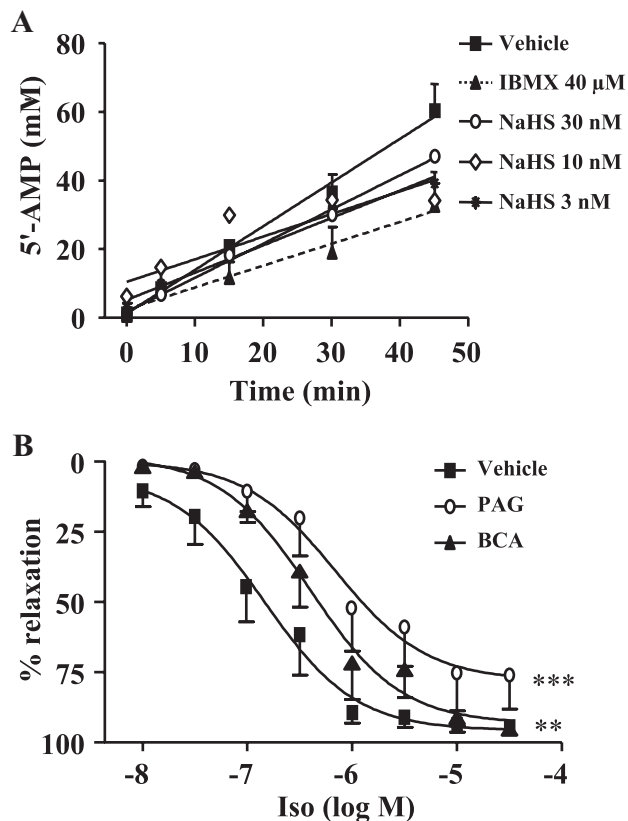


Figure 6. H₂S inhibits cAMP PDE activity. A, cAMP-degrading PDE activity was evaluated in the presence of the indicated concentration of NaHS or IBMX, using a commercially available cyclic nucleotide PDE kit at different time points. B, Aortic rings were incubated with the 10 mmol/L PAG or BCA for 15 and 60 minutes, respectively. Thereafter, aortic rings were contracted with PE (1 μ mol/L), and once a plateau was reached, cumulative-concentration response curves to isoproterenol were performed. $n=6$, ** $P<0.01$, *** $P<0.001$ from vehicle.

H₂S is produced in mammalian cells both through enzymatic and nonenzymatic pathways.^{4,5} The pyridoxal-5'-phosphate-dependent enzymes cystathionine β -synthase and cystathionine- γ -lyase (CSE) use the amino acid L-cysteine as a substrate to generate H₂S, with the latter being the major H₂S-producing enzyme in the vasculature. Additional pathways (eg, 3-mercaptopyruvate sulfurtransferase) have been shown to contribute to H₂S production by endothelial cells.¹³ In our experiments, incubation of rat aortic rings with L-cysteine elicited concentration-dependent relaxations that were inhibited by PAG and BCA, suggesting that L-cysteine is taken up by the tissue and converted to H₂S, leading to relaxation. In line with our findings, Cheng et al observed that incubation of the mesenteric bed with L-cysteine causes dilation that is blocked by CSE inhibition.¹¹ In our studies, inhibition of L-cysteine-induced relaxations were affected to a different extent by PAG and BCA, with the former exhibiting a greater inhibitory effect. Although both inhibit CSE, PAG is a noncompetitive/"suicide" inhibitor, whereas BCA is a competitive inhibitor^{14,15}; thus combining PAG and BCA would not be expected to yield an additional effect. Similarly to what was observed with L-cysteine, endothelium-dependent relaxations triggered by Ach were attenuated by

CSE inhibition. This finding is in agreement with that of Yang et al, who showed that cholinergic receptor stimulation in EC increases H₂S release by 3-fold; other calcium-elevating agents, such as A23187 and vascular endothelial growth factor, also stimulate release of H₂S from the endothelium.^{6,16} Most interestingly, BCA and PAG reduced the endothelium-independent relaxation elicited by exogenously applied NO. It has been shown that H₂S under some conditions inhibits the vasorelaxant effects of NO donors, probably because of a direct reaction between NO and H₂S and the formation of a nitrosothiol-like molecule.^{9,17,18} A similar rightward shift in the SNP effect was observed in mesenteric arteries harvested from CSE null mice, but it was reported not to reach statistical significance.⁶ The finding that inhibition of endogenously produced H₂S altered the response to exogenously added NO indicates that H₂S exerts its effects on vessel tone by modulating cGMP levels and cGMP signaling in smooth muscle cells and prompted us to investigate the effects of this agent on cGMP levels.

Intracellular cGMP levels reflect the difference between the rate of cGMP synthesis and breakdown, the former being regulated by guanylyl cyclases and the latter by phosphodiesterases.^{19,20} It has been reported that although H₂S binds with high affinity to heme,²¹ it does not appear to activate soluble guanylate cyclase²² and that H₂S-induced vasorelaxation is not inhibited by a soluble guanylate cyclase inhibitor.²³ Initially, we observed that addition of exogenous H₂S caused a concentration- and time-dependent increase in cGMP levels, whereas pharmacological inhibitors or knock-down of CSE reduced cGMP levels in cells and tissues. To determine whether the changes that we observed in smooth muscle cell cGMP levels were due to activation of soluble guanylate cyclase or inhibition of PDE activity, we incubated cells with a nonselective PDE inhibitor. Such pretreatment reduced the H₂S-stimulated increase in cGMP from 70-fold to less than 55% and suggested that H₂S and IBMX share a common target, supporting the hypothesis that H₂S inhibits PDE activity. Our hypothesis was further substantiated by the finding that H₂S ameliorated the reduction in cGMP levels brought about by overexpression of PDE5A. Finally, to obtain direct evidence for the inhibition of PDE activity by H₂S, we used a mixture of semipurified PDE isoforms in a commercially available cell-free assay. Results from these experiments confirmed that H₂S is a nonselective inhibitor of PDE activity. Although our results do not unravel the mechanism through which H₂S blocks PDE activity, at least 2 possibilities exist. PDEs are Zn-containing enzymes²⁴; Zn is coordinated with histidine and aspartic acid residues in the substrate binding pocket and its removal abolishes PDE activity.²⁴ At the same time, H₂S is known to bind Zn⁵ and alter the activity of Zn-dependent enzymes. A second mechanism through which H₂S might regulate PDE activity is through sulfhydrylation. Recently, mass spectrometric analysis revealed the attachment of an additional sulfur to the thiol (-SH) groups of cysteines, yielding a hydropersulfide (-SSH) moiety²⁵ This posttranslational modification affects 10% to 25% of some proteins that include, but are not limited to GAPDH, β -tubulin, and actin. Most importantly, sulfhydrylation alters the proteins' function, as it increases GAPDH

activity by 7-fold and it enhances actin polymerization. It is therefore possible that H₂S modifies some of the critical cysteine residues that regulate PDE activity.

On the basis of the above, the mechanism through which H₂S promotes vasorelaxation would likely depend on the relative expression of K_{ATP} channels, the PDE isoform expressed, and the amount of H₂S present in the microenvironment. For example, tissues expressing K_{ATP} channels and no or low PDE levels would be expected to dilate in a glibenclamide-inhibitible manner. The molecular basis for K_{ATP} channel activation by H₂S was recently proposed to require the cysteine residues C6 and C26 in the extracellular loop of the SUR1 subunit,²⁶ which most likely become sulfhydrylated. On the other hand, tissues expressing high PDE5 levels and abundant amounts of PKG, but no K_{ATP} channels, would dilate in a cGMP-dependent manner. Because H₂S was found to also inhibit cAMP breakdown and to modulate the relaxation to the cAMP-elevating agent isoproterenol, H₂S responses in cells containing high amounts of cAMP-specific PDEs would be expected to be mediated mainly by PKA. In agreement with this hypothesis, in porcine pulmonary artery endothelial cells, inhibition of PKA but not PKG blocked the effects of NaHS on gp91(phox) expression.²⁷ Another important observation that needs to be kept in mind is that PKG activates K⁺ channels, leading to hyperpolarization and relaxation.^{28,29} This latter finding allows us to speculate that in cells and tissues in which H₂S responses are mediated by K⁺ channels, there might be both a cGMP-independent and a cGMP/PKG-dependent component.

In conclusion, we have demonstrated that H₂S causes vasorelaxation by acting as a nonselective endogenous PDE inhibitor that boosts cyclic nucleotide levels in tissues. Interactions between the H₂S and NO has long been suspected to occur. The 2 gasotransmitters have been proposed to interact in multiple ways, ranging from regulation of each other's expression or activity to direct chemical reaction.^{4,9} In many cases, conflicting results have been obtained. For example, NO has been shown to enhance or reduce the effects of H₂S on vascular tone.⁴ Our findings establish the existence of cross-talk between NO and H₂S. Moreover, our data will aid in reconciling conflicting or difficult-to-explain observations regarding H₂S-induced vasorelaxation.

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Disclosures

Dr Giuseppe Cirino is a member of the Scientific Advisory Board of Antibe Therapeutics (Toronto, Ontario, Canada).

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Hydrogen Sulfide Is an Endogenous Inhibitor of Phosphodiesterase Activity

Mariarosaria Bucci, Andreas Papapetropoulos, Valentina Vellecco, Zongmin Zhou, Anastasia Pyriochou, Charis Roussos, Fiorentina Roviezzo, Vincenzo Brancaleone and Giuseppe Cirino

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SUPPLEMENT MATERIAL

MATERIALS AND METHODS

Aortic tissue preparation

Male Wistar rats 8-10 weeks of age were sacrificed and thoracic aortas were rapidly dissected and cleaned from fat and connective tissue. Rings (2-3 mm in length) were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O₂ - 5% CO₂) Krebs solution at 37°C, mounted to isometric force transducers (type 7006, Ugo Basile, Comerio, Italy) and connected to a Graphtec linear recorder (WR3310, Japan). The composition of the Krebs solution was as follows: NaCl 118 mM, KCl 4.7 mM, MgCl₂ 1.2 mM, KH₂PO₄ 1.2 mM, CaCl₂ 2.5 mM, NaHCO₃ 25 mM and glucose 10.1 mM. Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 minutes during which tension was adjusted, when necessary, to 0.5 g and bathing solution was periodically changed. In a preliminary study, a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. Our studies were carried out in accordance with the Declaration of Helsinki and conform to the European Community guidelines for the use of experimental animals.

Isolated aortic rings experimental protocol

After about 60 minutes of equilibration period, rings were initially challenged with phenylephrine (PE, 1 μM) until the responses were reproducible. In order to verify the integrity of the endothelium, acetylcholine (ACh) cumulative concentration-response curves (10 nM - 30 μM) were performed on PE-precontracted rings. Tissues that relaxed less than 80% were discarded. In a separate series of experiments, rings were

pre-contracted with PE (1 μ M) and once a plateau was reached L-cysteine (L-cys, 1 μ M - 10 mM), acetylcholine (Ach, 10 nM - 30 μ M) or sodium nitroprusside (SNP, 1 nM – 3 μ M) cumulative concentration-response curves were performed. In some cases, aortic rings were exposed to two different inhibitors of CSE: PAG and BCA (1, 3 and 10 mM) for 15 and 60 minutes respectively, prior to performing the cumulative-concentration response curves to vasorelaxing agents. The concentration and the time of incubation of CSE inhibitors were determined in preliminary experiments (derived from similar tissue bath experiments reported in literature^{1,2}). None of the drugs used altered the contraction induced by PE. All chemicals were from Sigma-Aldrich (St Louis, MO) unless otherwise stated.

eNOS activity assay

The eNOS activity was monitored by conversion of [¹⁴C] L-arginine to [¹⁴C] L-citrulline³. Briefly, eNOS transfected COS-7 cells were lysed in homogenization buffer containing 50 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 20 mM NaF, 1 mM Na₄P₂O₇, 0.3 μ g/ml pepstatin A and 1 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ g/ml pefabloc SC, 1 μ g/ml sodium orthovanadate. Homogenized samples were centrifuged at 1,000 rpm for 10 minutes to remove insoluble cell particulates and assayed to normalize protein concentration. Approximately 800 μ g total protein was used per assay reaction and was diluted in a total volume of 1 ml of activity assay buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM EGTA, 100 nM calmodulin, 1 mM NADPH, 15 μ M (6R-tetrahydro-LM-biopterin, 8.6 μ M [L-arginine, 1.4 ¹⁴C] L-arginine (348 mCi/mmol) and 2.5 mM CaCl₂ plus or minus 1 mM N^ω-nitro-L-arginine methyl ester (L-NAME) or PAG (1, 10, 100 mM). All reaction samples were kept on ice until incubation at 37°C for 15 minutes. The reaction was stopped by

adding 1 ml of ice cold stop buffer containing 20 mM HEPES (pH 5.5), 2 mM EDTA and 2 mM EGTA to each sample. The samples were then passed through Dowex AG50WX8 cation exchange resin and the flow through was counted on a liquid scintillation counter.

Thin-layer chromatography (TLC)

TLC has been performed on Merck silica gel 60 F₂₅₄ plates with fluorescent indicator loading the following compounds: PAG; PAG + SNP (10 µM); PAG + SNAP (100 µM); BCA; BCA + SNP (10 µM) and BCA + SNAP (100 µM). Both inhibitors were used at concentration of 10 mM. All the compounds used were dissolved in distilled water. All samples were applied to the plates by means of a fine glass capillary. After drying the spots, the plates were developed in butanol/H₂O/acetic acid 12:4:4 as mobile phase at room temperature (20 ± 2°C). Ascending development was performed to a distance of 1 cm from the point of application. The developed plates were again dried at 60°C. The locations of the spots were detected by treatment of the plate surface with a solution of ninhydrin in ethanol (0.5%).

H₂S measurements

H₂S determination was performed according to Stipanuk and Beck⁴ with some modifications. Briefly, thoracic aortas were dissected, placed in sterile phosphate buffer solution and cleaned of fat and connective tissue. Rings, of the same size 2 - 3 mm in length were cut and placed in DMEM (containing penicillin 100 U/ml, streptomycin 100 µg/ml and L-glutamine 2 mM) for 30 minutes at 37°C in an incubator (Mod. BB6220, Heraeus Instruments, Germany) under humidified air (5 % CO₂ / 95 % O₂). Then, aortic rings were homogenized in a lysis buffer (potassium

phosphate buffer 100 mM pH = 7.4, sodium orthovanadate 10 mM and protease inhibitors) and the protein concentration was determined using Bradford assay (Bio-Rad Laboratories, Milano, Italy). The homogenates were added in a reaction mixture (total volume 500 μ l) containing piridoxal-5'-phosphate (2 mM, 20 μ l), L-Cysteine (10 mM, 20 μ l) and saline (30 μ l). The reaction was performed in parafilm tubes and initiated by transferring tubes from ice to a water bath that was set at 37°C. After 30min incubation, ZnAc (1%, 250 μ l) was added to trap evolved H₂S followed by TCA (10%, 250 μ l). Subsequently, DPD (20 mM, 133 μ l) in 7.2 M HCl and FeCl₃ (30 mM, 133 μ l) in 1.2 M HCl were added. Twenty minutes later absorbance values were measured at a wavelength of 650 nm. When CSE inhibition was desired, aortic homogenates were kept on ice and exposed to PAG or BCA (10 mM) for 15 minutes before addition of L-cysteine and pyridoxal 5'-phosphate and then transferred to a water bath at 37°C. The H₂S concentration of each sample was calculated against a calibration curve of sodium hydrosulphide (NaHS; 3.12 – 250 μ M) and results were expressed as nmol H₂S/mg protein.

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from 2-4 fresh umbilical cords⁵ and grown on culture dishes (Corning-Costar Inc., Corning, NY) in M199 medium supplemented with 15% fetal calf serum (Life Technologies GIBCO-BRL, Paisley, UK), 50 U/ml penicillin and 50 μ g/ml streptomycin (Applichem, Darmstadt, Germany), 50 μ g/ml gentamycin, 2.5 μ g/ml amphotericin B, 5 U/ml sodium heparin (Biochrom AG, Berlin, Germany) and 150-200 μ g/ml endothelial cell growth supplement (ECGS) made from bovine brain. HUVEC between passages 1 and 2 were used for all experiments. Rat aortic smooth muscle cells (RASMC) were

isolated from 12 to 14 weeks old male Wistar rat as previously described⁶ and cultured in DMEM medium containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum and antibiotics.

cGMP determination in rat aortic tissue and cultured smooth muscle cells

Aortic rings were placed in Krebs solution at 37°C and incubated for 30min. Then vehicle, PAG or BCA (10 mM) were added for 15' and 60' respectively. Rings were stimulated with SNP (1 μM) and after 15min tissues were rapidly blotted and quick-frozen in liquid nitrogen. Samples were homogenized in eight volumes of buffer (containing 5% trichloroacetic acid) per gram of tissue and centrifuged at 1,500 × g for 10min. The supernatant was carefully removed and used in the next step. Residual trichloroacetic acid was removed by extraction into five volumes of water-saturated diethyl ether (repeated twice for a total of 3 extractions). Any residual ether was removed by warming the samples at 70°C for 5 min. The samples were then processed according to the instructions provided with the kit. Data are expressed as pmol/mg of wet tissue; each sample was run in duplicate.

For cGMP determination in cultured cells, after the pre-treatment, cells were washed twice with Hanks' balanced salt solution and incubated for 5 min (unless otherwise indicated) with NaHS. In some experiments cells were pre-treated with isobutylmethylxanthine (IBMX). To extract cGMP, cells were lysed in 0.1 N HCl and cGMP content was measured in the extracts using a commercially available enzyme immunoassay kit (Assay Designs; Ann Arbor, MI) following the manufacturer's instructions

Transfections

African green monkey kidney COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). The cells were plated in 24-well plates at a density of 5×10^4 per well and allowed to grow overnight. Cells were then transfected with appropriate plasmids using ExGen 500 in vitro transfection reagent (Fermentas, Ontario Canada) according to the manufacturers' instructions. The total of 1 μ g DNA (0.4 μ g of rat sGC α 1/ β 1 with 0.2 μ g of β -gal or human pDE5A cDNA) and 3.3 μ l ExGen 500 were used for each well. cGMP levels or expression of proteins was measured 48hr hr after transfection. For transfections of HUVEC with the control or CSE siRNA (Ambion, Austin, TX) the following protocol was used. Cells were plated in a 24-well plate and allowed to reach 75% confluence. The medium was then replaced and the transfection reagent (jetPEI; Polyplus Transfection; Illkirch Cedex, France) 2 μ L/5 nM siRNA was prepared in serum-free medium and added to the cells. Plates were then agitated vigorously to disperse siRNA evenly. Twenty-four hours post-transfection, cells were used for cGMP measurements or were lysed to determine protein levels.

Western Blotting

Cells were lysed in a buffer containing 1% Triton 100-X, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na_3VO_4 , 0.5% deoxycholic acid, 0.1% SDS, 10 μ M aprotinin, 10 μ M leupeptin, 10 μ M pepstatin A and 100 μ M PMSF (All of these reagents were from Sigma). Cellular debris was pelleted (12,000 \times g, 10 min), supernatants were then collected and protein concentration was subsequently determined. Lysates were subjected to SDS-PAGE on 10% polyacrylamide gels and transferred to PVDF membranes (Macherey-Nagel

(Düren, Germany). Following transfer, membranes were blocked with 5% dry milk in TBST (TBS with 0.2% Tween) for one hour at room temperature. Blots were then incubated overnight with the primary antibody diluted in TBST at 4 °C (monoclonal anti-actin antibody was from Chemicon (Temecula, CA, USA); the antibody against the sGC β 1 subunit was purchased from Caymen Chemical (Ann Arbor, MI, USA); The antibody against the myc-tagged sGC α 1 subunit, monoclonal anti-c-Myc (9E10) antibody was Sigma-Aldrich (St. Louis, MO); the PDE5 antibody was obtained from Cell Signaling Technologies (Beverly, MA, USA). Subsequently, the blots were incubated with secondary antibody for 2 hours at room temperature. Immunoreactive proteins were detected by chemiluminescence using the SuperSignal reagent kit (Rockford, IL, USA).

Phosphodiesterases (PDE) activity assay

The ability of NaHS to modulate PDE activity was evaluated using a colorimetric cyclic nucleotide phosphodiesterase assay kit (Biomol Green Quantizyme assay system AK-800, BIOMOL International). Briefly, the rationale of this assay relies on the cleavage of cGMP and/or cAMP by a cyclic nucleotide PDE. The 5'-GMP and 5'AMP released were further cleaved into the nucleosides and the phosphate released is quantified using a biomol green reagent in a modified Malachite Green assay. A non specific cyclic nucleotide PDE inhibitor, 3-isobutyl-1-methylxanthine (IBMX) was included as a test control for inhibitor screening. Different concentrations of NaHS (3, 10, 30 nM) were tested and compared to IBMX (40 μ M); the experimental procedure was performed following the manufacturer's instructions.

References

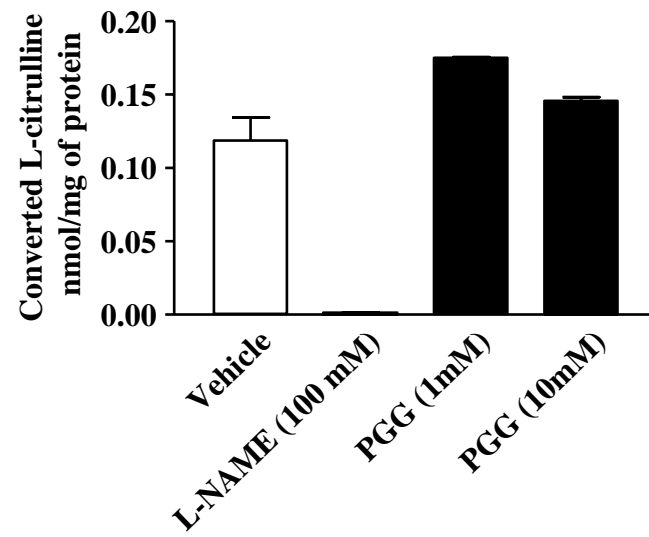
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Legends

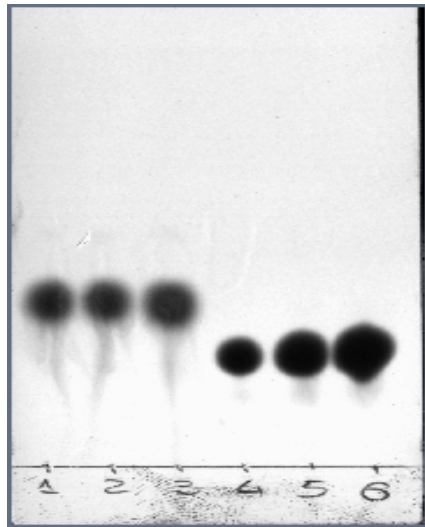
I. A. PGG did not affect eNOS activity measured as conversion of [¹⁴C] L-Arginine to [¹⁴C] L-citrulline. The eNOS inhibitor L-NAME was used as positive control. The figure is representative of three different experiments. B. PGG and BCA (10 mM) did not modify *hR_F* of SNP and SNAP (10, 100 μM) in thin-layer chromatography. The figure is representative of three different experiments.

II. Blots depicting the expression of proteins that regulate cGMP levels in the transfected COS-7 cells and correspond to the treatments presented in Fig.5B. β-actin was used to ensure equal protein loading.

A



B



1. PGG (10 mM)
2. PGG (10 mM) + SNP (10 μ M)
3. PGG (10 mM) + SNAP (100 μ M)
4. BCA (10 mM)
5. BCA (10 mM) + SNP (10 μ M)
6. BCA (10 mM) + SNAP (100 μ M)

Figure I

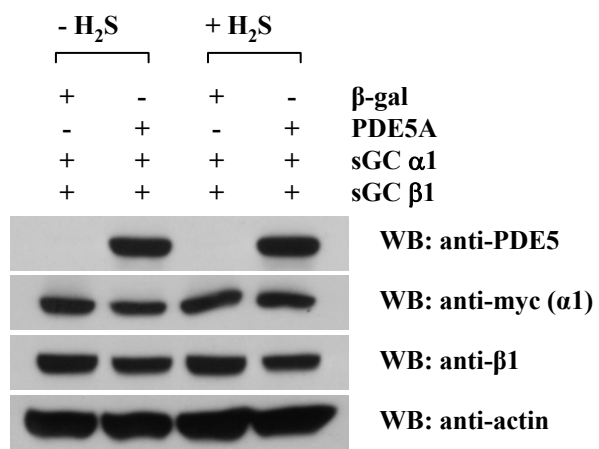


Figure II

Table SI

| Treatment | 5' GMP mM (30 min) | 5' AMP mM (45 min) |
|------------------|---------------------------|---------------------------|
| vehicle | 24.3±3.18 | 60.4±7.73 |
| IBMX (40 µM) | 17.6±6.4 *** | 32.7±9.92 *** |
| NaHS (30 nM) | 17.5±1.0 *** | 47.1±1.4 *** |
| NaHS (10 nM) | 14.8±6.2 *** | 34.2±0.9 *** |
| NaHS (3 nM) | 20.7±0.6 *** | 39.2±1.3*** |

PDE activity was evaluated in the presence of the indicated concentration of NaHS or IBMX, using a commercially available cyclic nucleotide phosphodiesterase kit. Data for a single time point (30min for 5'GMP and 45min for cAMP) is shown. *** = p<0.001