Hydrogen Sulfide and Vasodilation

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

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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. 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. 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 acetylcholine. Similarly, in knockout mice genetically engineered to lack enzymes for NO or prostacyclin synthesis, significant vasorelaxation to acetylcholine still occurs. 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. 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. 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 hypertensive in vivo, and their resistance blood vessels were substantively deficient in their EDHF responses when compared with those from wild-type (WT) littermate controls. H₂S has also been implicated as an EDHF. 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 pKₐ of ≈6.8 to 6.9, meaning at physiological

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pH it predominantly exists in the HS (thiolate anion) state. Such deprotonated thiols can rapidly react with oxidants to form disulfide or polysulfide species, likely via a transient sulfenic acid intermediate. These oxidized variants of H$_2$S instead of being reducing agents in fact have oxidant properties that are anticipated to catalyze thiol oxidation via thiol-disulfide exchange reactions. Because biological systems are exposed to molecular oxygen and also generate endogenous oxidants, when H$_2$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 H$_2$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 Cys42Ser were generated on a pure C57BL/6 background by TaconicArtemis as described before. Age and body weight–matched WT or PKGI 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. Age and body weight–matched WT or PKGI knockin mice were generated on a pure C57BL/6 background by TaconicArtemis as described before. 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.

**Polysulfide Formation**

A concentration of 100 mmol/L NaSH in degassed 100 mmol/L Tris-HCl, pH 8.8, was allowed to react with 250 μmol/L H$_2$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 H$_2$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 H$_2$O$_2$, it also depleted this oxidant (Figure 1B). Although the initial oxidant concentration in solution was initially approximately the same in both reactions (=250 μmol/L for either oxygen or H$_2$O$_2$), the rate of consumption of each markedly differed. The $t_{1/2}$ for NaSH-induced loss of oxygen was ≈10 minutes, whereas >90% of the H$_2$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. 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 N$_2$, the absorbance did not increase (Figure 1C). At the end of each of these protocols, H$_2$O$_2$ was added, achieving a final concentration of 100 μmol/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 μmol/L H$_2$O$_2$ added, the oxidant would logically have limited polysulfide formation. Indeed, when H$_2$O$_2$ concentration was incrementally increased in 100 μmol/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. Consistent with this rationale, commercially sourced potassium polysulfide induced PKG oxidation to the disulfide state (Figure 1E). This polysulfide-induced oxidation of PKG 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 H$_2$S from this donor. 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, vasodilation 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 physically 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...
of a sulfenic acid intermediate, we have provided no evidence of this mechanism and so alternatives remain possible. As the chelator DTPA did not alter the reaction of H$_2$S with oxygen or H$_2$O$_2$, it is perhaps reasonable to exclude a major role for transition metal impurities, such as iron participating in the oxygen or peroxide-mediated conversion of H$_2$S into polysulfides.

We also used spectrophotometry to monitor polysulfide formation in vitro, observing time-dependent polysulfide formation after an anoxic solution of NaSH is bubbled with oxygen, whereas maintained anoxia involving bubbling with N$_2$ did not. At the end of both protocols, H$_2$O$_2$ was added (and this rapidly and more markedly increased the polysulfide levels in both oxidized and anoxic samples). Because NaSH was 1000-fold more abundant than the 100 μmol/L H$_2$O$_2$ added, the oxidant would be anticipated to delimit polysulfide formation. Indeed, when H$_2$O$_2$ concentration was increased incrementally, we observed stepwise increases in polysulfides, consistent with oxidant being progressively less limiting as it was progressively added.

Polysulfides are capable of oxidizing protein thiols via thiol-disulfide exchange reactions, raising the possibility that they may induce PKG disulfide. Indeed, potassium polysulfide dose-dependently induced PKG disulfide formation. As rationalized above, oxidants, such as H$_2$O$_2$, likely induce the disulfide via a sulfenic intermediate, but the precise molecular intermediate will depend on the oxidant used. For example, our past studies have shown that transnitrosylating NO donors induce PKG disulfide, and this is probably via S-nitrosylation of Cys42 before a resolving action of Cys42 on the opposite chain of the homodimer. In the case of polysulfide-induced PKG disulfide formation, a short-lived adduct of H$_2$S with Cys42 is the anticipated intermediate. This disulfide bonding of H$_2$S to a protein has been referred to as sulfhydration, but sulfuration is a more appropriate alternative. Because this sulfurred intermediate is a disulfide, it should be readily

Figure 3. Vasodilation to NaSH or potassium polysulfide is deficient in knockin (KI) when compared with wild-type (WT) vessels. A, Mesenteric vessels were treated with NaSH, which caused a concentration-dependent protein kinase G (PKG) I disulfide formation. A concentration of 50 μmol/L H$_2$O$_2$, which was used as a positive control, also induced oxidation of PKG Iα. B and C, Mesenteric vessels from Cys42Ser PKG Iα KI or wild-type (WT) littermate control mice were mounted in a myography and constricted with u46619 and then their relaxation to cumulatively increasing concentrations of NaSH or potassium polysulfide was measured. This showed that mesenteries from KIs were deficient in their H$_2$S-dependent vasodilatory responses when compared with WT. *P<0.05.
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 once polysulfides are formed, whether they would react sufficiently rapidly with PKG in cells to mediate its oxidation. It remains possible that a catalyst accelerating the thiol-disulfide exchange reaction is contributing to the H$_2$S-induced oxidation observed.

Another consideration is how polysulfides would selectively oxidize PKG disulfides in vivo against a background abundance of many other thiols, especially from glutathione. The thiol in GSH has a high pK$_a$ of e9 and, therefore, will be mostly protonated at physiological pH and so is probably much less reactive than the Cys42 thiols of PKG. Indeed, when we monitored polysulfide-induced PKG oxidation, we found that the presence of 1 mmol/L glutathione, representing a 1000-fold molar excess over the kinase, only marginally attenuated PKG disulfide formation.

Overall, our in vitro findings demonstrate that polysulfides form when H$_2$S contacts oxidants, such as O$_2$ or H$_2$O$_2$. What we are not suggesting is that the high concentrations of H$_2$S used in these experiments reflect those found in vivo. The in vitro studies simply provide proof of concept that polysulfides form from H$_2$S and that their formation is limited by the relative molar abundance of oxidant. The high concentration of NaSH used in these studies was used because of the insensitivity of the spectrophotometric absorbance assay used to monitor polysulfide formation. Reasonably we can conclude the same chemical reactions also proceed at lower H$_2$S concentrations and also in cells where it is also exposed to O$_2$, H$_2$O$_2$, and other oxidant species that can promote sulfide formation.

NaSH in the presence of oxygen (so polysulfides will also be present) induced oxidation of recombinant PKG in vitro, consistent with the rationale and evidence presented above. An important question was whether these events occur in cells and tissues, which would provide a mechanism for H$_2$S-induced PKG activation. Indeed, we found that NaSH or polysulfide caused dose-dependent disulfide formation in PKG in smooth muscle cells. The disulfide-reducing agent TCEP abrogated polysulfide-induced PKG oxidation in cells. This is consistent with membrane-impermeant TCEP reversing exogenous disulfides back to the reduced H$_2$S state.
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 H$_2$S 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 H$_2$S. 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 $\approx$20% of the relaxation by these agents is mediated by PKG oxidation. Another important point to consider is that vasodilation to NaSH is observed at a lower concentration than disulfide PKG is observed. This possible disconnect is likely explained by the fact that disulfide PKG is likely rapidly recycled by thioredoxin as previously considered, preventing stable oxidation at lower concentrations. These findings are consistent with our previous studies, showing that H$_2$O$_2$-induced dilation of blood vessels was also significantly deficient in knockin mice, as was their response to an EDHF stimului. NO synthase null mice have hypertension, and their blood vessels are deficient in their vasodilator responses to an EDHF protocol. Similarly, NADPH oxidase 4 overexpressing mice have lower BP and their blood vessels are hyper-responsive to EDHF vasodilatory stimuli, such as acetylcholine. Oxidant-generating enzymes coupling to BP reduction can be fully rationalized within the context of the findings of this work because the oxidants produced would oxidize H$_2$S to polysulfides, which would then oxidatively activate PKG. Thus, the NO synthase null mice are hypertense and the NADPH oxidase 4 overexpressing transgensics that produce elevated levels of H$_2$O$_2$ 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 H$_2$S and above is pertinent to the physiological setting. NaSH has been used extensively in the study of H$_2$S responses in cells and tissues, especially vasodilation of blood vessels in organ bath studies. Indeed, such studies allowed H$_2$S to be identified as a vasodilator. If we accept NaSH as a vasodilator that can provide insight to the mechanism of physiological H$_2$S-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 H$_2$S is found at $>$100 μM in biological tissues, which is very unlikely. However, neither does it mean the observations made are irrelevant to the physiological mechanism of action of endogenously derived H$_2$S. The physiological concentration of reduced H$_2$S is a matter of debate, with reports differing by orders of magnitude. Although there have been some reports of using H$_2$S $>$100 μmol/L, the contemporary consensus is perhaps that sub or low μmol/L concentrations are more likely. However, it is important to be mindful that NaSH is used as a pharmacological tool, which has to cross the plasma membrane and then be transduced into a biological response, such as vasodilation. It is crucial to consider that H$_2$S at physiological pH exists primarily in its thiolate anion HS$^-$ state, which being charged does not cross the plasma membrane efficiently. This is in contrast to other vasodilatory biomolecules, such as NO or H$_2$O$_2$, which rapidly diffuse across membranes. However, when H$_2$S forms polysulfide, these species have less net charge and are membrane permeant. Furthermore, we should remember that H$_2$S is produced intracellularly, which we model by exogenous application of NaSH or polysulfide. Indeed, a limited time of this study is that the exogenous provision of NaSH, which is rather membrane impermeant, poorly models the endogenous intracellular enzymatic generation of H$_2$S. As explained, although the intracellular movement of exogenous H$_2$S 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 H$_2$S synthesizing may operate in concert with oxidant-generating enzymes to form bioactive persulfides or polysulfides. Indeed, it was notable that the EC$_{50}$ for polysulfide-induced vasodilation is $\approx$15 μmol/L, an order of magnitude lower than that typically observed for NaSH by us and others. Consequently, the concentration of intracellularly generated polysulfide that would induce oxidative activation of PKG could be a lot lower than the concentration of NaSH applied exogenously. Although NaSH is a commonly used tool, for the reasons explained, the concentration applied exogenously should not in any way be considered to reflect those produced endogenously. Another consideration is that disulfide formation in PKG is rapidly reversed by thioredoxin, making it difficult to detect oxidized kinase at lower H$_2$S 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 H$_2$S. 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 H$_2$S-mediated BP lowering is counterintuitively mediated by oxidative activation of PKG. Although oxidation of PKG in response to a reducing agent is counterintuitive at first sight, we have provided a molecular-level mechanistic explanation for this finding. Thus, H$_2$S in the presence of oxidants, such as molecule oxygen or H$_2$O$_2$, 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 H$_2$S. These findings help reconcile how despite it being a reducing agent H$_2$S 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

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 polysulfides, 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 systemically 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-containing species, which can then induce an activating disulfide in PKG.
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