Hydrogen Sulfide and Vasodilation

Protein Kinase G Iα Oxidation Paradoxically Underlies Blood Pressure Lowering by the Reductant Hydrogen Sulfide

Daniel Stubbert, Oleksandra Prysyazhna, Olena Rudyk, Jenna Scotcher, Joseph R. Burgoyne, Philip Eaton

See Editorial Commentary, pp 1196–1197

Abstract—Dysregulated blood pressure control leading to hypertension is prevalent and is a risk factor for several common diseases. Fully understanding blood pressure regulation offers the possibility of developing rationale therapies to alleviate hypertension and associated disease risks. Although hydrogen sulfide (H₂S) is a well-established endogenous vasodilator, the molecular basis of its blood-pressure lowering action is incompletely understood. H₂S-dependent vasodilation and blood pressure lowering in vivo was mediated by it catalyzing formation of an activating interprotein disulfide within protein kinase G (PKG) Iα. However, this oxidative activation of PKG Iα is counterintuitive because H₂S is a thiol-reducing molecule that breaks disulfides, and so it is not generally anticipated to induce their formation. This apparent paradox was explained by H₂S in the presence of molecular oxygen or hydrogen peroxide rapidly converting to polysulfides, which have oxidant properties that in turn activate PKG by inducing the disulfide. These observations are relevant in vivo because transgenic knockin mice in which the cysteine 42 redox sensor within PKG has been systemically replaced with a redox-dead serine residue are resistant to H₂S-induced blood pressure lowering. Thus, a primary mechanism by which the reductant molecule H₂S lowers blood pressure is mediated somewhat paradoxically by the oxidative activation of PKG. (Hypertension. 2014;64:1344-1351.)

Key Words: blood pressure ■ hydrogen sulfide ■ oxidation-reduction ■ PKGIα ■ vasodilation

Given the importance of blood pressure (BP) control in health and disease, it is important to elucidate the molecular mechanisms involved in its regulation fully because this new insight may aid the development of new therapies. Hydrogen sulfide (H₂S) is widely known to dilate blood vessels and lower BP.1,2 The importance of endogenously derived H₂S in the physiological context is emphasized by the finding that mice null for the H₂S synthesising enzyme cystathionine γ-lyase are basally hypertensive.3 However, despite this established role for H₂S in the physiological control of BP, the precise mechanism(s) by which it induces vasodilation remains incompletely understood.

The vasodilators nitric oxide (NO) and prostacyclin trigger well-defined pathways leading to BP lowering. However, even when these 2 pathways are fully blocked pharmacologically, significant residual vasorelaxation occurs in resistance blood vessels when they are treated with agents, such as acetylcysteine.4 Similarly, in knockout mice genetically engineered to lack enzymes for NO or prostacyclin synthesis, significant vasorelaxation to acetylcysteine still occurs.5 This remaining vasorelaxation, termed endothelium-derived hyperpolarizing factor (EDHF), is more prevalent in resistance than conduit blood vessels and is a major mechanism controlling BP. Oxidants, such as hydrogen peroxide (H₂O₂), are established components of EDHF in many vascular beds.6 Previously, we showed that H₂O₂ or an EDHF protocol causes the formation of a disulfide bond between the 2 α subunits of the protein kinase G (PKG) Iα homodimer.7,8 This disulfide activates PKG independently of the NO–cyclic guanosine monophosphate (cGMP) pathway, significantly explaining the molecular basis of EDHF. Consistent with these findings, a knockin mouse engineered to express only a C42S redox-dead version of PKG, was basally hypertense in vivo, and their resistance blood vessels were substantively deficient in their EDHF responses when compared with those from wild-type (WT) littermate controls.8 H₂S has also been implicated as an EDHF.9,10 However, because H₂S is a thiol-based reducing agent, this would seem broadly inconsistent with the substantive literature showing that oxidants induce vasodilation and that endogenous oxidant production significantly mediates EDHF-dependent BP lowering. Certainly, PKG disulfide formation as a mediator of H₂S-dependent vasodilation would seem illogical because H₂S is generally recognized for its ability to reduce disulfide bonds. Thus, it seems counterintuitive to suggest that H₂S may lower BP by inducing oxidation of PKG. However, this is a somewhat rudimentary oversimplification of the situation and ignores some important considerations of the redox chemistry of H₂S. H₂S has a pKᵢ of ≈6.8 to 6.9, meaning at physiological

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From the Cardiovascular Division, King’s College London, The British Heart Foundation Centre of Excellence, The Rayne Institute, St Thomas’ Hospital, London, United Kingdom.
Correspondence to Philip Eaton, Cardiovascular Division, King’s College London, The Rayne Institute, St Thomas’ Hospital, London, SE1 7EH, United Kingdom. E-mail philip.eaton@kcl.ac.uk
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pH it predominantly exists in the HS (thiolate anion) state.\textsuperscript{11,12} Such deprotonated thiols can rapidly react with oxidants to form disulfide or polysulfide species, likely via a transient sulfenic acid intermediate.\textsuperscript{13} These oxidized variants of $\text{H}_2\text{S}$ instead of being reducing agents in fact have oxidant properties that are anticipated to catalyze thiol oxidation via thioldisulfide exchange reactions. Because biological systems are exposed to molecular oxygen and also generate endogenous oxidants, when $\text{H}_2\text{S}$ is present, we anticipate its transformation into polysulfides, which in turn may induce oxidation of cellular thiols, including formation of disulfide PKG. Indeed, using integrated in vitro through in vivo approaches, we demonstrate that $\text{H}_2\text{S}$-dependent vasodilation and BP lowering is counterintuitively mediated by disulfide activation of PKG.

**Methods**

**Animal Study**

All procedures were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 in United Kingdom and were approved by an institutional review committee. Mice constitutively expressing PKGI$\alpha$ Cys42Ser were generated on a pure C57BL/6 background by TaconicArtemis as described before.\textsuperscript{4} Age and body weight–matched WT or PKGI$\alpha$ Cys42Ser knockin male mice were used in all studies. All animals had ad libitum access to standard chow and water. Mice were kept under specific pathogen-free conditions and under a 12-hour day/night cycle.

**Systemic BP Measurements**

Mean arterial pressure and the locomotor activity were assessed by telemetry in conscious freely moving mice as described before.\textsuperscript{5} Alzet osmotic minipumps were used to deliver NaSH at 23 mg/kg per day.

**Myography**

Second-order mesenteric arteries from WT or PKG knockin mice were mounted in a Danish Myo Technology tension myograph, stretched to the optimal pretension condition with Danish Myo Technology normalization module, and bathed in Krebs solution at 37°C with a 95% $\text{O}_2$:5% $\text{CO}_2$ environment. Vasotone measurements were made after wake-up with KCl (60 mmol/L) by determining the responses of U46619-contracted (0.1 mmol/L) mesenteric vessels to cumulatively increasing concentrations of NaSH (0.1–3 mmol/L) or potassium polysulfide ($\text{K}_2\text{S}_x$, where $x$ is 2–8; Sigma Aldrich).

**Monitoring PKG $\alpha$ Disulfide Dimerization**

Recombinant PKG $\alpha$ (Millipore) in 100 mmol/L Tris-HCl pH 7.4 was exposed to potassium polysulfide (Sigma Aldrich) or NaSH (Alfa Aesar) or diamide (Sigma Aldrich) for 20 minutes before analysis by Western immunoblotting to determine the kinase redox state. In some experiments, reduced glutathione (Sigma) at a final concentration of 1 mmol/L was included in the reaction mixtures. Rat aortic smooth muscle cells or segments of mesenteric arteries were exposed to NaSH or $\text{H}_2\text{O}_2$ (Sigma Aldrich) or in some experiments to GYY4137 (1 mg/mL). In some experiments, tris (2-carboxyethyl) phosphine (TCEP from Thermo Scientific) at a final concentration of 0.5 mmol/L was used. Western immunoblotting was then used to determine the redox state of the kinase as described previously,\textsuperscript{6} with maleimide (100 mmol/L) used in preparation buffers to alkylate thiols and prevent thiol disulfide exchange. Antibody ADIKAP-PK005 (Enzo Life Science) was used to probe blots for PKG $\alpha$. Horseradish peroxidase–linked secondary antibody (Dako) and ECL reagent (GE Healthcare) were used. Digitized immunoblots were analyzed quantitatively with a Gel-Pro Analyzer 3.1. The percentage of PKG $\alpha$ disulfide dimer was quantified from a total PKG $\alpha$ protein expression.

**Reaction of NaSH With Oxygen or $\text{H}_2\text{O}_2$**

NaSH was dissolved in 100 mmol/L Tris-HCl, pH 8.8. Immediately after dissolution, an oxygen probe (Ocean Optic NeoFox) was submerged in the solution and the surface was covered to limit diffusion of oxygen into the buffer. The data from the probe were recorded on a computer using the NeoFox view software. $\text{H}_2\text{O}_2$ consumption was measured using the PeroxiDetect kit colorimetric assay (Sigma Aldrich). A concentration of 100 mmol/L NaSH in degassed 100 mmol/L Tris-HCl, pH 8.8, was allowed to react with 250 mmol/L $\text{H}_2\text{O}_2$ for varying amounts of time before being sampled. In some experiments, diethylene triamine pentaacetic acid (DTPA from Fluka) at a final concentration of 1 mmol/L was present.

**Results**

To investigate the putative oxidant reactivity of $\text{H}_2\text{S}$, NaSH was added to an aqueous buffer and the oxygen concentration was observed to decrease in a time-dependent manner (Figure 1A). When NaSH was added to an anoxic solution of $\text{H}_2\text{O}_2$, it also depleted this oxidant (Figure 1B). Although the initial oxidant concentration in solution was initially approximately the same in both reactions ($\approx$250 mmol/L for either oxygen or $\text{H}_2\text{O}_2$), the rate of consumption of each markedly differed. The $t_{1/2}$ for NaSH-induced loss of oxygen was $\approx$10 minutes, whereas $>90\%$ of the $\text{H}_2\text{O}_2$ was depleted within the first seconds of initiating the reaction. The presence of the metal cation chelating agent DTPA did not modulate the reaction profiles shown in Figure 1A and 1B.

As NaSH consumes oxidant, it was anticipated that reaction products including polysulfides form, which can be monitored spectrophotometrically in vitro.\textsuperscript{14} Time-dependent polysulfide formation was observed after an anoxic solution of NaSH was bubbled with oxygen, whereas when anoxia was maintained by bubbling instead with $\text{N}_2$, the absorbance did not increase (Figure 1C). At the end of each of these protocols, $\text{H}_2\text{O}_2$ was added, achieving a final concentration of 100 mmol/L. This intervention rapidly and more markedly increased the polysulfide levels in both the oxic and the anoxic samples (Figure 1C). Because NaSH was 1000-fold more abundant than the 100 mmol/L $\text{H}_2\text{O}_2$ added, the oxidant would logically have limited polysulfide formation. Indeed, when $\text{H}_2\text{O}_2$ concentration was incrementally increased in 100 mmol/L steps, a progressive increase in polysulfide levels was observed (Figure 1D), which is expected as the oxidant was progressively less limiting in the reaction and allowed more product to form. Polysulfides are anticipated, via thiol-disulfide exchange reactions, to induce disulfide formation in reduced target proteins.\textsuperscript{14,15} Consistent with this rationale, commercially sourced potassium polysulfide induced PKG oxidation to the disulfide state (Figure 1E). This polysulfide-induced oxidation of PKG $\alpha$ oxidation was only partially attenuated when 1 mmol/L glutathione was included in the reaction mixture (Figure 1F).

Figure 2A shows that NaSH in the presence of oxygen can induce oxidation of recombinant PKG in vitro. Although this finding is perhaps unexpected because NaSH is a disulfide reductant, it inducing PKG disulfide is consistent with the fact that in the presence of oxygen this reducing agent forms polysulfides (Figure 1C). These nascent polysulfide species then induce kinase oxidation (Figure 1E). NaSH or polysulfide caused dose-dependent disulfide formation in PKG in smooth muscle cells (Figure 2B and 2C). This also occurred in smooth muscle cells exposed to GYY4137 (Figure 2D), albeit the extent of kinase oxidation was less, which is consistent with the slow release of $\text{H}_2\text{S}$ from this donor.\textsuperscript{16} PKG disulfide formation in smooth muscle cells induced by exogenously
Figure 1. H2S reacts with oxidant to form polysulfides that can oxidise protein kinase G (PKG). A and B. When NaSH was added to an oxygenated solution, it lowered the oxygen concentration in a time-dependent manner. NaSH similarly lowered the concentration of H2O2 in solution, but this reaction occurred much faster (seconds instead of minutes) than the corresponding reaction with oxygen shown in A. These reactions between NaSH and oxygen or H2O2 were unaffected by the presence of the metal cation chelating agent diethylene triamine pentaacetic acid (DTPA). C. Polysulfides were anticipated to form when NaSH reacts with oxygen or H2O2, and we monitored their potential formation spectrophotometrically. Indeed, we observed time-dependent polysulfide formation when NaSH was introduced to an oxygenated solution bubbled with oxygen. In contrast, when NaSH was introduced to a solution bubbled with nitrogen to maintain an anoxic environment, polysulfides were not formed. When H2O2 was added to a final concentration of 100 μmol/L to each of these solutions, there was a rapid increase in polysulfide levels in both. D. Successive additions of H2O2 (to increase its concentration in 100 μmol/L increments) to an anoxic solution of NaSH lead to a progressive linear in polysulfide formation. E. Reduced recombinant PKG Iα was treated with diamide, which promoted interprotein disulfide formation. Potassium polysulfide also induced PKG Iα disulfide and this occurred in a concentration-dependent manner. F. Concentration-dependent polysulfide-induced recombinant PKG Iα oxidation was only partially attenuated by 1 mmol/L glutathione, despite this low molecular weight thiol being present at a 1000-fold higher concentration than the kinase. *P<0.05.
applied polysulfide was abrogated by coadministration of TCEP (Figure 2E).

NaSH also induced dose-dependent oxidation of PKG disulfide in mesenteric arteries (Figure 3A). Consistent with this, vasodilatation of mesenteric vessels to H$_2$S was deficient in the PKG redox-dead knockin relative to WT littermate controls as shown in (Figure 3B). Furthermore, vasorelaxation to potassium polysulfide was also deficient in knockin vessels when compared with WT (Figure 3C). We could not precisely define the potassium polysulfide concentration because the reagent used is mixture of sulfide of variable chain lengths (ie, K$_2$S$_x$, where x is 2–8). The concentrations defined in Figure 3C were calculated based on the average sulfide chain being pentameric.

We investigated the role of H$_2$S-induced PKG in vivo by comparing the responses of WT and C42S knockin mice to NaSH administered by osmotic minipump. Figure 4A shows a temporal comparison of the mean arterial BP of both genotypes before and after the administration of NaSH. The trace shows that administration of NaSH decreased nocturnal (ie, when the mice are physiologically active) BP in WT, whereas the knockin was resistant to this hypotensive response. The temporal BP traces are complex as expected because the knockin mice are basally hypertensive when compared with WT and the diurnal variation in BP, as well as the transient stress response after surgical-implantation under anesthesia of the osmotic minipump that delivers the H$_2$S. Despite this complexity, by assessing time-averaged data (Figure 4B), it is evident that after H$_2$S is supplied by osmotic minipump nocturnal BP is lowered in WT but not in knockin mice.

**Discussion**

Despite significant past and contemporary efforts to define how H$_2$S dilates blood vessels, our understanding remains far less than...
fides are also anticipated to deprotonate (HSS−) and reacts with oxidants to form a sulfenic acid, which in the case of H2S comes from our observation that NaSH depleted oxygen from a solution in a time-dependent manner.

Corroboration of this came from the observation that H2O2 is deprotonated from an anoxic solution when NaSH was added. However, the rate by which NaSH consumed H2O2 was much faster than that achieved by oxygen. As NaSH consumes oxidant, reaction products should logically be formed. Oxygen and H2O2 can react with thiolates to form a sulfenic acid, which in the case of HS− is anticipated to be HSOH. Because sulfenic acids are generally unstable and readily react with thiols that are abundant in cells H2S predominantly exists in solution and not as a gas as potentially implied often by biologically focussed reviews. Evidence for the putative oxidant reactivity of H2S comes from our observation that NaSH depleted oxygen from a solution in a time-dependent manner.

Although H2S is an electron donor that will reduce disulfide bonds because it has a pK of 6.8 to 6.9,11,12 at physiological pH it will be ≈80% deprotonated and so reactive with oxidants. This deprotonation also means that within cells H2S predominantly exists in solution and not as a gas as potentially implied often by biologically focussed reviews. Evidence for the putative oxidant reactivity of H2S comes from our observation that NaSH depleted oxygen from a solution in a time-dependent manner.

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resolved by an exchange reaction with the proximal thiol on the opposite chain of reduced PKG, which will generate the disulfide. Overall, it is evident that polysulfides are capable of inducing PKG oxidation and that H$_2$S in the presence of oxidants readily converts to polysulfide in vitro at least.

Although polysulfides are likely contributing to H$_2$S-dependent vasodilation via oxidation of PKG, additional mechanisms may also be participating. For example, H$_2$S could perhaps increase endogenous H$_2$O$_2$ generation or interact with reactive NO-derived species. Furthermore, endogenously generated oxidants, such as H$_2$O$_2$, may primarily react directly with the kinase to induce disulfide instead of via polysulfide formation. Although, because the pK$_a$ of H$_2$S is likely significantly lower than that of the PKG Cys42, the oxidant may selectively react with the former to generate polysulfides. Another consideration is that polysulfides may instead oxidize PKG disulfides formed by the Cys42 in the presence of oxidants readily converts to polysulfide in vitro at least.

Figure 4. H$_2$S lowers the blood pressure of wild-type (WT) but not of Cys42Ser protein kinase G (PKG) Iα knockin (KI) mice (A) The blood pressure of Cys42Ser PKG Iα KI or WT littermate control mice were monitored using radiotelemetry. Their blood pressure was monitored before or after administration of NaSH, which was delivered by osmotic minipumps implanted subcutaneously. B, NaSH administration reduced the blood pressure of WT, but not of KI, mice. *P<0.05. C, Overview of the principal findings of this study. H$_2$S is charged at physiological pH so cannot efficiently enter the cell efficiently because it is relatively impermeant to their plasma membrane. However, because the deprotonated HS$^-$ is oxidant reactive, in the presence of ubiquitous present oxygen, persulfides and polysulfides form. These disulfide species are relatively membrane permeable and so enter cells where it can oxidize PKG Iα to its disulfide-activated state to cause vasodilation. However, in cells expressing C42S redox-dead PKG Iα, as exist in the KI mouse, the kinase cannot be disulfide activated. This provides a molecular level explanation and evidence at the physiological for how H$_2$S can mediate vasodilation and blood pressure lowering.
which is not cell permeant because of its charge. GYY4137 similarly induced PKG disulfide in smooth muscle cells although the stoichiometry of oxidation was less than that observed with NaSH, consistent with this being a slow release H₂S donor. NaSH also induced dose-dependent oxidation of PKG disulfide in mesenteric arteries. This oxidation of PKG induced by NaSH is functionally important to the vasodilation caused by H₂S. We conclude that this as mesenteric vessels from C42S PKG redox-dead knockin were deficient in their relaxation to NaSH when compared with those from WT littermate controls. Furthermore, vasorelaxation to potassium polysulfide was also deficient in knockin vessels when compared with WT. However, the deficiency of knockin vessels to NaSH or polysulfide-dependent vasodilation suggested only ≈20% of the relaxation by these agents is mediated by PKG oxidation. Another important point to consider is that vasodilation to NaSH is observed at a lower concentration than disulfide PKG is observed. This possible disconnect is likely explained by the fact that disulfide PKG is likely rapidly recycled by thioredoxin as previously considered, preventing stable oxidation at lower concentrations. These findings are consistent with our previous studies, showing that H₂O₂-induced dilation of blood vessels was also significantly deficient in knockin mice, as was their response to an EDHF stimuli. NO synthase null mice have hypertension, and their blood vessels are deficient in their vasodilator responses to an EDHF protocol. Similarly, NADPH oxidase 4 overexpressing mice have lower BP and their blood vessels are hyper-responsive to EDHF vasodilatory stimuli, such as acetylcholine. Oxidant-generating enzymes coupling to BP reduction can be fully rationalized within the context of the findings of this work because the oxidants produced would oxidize H₂S to polysulfides, which would then oxidatively activate PKG. Thus, the NO synthase null mice are hypertense and the NADPH oxidase 4 overexpressing transgenics that produce elevated levels of H₂O₂ have lower BP. Throughout these studies, we found that excess of 100 μmol/L NaSH was required to induce PKG oxidation, reflecting the concentration needed to induce vasodilation, consistent with a possible causative association. A key consideration arising from these findings relates to whether 100 μmol/L H₂S and above is pertinent to the physiological setting. NaSH has been used extensively in the study of H₂S responses in cells and tissues, especially vasodilation of blood vessels in organ bath studies. Indeed, such studies allowed H₂S to be identified as a vasodilator. If we accept NaSH as a vasodilator that can provide insight to the mechanism of physiological H₂S-dependent vasodilation, we should consider the concentrations needed to achieve vessel relaxation, as reported in studies utilizing it. The majority of studies report no significant vasorelaxation until NaSH is applied in excess of 100 μmol/L, in line with the observations here. This does not mean we should conclude H₂S is found at >100 μM in biological tissues, which is very unlikely. However, neither does it mean the observations made are irrelevant to the physiological mechanism of action of endogenously derived H₂S. The physiological concentration of reduced H₂S is a matter of debate, with reports differing by orders of magnitude. Although there have been some reports of using H₂S >100 μmol/L, the contemporary consensus is perhaps that sub or low μmol/L concentrations are more likely. However, it is important to be mindful that NaSH is used as a pharmacological tool, which has to cross the plasma membrane and then be transduced into a biological response, such as vasodilation. It is crucial to consider that H₂S at physiological pH exists primarily in its thiolate anion HS⁻ state, which being charged does not cross the plasma membrane efficiently. This is in contrast to other vasodilatory biomolecules, such as NO or H₂O₂, which rapidly diffuse across membranes. However, when H₂S forms polysulfide, these species have less net charge and are membrane permeant. Furthermore, we should remember that H₂S is produced intracellularly, which we model by exogenous application of NaSH or polysulfide. Indeed, a limitation of this study is that the exogenous provision of NaSH, which is rather membrane impermeant, poorly models the endogenous intracellular enzymatic generation of H₂S. As explained, although the intracellular movement of exogenous H₂S is enhanced when it becomes oxidized to form persulfides or polysulfides, this conversion can be limited by availability of oxidant required for this reaction. It is possible that H₂S synthesizing may operate in concert with oxidant-generating enzymes to form bioactive persulfides or polysulfides. Indeed, recently it was shown that the H₂S-generating enzymes, cystathionine β-synthase and cystathionine γ-lyase, generated high concentration of persulfides (>100 μmol/L) and polysulfides. This would further highlight the limitations of using exogenous H₂S, and perhaps suggest that our experiments with polysulfides are more relevant physiologically. Indeed, it was notable that the EC₅₀ for polysulfide-induced vasodilation is ≈15 μmol/L, an order of magnitude lower than that typically observed for NaSH by us and others. Consequently, the concentration of intracellularly generated polysulfide that would induce oxidative activation of PKG could be a lot lower than the concentration of NaSH applied exogenously. Although NaSH is a commonly used tool, for the reasons explained, the concentration applied exogenously should not in any way be considered to reflect those produced endogenously. Another consideration is that disulfide formation in PKG is rapidly reversed by thioredoxin, making it difficult to detect oxidized kinase at lower H₂S or oxidant concentrations.

Overall, it was evident that isolated resistance blood vessels from knockin mice were relatively insensitive in their vasodilatory response to NaSH or potassium polysulfide when compared with WT. On this basis, it was rational to suggest that a deficient response of knockin would also likely be evident in vivo in terms of BP lowering by H₂S. Indeed, this hypothesis was found to be correct because NaSH decreased BP in WT, whereas the knockin was resistant to this hypotensive response.

Perspectives

In summary, we have shown that H₂S-mediated BP lowering is counterintuitively mediated by oxidative activation of PKG. Although oxidation of PKG in response to a reducing agent is counterintuitively at first sight, we have provided a molecular-level mechanistic explanation for this finding. Thus, H₂S in the presence of oxidants, such as molecule oxygen or H₂O₂, rapidly forms polysulfides, which can promote the oxidation of PKG via thiol-disulfide exchange reactions. The importance of these events in vivo is substantiated by C42S PKG redox-dead knockin being resistant to BP-lowering actions of H₂S. These findings help reconcile how despite it being a reducing agent H₂S can act as an EDHF, which is more typically associated with oxidant molecules. Figure 4C provides a summary overview of the findings from these studies.
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Disclosures
None.

References
12. Olson KR. A practical look at the chemistry and biology of hydrogen sul-
13. Poole LB, Karplus PA, Claiborne A. Protein sulfenic acids in redox signal-
29. Tang G, Yang G, Jiang B, Ju Y, Wu L, Wang R. H2S is an endo-

Novelty and Significance

What Is New?

• Blood pressure lowering induced by the reductant H2S is counterintuitively mediated by oxidative activation of protein kinase G (PKG).
• This apparent paradox, which a reductant counterintuitively induced oxidation of a protein, was explained by H2S in the presence of oxidant-
    forming polysulphides, which then oxidatively activate PKG.

What Is Relevant?

• These observations are relevant in vivo because a transgenic knockin mice in which the cysteine 42 redox sensor within PKG has been sys-
    temically replaced with a redox-dead serine residue are resistant to H2S-induced blood pressure lowering.

• Fully understanding blood pressure regulation offers the possibility of developing rationale therapies to alleviate hypertension and associated disease risks.

Summary

A primary mechanism by which the reductant molecule H2S dilates blood vessels and lowers blood pressure is mediated counterintuitively by the oxidative activation of PKG. This apparent paradox can be rationalized by understanding that the by H2S rapidly converts in the presence of oxygen or hydrogen peroxide to form a disulfide-cont-
    aining species, which can then induce an activating disulfide in PKG.
Protein Kinase G Iα Oxidation Paradoxically Underlies Blood Pressure Lowering by the Reductant Hydrogen Sulfide

Daniel Stubbert, Oleksandra Prysyazhna, Olena Rudyk, Jenna Scotcher, Joseph R. Burgoyne and Philip Eaton

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