Nitric oxide, the classic endothelium-derived relaxing factor (EDRF), acts through cyclic GMP and calcium without notably affecting membrane potential. A major component of EDRF activity derives from hyperpolarization and is termed endothelium-derived hyperpolarizing factor (EDHF). Hydrogen sulfide (H2S) is a prominent EDHF, since mice lacking its biosynthetic enzyme, cystathionine γ-lyase (CSE), display pronounced hypertension with deficient vasorelaxant responses to acetylcholine.

**Objective:** The purpose of this study was to determine if H2S is a major physiological EDHF.

**Methods and Results:** We now show that H2S is a major EDHF because in blood vessels of CSE-deleted mice, hyperpolarization is virtually abolished. H2S acts by covalently modifying (sulfhydrating) the ATP-sensitive potassium channel, as mutating the site of sulfhydration prevents H2S-elicited hyperpolarization. The endothelial intermediate conductance (IKCa) and small conductance (SKCa) potassium channels mediate in part the effects of H2S, as selective IKCa and SKCa channel inhibitors, charybdotoxin and apamin, inhibit glibenclamide-insensitive, H2S-induced vasorelaxation.

**Conclusions:** H2S is a major EDHF that causes vascular endothelial and smooth muscle cell hyperpolarization and vasorelaxation by activating the ATP-sensitive, intermediate conductance and small conductance potassium channels through cysteine S-sulfhydration. Because EDHF activity is a principal determinant of vasorelaxation in numerous vascular beds, drugs influencing H2S biosynthesis offer therapeutic potential. (Circ Res. 2011;109:00-00.)

**Key Words:** hydrogen sulfide ■ EDHF ■ hyperpolarization ■ potassium channel ■ sulfhydration

Multiple molecular mechanisms regulate blood vessel relaxation, with nitric oxide (NO) well established as a mediator of endothelium-dependent vasorelaxation (endothelium-derived relaxing factor, EDRF).1,2 Whereas NO acts by both stimulating cyclic GMP (cGMP) levels and in a cGMP-independent manner to influence calcium disposition and sensitivity,4 blood vessel relaxation and tone are also prominently mediated by endothelium-dependent hyperpolarization.5–7 Numerous substances have been advanced as putative endothelium-derived hyperpolarizing factors (EDHFs) including metabolites of arachidonic acid from cyclooxygenase, prostacyclin (PGI2), epoxygenesatrienoic acids (EETs) derived from cytochrome P450, lipooxygenase [12-(s)-hydroxyeicosatetraenoic acid (12-S-HETE)], reactive oxygen species, hydrogen peroxide (H2O2), potassium ions (K+), vasoactive peptides, as well as NO itself.5–8 It has also been suggested that EDHF function may be mediated through direct coupling between endothelial and smooth muscle cells by myoendothelial gap junctions composed of connexins.5–7

Recently, hydrogen sulfide (H2S) has been shown to be a major EDHF, formed in vascular endothelial cells from cysteine by cystathionine γ-lyase (CSE), which is calcium-calmodulin dependent.9 Whereas CSE appears to play a significant role in the cardiovascular system, 2 other enzymes have also been shown to generate H2S in various tissues, namely cystathionine β-synthase (CBS) and 3-mercaptopryruvate sulfurtransferase (3-MST). In blood vessels however, CBS appears to play a negligible role in the production of H2S,10 whereas the precise role of 3-MST has yet to have been defined despite its presence in vascular endothelium.11

Original received January 4, 2011; revision received September 22, 2011; accepted September 27, 2011. In August 2011, the average time from submission to first decision for all original research papers submitted to Circulation Research was 16 days.

From the Solomon H. Snyder Department of Neuroscience (A.K.M., S.K.G., F.K.G., R.K.B., S.H.S.) and the Department of Anesthesiology/Critical Care Medicine (G.S., J.S., S.M.J., A.K.B., V.M.B., D.E.B.) Johns Hopkins University School of Medicine, Baltimore, MD; the Department of Biology, Lakehead University, Thunder Bay, Ontario, Canada (R.W.); and the Department of Biophysics and Biophysical Chemistry (M.A.), the Department of Biomedical Engineering (D.E.B.), the Departments of Pharmacology and Molecular Sciences (S.H.S.), and the Department of Psychiatry (S.H.S.), Johns Hopkins University School of Medicine, Baltimore, MD.

A.K.M. and G.S. contributed equally to this work.

Correspondence to Solomon H. Snyder, MD, Department of Psychiatry, Johns Hopkins University School of Medicine, Baltimore, MD 21205. E-mail dberkowi1@jhmi.edu or ssnyder@jhmi.edu

© 2011 American Heart Association, Inc.

Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.111.240242
Acetylcholine-mediated blood vessel relaxation, however, is markedly reduced in CSE-deleted mice, which manifest increased blood pressure comparable to levels in mice lacking endothelial NO synthase (NOS).9,12 Using genetic deletion of CSE and other approaches, we now show that H₂S is a major EDHF acting by chemically modifying sulfhydryl groups—sulfhydration—of potassium channels.

**Methods**

**Myograph Measurements of Vascular Tension**

The segments (1–1.5 mm in length) of mesenteric arteries or aortas from 8- to 12-week-old male animals were used for myograph measurements of vascular tension as described before.13 Briefly, the mice were heparinized 1 hour before euthanasia. Once euthanized, the arteries were carefully excised and cleaned from the surrounding fat and placed in an ice-cold Krebs-Ringer bicarbonate solution (pH 7.4, 95% O₂ and 5% CO₂) at 37°C and incrementally oxygen gas (95% O₂ and 5% CO₂) at 37°C and incrementally bubbling with continuous of dextrose). The vessels were then carefully placed in the multiwire myograph system DMT 610 mol/L bubbling with continuous of oxygen gas (95% O₂ and 5% CO₂) at 37°C and incrementally.

**Vessel Diameter Measurements**

Vessels were prepared as described above, cannulated at both ends with glass micropipettes (80–100 μm), secured with nylon monofilament suture, and placed in a microvascular chamber (Living Systems, Burlington, VT). Vessels were studied in the absence of flow and maintained at a constant transmural pressure of 70 mm Hg as described before.14 The chambers were superfused with Krebs-Ringer bicarbonate solution at pH 7.4, loaded with 100 mmol/L DibAC₇(3) dye (Molecular Probes, Carlsbad, CA) or fluorometric imaging plate reader red dye (Molecular Devices, Sunnyvale, CA) and maintained in the dark for 30 minutes. The majority of the experiments were conducted using the DibAC₇ dye unless otherwise indicated. Each tissue was then mounted under a fluorescent microscope (Nikon Eclipse 80i Microscope with Roper Scientific Camera) and the set system at an exposure time of 100 ms with a sampling rate of 3 images per second. FITC filter (fluorescein isothiocyanate) was used because the dye has an excitation of 488 nm and a peak emission of 518 nm. Changes in fluorescence intensities were then recorded with addition of various drugs in small volumes without disturbing the focus. A similar process was used for cultured cells, but the FlexStation-3 fluorescence microplate reader system (Molecular Devices, Sunnyvale, CA) was used instead.

**S-Sulfhydration (Modified Biotin Switch) Assay**

The assay was carried out as described previously,20 but with modifications. Briefly, arteries or cells treated with appropriate stimulants such as NaHS or acetylcholine were homogenized in HEN buffer (250 mmol/L HEPES-NaOH, pH 7.7, 1 mmol/L EDTA, 0.1 mmol/L Neocuproine) supplemented with 100 μmol/L deferroxamine (DFO) and centrifuged at 13 000g for 30 minutes at 4°C. Lysates (240 μg) were added to blocking buffer (HEN buffer plus 25% SDS and 20 mmol/L methyl methanethiosulfonate [MMTS]) at 50°C for 20 minutes with frequent vortexing. The MMTS was then removed by acetone and the proteins precipitated at −20°C for 20 minutes. After acetone removal, the proteins were resuspended in HENS (HEN + SDS) buffer. To the suspension was added 4 mmol/L biotin-γ-[6-(biotinamido)hexyl]-3-[2-pyridyldithio]propionamide (HPDP) in DMSO without ascorbic acid. After incubation for 4 hours at 25°C, biotinylated proteins were precipitated by streptavidin-agarose beads, which were then washed with HENS buffer. The biotinylated proteins were eluted by SDS-PAGE sample buffer and subjected to Western blot analysis.

**CSE Activity Assays**

CSE protein was purified and its activity assayed using the tissue homogenate method as described previously,21 with the exception of a preincubation step with 100 mmol/L S-nitroso-glutathione (GSNO) at 37°C for 2 hours.

**Shear Stress Experiments**

Human aortic endothelial cells (HAEC) were grown to ~80% confluence and subjected to a laminar shear stress of 20 dyn/cm² for 24 hours, using a cone-and-plate viscometer as described earlier.22,23 The cells were then scrapped for CSE activity assay.

Additional Methods can be found as an Online Supplement available at [http://circres.ahajournals.org](http://circres.ahajournals.org).

**Results**

**Cholinergic Vasorelaxation and Hyperpolarization are Significantly Reduced in CSE⁻/⁻ and Glibenclamide-Treated Vessels**

We confirm the importance of H₂S in mediating muscarinic cholinergic-dependent vasorelaxation of the smaller mesenteric artery (diameter of 80–200 μm in mice) and larger aorta (diameter of 350–450 μm in mice), using force-tension myography and vessel diameter measurements (Figure 1A and Online Figures I, A, and IV, A). We eliminated influences of NOS and cyclooxygenase (COX) products by

---

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>CSE</td>
<td>cystathionine γ-lyase</td>
</tr>
<tr>
<td>DIBAC</td>
<td>bis-(1,3-dibutylbarbituric acid/triethylenemethine oxonol</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>Eₘ</td>
<td>membrane potential</td>
</tr>
<tr>
<td>H₂S</td>
<td>hydrogen sulfide</td>
</tr>
<tr>
<td>IKᵥₑ</td>
<td>intermediate conductance potassium channel</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-NG-nitroarginine methyl ester</td>
</tr>
<tr>
<td>NaHS</td>
<td>sodium hydrogen sulfide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>SKᵥₑ</td>
<td>small conductance potassium channel</td>
</tr>
</tbody>
</table>
treatment with appropriate inhibitors (L-NAME 100 μmol/L and indomethacin 10 μmol/L, respectively). L-NAME nearly abolishes NO generation in both wild-type and CSE knockout vessels (Online Figure II), which display similar basal NO productions (Online Figure II). In the mesenteric arteries, the overall cholinergic relaxation, of which about 75–80% is NOS/COX independent, is reduced by 60% in CSE deleted animals (Figure 1A). Conversely, in the aorta, cholinergic relaxation appears to be primarily NOS/COX dependent and is reduced by less than 25% in CSE knockout vessels treated with NOS/COX inhibitors (Online Figure IV, A). To study the effects of H2S on cellular membrane potential, we used 2 potentiometric fluorescent probes (Online Figure III): (1) DiBAC, a probe with slow response time, and (2) fluorometric imaging plate reader, a newer dye with increased sensitivity and rapid response time. Although the authors acknowledge that electrophysiological techniques such as whole-cell patch clamp are the gold standard for investigating channel function, the use of fluorescent voltage-sensitive dyes to interrogate channels in a rapid, high throughput and economical manner is rapidly emerging. Indeed, studies have shown dye responses to ligand-evoked activation of potassium channels to be comparable with whole-cell patch clamp measurements.17–19 Using the dyes, we find that cholinergic relaxation is associated with pronounced hyperpolarization of about 13–16 mV in mesenteric arteries and about 6–8 mV in the aorta (Figure 1B and Online Figure IV, B). For the NOS/COX independent system, CSE deletion virtually abolishes hyperpolarization. The importance of potassium channels for cholinergic vasorelaxation is evident in that vasorelaxation is markedly reduced in the presence of 30 mmol/L KCl, which fully blocks all potassium channels (Online Figure I, B). Several potassium channels have been implicated in vasorelaxation, with the ATP-sensitive channels closely linked to H2S and EDHF.24,25 The channel inhibitor glibenclamide reduces hyperpolarization about 65% (Figure 1B and Online Figure IV, B). Thus, cholinergic vasorelaxation primarily reflects H2S hyperpolarizing cells through the ATP-sensitive potassium channels. Cholinergic vasorelaxation in mouse mesenteric artery (Figure 1C) as well as vasorelaxation and hyperpolarization in rat mesenteric artery (Figure 1D and Online Figure V) are also largely independent of NOS and COX and prevented by glibenclamide. The same is true for hyperpolarization in rat aorta, even though overall vasorelaxation is dependent on the NOS/COX system (Online Figure IV, C and D). In addition, the CSE inhibitor prepar-
Glycylglycine significantly reduces cholinergic hyperpolarization in mesenteric arteries (Figure 1D). Since elevated reactive oxygen species (ROS) might contribute to endothelium dysfunction, we measured differences in ROS levels in the vessels of wild-type and CSE knockout mice. We did not however observe any significant difference in basal ROS production between wild-type and knockout arteries (Online Figure VI).

KCl and Glibenclamide Markedly Diminish H$_2$S Vasorelaxation and Hyperpolarization in Intact and Endothelium-Denuded Mesenteric Arteries

The vasorelaxing and hyperpolarizing actions of applied H$_2$S involve potassium channels, since they are blocked by 30 mmol/L KCl, which fails to alter NO responses (Figure 2A and 2B and Online Figure VIII, A and B). The H$_2$S-mediated vasorelaxation is not affected by changes in the buffer oxygen concentration as relaxation is comparable in buffer bubbled with 95% oxygen and HEPES buffer containing the ambient 21% oxygen (Online Figure VII). H$_2$S acts primarily through ATP-sensitive potassium channels, as glibenclamide (5 μmol/L) markedly reduces the H$_2$S precursor sodium hydrogen sulfide (NaHS)-elicited vasorelaxation and hyperpolarization (Figure 2A and 2B and Online Figure VIII, A and B). In contrast, glibenclamide fails to influence relaxation in response to the NO donor sodium nitroprusside (1 μmol/L). NO but not H$_2$S mediated vasorelaxation is prevented by the cGMP pathway inhibitors ODQ (sGC inhibitor) and KT5823 (PKG inhibitor) (Online Figure IX). Since charybdotoxin and apamin also inhibit a component of the H$_2$S induced vasorelaxation (Figure 2A), IKCa and SKCa channels may in part mediate the effects of H$_2$S, consistent with the findings of Wang et al. The combination of glibenclamide and charybdotoxin/apamin abolishes all H$_2$S-mediated vasorelaxation (Figure 2A).
H₂S is generated by CSE in the endothelium of blood vessels, and, like NO, diffuses to the adjacent smooth muscle to elicit vasorelaxation. We confirm that the actions of H₂S-induced vasorelaxation through the ATP-sensitive potassium channels reflect direct influences on the vascular smooth muscle, as in endothelium-denuded mesenteric artery, NaHS relaxation is abolished by glibenclamide, which fails to alter effects of NO (Figure 2C). H₂S can also hyperpolarize endothelial cells, as primary cultures of wild-type but not CSE knockout mouse aortic endothelial cells are hyperpolarized on acetycholine stimulation (Figure 2D). This effect is mediated not by ATP-sensitive potassium channels, but by the combination of IKCa/SKCa channels, as hyperpolarization is completely blocked by charybotoxin/apamin (Figure 2D). In addition, in cultured human endothelial cells (HAECs), H₂S-mediated hyperpolarization is unaffected by either glibenclamide or the BKca channel blocker iberiotoxin, but is significantly diminished by the IKca channel blocker TRAM-34 (Figure 2E). We have previously demonstrated that chemical stimulation of endothelial cells with ACh or the Ca²⁺ ionophore A23187 increases CSE activity. We now observe an increase in CSE activity in cultured HAECs after shear stress suggesting that H₂S, and hence EDHF activity, can be induced not only by cholinergic means, but also by a physiological mechanical stimulus (Online Figure X).

**Physiological Sulphhydration of Kir 6.1-C43 Activates the Channel**

**Causing Hyperpolarization**

Because sulphhydration appears to be a principal means whereby H₂S signals, we wondered whether vasorelaxation reflects sulphhydration of its target potassium channels. Both the Kir 6.1 subunit of ATP-sensitive potassium channels overexpressed in HEK293 cells and IKca channels from human aortic endothelial cells are sulphhydrated by NaHS in a DTT-sensitive fashion (Figure 3A and Online Figure XI). Kir 6.1 is basally sulphhydrated in cells overexpressing wild-type CSE but not in cells lacking CSE or containing catalytically-inactive CSE (Figure 3B). Cholinergic stimulation of mouse aorta enhances sulphhydration of Kir 6.1 in wild-type but not CSE mutant mice (Figure 3C).

To link sulphhydration to channel function, we overexpressed Kir 6.1 in HEK293 cells in which NaHS-elicted hyperpolarization is blocked by glibenclamide, just as in blood vessels (Figure 3D). To identify the sulphhydrated cysteine residue, we modeled Kir 6.1 on the basis of the established structure of the highly homologous Kir 3.1 (Figure 3E). Kir 6.1 has 9 cysteines with cysteine-43 (C43), which lies close to the surface, responding selectively to oxidative insults. C43 is the principal target of sulphhydration in Kir 6.1, as sulphhydration of the channel is abolished with C43S mutation (Figure 3F, inset). NaHS-elicted hyperpolarization is significantly reduced in Kir 6.1-C43S mutants, but responses to the channel opener cromakalim remain preserved (Figure 3F and Online Figure XII, A and B). Thus, H₂S vasorelaxation reflects hyperpolarization mediated by the opening of Kir 6.1 channels through their sulphhydration at C43. The channel openers pinacidil and cromakalim elicit hyperpolarization comparable to NaHS in HEK293 cells (Online Figure XII, C).

**Sulphhydration Augments ATP-Sensitive Potassium Channel Activity by Reducing Kir 6.1-ATP Binding and Enhancing Kir 6.1-PIP2 Binding**

Physiological activation of the ATP-sensitive potassium channels is elicited by binding of its Kir subunits to the phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2) with concomitant reductions in binding to the inhibitor ATP. We wondered whether the regulation by H₂S of Kir 6.1 stems from influences on its binding to ATP and PIP2, since cysteine-43 appears to be located within the ATP binding region and adjacent to the PIP2 binding region of Kir 6.1 (Figure 4A and 4B and Online Figure XIII, A). In HEK293 cells, NaHS reduces ATP-Kir 6.1 binding (Figure 4C). Confirming that ATP-Kir 6.1 binding involves the sulphhydrated C43, we observed significantly more ATP binding to Kir 6.1-C43S mutants on treatment with NaHS compared with the wild-type Kir 6.1 (Figure 4D). Unlike its influences on ATP-Kir 6.1 interactions, NaHS markedly augments PIP2-Kir 6.1 binding (Figure 4E). In cells overexpressing wild-type active CSE, PIP2 binds Kir 6.1 with minimal binding in cells lacking CSE or containing the catalytically inactive enzyme (Online Figure XIV). Finally, we observed substantial reductions of PIP2 binding to Kir 6.1-C43S mutants (Figure 4F).

**Discussion**

In summary, our findings establish H₂S as a principal mediator of EDHF activity, as it satisfies all the major requirements of an EDHF candidate (Online Table I). EDHF activity is virtually abolished in two major vascular beds of CSE deleted mice. EDHF, like H₂S, is produced by vascular endothelial cells on cholinergic stimulation in a calcium-calmodulin-dependent manner and both directly activate endothelial potassium channels, hyperpolarizing the cells while diffusing to adjacent smooth muscle cells where they function in a similar capacity. EDHF appears to function by covalently modifying cysteine residues of its targets, as reducing agents such as DTT reverse its effects. H₂S also functions by sulphhydrating cysteine residues of key potassium channels in a DTT-sensitive manner. Hyperpolarization of endothelial and smooth muscle cells by H₂S and EDHF leads to vasorelaxation that is independent of the NO-cGMP pathway. Unlike NO, which signals primarily in larger conductance vessels, EDHF activity is notably predominant in smaller vascular beds, the resistance blood vessels that determine blood pressure. This fits with our observations of a greater role for H₂S in the mesenteric artery, a resistance vessel, than in the aorta, which displays more prominent NO-mediated relaxation. Recently, H₂S has been shown to be an important endogenous vasorelaxant in smaller cerebral arteries. NO can inhibit the synthesis and release of EDHF, which might explain the prominence of EDHF in mesenteric arteries whose levels of eNOS, and therefore NO production, are less compared with the aorta. We find that
NO can directly inhibit CSE activity in vitro with an IC$_{50}$ of approximately 100 nmol/L (Online Figure XV).

It is important to note, however, that mediators beyond EDHF and EDRF do play significant vasoregulatory roles in different arteries. For example, studies have shown that CO plays an important role in renal vasoregulation, although the molecular mechanism of which has not entirely been worked out. Endothelium-dependent potassium channel activity does not appear to be involved in guinea pig uterine artery relaxation. H$_2$O$_2$ dilates coronary vasculature through a redox mechanism involving thiol oxidation through p38 map kinase. Although the variation in histology and physiology of vessels among different species appears to preclude the existence of a universal set of vasoregulatory molecules, EDHF or EDRF have nonetheless been repeatedly demonstrated to be the principal mechanism by which vascular tone is regulated.

Figure 3. Physiological sulfhydration of Kir 6.1-cysteine-43 activates the channel causing hyperpolarization. A, H$_2$S (100 μmol/L) sulfhydrates (SHY) Kir 6.1 overexpressed in HEK293 cells, an effect reversed by DTT (1 mmol/L, n=4). B, Kir 6.1 is basally sulfhydrated in cells overexpressing catalytically active wild-type (wt) CSE but not in cells lacking CSE or containing catalytically inactive mutant CSE (mut; n=4). C, Cholinergic stimulation of mouse aorta enhances sulfhydration of Kir 6.1 in wild-type but not CSE knockout (ko) mice (n=3). D, H$_2$S-elicited (100 μmol/L) hyperpolarization in HEK293 cells overexpressing Kir 6.1 is substantially reduced by glibenclamide (5 μmol/L, n=7). E, Model of Kir 6.1 homotetramer based on the established structure of Kir 3.1 with surface residue cysteine-43 highlighted in yellow. F, H$_2$S-mediated (300 μmol/L) sulfhydration (inset) and hyperpolarization are absent in HEK293 cells overexpressing C43S mutant Kir 6.1 (n=12). Quantitative densitometric analysis is also shown for A through C. All results are mean±SEM (**P<0.001).

Although some studies indicate that circulating H$_2$S levels in the vasculature are less than 1 μmol/L, there are numerous studies that show much larger concentrations of H$_2$S ranging from 30–300 μmol/L in blood vessels as well as in numerous other tissues including the heart, lung, brain, liver, and kidney. Presumably, this generation of H$_2$S by different tissues (particularly the liver) contributes to circulating plasma levels in the 30–300 μmol/L range. This may result in perfusion of the entire body with significant H$_2$S concentrations. Our utilization of 100 μmol/L NaHS is in keeping with physiological concentration of the gas to which blood vessels might well be exposed.

Of the numerous substances that have been explored as potential mediators of EDHF, including potassium ions, lipoxygenase products, hydrogen peroxide, CNP (C-type natriuretic peptide), cytochrome P450-derived EETs, and even NO itself, there are few studies utilizing mutant mice
indicating a physiological role for them as EDHF mediators. eNOS/COX-1 double knockout mice display reduced endothelium-dependent vasodilation but no significant attenuation of membrane potential change.51 Epoxide hydrolase knockouts manifest elevated EETs and hypotension, but no available membrane potential data support these molecules as EDHF.52 In contrast, the profoundly diminished vasorelaxation and hyperpolarization of CSE knockouts establishes H2S as a major EDHF. It is nonetheless possible that these other EDHF candidates may play important roles in modulating the formation or actions of H2S. As our studies have been confined to rodents, we do not know if they apply fully to human vasculature.5,7

Figure 4. Sulfhydration augments ATP-sensitive potassium channel activity by reducing Kir 6.1-ATP binding and enhancing Kir 6.1-PIP2 binding. A, Model of Kir 6.1 with cysteine-43 highlighted in yellow as well as ATP interacting residues (R51, G54, R195, and R211) highlighted in violet.31,32 B, Model of Kir 6.1 with cysteine-43 highlighted in yellow and PIP2 interacting residues (R55, K68, R186, R187, R216, and R310) in slate blue.29,30 C, Sulfhydration of Kir 6.1 in HEK293 cells reduces its interaction with ATP. n=4. D, Kir 6.1-ATP interaction is substantially enhanced in H2S (100 μmol/L)-treated HEK293 cells overexpressing Kir 6.1-C43S mutant. n=3. E, Sulfhydration of Kir 6.1 in HEK293 cells markedly augments its binding to PIP2. n=3. F, Kir 6.1-PIP2 interaction is significantly reduced in H2S (100 μmol/L)-treated HEK293 cells overexpressing Kir 6.1-C43S mutant. n=4. Quantitative densitometric analysis is also shown for C through F. All results are mean±SEM (*P<0.05, **P<0.01 and ***P<0.001).

Sulfhydration is a physiological modification of cysteines in H2S target proteins analogous to S-nitrosylation by NO.21,53 S-nitrosylation most often inhibits the function of its targets, while sulfhydration predominantly enhances activity.21,53 The importance of sulfhydration is indicated by the large proportion of proteins that are sulfhydrated and the considerable extent of sulfhydration, 10–25% for some major liver proteins including actin, β-tubulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).21 The process of sulfhydration reflects the formation of a persulfide bond, which is an oxidative reaction. H2S is sometimes referred to as a reducing agent. Like many other substances, however, the redox potential of H2S enables it to act both as a reducing as well as an oxidizing agent. Some well-known examples of dual role substances are cysteine and glutathione, which despite being recognized as reducing agents, mediate the oxidizing processes of cysteinylolation and glutathionylation of proteins, respectively.54 These modifications essentially ap-
pear to follow a similar chemistry as sulfhydration. This contrasts with substances such as DTT, which are very strong reducing agents and not likely to have oxidizing functions.55

Evidence that Kir 6.1 is physiologically sulfhydrated includes the demonstration of its sulfhydration basally as well as elicited by cholinergic simulation and H2S donors with sulfhydration abolished in CSE deleted tissues. Moreover, we established that sulfhydration involves a single cysteine, cysteine-43, whose mutation abolishes sulfhydration and the subsequent H2S-mediated hyperpolarization. Previously, we demonstrated sulfhydration of several dozen proteins, with the modification confirmed in vivo by mass spectrometry.21 The very low abundance of Kir 6.1 in vascular tissue, however, renders a mass spectrometric analysis not feasible.

Activation of Kir 6.1 is known to reflect its dissociation with ATP28 and binding to PIP2,29 which we also observe after sulfhydration at cysteine-43. As sulfhydration renders cysteines more electronegative, the modification at cysteine-43, which lies within the electropositive ATP binding region, might electrostatically hinder channel binding to ATP in addition to causing steric hindrance (Online Figure XIII, B). Since the PIP2 binding region lies adjacent to the ATP binding region, preclusion of ATP binding may provide PIP2 greater access to its binding site on the channel leading to enhanced channel activity.

Several studies suggest that myoendothelial gap junctions composed of connexins transduce endothelial to vascular smooth muscle hyperpolarization.56-57 Connexin 40–deleted mice, which lack the myoendothelial gap junctions, are hypertensive.56 Furthermore, inhibitors of gap junction attenuate smooth muscle hyperpolarization in rat mesenteric artery but have no effect on endothelial hyperpolarization.57 H2S stimulates endothelial IKₑ/Kᵄₑ as well as smooth muscle ATP-sensitive potassium channels leading to hyperpolarization and vasorelaxation (Online Figure XVI). Given the clear implications of gap junctions regulating smooth muscle hyperpolarization, it is likely that H2S diffuses from endothelial to smooth muscle cells through gap junctions to sulfhydrate the cytosolic cysteine-43 of smooth muscle ATP-sensitive potassium channels. These potential mechanisms, however, remain to be explored.

What are the physiological and pathophysiologic consequences of these observations? It is clear that deletion of these potassium channels,58 as well as application of potent and selective channel inhibitors such as glibenclamide,59,60 causes hypertension similar to our earlier observations with CSE deleted animals.9 Recently, Ishii et al have shown that deletion of CSE does not significantly alter blood pressure in mice.61 It is important to note, however, that in this instance blood pressure was measured using the tail-cuff method which is not only less precise compared with the more invasive catheter measurements conducted by our laboratories,62 but also leads to highly variable measurements hindering proper analysis of the data. On the other hand, in addition to the data presented in this study on the CSE inhibitor propargylglycine, there is now clear evidence that selective CSE inhibitors, as well as pathological conditions such as intermittent hypoxia in which H2S is diminished, significantly increase vascular myogenic tone and therefore raise blood pressure.62

Thus, the finding that H2S is a major EDHF of resistance blood vessels that regulate blood pressure, as well as its novel mechanism of action may have important therapeutic implications. Drugs altering CSE activity or H2S-mediated channel sulfhydration may be beneficial in treating diverse vascular disorders including hypertension.

Acknowledgments

We thank Drs Yoshi Kurachi and Hiroshi Hibino (Osaka University, Japan) for providing the SUR2B cDNA construct and Drs Victor Miriel, David Yue, and Manu Ben Johny for advice on the membrane potential experiments.

Sources of Funding

This study was supported by a National Institutes of Health National Research Service Award (1 F30 MH07419-01A2) to A.K.M.; American Heart Association Postdoctoral Fellowship Award (10POST4010028) to G.S.; operating grants of Canadian Institutes of Health Research to R.W.; NIH/NHLBI ROI grant (HL105296-02) to D.E.B.; and NIH USPHS grant (MH118501) and Research Scientist Award (DA00074) to S.H.S.

Disclosures

None.

References

15. Chotani MA, Flavahan S, Mitra S, Daunt D, Flavahan NA. Silent alpha(2C)-adrenergic receptors enable cold-induced vasoconstriction in


What Is Known?

• Hydrogen sulfide (H$_2$S) is a gaseous signaling molecule. It is synthesized by cystathionine γ-lyase (CSE), which is confined predominantly to the vascular endothelium.

• Mice lacking H$_2$S are hypertensive and demonstrate impaired endothelium-dependent vasorelaxation. Thus, H$_2$S acts as an endothelium-derived relaxing factor that mediates vascular relaxation and lowers blood pressure.

• The effects of H$_2$S, unlike those of nitric oxide, are mediated, in part, by the activation of the ATP-sensitive potassium channels (K$_{ATP}$) but are independent of cyclic GMP.

What New Information Does This Article Contribute?

• H$_2$S causes a redox-sensitive posttranslational modification, sulfhydration, of a single cysteine, C43, in the Kir 6.1 subunit of the K$_{ATP}$ channel.

• H$_2$S-mediated sulfhydration enhances Kir 6.1 activity by reducing Kir 6.1-ATP binding and increasing Kir 6.1-PIP2 binding.

• Hence, cholinergic, endothelium-dependent vasorelaxation and hyperpolarization are significantly reduced in vessels in which CSE is inhibited, in vessels from CSE-/- mice, or in which the K$_{ATP}$ channel has been inhibited.

• Sulfhydration of the calcium-dependent intermediate conductance potassium channel (IK$_{ca}$) contributes to H$_2$S-dependent hyperpolarization of endothelial cells.

Emerging evidence suggests that H$_2$S is an important gaseous signaling molecule in the vascular system, where it is produced by the endothelial enzyme CSE. It mediates vasorelaxation in part by activating vascular smooth muscle K$_{ATP}$ channels. We found that cholinergic vasorelaxation and hyperpolarization are markedly reduced in CSE-/- and glibenclamide-treated vessels, indicating that H$_2$S is a major endothelium-derived hyperpolarizing factor that causes vascular endothelial and smooth muscle cell hyperpolarization and vasorelaxation; since H$_2$S mediates its effect by a novel redox-sensitive thiol-dependent, posttranslational modification of proteins by sulfhydration. Indeed, the Kir 6.1 K$_{ATP}$ subunit C43S mutant expressed in HEK293 cells abolishes sulfhydration and significantly reduces H$_2$S mediated hyperpolarization. Sulfhydration of C43 in the Kir 6.1 subunit of the K$_{ATP}$ channel reduces ATP binding and enhances PIP2 binding, a process that leads to channel activation. Finally, H$_2$S also leads to sulfhydration and hyperpolarization of endothelial cells through the IK$_{ca}$ and SK$_{ca}$ channels. These findings suggest that H$_2$S is an important endothelium-derived hyperpolarizing factor; therefore, dysregulation of this pathway may be critical step in the development of vascular diseases such as hypertension.
Hydrogen Sulfide as Endothelium-Derived Hyperpolarizing Factor Sulfhydrates
Potassium Channels
Asif K. Mustafa, Gautam Sikka, Sadia K. Gazi, Jochen Steppan, Sung M. Jung, Anil K. Bhunia, Viachaslau M. Barodka, Farah K. Gazi, Roxanne K. Barrow, Rui Wang, L. Mario Amzel, Dan E. Berkowitz and Solomon H. Snyder

Circ Res. published online October 6, 2011;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2011/10/06/CIRCRESAHA.111.240242

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/10/06/CIRCRESAHA.111.240242.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
Supporting Methods

Animals

Biochemical experiments involving Wistar rats and C57B/L6 mice were performed on arteries removed from 8-12-week-old male animals. Animals were maintained on a 12 h light/dark cycle at a room temperature of 25ºC, with free access to food and water. All animal-use procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. CSE deleted mice were generated and genotyped as described previously. A conventional mouse CSE gene knockout procedure was performed with assistance from inGenious Targeting Laboratory, Inc. (Stonybrook, NY). A Bacterial Artificial Chromosome (BAC) Library containing a 129SvEv mouse strain genomic DNA was screened with a CSE gene probe (Genbank accession number NT_078406). A ~14.6 kb region containing CSE 5’ untranslated region to exon 4 was obtained from positively identified BAC clone and subcloned into backbone of a homemade gene targeting vector. To introduce a neomycin resistance gene (neo') cassette that flanks CSE exon 1 (including the ATG start codon), exon 2, and exon 3, a selection cassette flanked by BsiWI endonuclease sites was firstly inserted by homologous recombination (1). The selection cassette was then removed by cutting with BsiWI and the neo' cassette digested with KpnI was ligated in antisense orientation to the CSE targeting vector. The CSE 5’ untranslated region served as the long homology arm which fused with 3’ end of the neo' cassette. The CSE gene sequence between exon 3 and exon 4 served as the short homology arm, which fused its 5’ end with 5’ end of the antisense oriented neo' cassette. The vector construct was confirmed with both restriction analyses (BamHI and/or HindIII) and bi-directional DNA sequencing using T7 (5'-ATTATGCTGAGTGA TATCCCTCT-3'), N7 (5'-ATGTGTCAGTTTCATAGCCTGAAG-3') and N1 (5'-TGC GAGGCCAGAGGCCAGTTGTAGC-3'), P6 (5'-ATTTAGGTGACACTATAGAAC TC-3') sequencing primers. CSE gene targeting vector was linearized with an appropriate endonuclease and transfected into the 129SvEv-derived embryonic stem (ES) cells with electroporation. ES cells were then selected with G418 (300 µg ml⁻¹) for two weeks. About 300 G418 resistant cell clones were subjected to a PCR base screening using N1/A1 primers (A1: 5’-TCAGTCCAAATTCAGATGCCACCC-3’). Positive cell clones were expanded to microinject into C57BL/6J mice blastocysts, and then planted back to a surrogate mother. Two litters of chimeras carrying disrupted CSE were estimated by coat colors (i.e. C57BL/6J is black and 129SvEv is agouti in color). The chimeras were chosen for mating to generate F1. Two males and 8 females were identified as heterozygous CSE knockout mice (CSE+/-) and they were set up to generate F2 mice. Unless otherwise stated, the fourth generation of male homozygous and heterozygous CSE knockout (CSE-/- and CSE+/-) offspring and age-matched male wild-type littermates (CSE+/+) on C57BL/6J × 129SvEv background were used.

Reagents

All reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. The stock solution of NaHS as precursor of H₂S was used.²

Cell Culture

Endothelial cells were isolated as described before.³ Briefly, the aorta was dissected out with fat and connecting tissue removed using fine forceps under a stereoscopic microscope. The cleaned aorta was washed with sterile DMEM and one end tied. Next, the aorta was filled with 2 mg/ml collagenase type II solution and incubated at 37ºC for 45 min. At the end of the incubation, endothelial cells were removed from the aorta by flushing with 5 ml of DMEM containing 20% fetal bovine serum and cultured in 35 mm collagen type I-coated Petri dish. To remove smooth muscle cells, medium was replaced with endothelial cell medium (Sciencell, Carlsbad, CA) 2 h after incubation. Finally, endothelial cells were purified using Dynabeads CD31 (Invitrogen, Carlsbad, CA). Cells have been previously shown to be 98% pure by flow cytometry for CD31 and Dil-Ac-LDL incorporation.
HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in a humid atmosphere of 95% O2 and 5% CO2 at 37ºC in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine (300 µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were co-transfected with the Kir6.1 (Open Biosystems, Huntsville, AL) and SUR2B (Osaka University, Japan) subunits of the K\textsubscript{ATP} channel using Polyfect from Qiagen (Valencia, CA) as per manufacturer’s protocol.

**Site-directed Mutagenesis**

The QuickChange site-directed mutagenesis system (Stratagene, La Jolla, CA) was employed per manufacturer’s instructions. Catalytically-inactive mutant CSE was generated with mutations of the following active site residues to glycine: L90/Y113/N160/D186/S208/K211. Mutant constructs were verified via automated sequencing by the Johns Hopkins Core Facility.

**Western Blotting**

Briefly, we prepared the protein samples by boiling for 5 min in LDS + betamercaptoethanol. We then loaded the samples into 4-12% Bis-Tris gels and ran at 100 V for 1 h. We then transferred the protein onto nitrocellulose papers at 400 mA for 1 h following which we blocked the membrane with 10% milk solution in TBST for 30 min. We then replaced the solution with primary antibody in 5% milk solution and incubated overnight at 4°C. The next day, we washed the membrane with TBST and incubated with secondary antibody in 5% milk solution for 1 h at room temperature. Following further washes with TBST, we incubated the membrane with chemiluminescent substrates and developed film in the dark room.

**NO Generation Assays**

NO formation was assessed as described previously. Briefly, aorta from wild-type and CSE-/- mice were isolated just as in the myograph experiments and treated with or without 100 µM L-NAME. The vessels were then incubated for 5 min with 5 µM 4-amino-5-methylamino-2'7'-difluorofluorescein diacetate (DAF-FM DA) (Invitrogen, Carlsbad, CA), a specific dye that emits fluorescence intracellularly only upon interaction with NO. The vessels were then gently washed with fresh HEPES solution and subjected to immunofluorescence microscopy with excitation wavelength at 495 nm and emission wavelength at 515 nm for 15 min with signal recording for 0.5 sec every 30 sec.

**ATP Binding Assays**

ATP-Agarose beads (Sigma, St. Louis, MO) were reconstituted as per manufacturer’s protocol and incubated with 100 µg of cell extracts in reaction buffer containing 20 mM Tris, pH 7.7 and 1% Triton X-100 for 2 h at 4°C. The beads were then washed 3 times with 1 ml wash buffer containing 20 mM Tris, pH 7.7 and 250 mM NaCl, mixed with LDS loading buffer and processed for western blotting using anti-Kir 6.1 antibody from Santa Cruz Biotech (Santa Cruz, CA). Densitometric analysis was carried-out using the software EagleSight (Stratagene, La Jolla, CA).

**Liposomal Assays**

Liposomal assays were carried out using pre-made biotin-tagged PolyPIPosomes containing PIP2 (Echelon Biosciences, Salt Lake City, UT). 2 µl of PolyPIPosomes were incubated overnight with 100 µg of cell or organ extracts in reaction buffer containing 0.12 M NaCl, 1 mM EGTA, 0.2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5 mM KCl, 20 mM HEPES pH 7.4 at 4°C. The next day, 30 µl of Neutravidin beads were added to pull-down the lipid-protein mixtures for 1 h at 25°C. Following 5 washes with 1 ml reaction buffer as detailed above, the beads were mixed with LDS loading buffer and processed for western blotting using anti-Kir 6.1 antibody. Densitometric analysis was carried-out using the software EagleSight (Stratagene, La Jolla, CA).

**Structural Modeling of ATP-sensitive Potassium Channel**
The homology model of the mouse Kir6.1 channel was developed using the structure of the homologous mouse Kir3.1 (PDB ID 2QKS) as a template. The model was built based on an initial sequence alignment obtained with the program BLAST (34% identity; 58% similarity) that was manually corrected to ensure that all deletions and insertions occurred in non-helical regions of the molecule. This initial alignment was used to build a preliminary model using the program MODELLER 8 v.2 (Sali and Blundell, 1993). This model was displayed in the program QUANTA (Accelrys Inc., San Diego, CA) and manually modified to improve side chain interactions. A small number of insertions were built based on the fragments database using the program ‘O’ (Alwyn Jones). Several rounds of small sequence alignment changes and manual rebuilding followed by rebuilding in MODELLER yielded, after energy minimization, the final model. The program PyMOL (Schrodinger LLC, New York, NY) was used to generate the model figures.

Reactive Oxygen Species (ROS) Analysis
Vascular endothelial ROS was measured using \( \text{O}_2^- \)-sensitive fluorescent probe to superoxide, dihydroethidine (DHE, 1 µmol / L, 10 min) as described previously. Images were acquired using a Nikon TE-200 epi-fluorescence microscope (60X objective).

Statistical Analysis
All data are expressed as mean ± SEM with \( p \) values analyzed by unpaired Student’s \( t \)-test. ANOVA with Bonferroni post test was used to compare 3 or more groups where appropriate (dose-response curves). *\( p < 0.05 \), **\( p < 0.01 \) and ***\( p < 0.001 \).

Supporting References


**Supporting Figure Legends**

**Online Figure I. Cholinergic vasorelaxation is diminished in CSE knockout and KCl treated mesenteric arteries.** (A) Muscarinic cholinergic-dependent vasorelaxation of the smaller mesenteric artery (diameter between 80 and 100 μm), monitored by changes in vessel diameter, is markedly diminished in CSE knockout mice compared to wild-type controls. Samples were treated with L-NAME (100 μM) and indomethacin (10 μM). n = 15. (B) Cholinergic vasorelaxation in rat mesenteric artery is markedly reduced in the presence of 30 mM KCl. n = 8. All results are mean ± SEM.

**Online Figure II. NO generation is nearly abolished in L-NAME treated wild-type and CSE knockout arteries, which display similar basal NO productions.** L-NAME (100 μM) treatment eliminates NO generation in wild-type and CSE knockout aorta, as measured by the changes in fluorescence intensity, in arbitrary fluorescence units (δFU) per minutes, of the NO-specific dye DAF-FM DA. There is also no difference in basal NO production between wild-type and knockout arteries. n = 22. All results are mean ± SEM (**p < 0.001).

**Online Figure III. Computation of membrane potentials from changes in DiBAC fluorescence intensities.** (A) Changes in DiBAC fluorescence intensity in arbitrary fluorescence units (δFU) plotted over membrane potentials (E_m) at different extracellular potassium concentrations display a linear relationship with a slope of 18.5 δFU / E_m. The E_m values are computed using the Nernst and the Goldman-Hodgkin-Katz equations. n = 11. (B) Changes in FLIPR fluorescence intensity in arbitrary fluorescence units (δFU) plotted over membrane potentials (E_m) at different extracellular potassium concentrations display a linear relationship with a slope of 0.07 δFU / E_m. The E_m values are computed using the Nernst and the Goldman-Hodgkin-Katz equations. n = 6. (C) Example of how a change in membrane potential is calculated based on alterations in DiBAC fluorescence intensity. Example shows calculations of an E_m change of approximately 13 mV in a mouse mesenteric artery treated with acetylcholine (100 μM) bringing the resting membrane potential (E_0) to approximately -77 mV. For arterial smooth muscle cells, E_0 has previously been reported to be approximately -64 mV. (D) Representative tracing of H2S (100 μM) hyperpolarization in mouse mesenteric artery over time shows a maximum change of approximately -15 mV measured using DiBAC. All results are mean ± SEM.
Online Figure IV. **Cholinergic vasorelaxation and hyperpolarization are significantly reduced in CSE knockout and glibenclamide treated aorta.** (A) Muscarinic cholinergic-dependent vasorelaxation of the aorta is markedly diminished in CSE knockout mice compared to wild-type controls. The NOS and COX enzymes were inhibited by treatment with L-NAME (100 μM) and indomethacin (10 μM) respectively. n = 16. (B) CSE knockout almost completely abolishes the cholinergic-dependent hyperpolarization in the aorta. Treatment of wild-type aorta with glibenclamide reduces the hyperpolarization by about 60%. Some of the samples were treated with L-NAME (100 μM) and indomethacin (10 μM) as indicated. n = 18. (C) Cholinergic vasorelaxation is diminished in rat aorta treated with glibenclamide (5 μM) in the presence of L-NAME (100 μM) and indomethacin (10 μM). n = 15. (D) Acetylcholine-mediated hyperpolarization is reduced by about 65% in rat aorta treated with glibenclamide (5 μM). L-NAME (100 μM) and indomethacin (10 μM) do not influence membrane hyperpolarization. n = 12. All results are mean ± SEM (**p < 0.01 and ***p < 0.001).

Online Figure V. **Cholinergic vasorelaxation is significantly reduced in glibenclamide treated mesenteric arteries.** Cholinergic vasorelaxation is markedly diminished in rat mesenteric arteries treated with glibenclamide (5 μM) in the presence of L-NAME (100 μM) and indomethacin (10 μM). n = 18.

Online Figure VI. **Reactive oxygen species (ROS) levels are not altered in CSE knockout arteries.** There is no difference in ROS levels between wild-type and CSE deleted arteries. n = 7. All results are mean ± SEM (***p < 0.001).

Online Figure VII. **H₂S-mediated vasorelaxation is not affected by changes in buffer oxygen concentration.** The H₂S vasorelaxation is not affected by changes in the buffer oxygen concentration as relaxation is comparable in buffer bubbled with 95% oxygen and HEPES buffer containing the ambient 21% oxygen. n = 6.

Online Figure VIII. **KCl and glibenclamide markedly diminish H₂S vasorelaxation and hyperpolarization in aorta.** (A) H₂S (100 μM) vasorelaxation of rat aorta is completely blocked by 30 mM KCl and 75% reduced by glibenclamide (5 μM). Neither treatment have any effect on SNP (1 μM) vasorelaxation. n = 14. (B) H₂S (100 μM) hyperpolarization of rat aorta is completely blocked by 30 mM KCl and is reduced by about 60% with glibenclamide (5 μM). SNP (1 μM) does not induce hyperpolarization. n = 19. All results are mean ± SEM (**p < 0.01 and ***p < 0.001).

Online Figure IX. **Cyclic GMP inhibitors abolish NO, but not H₂S, mediated vasorelaxation in aorta.** (A) NO-mediated (SNP, 1 μM) vasorelaxation of the aorta is abolished following treatment with ODQ (0.3 μM) and KT5823 (1 μM), inhibitors of cGMP. There was no effect to H₂S-mediated relaxation. n = 8. (B) Representative tracing of H₂S and NO-mediated vasorelaxation with ODQ and KT5823 shows complete inhibition of the NO, but not the H₂S, effect. All results are mean ± SEM (***p < 0.001).

Online Figure X. **H₂S generation by CSE is enhanced by shear mechanical stress (SS) in cultured endothelial cells.** (A) Shear mechanical stress of cultured human aortic endothelial cells (HAEC) display greater H₂S production. n = 4. (B) CSE protein levels do not change with shear stress. All results are mean ± SEM (**p < 0.001).

Online Figure XI. **H₂S sulfhydrates IKCa channel.** H₂S (100 μM) sulfhydrates (SHY) IKCa channel in primary human aortic endothelial cells (EC), an effect reversed by DTT (1 mM). n = 3. Quantitative densitometric analysis is also shown. All results are mean ± SEM (**p < 0.001).
Online Figure XII. Sulfhydration of Kir 6.1-cysteine-43 activates the channel causing hyperpolarization. (A) H₂S (300 μM) activates wt, but not C43S, Kir 6.1 causing hyperpolarization of HEK293 cells as measured by the FLIPR dye. n = 19. (B) The ATP-sensitive potassium channel opener cromakalim (30 μM) equally activates both wt and C43S Kir 6.1. n = 7. (C) The channel openers pinacidil (100 μM) and cromakalim (30 μM) elicit hyperpolarization in HEK293 cells comparable to H₂S (300 μM). n = 7.

Online Figure XIII. Molecular modeling of Kir6.1 shows sulfhydration target cysteine-43 to reside within the electropositive ATP binding region and adjacent to the PIP2 interacting site. (A) Model of Kir 6.1 with cysteine-43 highlighted in yellow as well as ATP and PIP2 interacting residues highlighted in violet and stale blue respectively. (B) Model of Kir 6.1 monomer with cysteine-43 highlighted in yellow and superimposed surface electrostatic charges. Bluer regions are electropositive whereas regions in red are more electronegative. Cysteine-43 lies within the electropositive ATP binding region and adjacent to the PIP2 binding region.

Online Figure XIV. H₂S generated by CSE sulfhydrates Kir 6.1 in HEK293 cells. Kir 6.1 is basally sulfhydrated in HEK293 cells overexpressing the catalytically-active wild-type (wt) CSE but not in cells lacking CSE or containing catalytically-inactive mutant (mut) CSE. n = 3. Quantitative densitometric analysis is also shown. All results are mean ± SEM (**p < 0.01).

Online Figure XV. NO directly inhibits CSE in vitro. GSNO (100 nM) inhibits CSE activity in vitro by nearly 50%. n = 8. All results are mean ± SEM (**p < 0.01).

Online Figure XVI. Model for the physiologic vasorelaxation by H₂S. Stimulation of muscarinic acetylcholine receptors on endothelial cells activates Ca²⁺-calmodulin, which in turn binds to and stimulates cystathionine γ-lyase (CSE) to produce H₂S. H₂S then activates small and intermediate conductance potassium channels in endothelial cells and diffuses into smooth muscle cells activating ATP-sensitive potassium channels. The overall action of H₂S on all three potassium channels lead to endothelial and smooth muscle hyperpolarization and vasorelaxation.

Supporting Table Legend

Online Table I. Comparison between leading EDHF candidates based on established characteristics of EDHF. Direct comparison between some of the leading EDHF candidates shows that only H₂S possesses all the characteristics required of an EDHF. Calmodulin (CaM), cyclic GMP (cGMP).
Online Figure II
Online Figure III

(A) DiBAC

Change in Fluorescence Units (FU) vs. Membrane potential - $E_m$ (mV)

Slope ($\Delta F_U / \Delta E_m$) = 18.5 $\Delta F_U / mV$

(B) FLIPR

Change in Fluorescence Units (FU) vs. Membrane potential - $E_m$ (mV)

Slope ($\Delta F_U / \Delta E_m$) = 0.07 $\Delta F_U / mV$

(C) 

1. Avg. $\Delta F_U$ following Ach treatment = -240.1 FU
2. Conversion ratio between $\Delta F_U$ and $\Delta E_m$ = 18.5 FU / mV
3. Calculated $\Delta E_m$ following Ach treatment = (1) / (2) = -240.1 FU / 18.5 FU / mV = -13 mV
4. Estimated resting membrane potential ($E_0$) = -64 mV
5. Final $E_m$ = $E_0 + \Delta E_m$ = -64 mV + -13 mV = -77 mV

(D) Change in $E_m$ (mV) vs. Time (min)

- Black line: NaHS
- Gray line: Control

Red arrow indicates a significant change at 1 minute.
Online Figure V

![Graph showing relaxation (%) against Ach (M) with different conditions: Glibnd - NCI, Glibnd + NCI, Glibnd + NCI.](image)
Online Figure VII
Online Figure X

A

B

CSE activity (% of control)

- Shear Stress

***

GAPDH Load

CSE

SS

HAEC
Online Figure XI
Online Figure XII

A

B

C

Change in $E_m$ (mV)

Change in $E_m$ (mV)

Change in $E_m$ (mV)

Time (sec)

Time (sec)

Time (sec)

H$_2$S

Cromakalim

Pimozide

Cromakalim

H$_2$S

wt Kir6.1

wt Kir6.1

C43S Kir6.1

C43S Kir6.1
Online Figure XIII

**A**

**B**

ATP binding region

PIP2 binding region
Online Figure XV
Online Table I

<table>
<thead>
<tr>
<th>Characteristics of EDHF</th>
<th>H₂S</th>
<th>NO/PGI₂</th>
<th>EETs</th>
<th>H₂O₂</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Independent of NO-cGMP pathway</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>?</td>
<td>✓</td>
</tr>
<tr>
<td>Endothelial derived</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Stimulated by acetylcholine</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Ca²⁺-CaM dependent</td>
<td>✓</td>
<td>✓</td>
<td>x</td>
<td>?</td>
<td>x</td>
</tr>
<tr>
<td>Activates endothelial potassium channels</td>
<td>✓</td>
<td>x</td>
<td>✓</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Hyperpolarizes endothelial cells</td>
<td>✓</td>
<td>x</td>
<td>✓</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Diffuses to adjacent smooth muscle cells</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Activates smooth muscle potassium channels</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Hyperpolarizes smooth muscle cells</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Relaxes blood vessels</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Effect most prominent in smaller resistance arteries</td>
<td>✓</td>
<td>x</td>
<td>✓</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>NO directly inhibits synthesis/release</td>
<td>✓</td>
<td>x</td>
<td>?</td>
<td>x</td>
<td>?</td>
</tr>
<tr>
<td>Functions by covalently modifying cysteine residues</td>
<td>✓</td>
<td>x</td>
<td>?</td>
<td>✓</td>
<td>x</td>
</tr>
<tr>
<td>Effect reversed by reducing agents</td>
<td>✓</td>
<td>x</td>
<td>?</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>In vivo alterations affect hyperpolarization (e.g. knockout mice, biosynthetic or degrading enzyme inhibitors)</td>
<td>✓</td>
<td>x</td>
<td>x</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Knockout mice are hypertensive</td>
<td>✓</td>
<td>✓</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>