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Hydrogen Sulfide as Endothelial Derived Hyperpolarizing Factor Sulfhydrates Potassium Channels

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Abstract

Rationale—Nitric oxide, the classic endothelial derived relaxing factor (EDRF), acts via cyclic GMP and calcium without notably affecting membrane potential. A major component of EDRF activity derives from hyperpolarization and is termed endothelial derived hyperpolarizing factor (EDHF). Hydrogen sulfide (H_2S) is a prominent EDRF, 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 is to determine if H_2S is a major physiologic EDHF.

Methods and Results—We now show that H_2S is a major EDHF, as in blood vessels of CSE deleted mice hyperpolarization is virtually abolished. H_2S acts by covalently modifying (sulfhydrating) the ATP-sensitive potassium channel, as mutating the site of sulfhydration prevents H_2S-elicited hyperpolarization. The endothelial intermediate conductance (IK_{Ca}) and small conductance (SK_{Ca}) potassium channels mediate in part the effects of H_2S, as selective IK_{Ca}

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None.

and SK_{Ca} channel inhibitors, charybdotoxin and apamin, inhibit glibenclamide insensitive H_2S induced vasorelaxation.

Conclusions—H_2S 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. As EDHF activity is a principal determinant of vasorelaxation in numerous vascular beds, drugs influencing H_2S biosynthesis offer therapeutic potential.

Keywords

Hydrogen Sulfide; EDHF; Hyperpolarization; Potassium Channel; Sulfhydration

Introduction

Multiple molecular mechanisms regulate blood vessel relaxation with nitric oxide (NO) well established as a mediator of endothelial dependent vasorelaxation (endothelial derived relaxing factor, EDRF).[1–3] While 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 endothelial dependent hyperpolarization.[5–7] Numerous substances have been advanced as putative **E**ndothelial **D**erived **H**yperpolarizing **F**actors (**EDHF**s) including metabolites of arachidonic acid from cyclooxygenase, prostacyclin (PGI_2), epoxyeicosatrienoic acids (EETs) derived from cytochrome P450, lipoxygenase (12-(s)-hydroxyeicosatetraenoic acid (12-S-HETE)), reactive oxygen species, hydrogen peroxide (H_2O_2), 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, H_2S has been shown to be a major EDRF, formed in vascular endothelial cells from cysteine by cystathionine γ-lyase (CSE) which is calcium-calmodulin dependent.[9] While CSE appears to play a significant role in the cardiovascular system, two other enzymes have also been shown to generate H_2S in various tissues, namely cystathionine β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST). In blood vessels however, CBS appears to play a negligible role in the production of H_2S,[10] whereas 3-MST's precise role has yet to have been defined despite its presence in vascular endothelium.[11] 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] Utilizing genetic deletion of CSE and other approaches, we now show that H_2S 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 8–12-week-old male animals were used for myograph measurements of vascular tension as described before.[13] Briefly, the mice were heparinized 1 h before sacrifice. Once euthanized, the arteries were carefully excised and cleaned from the surrounding fat and placed in a Petri dish containing ice-cold Krebs-Ringer-bicarbonate solution at pH 7.4 (concentrations in mM: 118.3 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2* KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, and 11.1 dextrose). The vessels were then carefully placed in the multi-wire myograph system DMT 610M bubbling with continuous of oxygen gas (95% O_2 and 5% CO_2) at 37°C and

incrementally stretched for optimized contractility. Phenylephrine was then applied to pre-constrict the vessels, following which changes in vascular tension were recorded with application of different pharmacologic agents. In some experiments, endothelium removal was performed as described before.[14]

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 mmHg as described before.[15, 16] The chamber was superfused with Krebs-Ringer-bicarbonate solution, maintained at 37°C, pH 7.4, and gassed with 95% O_2 and 5% CO_2. The chamber was then placed on the stage of an inverted microscope (Nikon TMS-F) connected to a video camera (Panasonic CCTV camera). The vessel image was projected on a video monitor, and the internal diameter continuously determined by a video dimension analyzer (Living Systems Instrumentation) with BIOPAC data acquisition system (Santa Barbara, CA). Changes in vessel diameter were measured with application of different pharmacologic agents.

Membrane Potential Measurements

Membrane potentials were measured as described before[17–19] but with modifications. Briefly, vessels were prepared as above, fixed by pinning one end and cut open in the longitudinal plane. Each corner was pulled out enough, and pinned (0.125 mm diameter tungsten pins), such that the cellular layers remained intact. The vessels were maintained at 37°C in Krebs-Ringer-bicarbonate solution at pH 7.4, loaded with 100 nM $DiBAC_4(3)$ dye (Molecular Probes, Carlsbad, CA) or FLIPR red dye (Molecular Devices, Sunnyvale, CA) and maintained in the dark for 30 min. Majority of the experiments were conducted using the DiBAC dye unless otherwise indicated. Each tissue was then mounted under a fluorescent microscope (Nikon Eclipse 80i Microscope with Roper Scientific Camera) and the system set at an exposure time of 100 msec with a sampling rate of 3 images / sec. FITC filter (Fluorescein isothiocyanate) was used since 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 mM Hepes-NaOH, pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine) supplemented with 100 μM deferoxamine (DFO) and centrifuged at 13,000 × *g* for 30 min at 4°C. Lysates (240 μg) were added to blocking buffer (HEN buffer plus 25% SDS and 20 mM methyl methanethiosulfonate (MMTS)) at 50°C for 20 min with frequent vortexing. The MMTS was then removed by acetone and the proteins precipitated at −20°C for 20 min. After acetone removal, the proteins were resuspended in HENS (HEN + SDS) buffer. To the suspension was added 4 mM biotin-*N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (HPDP) in DMSO without ascorbic acid. After incubation for 4 h 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 pre-incubation step with 100 nM *S*-nitroso-glutathione (GSNO) at 37°C for 2 h.

Shear Stress Experiments

Human aortic endothelial cells (HAEC) were grown to ~80% confluence and subjected to a laminar shear stress of 20 dynes / cm^2 for 24 h 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.ahajournls.org.

Results

Cholinergic vasorelaxation and hyperpolarization are significantly reduced in CSE−/− and glibenclamide treated vessels

We confirm the importance of H_2S 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, Online Figure IA and IVA). We eliminated influences of NOS and cyclooxygenase (COX) products by treatment with appropriate inhibitors (L-NAME 100 μM and indomethacin 10 μM 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 IVA). To study the effects of H_2S on cellular membrane potential, we used two potentiometric fluorescent probes (Online Figure III): 1) DiBAC, a probe with slow response time and 2) FLIPR, a newer dye with increased sensitivity and rapid response time. While the authors acknowledge that electrophysiologic techniques such as whole-cell patch clamping 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] Employing the dyes, we find that cholinergic relaxation is associated with pronounced hyperpolarization of about 13 to 16 mV in mesenteric arteries and about 6 to 8 mV in the aorta (Figure 1B and Online Figure IVB). 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 mM KCl which fully blocks all potassium channels. (Online Figure IB). Several potassium channels have been implicated in vasorelaxation, with the ATP-sensitive channels closely linked to H_2S and EDHF.[24, 25] The channel inhibitor glibenclamide reduces hyperpolarization about ~ 65% (Figure 1B and Online Figure IVB). Thus, cholinergic vasorelaxation primarily reflects H_2S hyperpolarizing cells via 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 IVC, D). In addition, the CSE inhibitor propargylglycine (PPG) 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_2S vasorelaxation and hyperpolarization in intact and endothelium-denuded mesenteric arteries

The vasorelaxing and hyperpolarizing actions of applied H_2S involve potassium channels, since they are blocked by 30 mM KCl, which fails to alter NO responses (Figure 2A, B and Online Figure VIIIA, B). The H_2S-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_2S acts primarily via ATP-sensitive potassium channels, as glibenclamide (5 μM) markedly reduces the H_2S precursor sodium hydrogen sulfide (NaHS)-elicited vasorelaxation and hyperpolarization (Figure 2A, B and Online Figure VIIIA, B). In contrast, glibenclamide fails to influence relaxation in response to the NO donor sodium nitroprusside (SNP, 1 μM). NO, but not H_2S, 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_2S induced vasorelaxation (Figure 2A), IK_{Ca} and SK_{Ca} channels may in part mediate the effects of H_2S, consistent with the findings of Wang *et al*..[24] The combination of glibenclamide and charybdotoxin/apamin abolishes all H_2S-mediated vasorelaxation (Figure 2A).

H_2S is generated by CSE in the endothelium of blood vessels, and, like NO, diffuses to the adjacent smooth muscle to elicit vasorelaxation.[9] We confirm that the actions of H_2S-induced vasorelaxation via the ATP-sensitive potassium channels reflect direct influences upon 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). H_2S can also hyperpolarize endothelial cells, as primary cultures of wild-type, but not CSE knockout, mouse aortic endothelial cells are hyperpolarized upon acetylcholine stimulation (Figure 2D). This effect is mediated not by ATP-sensitive potassium channels, but by the combination of IK_{Ca}/SK_{Ca} channels, as hyperpolarization is completely blocked by charybdotoxin/apamin (Figure 2D). In addition, in cultured human endothelial cells (HAECs), H_2S-mediated hyperpolarization is unaffected by either glibenclamide or the BK_{ca} channel blocker iberiotoxin, but is significantly diminished by the IK_{ca} 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.[9] Here, we now observe an increase in CSE activity in cultured HAECs following shear stress suggesting that H_2S, and hence EDHF activity, can be induced not only by cholinergic means, but also by a physiologic mechanical stimulus (Online Figure X).

Physiologic sulfhydration of Kir 6.1-C43 activates the channel causing hyperpolarization

Because sulfhydration appears to be a principal means whereby H_2S signals,[21] we wondered whether vasorelaxation reflects sulfhydration of its target potassium channels. Both the Kir 6.1 subunit of ATP-sensitive potassium channels overexpressed in HEK293 cells and IK_{ca} channels from human aortic endothelial cells are sulfhydrated by NaHS in a DTT-sensitive fashion (Figure 3A and Online Figure XI). Kir 6.1 is basally sulfhydrated 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 sulfhydration of Kir 6.1 in wild-type but not CSE mutant mice (Figure 3C).

To link sulfhydration to channel function, we overexpressed Kir 6.1 in HEK293 cells in which NaHS-elicited hyperpolarization is blocked by glibenclamide, just as in blood vessels (Figure 3D). To identify the sulfhydrated cysteine residue we modeled Kir 6.1 based on the established structure of the highly homologous Kir 3.1 (Figure 3E).[26] Kir 6.1 possesses nine cysteines with cysteine-43 (C43), which lies close to the surface, responding selectively to oxidative insults.[27] C43 is the principal target of sulfhydration in Kir 6.1, as sulfhydration of the channel is abolished with C43S mutation (Figure 3F inset). NaHS-elicited hyperpolarization is significantly reduced in Kir 6.1-C43S mutants, but responses to the channel opener cromakalim remain preserved (Figure 3F and Online Figure XIIA, B). Thus, H_2S vasorelaxation reflects hyperpolarization mediated by the opening of Kir 6.1 channels via their sulfhydration at C43. The channel openers pinacidil and cromakalim elicit hyperpolarization comparable to NaHS in HEK293 cells (Online Figure XIIC).

Sulfhydration augments ATP-sensitive potassium channel activity by reducing Kir 6.1-ATP binding and enhancing Kir 6.1-PIP2 binding

Physiologic activation of the ATP-sensitive potassium channels is elicited by binding of its Kir subunits to the phospholipid phosphatidylinositol (4,5)-bisphosphate (PIP2)[28] with concomitant reductions in binding to the inhibitor ATP.[29] We wondered whether the regulation by H_2S 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 channels (Figure 4A, B and Online Figure XIIIA).[29–32] In HEK293 cells, NaHS reduces ATP-Kir 6.1 binding (Figure 4C). Confirming that ATP-Kir 6.1 binding involves the sulfhydrated C43, we observe significantly more ATP binding to Kir 6.1-C43S mutants upon treatment with NaHS compared to 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 observe substantial reductions of PIP2 binding to Kir 6.1-C43S mutants (Figure 4F).

Discussion

In summary, our findings establish H_2S 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 H_2S, is produced by vascular endothelial cells upon 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.[5–7] EDHF appears to function by covalently modifying cysteine residues of its targets, as reducing agents such as DTT reverse its effects.[33] H_2S also functions by sulfhydrating cysteine residues of key potassium channels in a DTT-sensitive manner. Hyperpolarization of endothelial and smooth muscle cells by H_2S and EDHF leads to vasorelaxation that is independent of the NO-cGMP pathway.[34] 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.[5–7, 34, 35] This fits with our observations of a greater role for H_2S in the mesenteric artery, a resistance vessel, than in the aorta, which displays more prominent NO-mediated relaxation. Recently, H_2S has been shown to be an important endogenous vasorelaxant in smaller cerebral arteries.[36] NO can inhibit the synthesis and release of EDHF,[37] which might explain the prominence of EDHF in mesenteric arteries whose levels of eNOS, and therefore NO production, are less compared to the aorta.[38] We find that NO can directly inhibit CSE activity *in vitro* with an IC_{50} of approximately 100 nM (Online Figure XV).

It is important to note however that mediators beyond EDHF and EDRF do play significant vaso-regulatory roles in different arteries. For example, studies have shown that CO plays an important role in renal vaso-regulation, although the molecular mechanism of which has not entirely been worked-out.[39] Endothelial-dependent potassium channel activity does not appear to be involved in guinea-pig uterine artery relaxation.[40] H_2O_2 dilates coronary vasculature through a redox mechanism involving thiol oxidation via p38 map kinase.[41] Although the variation in histology and physiology of vessels amongst different species appears to preclude the existence of a universal set of vaso-regulatory molecules, EDHF or EDRF have nonetheless been repeatedly demonstrated to be the principal mechanism by which vascular tone is regulated.

While some studies indicate that circulating H_2S levels in the vasculature are less than 1 μM[42] there are numerous studies that show much larger concentrations of H_2S ranging from 30 to 300 μM in blood vessels as well as in numerous other tissues including the heart, lung, brain, liver and kidney.[10, 43–50] Presumably, this generation of H_2S by different tissues (particularly the liver) contributes to circulating plasma levels in the 30 to 300 μM range. This may result in perfusion of the entire body with significant H_2S concentrations. Our utilization of 100 μM NaHS is in keeping with physiologic 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,[6] there are few studies utilizing mutant mice indicating a physiologic role for them as EDHF mediators. eNOS/COX-1 double knockout mice display reduced endothelial 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 H_2S as a major EDHF. It is nonetheless possible that these other EDHF candidates may play important roles in modulating the formation or actions of H_2S. As our studies have been confined to rodents, we do not know if they apply fully to human vasculature. However, vascular regulation is generally similar in humans and rodents.[5, 7]

Sulfhydration is a physiologic modification of cysteines in H_2S 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. H_2S is sometimes referred to as a reducing agent. Like many other substances however, the redox potential of H_2S 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 cysteinylation and glutathionylation of proteins respectively.[54] These modifications essentially appear 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 H_2S 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 H_2S-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 ATP[29] and binding to PIP2[28] which we also observe following 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 XIIIB). 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] H_2S stimulates endothelial IK_{ca}/SK_{ca} 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 H_2S diffuses from endothelial to smooth muscle cells via 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 physiologic 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 to the more invasive catheter measurements conducted by our laboratories,[9] but also leads to highly variable measurements hindering proper analysis of the data. On the other hand, in addition to the data presented here on the CSE inhibitor PPG, there is now clear evidence that selective CSE inhibitors, as well as pathologic conditions such as intermittent hypoxia in which H_2S is diminished, significantly increase vascular myogenic tone, and therefore raise blood pressure.[62]

Thus, the finding that H_2S 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 H_2S-mediated channel sulfhydration may be beneficial in treating diverse vascular disorders including hypertension.

Novelty and Significance

What Is Known?

- Hydrogen Sulfide (H_2S) is a gaseous signaling molecule. It is synthesized by cystathionine γ-lyase (CSE), which is confined predominantly to the vascular endothelium.
- Mice lacking H_2S are hypertensive and demonstrate impaired endothelial-dependent vasorelaxation. Thus, H_2S acts as an Endothelial Derived Relaxing Factor (EDRF) that mediates vascular relaxation and lowers blood pressure.

- The effects of H_2S, unlike those of NO, 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_2S causes a redox sensitive post-translational modification, sulfhydration, of a single cysteine, C43, in the Kir 6.1 subunit of the K_{ATP} channel.
- H_2S-mediated sulfhydration enhances Kir 6.1 activity by reducing Kir 6.1-ATP binding and increasing Kir 6.1-PIP2 binding.
- Hence, cholinergic, endothelial-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_2S-dependent hyperpolarization of endothelial cells.

Emerging evidence suggests that H_2S is an important gaseous signaling molecule in the vascular system, where it is produced by the endothelial enzyme cystathionine γ-lyase. 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_2S is a major Endothelial Derived Hyperpolarizing Factor (EDHF) that causes vascular endothelial and smooth muscle cell hyperpolarization and vasorelaxation since H_2S mediates its effect by a novel redox sensitive thiol-dependent post-translational 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_2S 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_2S also leads to sulfhydration and hyperpolarization of endothelial cells through the IK_{ca} and SK_{ca} channels. These findings suggest that H_2S is an important EDHF; therefore, dysregulation of this pathway may be critical step in the development of vascular diseases such as hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

H_2S	Hydrogen Sulfide
NaHS	Sodium Hydrogen Sulfide
EDHF	Endothelial Derived Hyperpolarizing Factor
EDRF	Endothelial Derived Relaxing Factor
CSE	Cystathionine γ-lyase
IK_{ca}	Intermediate conductance potassium channel
SK_{ca}	Small conductance potassium channel
NOS	Nitric Oxide Synthase
COX	Cyclooxygenase

L-NAME	L-NG-Nitroarginine methyl ester
PPG	Propargylglycine
DiBAC	Bis-(1,3-dibutylbarbituric acid)trimethine oxonol
FLIPR	Fluorometric Imaging Plate Reader
PIP2	Phosphatidylinositol (4,5)-bisphosphate
E_m	Membrane potential
NCI	NOS/COX Inhibitors
Ach	Acetylcholine
SNP	Sodium Nitroprusside

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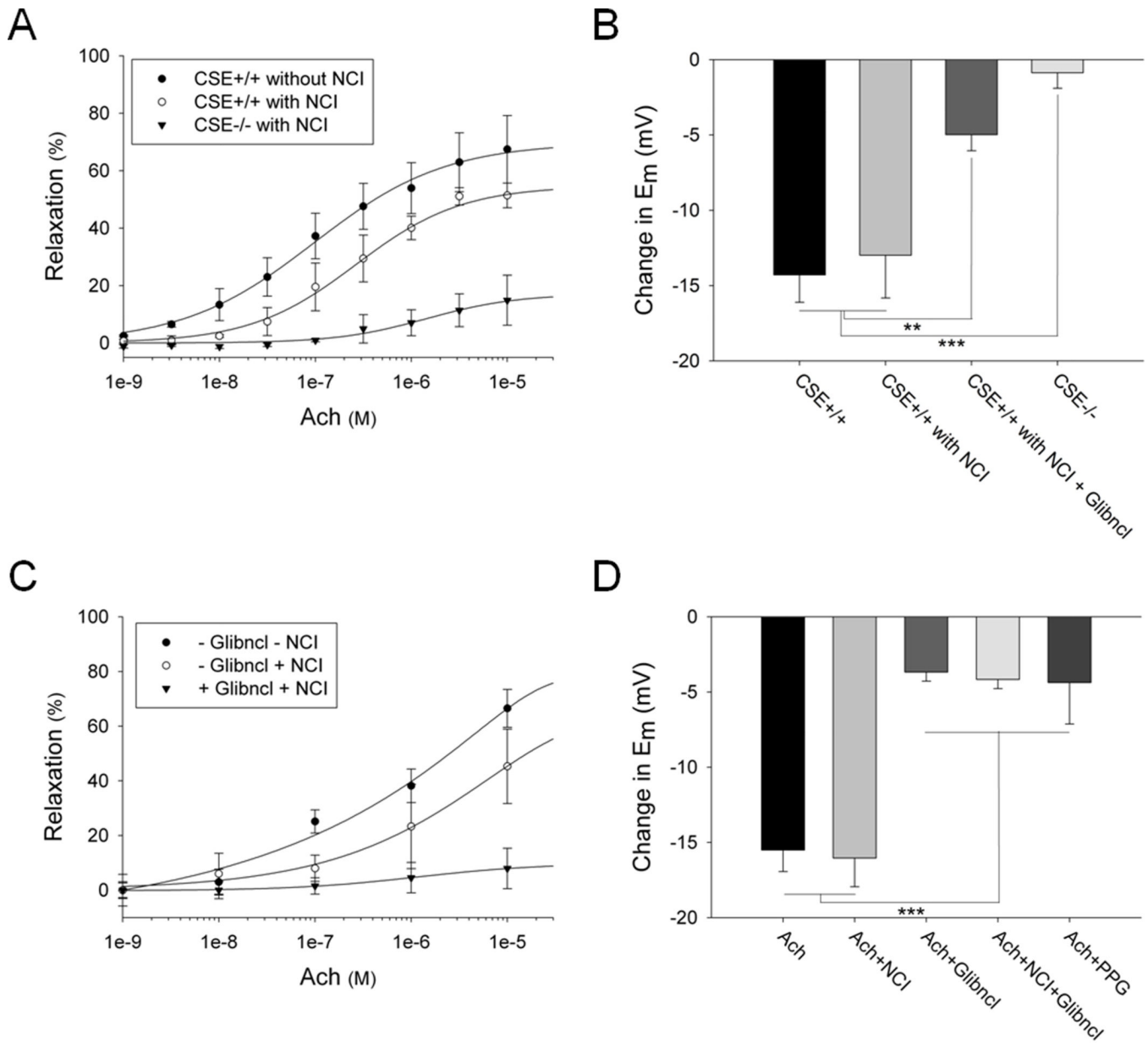

Figure 1. Cholinergic vasorelaxation and hyperpolarization are significantly reduced in CSE knockout and glibenclamide treated mesenteric arteries

(A) Muscarinic cholinergic-dependent vasorelaxation of the mesenteric artery, measured using force-tension myography, 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. NOS/COX inhibitors (NCI), Acetylcholine (Ach). n = 15. **(B)** CSE deletion almost completely abolishes the cholinergic-dependent hyperpolarization in mesenteric arteries. Treatment of wild-type mesenteric arteries with glibenclamide (5 μM) reduces the hyperpolarization by about 65%. Some of the samples were treated with L-NAME (100 μM) and indomethacin (10 μM) as indicated. The changes in membrane potential (E_m) were measured with the voltage-sensitive dyes DiBAC and FLIPR. Acetylcholine was used at 10 μM. n = 24. **(C)** Cholinergic vasorelaxation is markedly diminished in mouse mesenteric arteries treated with

glibenclamide (5 μM) in the presence of L-NAME (100 μM) and indomethacin (10 μM). n = 6. **(D)** Acetylcholine-mediated hyperpolarization is significantly reduced in rat mesenteric arteries treated with glibenclamide (5 μM) or propargylglycine (PPG) (10 μM). L-NAME (100 μM) and indomethacin (10 μM) do not influence membrane hyperpolarization. n = 13. All results are mean ± SEM (**$p < 0.01$ and ***$p < 0.001$).

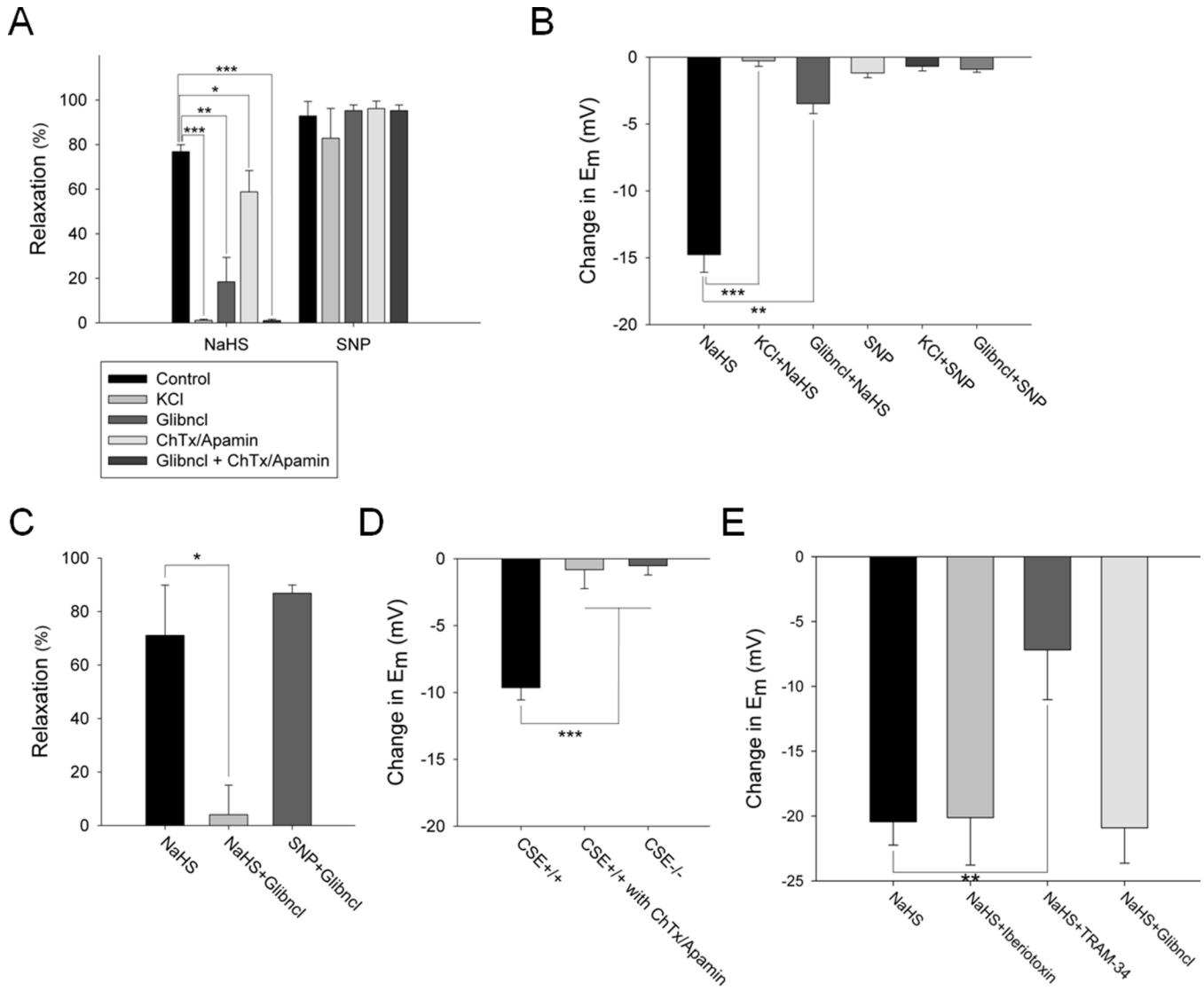

Figure 2. KCl and glibenclamide markedly diminish H_2S vasorelaxation and hyperpolarization in intact and endothelium-denuded mesenteric arteries

(A) H_2S (100 μM) vasorelaxation of rat mesenteric arteries is completely blocked by 30 mM KCl, is reduced by 75% with glibenclamide (5 μM) alone, 25% with combination of charybdotoxin (ChTx) (1 μM) and apamin (5 μM) and almost 100% with glibenclamide and ChTx/Apamin. SNP (1 μM) vasorelaxation is not affected by any of the potassium channel inhibitors. n = 20. **(B)** H_2S (100 μM) hyperpolarization of rat mesenteric arteries is completely blocked by 30 mM KCl and is reduced by about 75% with glibenclamide (5 μM). SNP (1 μM) does not induce hyperpolarization. n = 13. **(C)** H_2S (100 μM) vasorelaxation in endothelium-denuded rat mesenteric artery is almost completely abolished by glibenclamide (5 μM), which fails to alter effects of SNP (1 μM). n = 6. **(D)** H_2S hyperpolarizes endothelial cells, as seen in primary cultures of wild-type, but not CSE knockout, mouse aortic endothelial cells stimulated with acetylcholine. Treatment with ChTx/apamin completely abolishes the H_2S effect. n = 10. **(E)** H_2S hyperpolarizes human aortic endothelial cells (HAEC) treated with Iberiotoxin (0.5 μM) or glibenclamide (0.1 μM), but not TRAM-34 (10 nM). n = 8. All results are mean ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).

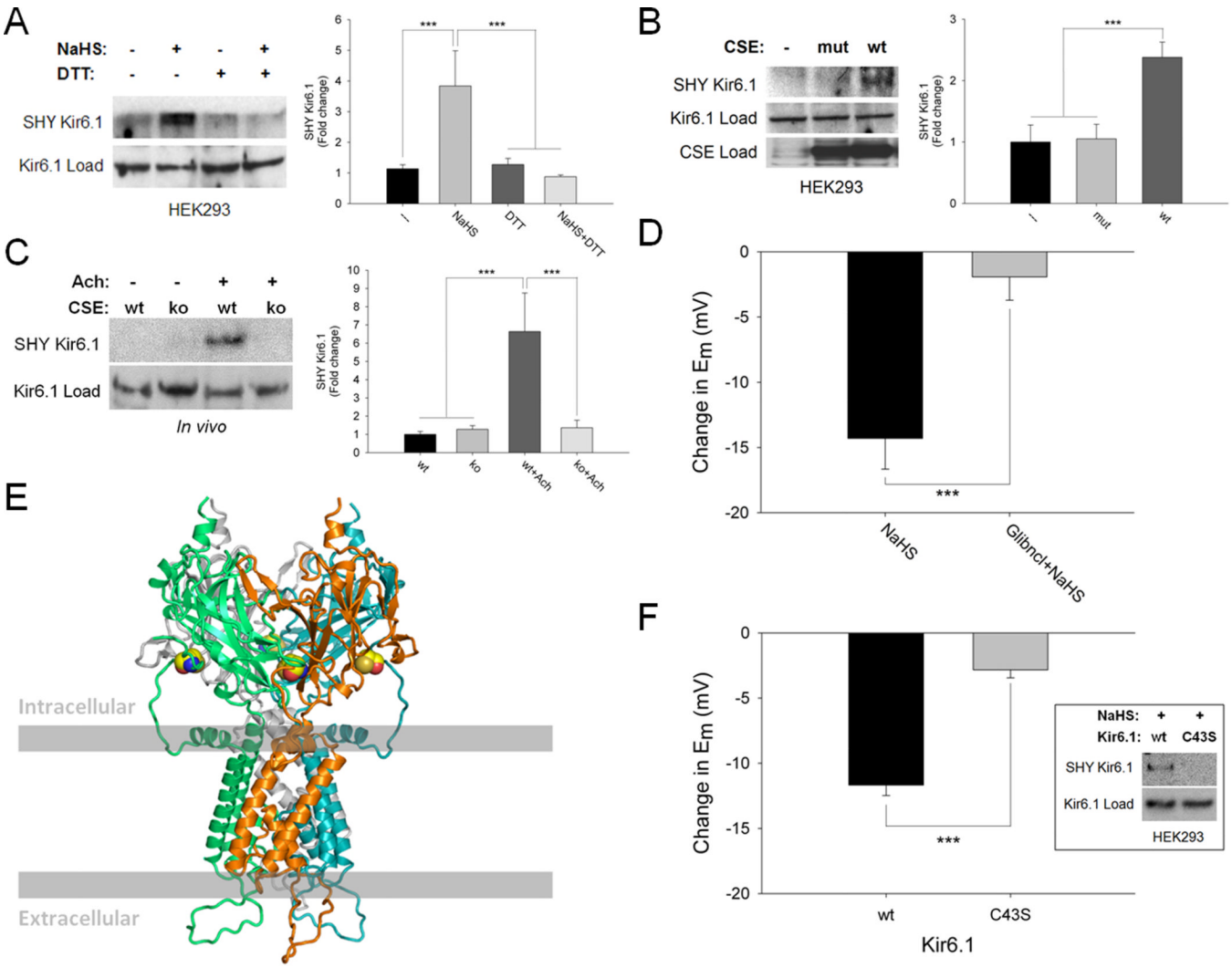

Figure 3. Physiologic sulfhydration of Kir 6.1-cysteine-43 activates the channel causing hyperpolarization

(A) H_2S (100 μM) sulfhydrates (SHY) Kir 6.1 overexpressed in HEK293 cells, an effect reversed by DTT (1 mM). 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_2S (100 μM)-elicited hyperpolarization in HEK293 cells overexpressing Kir 6.1 is substantially reduced by glibenclamide (5 μM). 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_2S (300 μM)-mediated sulfhydration (inset) and hyperpolarization are absent in HEK293 cells overexpressing C43S mutant Kir 6.1. n = 12. Quantitative densitometric analysis is also shown for Figure 3A–C. All results are mean ± SEM (***$p < 0.001$).

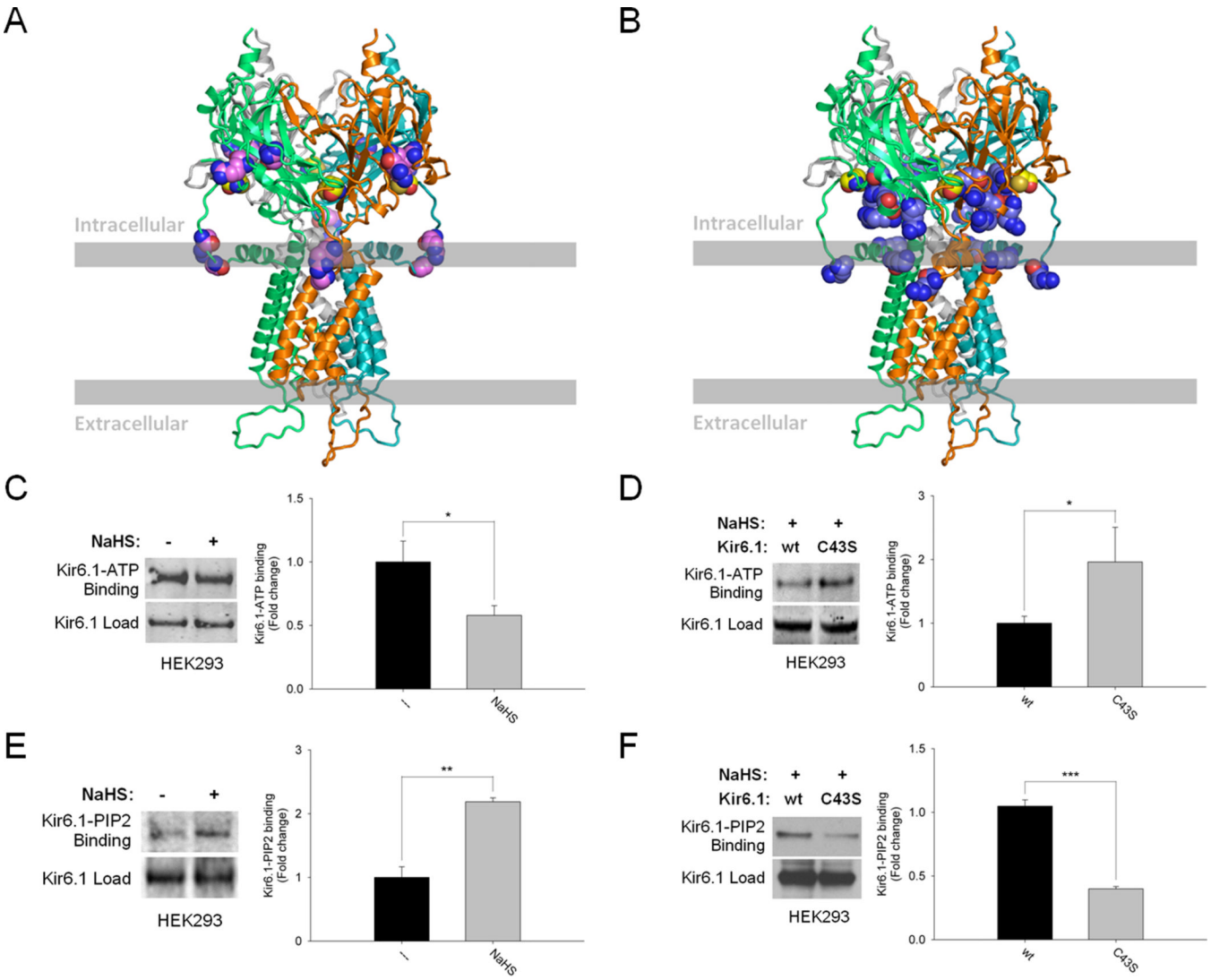

Figure 4. Sulfhydration augments ATP-sensitive potassium channel activity by reducing Kir 6.1-ATP binding and enhancing Kir 6.1-PIP2 binding

(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 stale 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 H_2S (100 μM)-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 H_2S (100 μM)-treated HEK293 cells overexpressing Kir 6.1-C43S mutant. n = 4. Quantitative densitometric analysis is also shown for Figure 4C–F. All results are mean ± SEM (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).