Thiosulfate: a readily accessible source of hydrogen sulfide in oxygen sensing

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Olson KR, DeLeon ER, Gao Y, Hurley K, Batz VS, Stoy GF. Thiosulfate: a readily accessible source of hydrogen sulfide in oxygen sensing. Am J Physiol Regul Integr Comp Physiol 305: R592–R603, 2013.—H₂S derived from organic thiol metabolism has been proposed serve as an oxygen sensor in a variety of systems because of its susceptibility to oxidation and its ability to mimic hypoxic responses in numerous oxygen-sensing tissues. Thiosulfate, an intermediate in oxidative H₂S metabolism can alternatively be reduced and generate H₂S. We propose that this contributes to the H₂S-mediated oxygen-sensing mechanism. H₂S formation from thiosulfate in buffers and in a variety of mammalian tissues and in lamprey dorsal aorta was examined in real time using a polarographic H₂S sensor. Inferences of intracellular H₂S production were made by examining hypoxic pulmonary vasoconstriction (HPV) in bovine pulmonary arteries under conditions in which increased H₂S production would be expected and in mouse and rat aortas, where reducing conditions should mediate vasorelaxation. In Krebs-Henseleit (mammalian) and Cortland (lamprey) buffers, H₂S was generated from thiosulfate in the presence of the exogenous reducing agent, DTT, or the endogenous reductant dihydroxyoic acid (DHLA). Both the magnitude and rate of H₂S production were greatly increased by these reductants in the presence of tissue, with the most notable effects occurring in the liver. H₂S production was only observed when tissues were hypoxic; exposure to room air, or, injecting oxygen inhibited H₂S production and resulted in net H₂S consumption. Both DTT and DHLA augmented HPV, and DHLA dose-dependently relaxed precontracted mouse and rat aortas. These results indicate that thiosulfate can contribute to H₂S signaling under hypoxic conditions and that this is not only a ready source of H₂S production but also serves as a means of recycling sulfur and thereby conserving biologically relevant thiols.

oxygen sensing; gasotransmitter; mitochondria

THERE IS INCREASING EVIDENCE that hydrogen sulfide (H₂S) functions as an oxygen sensor in many tissues, including the vasculature (20, 24, 25, 28, 35), chemoreceptor cells, such as neural epithelial cells in the fish gill (26) and type I glomus cells in the carotid body (18, 29, 38, 39), adrenal chromaffin cells (30), nonvascular smooth muscle (5, 6), and even activating hypoxia-inducible factor-1 (HIF-1) (15). In our original model of H₂S-mediated O₂ sensing, we presumed that H₂S was constitutively synthesized in the cell cytoplasm and oxidized in the mitochondrion and that the balance between production and oxidation, and, hence, the level of biologically active H₂S, was, thereby, directly coupled to mitochondrial oxygen availability (24). The observation that the cytoplasm of the ancestral eukaryotic cell is believed to originate from the endosymbiosis of sulfur-reducing Archea and the mitochondria from sulfide oxidizing α-proteobacterium (33) provided anecdotal support for this model. Evidence supporting this mechanism has been recently summarized (23, 27).

In our model, it was initially assumed that cysteine was the primary source of H₂S biosynthesis, as H₂S can be generated from two well-characterized enzymes, cystathione β-synthase (CBS) and cystathione γ-lyase (CSE). However, other studies have shown that a variety of thiols, such as glutathione, homocysteine, cystathionine, and methionine can either produce H₂S directly or augment the hypoxic response (1, 20, 28), and another pathway for H₂S production from cysteine involving cysteine aminotransferase and 3-mercaptoppyruvate sulfur transferase (3-MST) has recently been described (34). It has also been generally assumed that mitochondrial catalysis of H₂S in vertebrates proceeds through a series of oxidation steps with thiosulfate (S₂O₅²⁻) as an intermediate and sulfate (SO₄²⁻) as the ultimate excretory product. However, Villarejo and Westley (44) observed that in the presence of the endogenous reductant, dihydroxyoic acid (DHLA), H₂S was produced from thiosulfate by the enzyme rhodanase. This has recently been confirmed in neural tissue (21). Because the mitochondrial matrix becomes selectively reduced during hypoxia (46) and because this would favor formation of H₂S from thiosulfate, it seems reasonable to assume that during hypoxia H₂S could be generated from the thiosulfate pool and immediately contribute to the O₂-sensing process. This would not only provide a readily available mechanism for rapidly increasing intracellular H₂S, it would also conserve organic thiols. To our knowledge, this has not been experimentally verified in the oxygen-sensing tissues.

In the present study, we examined the possibility that H₂S could be regenerated from thiosulfate by tissue and that this should augment the hypoxic response. To accomplish this, we first measured H₂S production from thiosulfate in the presence of the exogenous reductant, DTT, in a variety of tissues using an amperometric (polarographic) H₂S sensor constructed in our laboratory. This enabled us to measure H₂S production in real-time and under physiological conditions (47). We then examined the effect of thiosulfate and DTT on hypoxic vasoconstriction (HPV) of bovine pulmonary arteries using thin-wire myography. To further examine these processes, we replaced DTT with DHLA, an endogenous reducing compound found in the mitochondria (21) and measured H₂S production in select tissues, as well as its effects on HPV and on relaxation of precontracted mouse and rat thoracic aortas, where hypoxia and H₂S produce vasorelaxation (24). In previous experiments, we also noticed that precontracting bovine pulmonary arteries with the thromboxane A₂ mimetic, U-46619, significantly augmented HPV, and this dominated the response to the extent that the contribution of organic sulfur-donating molecules (i.e., cysteine, glutathionine, and cysteine plus α-ketoglutarate) be-
came less evident (28). We proposed that prestimulation so activated the downstream signaling that it obscured the initial \(O_2\)-sensing events. To verify this, the effects of prestimulation on the hypoxic responses to DTT were also examined.

**MATERIALS AND METHODS**

**Animals**

Adult Lobund-Wistar rats (0.35–0.5 kg) and mice (~25 g, from a variety of strains) were maintained at the University of Notre Dame, Friedemann Life Science Center and kept on a 12:12-h light-dark cycle with access to food and water ad libitum. These animals were being culled from breeding stocks and were not specifically killed for our experiments. They were euthanized with \(CO_2\) followed by cervical dislocation, and the tissues were removed and placed in cold (4°C) Krebs-Henseleit buffer and stored in the refrigerator.

Bovine (Bos taurus) lung and heart were collected shortly after the animals were killed at a local slaughterhouse. The tissues were placed in cold (4°C) Krebs-Henseleit buffer and transported back to the laboratory. Pulmonary arteries (0.75–1.5-mm diameter) were dissected free from the lung tissue and stored in buffer in the refrigerator. Tissue for \(H_2S\) production studies was cut into small pieces and stored in the refrigerator in buffer.

Female sea lamprey (Petromyzon marinus, 150–450 g) were trapped in streams feeding into the Great Lakes during the spring-summer spawning season and airlifted to the Indiana University School of Medicine-South Bend, where they were maintained in 500-liter rectangular tanks with aerated, flowing well water (15°C) and exposed to a 12:12-h light-dark photoperiod. These fish no longer fed. They were anesthetized in benzocaine (1:5,000, wt/vol) in ice water, and the dorsal aortas were dissected out and placed in Cortland buffer at 4°C until use. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

**Tissue \(H_2S\) Production in the Presence of DTT**

Mammalian tissues were minced with scissors and then homogenized in buffer (1:9; wt:vol) on ice for 15 s using an Ultra Turrax homogenizer (IKA, Wilmington, NC) that was activated by sparging for 30 min with humidified nitrogen. One and one-half milliliters of homogenate was then placed in an ice bath and deoxygenated by sparging for 30 min with humidified nitrogen. One and one-half milliliters of homogenate was then placed in a reaction chamber (see Metabolism Chamber and \(H_2S\) Measurement) that was fitted with a plunger to remove head-space gas and prevent \(H_2S\) volatilization which would otherwise occur (2). The homogenate was gently stirred and allowed to warm to room temperature for 30 min before an experiment was started. \(H_2S\) gas was monitored with an amperometric (polarographic) sensor (see Metabolism Chamber and \(H_2S\) Measurement). Tissue-free buffer, used as a control, was processed similarly. GSH, DTT, and sodium thiosulfate (\(Na_2S_2O_3\)) were then added at intervals to produce a final concentration of either 1 mM or 10 mM. Initially, the order of addition of these compounds was random; however, it became apparent that \(H_2S\) was only produced after both DTT and thiosulfate were added. In subsequent experiments, the order was standardized: GSH, DTT, thiosulfate, or DTT, thiosulfate. Timing of the intervals was largely dependent on production; GSH produced only a short transient increase in \(H_2S\), and \(H_2S\) production after the addition of DTT was somewhat longer. Because \(H_2S\) production was maintained for a considerable period after the addition of thiosulfate, values were typically recorded for longer periods (30–60 min). To verify that \(H_2S\) production from thiosulfate was an oxygen-dependent process, the chamber was opened to the atmosphere, or ~100 \(\muL\) of oxygen was injected into the reaction chamber at the end of the experiment. Lamprey tissues were treated as above, except that the studies were performed in Cortland buffer.

**Tissue \(H_2S\) Production in the Presence of Dihydrolipoic Acid**

DHLA was prepared as described below. The effects of DHLA on \(H_2S\) production from thiosulfate were examined in rat and mouse liver and rat and bovine lung. The general protocol was similar to that described above for DTT with the following modifications. Two DHLA concentrations (100 \(\muM\) and 1 \(mM\)) and one thiosulfate concentration (1 \(mM\)) were examined, as preliminary experiments suggested this was adequate for \(H_2S\) generation. The order of addition thiosulfate vs. DHLA was reversed in half of the experiments to confirm that both were necessary for \(H_2S\) production. In all experiments the response to 100 \(\muM\) DHLA was allowed to plateau, and then 1 \(mM\) DHLA was added. Because the response to 100 \(\muM\) DHLA was inconsistent and because there were no significant effects of the order of thiosulfate and DHLA treatment, the results were pooled for comparisons between buffer and tissue.

**Metabolism Chamber and \(H_2S\) Measurement**

The metabolism chamber (28) was constructed in-house and consisted of a lost-wax-cast soda lime glass inner chamber with a side-port for the \(H_2S\) sensor and surrounded by an acrylic water jacket. A polyvinylidene difluoride (PVDF) stopper with a viton O-ring tightly fit into the opening from above. A small hole drilled through the stopper permitted venting the headspace air when the stopper was lowered into the chamber and provided an access port for injection of drugs or air bubbles with a Hamilton syringe. The chamber was placed on a magnetic stirrer and stirred with either a standard Teflon micro stir bar or a PVDF-coated stirbar (28).

The \(H_2S\) sensor was constructed in-house after Doeller et al. (4), with modifications, as described previously (47). This sensor has a sensitivity of 14 nM \(H_2S\) gas (~100 nM total sulfide). The advantages of this sensor are that it can be used with otherwise unadulterated tissue, and it rapidly responds to changes in \(H_2S\) (63% response in 8 s). The sensor was connected to an TBR 4100 Free Radical Analyzer (World Precision Instruments, Sarasota, FL) with 100 mV polarizing voltage. Data were archived on a laptop PC with software provided by the manufacturer and exported into Microsoft Excel. The sensor was calibrated with sodium sulfide nonahydrate. Crystals were placed in a glass syringe, which was then sealed with a rubber stopper and sparged with \(N_2\). \(N_2\)-sparged buffer was then injected into the syringe through the stopper to dissolve the \(Na_2S\). Stock solutions were made fresh daily.

**Protocol**

In preliminary experiments, we observed that \(H_2S\) production was greatest after both DTT and thiosulfate were added but that production was less dependent on the order of addition of these compounds. In subsequent experiments with mammalian tissues, we selected two protocols; the addition of GSH first followed by DTT and then thiosulfate and in the second protocol, the GSH was omitted. Effects of DTT and thiosulfate were also examined in lamprey aortas. GSH was omitted from these studies after pilot studies indicated it did not contribute to \(H_2S\) production. \(H_2S\) concentration was recorded continuously after the addition of each compound, and the subsequent compound was not added until \(H_2S\) production from the previous one had appeared to plateau. The time for \(H_2S\) production to plateau after \(GSH\) addition was relatively short, typically within 5 to 10 min; the time for the \(H_2S\) plateau after DTT was generally 10–15 min. \(H_2S\) concentration continued to increase, often for over 1 h, after thiosulfate addition, and these experiments often did not plateau. Similar responses were observed when DHLA was substituted for DTT. Therefore, the values for \(H_2S\) production after thiosulfate addition likely underestimate the total \(H_2S\) generative capacity.

**Vessel Myography**

Bovine pulmonary arteries were cut into 3–8 mm-long segments and mounted on 280-\(\mu\)m-diameter stainless-steel wire hooks and suspended in 5-ml water-jacketed smooth muscle baths filled with...
buffer at 37°C. They were aerated with 95% air-5% CO₂. One hook was stationary; the other was connected to the force transducer. In initial experiments, the apparatus consisted of a Grass model FT03C force-displacement transducer (Grass Instruments, West Warwick, RI) and Grass Model 7E or 7F polygraphs (Grass Instruments), and data were archived on a PC computer using SoftWire A-D conversion (Measurement Computing, Middleboro, MA). In subsequent experiments iWorx Systems (Dover, NH) force transducers (FT-302) and data loggers (308T) were used, and the data were archived on PCs. The transducers were calibrated prior to each experiment to detect changes as small as 5 mg.

Baseline (resting) tension (~1,000 mg) was applied to the vessels for 0.5–1 h prior to experimentation. In a typical experiment, vessels were contracted twice with 80 mM KCl, and resting tension was re-established after each contraction during a 30–45 min rinse (4×) period. Hypoxic HPV was produced by gassing with 95% N₂-5% CO₂. This typically lowers P₀₂ to <5 mmHg within 1 min.

Protocol. Previous studies in our laboratory have shown that the force of a HPV significantly increases between the first and second hypoxic exposure, whereas there is less of an increase between subsequent hypoxic treatments. This enabled us to use each vessel as its own control with the second hypoxia as the reference (pretreatment) and comparisons to a third or fourth HPV (posttreatment).

To examine the effects of pretreatment on hypoxic responses, vessels were precontracted with the thromboxane A₂ mimetic, U-46619 (10⁻⁸ or 10⁻⁶ M). The effects of DTT (1 μM–10 mM) and/or DDT plus 1 or 10 mM thiosulfate on the hypoxic responses of otherwise unstimulated vessels or vessels precontracted with 10⁻⁶ M U-46619 were examined 15–20 min after the drugs were added. The effects of DHLA on HPV were examined in otherwise unstimulated vessels 20 min after the addition of either 10 or 100 μM DHLA.

Thoracic aortas from mice and rats were prepared similar to that for bovine pulmonary arteries. To examine the effect of DHLA, vessels were precontracted with 10⁻⁷ M U-46619, and increasing concentrations of DHLA were added in 10-fold increments from 10⁻⁶ to 10⁻³ M.

Preparation of Dihydrolipoic Acid

Dihydrolipoic acid (DHLA) was prepared after the method of Volini and Wesley (45) as modified by Mikami et al. (21). Briefly, 0.4126 g of α-lipoic acid was dissolved in 10 ml of 0.2 M Tris base and 2 M sodium borohydride (NaBH₄) and incubated at room temperature for 15–30 min to reduce the α-lipoic acid to DHLA. The solution was then acidified by adding 85% phosphate solution on ice until the DHLA precipitated (pH ≈ 5.0). Acidification converts excess BH₄ into borate. The DHLA solution in the phosphate-borate buffer was adjusted back to pH 7.3 by adding Tris base and diluted to 20 ml with 0.15 M Tris·HCl (pH 7.3).

Buffers. The following buffers were used: Krebs-Henseleit (in mM): 115 NaCl, 2.5 KCl, 2.46 MgSO₄, 2 CaCl₂·2H₂O, 5.6 glucose, 1.38 NaH₂PO₄, and 25 NaHCO₃, at pH 7.4; HEPES (in mM): 145 NaCl, 3 KCl, 0.57 MgSO₄, 2 CaCl₂, 5 glucose, 3 HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid] acid, 7 HEPES sodium salt (HEPES-Na), at pH 7.8; potassium phosphate: 100 mM KH₂PO₄/K₂HPO₄; Cortland buffer (in mM): 124 NaCl, 3 KCl, 0.57 MgSO₄·7H₂O, 2 CaCl₂·2H₂O; 0.09 NaH₂PO₄, 1.8 NaHPO₃, 12 NaHCO₃, 5.5 glucose, at pH 7.8.

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Data Analysis

Statistical significance was determined using Student’s t-test, paired t-test, or ANOVA and the Holm-Sidak test using SigmaStat (Systat Software, San Jose, CA). Results are given as means ± SE; significance was assumed when P ≤ 0.05.

RESULTS

H₂S Production from Thiosulfate and DTT

H₂S production in buffer. H₂S production was observed in Krebs-Henseleit buffer following the addition of glutathione, DTT, and thiosulfate (Fig. 1A; Tables 1 and 2). The amount of H₂S produced from glutathione alone was relatively small (~0.1%), whereas around 10 times as much H₂S was produced by the addition of DTT. The H₂S production from DTT plateaued within several minutes (Fig. 1A), suggesting that this was a contaminant of DTT rather than actual H₂S production in buffer. The addition of thiosulfate to the DTT further increased.
H₂S concentration, and this did not rapidly plateau, suggesting that the H₂S was continually evolving from thiosulfate. Omission of glutathione did not significantly affect H₂S production from either the DTT or thiosulfate, indicating that glutathione does not contribute to H₂S production in buffer. Similar effects were observed upon the addition of DTT and thiosulfate to Courtland buffer, indicating that H₂S production was independent of the medium used. Furthermore, reducing the DTT concentration to 100 μM and 10 μM while maintaining 1 mM thiosulfate in Courtland buffer progressively decreased H₂S production from both DTT and thiosulfate, indicating that the reducing conditions provided by DTT were important in generation of H₂S from thiosulfate.

H₂S Production by Mammalian Tissues

The effects of sequential additions of either 1 or 10 mM GSH, DTT, and thiosulfate, or 1 or 10 mM DTT and thiosulfate on H₂S production by homogenized tissues are tabulated in Tables 1 and 2, and representative traces of the effects of 10 mM GSH, DTT, and TS are shown in Fig. 1, B–E. Typically, the responses were similar to those observed in buffer, albeit at greater rates of H₂S production after the addition of DTT and thiosulfate. The addition of 1 or 10 mM GSH to homogenized tissue either failed to generate H₂S, or produced a slight increase in H₂S that plateaued within 10 min (Fig. 1, B and C). The addition of DTT produced a further increase in H₂S, and this also typically plateaued within 10–15 min (Fig. 1, B–E). H₂S production was greatly enhanced when thiosulfate was added after DTT, and H₂S concentrations frequently continued to increase for up to 1 h (Fig. 1, B–E). Relatively little H₂S production from thiosulfate was observed when the thiosulfate was added prior to DTT; however, after adding DTT, there was an initial rapid increase in H₂S, which was then followed by a slower but prolonged increase in H₂S production (Fig. 1F). This had the appearance of an initial DTT effect followed by H₂S production from thiosulfate, but it did not appreciably change the total amount of H₂S produced. As might be expected, the greatest rate of H₂S production and maximum H₂S concentrations was observed in liver homogenates.

Venting the reaction chamber near the end of the experiment produced an immediate fall in H₂S concentration, indicative of tissue oxidation and volatilization (Fig. 1, C and E). Injection of a 100-μl bubble of oxygen into the reaction chamber also produced a slight but rapid drop in H₂S concentration (not shown). Given the considerable amount and rate of H₂S production, the oxygen bubble had less of an effect on H₂S production than venting the chamber.

Table 1. Maximum or near-maximum H₂S production in tissues incubated with sequential addition of 1 mM glutathione, 1 mM dithiothreitol, and 1 mM sodium thiosulfate (Na₂S₂O₃) or 10 mM DTT and 10 mM Na₂S₂O₃

<table>
<thead>
<tr>
<th>n</th>
<th>GSH (max)</th>
<th>DTT (max)</th>
<th>Na₂S₂O₃ (max)</th>
<th>Na₂S₂O₃ (rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>15</td>
<td>0.3 ± 0.04</td>
<td>1.7 ± 0.2*</td>
<td>0.8 ± 0.2*</td>
</tr>
<tr>
<td>Rat lung</td>
<td>7</td>
<td>0.1 ± 0.04**</td>
<td>3.7 ± 1.0*</td>
<td>11.5 ± 7.1**</td>
</tr>
<tr>
<td>Mouse lung</td>
<td>5</td>
<td>0.2 ± 0.1</td>
<td>1.1 ± 0.4</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Bovine heart</td>
<td>3</td>
<td>0.6 ± 0.1**</td>
<td>3.8 ± 1.2**</td>
<td>2.6 ± 0.7**</td>
</tr>
<tr>
<td>Rat liver</td>
<td>12</td>
<td>4.9 ± 2.9</td>
<td>12.5 ± 4.8</td>
<td>200.7 ± 60.8**</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>4</td>
<td>0.2 ± 0.1</td>
<td>3.3 ± 1.5</td>
<td>42.2 ± 30.4**</td>
</tr>
<tr>
<td>Buffer</td>
<td>14</td>
<td>2.3 ± 0.5</td>
<td>5.0 ± 0.1**</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>Bovine lung</td>
<td>4</td>
<td>6.8 ± 1.4**</td>
<td>3.9 ± 0.7**</td>
<td>7.9 ± 2.3**</td>
</tr>
<tr>
<td>Rat lung</td>
<td>3</td>
<td>11.0 ± 3.6**</td>
<td>38.1 ± 12.6**</td>
<td>26.6 ± 7.7***</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>5</td>
<td>3.3 ± 1.6</td>
<td>172.0 ± 76.6**</td>
<td>75.9 ± 27.4***</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = number of animals. Max, maximum H₂S produced expressed as μmole; rate, rate of H₂S production from Na₂S₂O₃ expressed as μmoles/g tissue/1 min. Because 0.15 g of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. B, BB, and BB are significantly different from respective buffer; G, GG, GGG are significantly different from respective GSH, and D, DD, and DDD show significant difference between DTT and Na₂S₂O₃ at P < 0.05, 0.01, and 0.001, respectively. * ** *** P < 0.05, 0.01, 0.001, respectively.

Table 2. Maximum or near-maximum H₂S production in tissues incubated with sequential addition of 10 mM GSH, 10 mM DTT, and 10 mM sodium thiosulfate (Na₂S₂O₃) or 10 mM DTT and 10 mM Na₂S₂O₃

<table>
<thead>
<tr>
<th>n</th>
<th>GSH (max)</th>
<th>DTT (max)</th>
<th>Na₂S₂O₃ (max)</th>
<th>Na₂S₂O₃ (rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>11</td>
<td>1.4 ± 0.5</td>
<td>18.5 ± 5.9**</td>
<td>36.7 ± 7.3**</td>
</tr>
<tr>
<td>Bovine lung</td>
<td>5</td>
<td>27.8 ± 16.6**</td>
<td>40.9 ± 7.6**</td>
<td>651.4 ± 150.2**</td>
</tr>
<tr>
<td>Rat lung</td>
<td>7</td>
<td>1.8 ± 1.5</td>
<td>31.0 ± 11.0**</td>
<td>429.4 ± 203.7**</td>
</tr>
<tr>
<td>Mouse lung</td>
<td>5</td>
<td>0.7 ± 0.4</td>
<td>30.3 ± 9.4*</td>
<td>197.9 ± 39.0**</td>
</tr>
<tr>
<td>Bovine heart</td>
<td>3</td>
<td>11.5 ± 3.6**</td>
<td>53.6 ± 0.5*</td>
<td>68.7 ± 4.5**</td>
</tr>
<tr>
<td>Rat liver</td>
<td>12</td>
<td>18.4 ± 8.4</td>
<td>55.9 ± 18.4</td>
<td>258.7 ± 91.4**</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>5</td>
<td>5.0 ± 2.6</td>
<td>27.2 ± 12.8</td>
<td>101.3 ± 70.0</td>
</tr>
<tr>
<td>Buffer</td>
<td>11</td>
<td>22.6 ± 4.1</td>
<td>147.7 ± 37.4**</td>
<td>19.1 ± 3.9</td>
</tr>
<tr>
<td>Bovine lung</td>
<td>4</td>
<td>94.5 ± 27.2**</td>
<td>315.5 ± 11.3**</td>
<td>279.1 ± 91.3***</td>
</tr>
<tr>
<td>Rat liver</td>
<td>3</td>
<td>113.3 ± 16.8**</td>
<td>616.7 ± 116.1**</td>
<td>75.9 ± 27.4***</td>
</tr>
<tr>
<td>Rat liver</td>
<td>2</td>
<td>82.1 ± 33.4**</td>
<td>72.4 ± 243.7</td>
<td>1154.3 ± 287.5</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>2</td>
<td>161.7 ± 50.2**</td>
<td>1278.9 ± 131.6**</td>
<td>1296.5 ± 24.8***</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = number of animals. Max, maximum H₂S produced expressed as μmole; rate, rate of H₂S production from Na₂S₂O₃ expressed as μmoles/g tissue/1 min. Because 0.15 g of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. B, BB, and BB are significantly different from respective buffer; G, GG, GGG are significantly different from respective GSH, and D, DD, and DDD show significant difference between DTT and Na₂S₂O₃ at P < 0.05, 0.01, and 0.001, respectively.
THIOSULFATE, H₂S, AND O₂ SENSING

Table 3. H₂S production in Cortland buffer and lamprey aorta

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>DTT</th>
<th>Na₂S₂O₃</th>
<th>Na₂S₂O₃ (rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortland buffer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM DTT/1 mM TS</td>
<td>4</td>
<td>2.98 ± 0.91</td>
<td>0.47 ± 0.24²</td>
<td>0.27 ± 0.07²</td>
</tr>
<tr>
<td>1 mM TS/1 mM DTT</td>
<td>3</td>
<td>3.74 ± 0.36</td>
<td>0.00</td>
<td>0.07 ± 0.07²</td>
</tr>
<tr>
<td>2 mM DTT/2 mM TS</td>
<td>3</td>
<td>5.88 ± 0.9</td>
<td>1.08 ± 0.43²</td>
<td>0.27 ± 0.07²</td>
</tr>
<tr>
<td>10 mM DTT/10 mM TS</td>
<td>3</td>
<td>30.74 ± 9.97</td>
<td>47.05 ± 20.37</td>
<td>7.67 ± 4.3</td>
</tr>
<tr>
<td>10 μM DTT/1 mM TS</td>
<td>3</td>
<td>0.90 ± 0.16</td>
<td>0.08 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>100 μM DTT/1 mM TS</td>
<td>4</td>
<td>19.21 ± 3.45</td>
<td>1.93 ± 0.33²</td>
<td></td>
</tr>
<tr>
<td>Lampey aorta</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM DTT/1 mM TS</td>
<td>4</td>
<td>4.12 ± 1.4</td>
<td>35.8 ± 6.07²</td>
<td>2.80 ± 0.8³</td>
</tr>
<tr>
<td>1 mM TS/1 mM DTT</td>
<td>3</td>
<td>20.77 ± 8.31</td>
<td>30.52 ± 15.8</td>
<td>3.47 ± 0.7³</td>
</tr>
<tr>
<td>2 mM DTT/2 mM TS</td>
<td>4</td>
<td>45.84 ± 10.36</td>
<td>85.26 ± 28.52</td>
<td>8.80 ± 2.07</td>
</tr>
<tr>
<td>10 mM DTT/10 mM TS</td>
<td>6</td>
<td>19.11 ± 3.51</td>
<td>28.52 ± 8.80</td>
<td></td>
</tr>
<tr>
<td>Values are expressed as means ± SE; n = number of animals. TS, thiosulfate; Rate, rate of H₂S production from Na₂S₂O₃ expressed as µmol·g tissue⁻¹·min⁻¹. Because 0.15 g of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. B and BB are significantly different from respective buffer; D and DD show significant difference between DTT and Na₂S₂O₃.</td>
<td></td>
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</tbody>
</table>

H₂S Production by Lamprey Aorta

The addition of DTT to homogenized lamprey aorta produced an initial increase in H₂S production that was similar to DTT addition to buffer; however, subsequent addition of thiosulfate now produced a steady increase in H₂S concentration that continued for well over an hour. The responses of lamprey aorta (Table 3) were similar to those observed with mammalian tissues. There was no noticeable H₂S production when thiosulfate was added to buffer prior to DTT, whereas the addition of thiosulfate to homogenized aorta prior to DTT considerably increased H₂S production. As with mammalian tissues, the increases in H₂S correlated with the concentrations of DTT and thiosulfate (Fig. 2).

H₂S Production from Thiosulfate and DHLA

H₂S production in buffer. The addition of 1 mM DHLA, and occasionally 100 µM DHLA, produced an immediate increase in H₂S; subsequent addition of 1 mM thiosulfate did not appear to augment H₂S production (Fig. 3A). When the order of addition was reversed, again thiosulfate had no effect on H₂S production, 100 µM DHLA slightly increased H₂S, and 1 mM DHLA produced a significant rise in H₂S (Fig. 3B). These results suggest that H₂S production was the result of spontaneous H₂S formation from DHLA and not due to DHLA-mediated reduction of thiosulfate. The maximum H₂S concentration and rate of formation are listed in Table 4. Significantly, more H₂S was formed in buffer from 1 mM DHLA than was formed from 1 mM DTT, or 1 mM DTT, and 1 mM thiosulfate combined (refer to Tables 1 and 4).

Fig. 2. Dose-dependent effect of DTT or TS after DTT on H₂S production in Cortland buffer or lamprey aorta measured in real time with the polarographic electrode. *Significantly different from respective buffer. #Significantly different from DTT in tissue; mean ± SE; n = 3–6.

Fig. 3. Polarographic, real-time measurements of H₂S production after the additions of dihydrolipoic acid (DHLA) and TS to buffer (A and B) or mouse liver (C and D) in a closed, anoxic chamber. In buffer, 100 µM DHLA produced a slight increase in H₂S, and 1 mM DHLA produced a rapid increase in H₂S. TS did not affect H₂S production when added either before or after DHLA. Maximal H₂S production in mouse liver required both TH and DHLA as the initial addition of TS did not increase H₂S (C) and when DHLA was added first (D), there was only a slight increase in H₂S. H₂S production by liver was an order of magnitude greater than H₂S production in buffer (compare scales in A and B to C and D). Opening the chamber to the atmosphere (air; C and D) rapidly decreased H₂S concentration due to the combined effect of tissue oxidation and volatilization.
Table 4. Combined data for maximum or near-maximum H₂S production in Krebs-Henseleit buffer or mammalian tissues incubated with sequential addition of 100 μM DHLA, 1 mM DHLA, and 1 mM Na₂S₂O₃, or 1 mM Na₂S₂O₃ followed by 100 μM and then 1 mM DHLA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Maximum H₂S Produced</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>10</td>
<td>5.5 ± 1.0</td>
</tr>
<tr>
<td>Rat liver</td>
<td>8</td>
<td>150.1 ± 21.0&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat lung</td>
<td>5</td>
<td>45.5 ± 12.2&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>7</td>
<td>62.5 ± 24.7&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bovine lung</td>
<td>4</td>
<td>46.3 ± 24.9&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = number of animals. DHLA, dihydrolipoic acid; Max, maximum H₂S produced expressed as μmol; rate, rate of H₂S production expressed as μmol·g tissue⁻¹·min⁻¹. Because 0.15 g of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. B, BB, and BBB are significantly different from respective buffer, at P < 0.05, 0.01, and 0.001, respectively.

H₂S production in tissue. The effects of thiosulfate and DHLA on H₂S production by rat and mouse liver and rat and bovine lung are tabulated in Table 4, and representative traces of H₂S production in mouse liver are shown in Fig. 3, C and D. Significantly more H₂S was produced by incubating tissues with thiosulfate and DHLA than was formed by these compounds in buffer alone, whereas only rat liver produced H₂S at a significantly faster rate than buffer. Furthermore, unlike that observed in buffer, maximal H₂S production from DHLA alone was negligible compared with DHLA plus thiosulfate, irrespective of the order of addition of these compounds. There was no difference in the maximum amount of H₂S formed from thiosulfate and either DTT or DHLA in any tissue (refer to Tables 1 and 4).

Effects of DTT and Thiosulfate on Bovine Hypoxic Pulmonary Vasoconstriction

Effects of prestimulation. As shown in Fig. 4, the magnitude of hypoxic pulmonary vasoconstriction (HPV) increases with consecutive hypoxic stimuli in otherwise unstimulated vessels but only slightly increases in vessels prestimulated with the thromboxane mimic, U-46619. However, the magnitude of HPV is considerably, and dose-dependently, increased by pre-stimulation with U-46619.

Effects of DTT and Thiosulfate on HPV in Unstimulated and Prestimulated Vessels

The effects of DTT and thiosulfate on HPV in otherwise unstimulated bovine pulmonary arteries are shown in Fig. 5A (0 U-46619). Low concentrations (1 mM) of DTT did not significantly increase HPV, whereas 10 mM DTT augmented the second and, to a lesser extent, the third HPV. Adding thiosulfate did not significantly change the DTT response, except it appeared to reverse the relaxation of the second hypoxia in unstimulated vessels at 1 mM.

Substantially different responses to DTT and thiosulfate were observed in vessels precontracted with 10⁻⁶ M U-46619 (Fig. 5B). Here, both 1 and 10 mM DTT greatly enhanced the magnitude of the initial HPV, whereas the magnitude of the second and third HPV decreased considerably (a net relaxation was observed in the second HPV treated with 10 mM DTT). When thiosulfate was added with DTT, the magnitude of the second and third HPV was reduced compared with the DTT alone.

The dose dependence of the DTT effects on HPV in unstimulated and prestimulated bovine pulmonary arteries is shown in Fig. 6, A and B, respectively. DTT (1 μM - 10 μM) did not affect HPV in otherwise unstimulated vessels, whereas it significantly increased HPV at concentrations of 100 μM and above. Similarly, DTT from 1 μM to 100 μM had no effect on HPV in prestimulated (10⁻⁶ M U-46619) vessels; however, it now inhibited HPV at 1 mM and produced a net relaxation at 10 mM. Addition of sulfite or sulfate (up to 10 mM) did not affect HPV (not shown).

Effects of DHLA on Bovine Hypoxic Pulmonary Vasoconstriction

The effects of 100 μM DHLA on HPV in bovine pulmonary arteries were more variable than the effects of DTT (Fig. 7). In some vessels DHLA alone had minimal effect on vessel tone...
during normoxia or on the HPV response (Fig. 7A). In other vessels, DHLA produced a modest contraction during normoxia and augmented the subsequent HPV (Fig. 7B). In many other vessels, DHLA substantially contracted the vessels and further augmented the HPV (Fig. 7C). A summary of the latter responses is shown in Fig. 7D. In ~10% of the vessels, 10 μM DHLA contracted normoxic vessels and also augmented HPV (not shown).

Effects of DHLA on Precontracted Mouse and Rat Aortas

The ability of DHLA to relax U-46619 precontracted mouse and rat aortas is shown in Fig. 8. At 10^{-5} M DHLA, the vessels were completely relaxed; the approximate EC_{50}s for these responses are around 30 μM (mouse) and 70 μM (rat).

DISCUSSION

We show in the present experiments that H2S is directly liberated from thiosulfate by the reducing agent DTT in buffer and that the presence of vertebrate tissues greatly enhances H2S production. Although the endogenous reductant, DHLA, does not spontaneously liberate H2S from thiosulfate in buffer, it does liberate H2S from thiosulfate in the presence of tissues with essentially the same efficacy as DTT. We also show that the addition of either DTT or DHLA to bovine pulmonary arteries increases the magnitude of hypoxic pulmonary vasoconstriction if these arteries are not prestimulated with another agonist. When pulmonary arteries are prestimulated with another agonist, DTT no longer augments hypoxic pulmonary vasoconstriction, indicating that prestimulation alters, or may conceal, the basic mechanism of hypoxic signaling. DHLA also directly contracts bovine pulmonary arteries and relaxes precontracted rat and mouse aortas. Both pulmonary and systemic vascular responses are consistent with the effects of exogenous H2S and hypoxia on these vessels. Although the exact concentration of DHLA in cells is somewhat questionable, it may be as high as 40 μM (16), which is within the range of vascular responses that we observed. Collectively, these studies suggest that DHLA releases H2S from endoge-

Fig. 5. Effects of DTT and thiosulfate (S_{2}O_{3}^{2-}) on consecutive (second, open bars; third, gray bars; fourth, black bars) hypoxic pulmonary vasoconstriction (HPV) in otherwise unstimulated (A) or 10^{-6} M U-46619 precontracted bovine pulmonary arteries (B). In otherwise unstimulated vessels, 1 and 10 mM DTT increased the magnitude of the third and/or fourth HPV, whereas in vessels precontracted with 10^{-6} M U-46619, DTT inhibited these contractions. The addition of thiosulfate did not significantly affect the DTT response. *Significantly different from second contraction, mean ± SE; number within parentheses denotes the number of vessels.
nous thiosulfate. In the mouse and rat aortas, the fact that exogenous thiosulfate did not enhance HPV is not surprising, as it is not readily taken up by cells, and, in fact, thiosulfate is often used as an indicator of extracellular volume (8). From these studies, we infer that H₂S can be endogenously generated from thiosulfate in cells, and this is an integral component of the mechanism of H₂S-mediated oxygen sensing in the vasculature.

Reduction of Thiosulfate to Form H₂S

Formation of H₂S from thiosulfate is favored by the oxidation states of the sulfur molecules in thiosulfate. Although the general calculation of the oxidation state of thiosulfate would suggest that both sulfur molecules are +2, which would render either one of them somewhat difficult to reduce back to H₂S (−2), this is not the case. Vairavamurthy et al. (43) have shown that these sulfur molecules actually exist in different oxidation states, such that the central sulfur that is bound to the three oxygen molecules has an oxidation state of +5, whereas the peripheral sulfur has an oxidation state of −1. The latter can, therefore, be considered a sulfane sulfur, i.e., the zero-valent form of sulfur which is attached to a molecule through a covalent bond between the zero-valent sulfur atom and other sulfur atoms. In the presence of a dithiol, such as DTT or DHLA, each sulfur in thiosulfate can undergo a 1 electron reduction, thereby forming sulfide (S = −2, i.e., H₂S and HS− at physiological pH) and sulfite (S = +4). In this process, DTT is oxidized and becomes a six-membered ring with an internal disulfide bond, while the DHLA becomes α-lipoic acid with a disulfide bond between the sulfurs at C6 and C8, forming a thiolute ring. In cells the α-lipoic acid can be reduced back to DHLA by reduced thioredoxin, NADPH or NADP⁺ (13).

Critical Role of Mitochondria in H₂S-Mediated Oxygen Sensing

In our initial hypothesis of H₂S-mediated oxygen sensing (24), we presumed that H₂S production was a cytoplasmic event and that oxidation of H₂S occurred in the mitochondria. This was supported by observations that, at the time, indicated a cytoplasmic presence for both CBS and the CSE, and the presence of H₂S-oxidizing enzymes, sulfur quinone oxidoreductase (SQR), sulfur transferase (ST), and sulfite oxidase (SO), in the mitochondria. This also had anecdotal support from an evolutionary perspective, as it was proposed that mitochondria originated from a sulfide oxidizing α-proteobacteria and the cytoplasm from sulfide reducing Archea (33). In addition SQR can be traced back to this initial endosymbiotic event (40). However, the present experiments suggest that the mitochondria are involved in both H₂S production and metabolism. This is consistent with the general opinion that because mitochondria are the primary oxygen consumers in cells, they are intimately involved in the oxygen-sensing process (37).

A number of observations suggest that H₂S is synthesized in the mitochondria. 1) As described in the introduction, 3-MST and other key enzymes are found in the mitochondria (17), and
hypoxia increases the reducing conditions of the mitochondrial matrix (46), which would favor liberation of H$_2$S from the sulfane sulfur on 3-MST. 2) Mitochondria are the site of thiosulfate synthesis, and our study shows that H$_2$S can be generated from thiosulfate under reducing conditions. 3) Stress has recently been shown to cause CSE translocation from the cytosol to the mitochondria, where it can take advantage of cysteine concentrations that are considerably higher (3 mM) than the surrounding cytosol (9). Although CSE translocation is not evident until 16 h after the initial stressor (9) and would not be expected to be involved in the initial hypoxic response, it could bias the overall rate of mitochondrial H$_2$S production.

Most inactivation of H$_2$S is also believed to occur in the mitochondria with the initial step catalyzed by SQR (11), although an alternative, as yet unidentified, pathway may be present in the brain (19). One H$_2$S molecule binds to each of two enzymes and two-electron oxidation of H$_2$S forms a sulfane sulfur (persulfide) complex with each enzyme. In the mechanism described by Hildebrandt and Grieshaber (11), these sulfane sulfurs are then transferred to a mobile carrier that has yet to be unequivocally identified. One of the sulfane sulfurs is delivered to mitochondrial sulfur dioxygenase (ETHE1), where it is oxidized to sulfite. The other sulfane sulfur is then transferred to sulfite by ST, thereby, forming thiosulfate ($S_2O_3^{2-}$). In most vertebrates, sulfite is further oxidized to sulfate (SO$_4^{2-}$) by SO and excreted. Jackson et al. (12), using purified recombinant human SQR generated in Escherichia coli, suggested the mobile sulfane sulfur carrier is sulfite (SO$_4^{2-}$), and sulfur is transported as thiosulfate. In their model, the sulfane sulfur of thiosulfate is transferred to glutathione by the enzyme ST forming glutathione persulfide (GSS$^-$. Glutathione is then regenerated by sulfur dioxygenase, which consumes molecular oxygen and results in the formation of a second sulfite, which can again act as the persulfide acceptor of SQR oxidation of H$_2$S. They also show that the metabolism of thiosulfate is catalyzed by thiosulfate reductase and consumes two equivalents of glutathione and results in the generation of H$_2$S, sulfite, and oxidized glutathione. Thus, in their scheme, sulfite is recycled in the oxidation of H$_2$S, and it can also be further oxidized to sulfate as a mechanism of sulfur excretion. Our results do not support the involvement of glutathione in H$_2$S production from thiosulfate but irrespective of the exact mechanism of sulfur transfer, thiosulfate appears to be a key intermediate.

$H_2S$, Thiolsulfate, and Oxygen Sensing

Figure 9 illustrates how thiosulfate can be integrated into the oxygen-sensing mechanism. Under normoxic conditions, H$_2$S produced from thiol (e.g., cysteine or homocysteine) metabolism is initially oxidized to thiosulfate and then to sulfite, and the latter is subsequently excreted in the urine. During hypoxia, the decrease in oxygen concentration prevents further oxidation of H$_2$S to thiosulfate (Fig. 9, pathway no. 1), while formation of H$_2$S from thiols continues because these processes are oxygen independent. This increases H$_2$S concentration. Furthermore, the thiosulfate that was previously formed can no longer be oxidized through sulfite to sulfate and, as the mitochondrial environment becomes more reduced by hypoxia (46), the concentration of endogenous reductants, such as DHLA, increases, and now, H$_2$S is regenerated from this previously formed thiosulfate. H$_2$S generation from thiosulfate has the added advantage of increasing the concentration of the H$_2$S signal without consuming additional thiols, and it is likely to be faster in onset than H$_2$S production from cysteine or other thiols. This minimizes the burden of oxygen sensing on overall sulfur metabolism. Of course, it is possible that CBS, CSE, or 3-MST are activated by hypoxia, but there is no evidence for this to date. Hypoxia may also activate these enzymes indirectly, as has been suggested in the carotid body glomus cells where hypoxia decreases carbon monoxide (CO) production, which, in turn, removes a CO-mediated inhibition of CSE (29).

Figure 9 shows a surprising number of pathways in H$_2$S metabolism, in addition to O$_2$ delivery to the mitochondrion at complex IV (Fig. 9, pathway no. 1) and thiosulfate (Fig. 9, pathways nos. 2 and 3) or other persulfide (Fig. 9, pathway no. 3) reduction, which involve oxygen and potentially contribute to H$_2$S-mediated oxygen sensing. Disruption of genes encoding proteins that catalyze these pathways, either in experimental animals or human mutations, in many instances provides evidence for concomitant increases in both H$_2$S and thiosulfate, thereby strengthening the hypothesis of H$_2$S-mediated oxygen sensing and the role of thiosulfate in this process.

The mitochondrial sulfur dioxygenase, ETHE1, uses molecular O$_2$ and water to oxidize the mobile persulfide from SQR to form sulfite (Fig. 9, pathway no. 4). ETHE1 deficiency in either experimental animals or humans prevents H$_2$S binding and is characterized by greatly elevated tissue H$_2$S and thiosulfate (3, 7, 10, 41). Because of the O$_2$ dependency of this reaction, hypoxia would be expected to achieve the same result.

Mitochondrial SO catalyzes the oxidation of sulfite to sulfate by transferring an atom of oxygen from water to sulfite, and in the process, the enzyme undergoes a two-electron reduction (31). The resultant electrons are then transferred from the enzyme to cytochrome c and on to complex IV, thereby coupling sulfite oxidation (and concentration) to oxygen availability (Fig. 9, pathway no. 5). As urinary thiosulfate is elevated in humans with SO deficiency (22), it is likely that hypoxia would achieve the same result.

One of the primary functions of the cytosolic enzyme cysteine dioxygenase (CDO) is to irreversibly catalyze the oxidation of cysteine to cysteinesulfinate (Fig. 9, pathway no. 6), which effectively detoxifies excess dietary or metabolic cysteine and eliminates the possibility of H$_2$S formation (36). Because CDO is dynamically regulated by cysteine concentration, whereas the H$_2$S-forming transsulfuration pathway is not (36), a reduction in CDO activity would be expected to increase cysteine concentration and by mass-action favor transsulfuration. Indeed, this is the case, as both thiosulfate and H$_2$S increase in CDO knockout mice, similar to that observed with ETHE1 deficiency (32, 42). As molecular O$_2$ is the only other substrate in dioxygenase-catalyzed reactions, it is likely that hypoxia will similarly impair cysteine oxidation and favor H$_2$S production. However, because this process depends on buildup of cysteine to produce H$_2$S, it probably places a long-term bias on H$_2$S-mediated O$_2$ sensing rather than contributing to the acute response. Anecdotally, this may partially explain how hypoxic responses are augmented by exogenous cysteine (cf. 23).
Oxygen-Sensing Mechanism is Obscured by Prestimulation

Precontracting pulmonary vessels with another agonist, such as U-46619, is often used to enhance HPV (37). We previously observed that the addition of sulfur donors, such as cysteine, oxidized and reduced glutathione, and cysteine plus α-ketoglutarate greatly increased the magnitude of HPV in bovine pulmonary arteries that were not precontracted, whereas there was little effect of these donors when the vessels were precontracted with U-46619 (28). We proposed that the strong stimulation by U-46619 overwhelmed any observable effect of H$_2$S. The present observation that DTT did not increase HPV shows that tissues have the ability to rescue sulfide from an otherwise catalytic pathway and integrate it into the oxygen-sensing mechanism. Mobilization of H$_2$S from thiosulfate may, otherwise catalytic pathway and integrate it into the oxygen-sensing mechanism, be another example of H$_2$S-mediated oxygen sensing, but it does not provide yet another example of H$_2$S-mediated oxygen sensing. It is becoming increasingly evident that organisms have had a long association with H$_2$S and that this molecule is woven into the fabric of many signaling systems. Perhaps the most ancient of these associations is the seemingly mutually exclusive relationship between oxygen and H$_2$S, and there is increasing evidence that cells exploit this relationship to detect and respond to hypoxia. The present work not only provides
ACKNOWLEDGMENTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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