Thiosulfate: a Readily Accessible Source of Hydrogen Sulfide in Oxygen Sensing

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ABSTRACT

H₂S derived from organic thiol metabolism has been proposed serve as an oxygen sensor in a variety of systems due to its susceptibility for 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 regenerate H₂S. We propose 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, dithiothreitol (DTT), or the endogenous reductant dihydrolipoic acid (DHLA). Both the magnitude and rate of H₂S production was 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.
**Introduction**

There is increasing evidence that hydrogen sulfide (H$_2$S) 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), non-vascular smooth muscle (5, 6) and even activation of hypoxia inducible factor 1 (HIF-1; 15). In our original model of H$_2$S-mediated O$_2$ sensing we presumed that H$_2$S 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 H$_2$S, 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 $\alpha$-protobacterium (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 H$_2$S biosynthesis as H$_2$S can be generated from two well characterized enzymes, cystathione $\beta$-synthase (CBS) and cystathione $\gamma$-lyase (CSE). However, other studies have shown that a variety of thiols such as glutathione, homocysteine, cystathionine, methionine can either produce H$_2$S directly or augment the hypoxic response (1, 20, 28) and another pathway for H$_2$S production from cysteine involving cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfur transferase (3-MST) has recently been described (34). It has also been generally assumed that mitochondrial catabolism of H$_2$S in vertebrates proceeds through a series of oxidation steps with...
thiosulfate ($S_2O_3^{2-}$) as an intermediate and sulfate ($SO_4^{2-}$) as the ultimate excretory product. However, Villarejo and Westley (44) observed that in the presence of the endogenous reductant, dihydrolipoic acid (DHLA), $H_2S$ 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 this would favor formation of $H_2S$ from thiosulfate, it seems reasonable to assume that during hypoxia $H_2S$ could be generated from the thiosulfate pool and immediately contribute to the $O_2$ sensing process. This would not only provide a readily available mechanism for rapidly increasing intracellular $H_2S$ it would also conserve organic thiols. To our knowledge, this has not been experimentally verified in oxygen sensing tissues.

In the present study, we examined the possibility that $H_2S$ could be regenerated from thiosulfate by tissue and that this should augment the hypoxic response. To accomplish this, we first measured $H_2S$ production from thiosulfate in the presence of the exogenous reductant, dithiothreitol (DTT) in a variety of tissues using an amperometric (polarographic) $H_2S$ sensor constructed in our laboratory. This enabled us to measure $H_2S$ 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 $H_2S$ production in select tissues as well as its effects on HPV and on relaxation of precontracted mouse and rat thoracic aortas where hypoxia and $H_2S$ produce vasorelaxation (24). In previous experiments, we also noticed that pre-contracting bovine pulmonary arteries with the thromboxane $A_2$ 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) became
less evident (28). We proposed that prestimulation so activated the downstream signaling that it
obscured the initial O₂-sensing events. To verify this, the effects of pre-stimulation on the
hypoxic responses to DTT were also examined.

**MATERIALS AND METHODS**

*Animals*

Adult Lobund-Wistar (LW) rats (0.35-0.5 kg) and mice (~25 g, from a variety of strains) were maintained at the University of Notre Dame, Friemann 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 CO₂ 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 was 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 dia) were dissected free
from the lung tissue and stored in buffer in the refrigerator. Tissue for H₂S 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 (IUSM-SB) where they were maintained in 500-liter
rectangular tanks with aerated, flowing well water (15°C) and exposed to a 12:12 h L:D photoperiod. These fish no longer feed. 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 approved by institutional IACUC review.

**Tissue H$_2$S production in the presence of dithiothreitol (DTT)**

Mammalian tissues were minced with scissors and then homogenized in buffer (1:9; W:V) on ice for 15 seconds using an Ultra Turrax model SDT homogenizer with a 10N probe (Teckmar, Cincinnati, OH). The homogenate was then placed in an ice bath and deoxygenated by sparging for 30 min with humidified nitrogen. One and one-half ml of homogenate was then placed in a reaction chamber (see below) that was fitted with a plunger to remove head-space gas and prevent H$_2$S 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$_2$S gas was monitored with an amperometric (polarographic) sensor (see below). Tissue-free buffer, used as a control, was processed similarly. Reduced glutathione (GSH), dithiothreitol (DTT) and sodium thiosulfate (Na$_2$S$_2$O$_3$) 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$_2$S 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$_2$S, H$_2$S production after addition of DTT was somewhat longer. Because H$_2$S production was maintained for a considerable period after addition of thiosulfate, values were typically recorded for longer periods (30-60 min). To verify that H$_2$S
production from thiosulfate was an oxygen-dependent process the chamber was opened to the atmosphere or approximately 100 μl 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$_2$S production in the presence of dihydrolipoic acid (DHLA)*

Dihydrolipoic acid (DHLA) was prepared as described below. The effects of DHLA on H$_2$S 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 μM and 1 mM) and one thiosulfate concentration (1 mM) were examined as preliminary experiments suggested this was adequate for H$_2$S generation. The order of addition thiosulfate versus DHLA was reversed in half of the experiments to confirm that both were necessary for H$_2$S production. In all experiments the response to 100 μM DHLA was allowed to plateau and then 1 mM DHLA was added. Because the response to 100 μM 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$_2$S 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$_2$S 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$_2$S 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$_2$S 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$_2$S (63% response in 8 s). The sensor was connected to an TBR 4100 Free Radical Analyzer (WPI, Sarasota FL) with 100 mV polarizing voltage. Data was 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$_2$S. Stock solutions were made fresh daily.

**Protocol.** In preliminary experiments we observed that H$_2$S 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; 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$_2$S production. H$_2$S concentration was recorded continuously after addition of each compound and
the subsequent compound was not added until H$_2$S production from the previous one had appeared to plateau. The time for H$_2$S production to plateau after GSH addition was relatively short, typically within 5 to 10 min; the time for the H$_2$S plateau after DTT was generally 10-15 min. H$_2$S concentration continued to increase, often for over 1 hr, 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$_2$S production after thiosulfate addition likely underestimate the total H$_2$S generative capacity.

**Vessel myography**

Bovine pulmonary arteries were cut into 3-8 mm-long segments and mounted on 280-μm-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% CO$_2$. 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, West Warwick, RI) and data was archived on a PC computer using SoftWire A-D conversion (Measurement Computing, Middleboro, MA). In subsequent experiments iWorx Systems Inc. (Dover, NH) force transducers (FT-302) and data-loggers (308T) were used and the data archived on PCs. The transducers were calibrated prior to each experiment to detect changes as small as 5 mg.

Baseline (resting) tension (approximately 1000 mg) was applied to the vessels for 0.5-1 hour prior to experimentation. In a typical experiment, vessels were contracted twice with 80 mM KCl and resting tension re-established after each contraction during a 30-45 min rinse (4x) period. Hypoxic pulmonary vasoconstriction (HPV) was produced by gassing with 95% N$_2$/5%
CO₂. This typically lowers Po₂ 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 prestimulation on hypoxic response 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 pre-contracted with 10⁻⁶ M U-46619 was examined 15-20 min after the drugs were added. The effects of DHLA on HPV were examined in otherwise unstimulated vessels 20 min after 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 tenfold increments from 10⁻⁶ to 10⁻³ M.

Preparation of dihydrolipoic acid (DHLA)

Dihydrolipoic acid (DHLA) was prepared after the method of Volini and Wesley, (45) as modified by Mikami and Kimura (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 approximately 5.0). Acidification converts excess BH₄ to 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.

Krebs-Henseleit (in mM): 115 NaCl, 2.5 KCl, 2.46 MgSO₄, 2 CaCl₂·2H₂O, 5.6 glucose, 1.38 NaH₂PO₄, and 25 NaHCO₃, 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), pH 7.8. Potassium phosphate: 100 mM KH₂PO₄/K₂HPO₄. Cortland (in mM): NaCl, 124; KCl, 3; MgSO₄·7H₂O, 0.57; CaCl₂·2H₂O, 2; NaH₂PO₄, 0.09; Na₂HPO₄, 1.8; NaHCO₃, 12; glucose, 5.5; pH 7.8.

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

Data Analysis.

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

RESULTS

H₂S production from thiosulfate and DTT

H₂S production in buffer
H$_2$S production was observed in Krebs Henseleit buffer following the addition of glutathione, DTT and thiosulfate (Fig. 1A; Tables 1, 2). The amount of H$_2$S produced from glutathione alone was relatively small (<0.1%), whereas around 10 times as much H$_2$S was produced by addition of DTT. The H$_2$S production from DTT plateaued within several minutes (Fig. 1A), suggesting that this was a contaminant of DTT rather than actual H$_2$S production in buffer. Addition of thiosulfate to the DTT further increased H$_2$S concentration and this did not rapidly plateau suggesting that the H$_2$S was continually evolving from thiosulfate. Omission of glutathione did not significantly affect H$_2$S production from either the DTT or thiosulfate indicating that glutathione does not contribute to H$_2$S production in buffer. Similar effects were observed upon addition of DTT and thiosulfate to Cortland buffer indicating that H$_2$S 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 H$_2$S production from both DTT and thiosulfate indicating that the reducing conditions provided by DTT were important in generation of H$_2$S from thiosulfate.

H$_2$S 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 H$_2$S 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. 1B-F. Typically, the responses were similar to those observed in buffer albeit at greater rates of H$_2$S production after addition of DTT and thiosulfate. Addition of 1 or 10 mM GSH to homogenized tissue either failed to generate H$_2$S, or produced a slight increase in H$_2$S that
plateaued within 10 min (Fig. 1B, C). Addition of DTT produced a further increase in H$_2$S and this also typically plateaued within 10-15 min (Fig. 1B-E). H$_2$S production was greatly enhanced when thiosulfate was added after DTT and H$_2$S concentrations frequently continued to increase for up to 1 hr (Fig. 1B-E). Relatively little H$_2$S production from thiosulfate was observed if the thiosulfate was added prior to DTT, however after adding DTT there was an initial rapid increase in H$_2$S which was then followed by a slower but prolonged increase in H$_2$S production (Fig. 1F). This had the appearance of an initial DTT effect followed by H$_2$S production from thiosulfate but it did not appreciably change the total amount of H$_2$S produced. As might be expected, the greatest rate of H$_2$S production and maximum H$_2$S concentrations were observed in liver homogenates.

Venting the reaction chamber near the end of the experiment produced an immediate fall in H$_2$S concentration indicative of tissue oxidation and volatilization (Fig. 1C, E). Injection of a 100 μl bubble of oxygen into the reaction chamber also produced a slight but rapid drop in H$_2$S concentration (not shown). Given the considerable amount and rate of H$_2$S production the oxygen bubble had less of an effect on H$_2$S production than venting the chamber.

**H$_2$S production by lamprey aorta**

Addition of DTT to homogenized lamprey aorta produced an initial increase in H$_2$S production that was similar to DTT addition to buffer, however, subsequent addition of thiosulfate now produced a steady increase in H$_2$S 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 H$_2$S production when thiosulfate was added to buffer prior to
DTT, whereas addition of thiosulfate to homogenized aorta prior to DTT considerably increased H$_2$S production. As with mammalian tissues the increases in H$_2$S correlated with the concentrations of DTT and thiosulfate (Fig. 2).

**H$_2$S production from thiosulfate and DHLA**

**H$_2$S production in buffer**

Addition of 1 mM DHLA, and occasionally 100 μM DHLA produced an immediate increase in H$_2$S; subsequent addition of 1 mM thiosulfate did not appear to augment H$_2$S production (Fig. 3A). When the order of addition was reversed, again thiosulfate had no affect on H$_2$S production, 100 μM DHLA slightly increased H$_2$S and 1 mM DHLA produced a significant rise in H$_2$S (Fig. 3B). These results suggests that H$_2$S production was the result of spontaneous H$_2$S formation from DHLA and not due to DHLA-mediated reduction of thiosulfate. The maximum H$_2$S concentration and rate of formation are listed in Table 4. Significantly more H$_2$S was formed in buffer from 1 mM DHLA than was formed from 1 mM DTT, or 1 mM DTT and 1 mM thiosulfate combined (Cf Tables 1 and 4).

**H$_2$S production in tissue**

The effects of thiosulfate and DHLA on H$_2$S production by rat and mouse liver and rat and bovine lung are tabulated in Table 4 and representative traces of H$_2$S production in mouse liver is shown in Fig. 3C, D. Significantly more H$_2$S was produced by incubating tissues with thiosulfate and DHLA than was formed by these compounds in buffer alone, whereas only rat liver produced H$_2$S at a significantly faster rate than buffer. Furthermore, unlike that observed in
buffer, maximal H$_2$S production from DHLA alone was negligible compared to DHLA plus thiosulfate, irrespective of the order of addition of these compounds. There was no difference in the maximum amount of H$_2$S formed from thiosulfate and either DTT or DHLA in any tissue (Cf Tables 1 and 4).

Effects of DTT and thiosulfate on bovine hypoxic pulmonary vasoconstriction

Effects of pre-stimulation

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 mimetic, 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 pre-contracted 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 were reduced compared to the DTT alone.

The dose-dependence of the DTT effects on HPV in unstimulated and prestimulated bovine pulmonary arteries is shown in Fig. 6A 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 affect on HPV in prestimulated (10^-6 M U-46619) vessels, however, it now inhibited HPV at 1 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 approximately 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 pre-contracted mouse and rat aortas is shown in
Fig. 8. At $10^{-3} \text{ M}$ DHLA the vessels were completely relaxed; the approximate EC$_{50}$s for these responses are around 30 $\mu\text{M}$ (mouse) and 70 $\mu\text{M}$ (rat).
We show in the present experiments that H$_2$S is directly liberated from thiosulfate by the reducing agent DTT in buffer and that the presence of vertebrate tissues greatly enhances H$_2$S production. Although the endogenous reductant, DHLA, does not spontaneously liberate H$_2$S from thiosulfate in buffer, it does liberate H$_2$S from thiosulfate in the presence of tissues with essentially the same efficacy as DTT. We also show that 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 H$_2$S 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 of responses we observed. Collectively these studies suggest that DHLA releases H$_2$S from endogenous 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 H$_2$S can be endogenously generated from thiosulfate in cells and this is an integral component of the mechanism of H$_2$S-mediated oxygen sensing in the vasculature.

Reduction of thiosulfate to form H$_2$S
Formation of H$_2$S 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 H$_2$S (-2), this is not the case. Vairadamurthy 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., H$_2$S and HS$^-$ at physiological pH) and sulfite (S = +4). In this process DTT is oxidized and becomes six-membered ring with an internal disulfide bond while the DHLA becomes lipoic acid with a disulfide bond between the sulfurs at C6 and C8 forming a thiolane ring. In cells the lipoic acid can be reduced back to DHLA by reduced thioredoxin, NADP or NADPH (13).

Critical role of mitochondria in H$_2$S-mediated oxygen sensing

In our initial hypothesis of H$_2$S-mediated oxygen sensing (24), we presumed that H$_2$S production was a cytoplasmic event and that oxidation of H$_2$S 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 H$_2$S oxidizing enzymes, SQR, ST and 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-protobacteria 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 H₂S from the sulfane sulfur on 3-MST. 2) mitochondria are the site of thiosulfate synthesis and our study shows that H₂S 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 H₂S production.

Most inactivation of H₂S is also believed to occur in the mitochondria with the initial step catalyzed by sulfur quinone oxidoreductase (SQR; 11), although an alternative, as yet unidentified pathway may be present in the brain (19). One H₂S molecule binds to each of two enzymes and two electron oxidation of H₂S 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 sulfur transferase (ST) thereby forming thiosulfate (S$_2$O$_3^{2-}$). In most vertebrates, sulfite is further oxidized to sulfate (SO$_4^{2-}$) by sulfite oxidase (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$_3^{2-}$) and sulfur is transported as thiosulfate. In their model, the sulfane sulfur of thiosulfate is transferred to glutathione by the enzyme sulfur transferase (ST) forming glutathione persulfide (GSS$^{\cdot}$). Glutathione is then regenerated by sulfur dioxygenase (SDO) which consumes molecular oxygen and results in 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 (GSSG). 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$_2$S, Thiosulfate 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 sulfate, 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 #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 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 #1) and thiosulfate (Fig. 9 pathways #2 and 3) or other persulfide (Fig. 9 pathway #3) reduction, that involve oxygen and can potentially contribute to H$_2$S-mediated oxygen sensing. Disruption of genes encoding proteins that catalyze these pathways, either in experimental animal 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 #4). ETHE1 deficiency in either experimental animals or humans prevents H\textsubscript{2}S binding and is characterized by greatly elevated tissue H\textsubscript{2}S and thiosulfate (3, 7, 10, 41). Because of the O\textsubscript{2} dependency of this reaction, hypoxia would be expected to achieve the same result.

Mitochondrial sulfite oxidase (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 2-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 #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 #6) which effectively detoxifies excess dietary or metabolic cysteine and eliminates the possibility of H\textsubscript{2}S formation (36). Because CDO is dynamically regulated by cysteine concentration whereas the H\textsubscript{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\textsubscript{2}S increase in CDO knockout mice, similar to that observed with ETHE1 deficiency (32, 42). As molecular O\textsubscript{2} is the only other substrate in dioxygenase-catalyzed reactions it is likely that hypoxia will similarly impair cysteine oxidation and favor H\textsubscript{2}S production. However, because this process depends on build up of cysteine to produce H\textsubscript{2}S it probably places a long-term bias on H\textsubscript{2}S-mediated O\textsubscript{2} sensing rather than contributing to the acute response. Anecdotally, this may partially explain how hypoxic
responses are augmented by exogenous cysteine (cf. 23).

The 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 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 10⁻⁶ U-46619 (28). We proposed that the strong stimulation by U-46619 overwhelmed any observable effect of H₂S. The present observation that DTT did not increase HPV when the vessels were precontracted with U-46619 is consistent with this hypothesis. These studies also illustrate the inherent problems associated with unraveling oxygen-sensing mechanisms in prestimulated vessels.

Perspectives

It is becoming increasingly evident that organisms have had a long association with H₂S 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 H₂S and there is increasing evidence that cells exploit this relationship to detect and respond to hypoxia. The present work not only provides yet another example of H₂S-mediated oxygen sensing but it shows that tissues have the ability to rescue sulfide from an otherwise catalytic pathway and integrate it into the oxygen sensing mechanism. Mobilization of H₂S from thiosulfate may in fact have a faster onset than H₂S production from organic thiols and by
recycling sulfur it clearly minimizes the need for their degradation.

ACKNOWLEDGMENTS

The authors would like to acknowledge the help of H. Kimura in synthesizing DHLA and the assistance of K.0'Donnell, L. Scalf, J. Shafer, V. Western, and W. Wolter of the Friemann Life Science Center, in obtaining tissues. This research was supported by National Science Foundation Grant No. IOS 1051627.
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47. Whitfield NL, Kreimier EL, Verdial FC, Skovgaard N and Olson KR. Reappraisal of H2S/sulfide concentration in vertebrate blood and its potential significance in ischemic...
Table 1. Maximum or near-maximum H$_2$S production in tissues incubated with sequential addition of 1 mM Glutathione (GSH), 1 mM dithiothreitol (DTT) and 1 mM sodium thiosulfate (Na$_2$S$_2$O$_3$) or 10 mM DTT and 10 mM Na$_2$S$_2$O$_3$.

<table>
<thead>
<tr>
<th></th>
<th>GSH (max)</th>
<th>DTT (max)</th>
<th>Na$_2$S$_2$O$_3$ (max)</th>
<th>Na$_2$S$_2$O$_3$ (rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (15)</td>
<td>0.3 ±0.04</td>
<td>1.7 ±0.2$^{GGG}$</td>
<td>0.8 ±0.2$^{G,DD}$</td>
<td>1.7 ±0.6</td>
</tr>
<tr>
<td>Rat Lung (7)</td>
<td>0.1 ±0.04$^{BB}$</td>
<td>3.7 ±1.0$^{B,GG}$</td>
<td>11.5 ±7.1$^{B}$</td>
<td>8.2 ±3.6$^{*}$</td>
</tr>
<tr>
<td>Mouse Lung (5)</td>
<td>0.2 ±0.1</td>
<td>1.1 ±0.4</td>
<td>0.6 ±0.2</td>
<td>1.6 ±0.5</td>
</tr>
<tr>
<td>Bovine Heart (3)</td>
<td>0.6 ±0.1$^{BB}$</td>
<td>3.8 ±1.2$^{BB}$</td>
<td>2.6 ±0.7$^{BBB,G}$</td>
<td>2.7 ±0.9</td>
</tr>
<tr>
<td>Rat Liver (12)</td>
<td>4.9 ±2.9</td>
<td>12.5 ±4.8</td>
<td>200.7 ±60.8$^{BBB,G,DDD}$</td>
<td>103.8 ±26.9$^{***}$</td>
</tr>
<tr>
<td>Mouse Liver (4)</td>
<td>0.2 ±0.1</td>
<td>3.3 ±1.5</td>
<td>42.2 ±30.4$^{BB}$</td>
<td>32.5 ±14.3$^{***}$</td>
</tr>
<tr>
<td>Tissue Type</td>
<td>Max (μmoles)</td>
<td>Rate (μmoles/g tissue/min)</td>
<td>Conditions</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------</td>
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<td>------------</td>
<td></td>
</tr>
<tr>
<td>Buffer (14)</td>
<td>2.3 ±0.5</td>
<td>0.5 ±0.1¹⁺ダーク</td>
<td>2.3 ±0.5</td>
<td></td>
</tr>
<tr>
<td>Bovine Lung (4)</td>
<td>6.8 ±1.4²⁺ダーク</td>
<td>3.9 ±0.7³⁺ダーク</td>
<td>7.9 ±2.3⁺⁺⁺</td>
<td></td>
</tr>
<tr>
<td>Rat Lung (3)</td>
<td>11.0 ±3.6³⁺ダーク</td>
<td>38.1 ±12.6³⁺ダーク</td>
<td>26.6 ±7.7⁺⁺⁺</td>
<td></td>
</tr>
<tr>
<td>Mouse Liver (5)</td>
<td>3.3 ±1.6</td>
<td>172.0 ±76.6³⁺ダーク, ¹⁺ダーク</td>
<td>75.9 ±27.4⁺⁺⁺</td>
<td></td>
</tr>
</tbody>
</table>

Max, maximum H₂S produced expressed as μmoles; rate, rate of H₂S production from Na₂S₂O₃ expressed as μmoles/g tissue/min. Because 0.15 grams of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. Mean ±SE, (N), number of animals. B, BB and BBB significantly different from respective buffer, G, GG, GGG significantly different from respective GSH and D, DD, DDD, significant difference between DTT and Na₂S₂O₃ at p < 0.05, 0.01 and 0.001, respectively.
<table>
<thead>
<tr>
<th></th>
<th>GSH (max)</th>
<th>DTT (max)</th>
<th>Na₂S₂O₃ (max)</th>
<th>Na₂S₂O₃ (rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer (11)</strong></td>
<td>1.4 ±0.5</td>
<td>18.5 ±5.9²</td>
<td>36.7 ±7.3³</td>
<td>6.6 ±0.7</td>
</tr>
<tr>
<td><strong>Bovine Lung (5)</strong></td>
<td>27.8 ±16.6³</td>
<td>40.9 ±7.6³</td>
<td>651.4 ±150.7²</td>
<td>149.8 ±36.2³²</td>
</tr>
<tr>
<td><strong>Rat Lung (7)</strong></td>
<td>1.8 ±1.5</td>
<td>31.0 ±11.0¹¹</td>
<td>429.4 ±203.7²³</td>
<td>50.4 ±30.7</td>
</tr>
<tr>
<td><strong>Mouse Lung (5)</strong></td>
<td>0.7 ±0.4</td>
<td>30.3 ±9.4¹¹</td>
<td>197.9 ±39.0²³³</td>
<td>75.3 ±20.7³²</td>
</tr>
<tr>
<td><strong>Bovine Heart (3)</strong></td>
<td>11.5 ±3.6³²³</td>
<td>53.6 ±0.5³²³</td>
<td>68.7 ±4.5³²³²</td>
<td>14.8 ±3.1³²²³</td>
</tr>
<tr>
<td><strong>Rat Liver (12)</strong></td>
<td>18.4 ±8.4</td>
<td>55.9 ±18.4²</td>
<td>258.7 ±91.4²³</td>
<td>79.9 ±24.2²¹²</td>
</tr>
<tr>
<td><strong>Mouse Liver (5)</strong></td>
<td>5.0 ±2.6</td>
<td>27.2 ±12.8²</td>
<td>101.3 ±70.0²</td>
<td>21.9 ±12.6</td>
</tr>
</tbody>
</table>

Table 2. Maximum or near-maximum H₂S production in tissues incubated with sequential addition of 10 mM Glutathione (GSH), 10 mM dithiothreitol (DTT) and 10 mM sodium thiosulfate (Na₂S₂O₃) or 10 mM DTT and 10 mM Na₂S₂O₃.
<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Max. (μmoles)</th>
<th>Rate (μmoles/g tissue/min)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (11)</td>
<td>22.6 ±4.1</td>
<td>147.7 ±37.4&lt;sup&gt;DD&lt;/sup&gt;</td>
<td>19.1 ±3.9</td>
</tr>
<tr>
<td>Bovine Lung (4)</td>
<td>94.5 ±27.2&lt;sup&gt;BBB&lt;/sup&gt;</td>
<td>315.5 ±11.3&lt;sup&gt;B, DDD&lt;/sup&gt;</td>
<td>95.1 ±6.8&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
<tr>
<td>Rat Lung (3)</td>
<td>113.3</td>
<td>616.7 ±116.1&lt;sup&gt;BBB, D&lt;/sup&gt;</td>
<td>279.1 ±93.1&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>±16.5&lt;sup&gt;BBB&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat Liver (2)</td>
<td>82.1</td>
<td>72.4 ±243.7 ±</td>
<td>±33.41&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>±</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Liver (2)</td>
<td>161.7</td>
<td>1278.9 ±131.6&lt;sup&gt;BBB, D&lt;/sup&gt;</td>
<td>1296.5</td>
</tr>
<tr>
<td></td>
<td>±50.2&lt;sup&gt;BBB&lt;/sup&gt;</td>
<td></td>
<td>±24.8&lt;sup&gt;BBB&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Max, maximum H₂S produced expressed as μmoles; rate, rate of H₂S production from Na₂S₂O₃ expressed as μmoles/g tissue/min. Because 0.15 grams of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. Mean ±SE, (N), number of animals. B, BB and BB significantly different from respective buffer, G, GG, GGG significantly different from respective GSH and D, DD, DDD, significant difference between DTT and Na₂S₂O₃ at p < 0.05, 0.01 and 0.001, respectively.
<table>
<thead>
<tr>
<th></th>
<th>DTT</th>
<th>Na₂S₂O₃</th>
<th>Na₂S₂O₃ (rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortland Buffer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mM DTT / 1 mM TS (4)</td>
<td>2.98 ±0.91</td>
<td>0.47 ±0.24D</td>
<td>0.27 ±0.07</td>
</tr>
<tr>
<td>1 mM TS / 1 mM DTT (3)</td>
<td>3.74 ±0.36</td>
<td>0.00</td>
<td>0.07 ±0.07</td>
</tr>
<tr>
<td>2mM DTT / 2 mM TS (3)</td>
<td>5.88 ±0.9</td>
<td>1.08 ±0.43DD</td>
<td>0.27 ±0.07</td>
</tr>
<tr>
<td>10 mM DTT / 10 mM TS (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 (3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100 μM DTT / 1 mM TS (4)</td>
<td>0.90 ±0.16</td>
<td>0.08 ±0.08</td>
<td>0</td>
</tr>
<tr>
<td><strong>Lamprey Aorta</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM DTT / 1 mM TS (4)</td>
<td>4.12 ±1.4</td>
<td>19.21 ±3.45BB,DD</td>
<td>1.93 ±0.33BB</td>
</tr>
<tr>
<td>1 mM TS / 1 mM DTT (3)</td>
<td>0</td>
<td>35.8 ±6.07</td>
<td>2.80 ±0.8B</td>
</tr>
</tbody>
</table>

Table 3. H₂S production in Cortland buffer and lamprey aorta.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Max (μmoles)</th>
<th>Rate (μmoles/g tissue/min)</th>
<th>Rate/0.15 (μmoles/g tissue/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mM DTT / 2 mM TS</td>
<td>20.77 ±8.31</td>
<td>30.52 ±15.8</td>
<td>3.47 ±0.7^B</td>
</tr>
<tr>
<td>10 mM DTT / 10 mM TS</td>
<td>45.84 ±10.36</td>
<td>85.26 ±28.52</td>
<td>8.80 ±2.07</td>
</tr>
</tbody>
</table>

Max, maximum H₂S produced expressed as μmoles; rate, rate of H₂S production from Na₂S₂O₃ expressed as μmoles/g tissue/min. Because 0.15 grams of tissue was used in each experiment, the rate of H₂S production in buffer was also divided by 0.15. Mean ±SE, (N), number of animals. B, BB and BB significantly different from respective buffer, D, DD, DDD, significant difference between DTT and Na₂S₂O₃ at p < 0.05, 0.01 and 0.001, respectively.
Table 4. Combined data for maximum or near-maximum H$_2$S production in Krebs Henseleit buffer or mammalian tissues incubated with sequential addition of 100 μM dihydrolipoic acid DHLA, 1 mM DHLA and 1 mM sodium thiosulfate (Na$_2$S$_2$O$_3$) or 1 mM Na$_2$S$_2$O$_3$ followed by 100 μM and then 1 mM DHLA.

<table>
<thead>
<tr>
<th></th>
<th>maximum H$_2$S produced</th>
<th>rate μmoles/g tissue/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (10)</td>
<td>5.5 ±1.0</td>
<td>3.0 ±0.6$^G^G$</td>
</tr>
<tr>
<td>Rat liver (8)</td>
<td>150.1 ±21.0$^{BBB}$</td>
<td>27.5 ±9.8$^{BB}$</td>
</tr>
<tr>
<td>Rat lung (5)</td>
<td>45.5 ±12.2$^{BBB}$</td>
<td>4.4 ±1.0</td>
</tr>
<tr>
<td>Mouse liver (7)</td>
<td>62.5 ±24.7$^B$</td>
<td>32.8 ±21.1</td>
</tr>
<tr>
<td>Bovine lung (4)</td>
<td>46.3 ±24.9$^B$</td>
<td>7.4 ±3.1</td>
</tr>
</tbody>
</table>

Max, maximum H$_2$S produced expressed as μmoles; rate, rate of H$_2$S production expressed as μmoles/g tissue/min. Because 0.15 grams of tissue was used in each experiment, the rate of H$_2$S production in buffer was also divided by 0.15. Mean ±SE, (N), number of animals. B, BB and BBB significantly different from respective buffer, at p < 0.05, 0.01 and 0.001.
FIGURE CAPTIONS

Figure 1. Polarographic, realtime measurements of H$_2$S production after additions of glutathione (GSH), dithiothreitol (DTT) and thiosulfate (TS) to buffer (A) or tissue (B-F) in a closed, anoxic chamber. A, H$_2$S production after sequential additions of 10 mM GSH, DTT and TS, to Krebs Henseleit buffer. B, C, H$_2$S production after sequential additions of 10 mM GSH, DTT and TS, to homogenized bovine lung or D, E, after 10 mM additions of DTT and TS to homogenized bovine lung. Glutathione had little effect on H$_2$S production, whereas the greatest increase in H$_2$S production was observed after addition of TS. Opening the chamber to the atmosphere (air: C, E) rapidly decreased H$_2$S concentration due to the combined effect of tissue oxidation and volatilization. F, as shown in the homogenized trout heart, when the order of TS and DTT addition was reversed, H$_2$S production only occurred after both compounds were added. H$_2$S production in buffer was only one-tenth of that in tissues; Note scale.

Figure 2. Dose-dependent effect of dithiothreitol (DTT) or thiosulfate (TS) after DTT on H$_2$S 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 plus or minus SE, $N = 3-6$.

Figure 3. Polarographic, realtime measurements of H$_2$S production after additions of dihydrolipoic acid (DHLA) and thiosulfate (TS) to buffer (A, B) or mouse liver (C, 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, D) rapidly decreased H₂S concentration due to the combined effect of tissue oxidation and volatilization.

**Figure 4.** Dose dependent effect of pre-stimulation with the thromboxane A₂ mimetic, U-46619 on consecutive hypoxic contractions of bovine pulmonary arteries. Vessels were exposed to four consecutive hypoxic stimuli and the force developed in the second (white bars), third (gray bars) and fourth (black bars) were compared either as a function of absolute tension per gram vessel (A) or normalized to the second contraction (B). Mean +SE, N = 6-9.

**Figure 5.** Effects of dithiothreitol (DTT) and thiosulfate (S₂O₃²⁻) on consecutive (second, white bars; third, gray bars; fourth, black bars) hypoxic pulmonary vasoconstriction (HPV) in otherwise unstimulated (A) or 10⁻⁶ M U-46619 pre-contracted 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. Addition of thiosulfate did not significantly effect the DTT response. *,
significantly different from second contraction, mean +/- SE, (N), number of vessels.

Figure 6. Dithiothreitol (DTT) dose-dependently increases the magnitude of hypoxic
vasoconstriction (HPV) in otherwise unstimulated bovine pulmonary arteries (A), whereas in
vessels pre-stimulated with the thromboxane A₂ mimetic, U-46619 (10⁻⁶ M), HPV is
progressively inhibited at 1 and 10 mM DTT. *, significantly different from control, mean +/-
SE, (N), number of vessels.

Figure 7. Effects of dihydrolipoic acid (DHLA) on hypoxic pulmonary vasoconstriction (HPV)
in otherwise unstimulated bovine pulmonary arteries. A - C; representative traces showing
variability of responses to DHLA (dashed arrow) and consecutive hypoxias (small arrows); A,
negligible response to DHLA and hypoxia; B, moderate response to DHLA and augmented
hypoxic response; C, DHLA-mediated contraction and augmented hypoxic response. D)
summary of responses of vessels exhibiting DLA contractions and augmented HPV. Vessels
were exposed to four consecutive 20 minute periods of hypoxia and normalized to the second
hypoxic exposure (% second N₂ contraction, white bars; third and fourth contractions are shown
as gray and black bars, respectively). Consecutive hypoxic exposures did not significantly
increase the magnitude of contraction in control vessels (CON), whereas DHLA (100 μM) added
after the second hypoxic treatment (DHLA + N₂) Significantly increased the magnitude of the
third and fourth hypoxia). Stippled bar shows magnitude of DHLA contraction compared to second hypoxia. Mean ±SE, N, number of vessels.

**Figure 8.** DHLA produces a dose-dependent relaxation of U-46619 (10^{-7} M) precontracted mouse (squares, dotted line) and rat (circles, solid line) thoracic aortas. Values are mean +/- SE; N = 5 (mouse) or 6 (rat) animals.

**Figure 9.** Pathways for H_2S production and degradation. H_2S is synthesized from homocysteine or cysteine by the cytosolic enzymes cystathionine β-synthase (CBS), cystathionine γ-lyase, or the sequential activity of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfur transferase (3-MST) in both the cytosol and mitochondria. Other potential mechanisms for H_2S biosynthesis have been described in invertebrates (dotted enclosure; Julian et al., 2002(14)) but have yet to be confirmed in mammals. d-amino acid oxidase (DAO) in peroxisomes can generate 3-mercaptopyruvate from d-cysteine which can be converted to in either cytosol or plasma by CAT/3-MST. Note that there is little evidence for the requirement for oxygen in H_2S biosynthesis. Oxidation of H_2S in the mitochondria (blue letters) is initiated by interaction with the enzyme sulfide:quinone oxidoreductase (SQR) producing two SQR persulfides that are then transferred to a mobile sulfide carrier (RSSH), one of which is oxidized to sulfite (SO_3^{2-}) by mitochondrial sulfur dioxygenase (ETHE1). Sulfur transferase (ST) transfers the other persulfide to form thiosulfate (S_2O_3^{2-}). H_2S may be regenerated from thiosulfate by 3-MST or rhodanase (Rde), or the thiosulfate may be oxidized to sulfate (SO_4^{2-}) by the sequential actions of thioredoxin reductase (TR) in combination with glutathione (GSH) and sulfur oxidase (SO). Oxidation of H_2S donates electrons to the respiratory chain (Q, III, IV) that has been shown in invertebrates and mammals to result in ATP production and increased O_2 consumption. An alternative oxidase (AOX) that oxidizes H_2S without producing ATP has been observed in invertebrates. H_2S can be regenerated from thiosulfate by 3-mercaptopyruvate sulfur transferase (3-MST) or rhodanase (Rde) in the presence of other reducing disulfides such as thioredoxin (Trx) or dihydrolipoic acid (DHLA). Circled numbers indicate actual or potential hypoxia-sensitive sites (see text for details).
Fig. 2

The graph shows the maximum H$_2$S produced (µmoles) as a function of DTT or Thiosulfate concentration (mM) in buffer and tissue. The * and # symbols indicate statistical significance compared to the control and another group, respectively.
Fig. 3

A

DHLA 100 μM ↓
DHLA 1 mM

B

DHLA 100 μM ↓
DHLA 1 mM

C

air ↓

D

air ↓

TS 1 mM ↓
DHLA 100 μM ↓
DHLA 1 mM ↓

TS 1 mM ↓
DHLA 1 mM
Fig. 4

A

Tension (mg)

U-46619 concentration (M)

0 10^{-9} 10^{-6}

B

% Second Contraction

U-46619 concentration (M)

0 10^{-9} 10^{-6}
Fig. 6

A

0 U-46619

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B

10^{-6} M U-46619

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* denotes significant difference compared to control.
Fig. 7

A

B

DHLA

C

DHLA

D

% Second N₂ Contraction

CON  DHLA  DHLA + N₂

(8)  *  

(14) *

200 mg

20 min
Fig. 9

Homocysteine + $\text{H}_2\text{S}$

L-Methionine → Homocysteine $\xrightarrow{\text{CBS}}$ Cystathionine $\xrightarrow{\text{CSE}}$ L-Cysteine

L-Cysteine $\xrightarrow{\text{CSE}}$ Hypotaurine

Cystathionine + $\text{H}_2\text{S}$

Cysteine + L-Serine + $\text{H}_2\text{S}$

Cysteine $\xrightarrow{\text{CBS}}$ Cysteinesulfinate + $\text{SO}_3^{2-}$

H2O + L-Serine + $\text{H}_2\text{S}$ → Pyruvate + $\text{NH}_3$

Cystine + RSH → Thiocysteine → Cysteine

$\text{H}_2\text{S}$ + CySR → Protein

D-Cysteine $\xrightarrow{\text{DAO}}$ Homolanthionine + $\text{H}_2\text{S}$

L-Cysteine $\xrightarrow{\text{CSE}}$ Thiocysteine

$\text{H}_2\text{O}$ + $\text{H}_2\text{S}$ → 3-Mercaptopyruvate

$\text{H}_2\text{O}$ + Pyruvate + $\text{NH}_3$ → 3-MST + $\text{H}_2\text{S}$

3-MST + $\text{SO}_3^{2-}$ $\xrightarrow{\text{ETHE1}}$ $\text{SO}_4^{2-}$

$\text{H}_2\text{S}$? $\text{H}_2\text{O}$ $\xrightarrow{\text{CAT}}$ a-ketoglutarate

Amino acid $\xrightarrow{\text{SQR}}$ FAD + $\text{SO}_3^{2-}$

$\frac{1}{2}\text{O}_2$ + $\text{H}_2\text{O}$ $\xrightarrow{\text{AOX}}$ $\text{Q}^+$ $\xrightarrow{\text{III}}$ $\text{Q}^0$ $\xrightarrow{\text{IV}}$ $\text{Q}^0$ + ATP $\xrightarrow{\text{II}}$ $\text{Q}^+$

3-MST-SS $\xrightarrow{\text{Trx DHPLA}}$ Trx

3-MST $\xrightarrow{\text{3-MST}}$ 3-Mercaptopyruvate

Pyruvate $\xrightarrow{\text{SQR}}$ FAD $\xrightarrow{\text{SQR}}$ Amino acid

$\text{H}_2\text{S}$ $\xrightarrow{\text{oxidation}}$ $\text{SO}_4^{2-}$