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Hydrogen Sulfide as an Oxygen Sensor

Kenneth R. Olson

Abstract

Significance: Although oxygen (O_2)-sensing cells and tissues have been known for decades, the identity of the O_2-sensing mechanism has remained elusive. Evidence is accumulating that O_2-dependent metabolism of hydrogen sulfide (H_2S) is this enigmatic O_2 sensor. ***Recent Advances:*** The elucidation of biochemical pathways involved in H_2S synthesis and metabolism have shown that reciprocal H_2S/O_2 interactions have been inexorably linked throughout eukaryotic evolution; there are multiple foci by which O_2 controls H_2S inactivation, and the effects of H_2S on downstream signaling events are consistent with those activated by hypoxia. H_2S-mediated O_2 sensing has been demonstrated in a variety of O_2-sensing tissues in vertebrate cardiovascular and respiratory systems, including smooth muscle in systemic and respiratory blood vessels and airways, carotid body, adrenal medulla, and other peripheral as well as central chemoreceptors. ***Critical Issues:*** Information is now needed on the intracellular location and stoichometry of these signaling processes and how and which downstream effectors are activated by H_2S and its metabolites. ***Future Directions:*** Development of specific inhibitors of H_2S metabolism and effector activation as well as cellular organelle-targeted compounds that release H_2S in a time- or environmentally controlled way will not only enhance our understanding of this signaling process but also provide direction for future therapeutic applications. *Antioxid. Redox Signal*. 22, 377–397.

> ''Nothing in Biology Makes Sense Except in the Light of Evolution''
>
> —*Theodosius Dobzhansky* (29)

Introduction

IN THE BEGINNING, there was no oxygen (O_2). Energy in the form of reducing equivalents flowed outward across the Earth's crust through ''pores,'' hydrothermal vents, and volcanoes. Much of this was sulfide, which was then oxidized in the mildly oxidizing atmosphere. Iron also traversed this boundary and its ability to associate with sulfur in the form of iron sulfur clusters enabled both the structural organization of compartment boundaries and the catalytic control of electron transfer (25). The energy could now be harnessed, and life had begun. For the next 3 billion years, organisms continued to evolve and develop more sophisticated methods to control this energy. During this period, rain leached sulfur from the land and as it flowed into the oceans the sulfur became reduced, creating a euxinic environment that was both sufidic and hypoxic (Fig. 1). Eukaryotic cells arose in this environment from the combination of a sulfide-reducing Archaea and a sulfide-oxidizing α proto-bacterium. This union arguably enabled sulfur cycling and energy transfer between the cytoplasm and mitochondrion (146). For 500 million years, eukaryotic cells continued to develop and thrive in this environment as evidenced by the fact that the oldest known microfossil was a sulfide-oxidizing organism (180). As photosynthesis began to create O_2-rich areas ''oases'' in the seas, organisms living in or around these environments now had to develop strategies to detoxify this unusually reactive gas. It was also soon realized that O_2's reactivity could be harnessed, and this culminated with O_2 as the ultimate electron acceptor in the electron transport chain. While the increase in ambient O_2 promoted oxidative metabolism, it presented another problem: reduced sulfide disappeared. Organisms were now obligated to change to carbon-based substrates as an energy source. On occasion, ambient concentrations of O_2 and hydrogen sulfide (H_2S) were again reversed and while this euxinic environment produced mass extinctions (51), it likely ensured the survival of organisms whose genome had not only retained, but also passed on the capacity

Department of Physiology, Indiana University School of Medicine—South Bend, South Bend, Indiana.

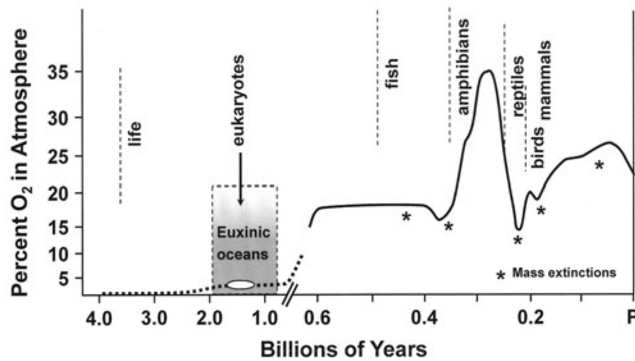


FIG. 1. Oxygen (O_2) and sulfide during evolution. Around 2.5 billion years ago, atmospheric O_2 began to increase and it was ~2% or ~15 mmHg (but undoubtedly much lower in the oceans) when the eukaryotes first appeared. Eukaryotic evolution continued for the next 500 million years in this sulfidic and very hypoxic (euxinic) environment. During this time, many of the metabolic pathways for sulfide metabolism were established and they continue to this day as the basis for O_2 sensing. Periodic reciprocal oscillations in O_2 and hydrogen sulfide (H_2S) probably contributed to mass extinctions (*). Drawn from Olson (121).

for sulfide metabolism. Remnants of this early life are present these days, even in modern aerobic eukaryotes, as sulfide is still preferred compared with carbon-based substrates in the electron transport chain [11, reviewed in Olson (121)].

O_2-Sensing Tissues

The vast majority of modern-day animals, and especially vertebrates, now depend on O_2 and monitoring ''sensing'' O_2 availability is key for survival. O_2 sensors can be divided into four ''reporting'' levels: external, internal, tissue (vascular), and intracellular.

External chemoreceptors monitor ambient O_2. Aquatic vertebrates are especially susceptible to ambient O_2 because of the lower solubility (1/30 that of air), low diffusivity (200,000 times slower), 60-fold higher viscosity, and wide swings in O_2 availability seasonally, daily, and spatially, even within a few meters (8). Chemoreceptor neuroepithelial cells (NEC) on the external surfaces of fish gills are employed to continuously monitor water partial pressure of oxygen (Po_2) (66, 109). Similar neuroepithelial-like cells are found in clusters (neuroepithelial bodies [NEB]) near airway bifurcations in lungs of newborn mammals where they may be important in the transition away from the relatively hypoxic uterine environment during and shortly after birth (72). External O_2 sensors other than NEB are relatively uncommon in terrestrial vertebrates. These are replaced by internal O_2 sensors that are better suited to monitor blood O_2 status and changes in O_2 availability (such as in borrows or with increasing altitude) if needed.

The first and second gill arches of fish are heavily invested with internal arterial-facing NEC, and these are the antecedents of glomus cells in the carotid body and aortic arch. The first gill arch and the mammalian carotid body arise from the third embryonic arch, and the second gill arch and aortic bodies arise from the fourth embryonic aortic arch; NEC and type I glomus cells of the carotid body are so similar at the ultrastructural level that there is little doubt of their lineage [reviewed in Jonz and Nurse (66)]. Mammalian adrenal medullary cells and homologous chromaffin cells in fish that line systemic veins secrete catecholamines in response to hypoxemia (119, 138) and may monitor tissue O_2 extraction. The adrenal medullary cells may be especially important in monitoring arterial O_2 in the newborn until the carotid bodies become fully functional (66).

The ability of blood vessels themselves to respond to O_2 is important in matching perfusion to metabolic demand or in the case of the respiratory organs to maintain normal ventilation/perfusion ratios. It is commonly assumed that to accomplish this, systemic vessels dilate in response to hypoxia and pulmonary vessels constrict (155) but this is obviously not consistent throughout vertebrates or even within mammals (129, 143).

Finally, it is evident that individual cells not only monitor their own O_2 status but also have biochemical ''contingency plans'' to adjust metabolic demand and energy utilization should O_2 levels fall. The scope of these contingency plans is highly variable from a few short minutes of survival in highly active mammalian tissues such as the brain and the heart to extended (weeks–months) anoxemia that is tolerated by lower vertebrates such as the crucian carp and turtle (48). Initial sensing and coping mechanisms reflected in response to ischemia/reperfusion injury and pre- and postconditioning are only briefly discussed here in the context of acute O_2 sensing. Chronic responses involving metabolic control are described in an excellent review by Clanton *et al.* (22) and are beyond the scope of the present discussion.

Acute O_2 Sensing

Despite intense interest, the identity of the O_2 ''sensor'' remains enigmatic. A number of mechanisms have been suggested, but none has achieved unequivocal support. Their pros and cons have been extensively reviewed (22, 37, 47, 54, 144, 155, 184, 187). The intent of the present review is to examine the evidence suggesting that the O_2-dependent metabolism of H_2S is an O_2 sensor.

H_2S and O_2 Sensing

There are a number of different opinions regarding how (or if) H_2S senses O_2 (Fig. 2). These include (i) direct inhibition of oxidative phosphorylation; (ii) as a downstream effector of a different O_2 sensor, for example, carbon monoxide (CO); or (iii) inverse coupling between O_2 availability and O_2-dependent metabolism of endogenously generated H_2S.

H_2S as a direct inhibitor of oxidative phosphorylation

It is well known that H_2S inhibits cytochrome C oxidase (CCO) and it has been proposed that this effect, while it mimics hypoxia, is merely an artifact of H_2S poisoning (15, 155, 181) (Fig. 2A). Indeed, H_2S concentrations above 20–40 μM inhibit complex IV (11). However, recent studies have shown that H_2S exerts a biphasic effect on respiration. H_2S stimulates respiration at concentrations of approximately 3 μM when complexes II–IV are intact, whereas the inhibitory effect is only mediated through complex IV at concentrations exceeding 30–100 μM (110). There are other

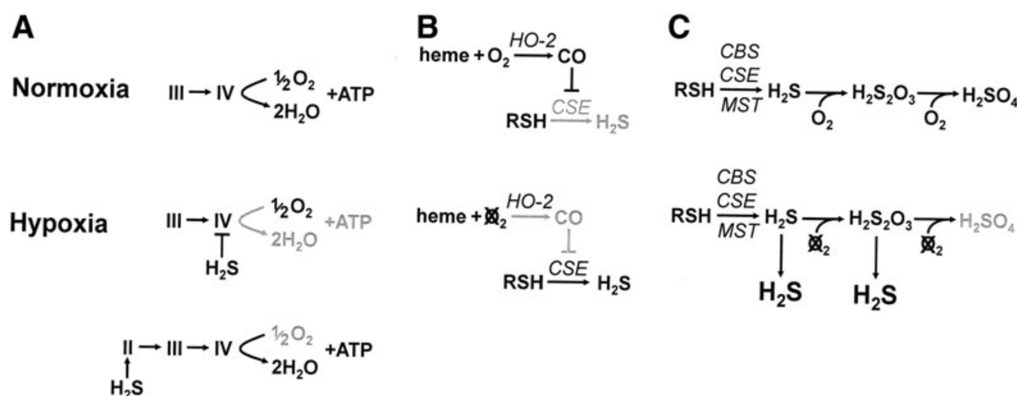


FIG. 2. Mechanisms of H_2S signaling in hypoxia. (A) Exogenous H_2S reversibly inhibits complex IV, thereby mimicking hypoxia. Elevated H_2S could also feed electrons into complex II and by driving oxidative phosphorylation, deplete cellular O_2. **(B)** H_2S as a downstream effector of carbon monoxide (CO) mediated O_2 sensing. In normoxia, CO is constitutively produced from heme by hemeoxygenase 2 (HO-2) and inhibits cystathionine γ-lyase (CSE), thereby preventing H_2S production from thiols (RSH). CO can no longer be produced during hypoxia, and the inhibitory effect on CSE is lost while permitting H_2S biosynthesis. **(C)** Direct inverse coupling between H_2S synthesis and O_2 availability. In normoxia, H_2S is constitutively generated from RSH in the transsulfuration pathway by CSE, cystathionine β-synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (3-MST) and oxidized to thiosulfate ($H_2S_2O_3$) and then sulfate (H_2SO_4), which is then excreted. Hypoxia does not affect H_2S production from RSH, but it prevents oxidation of H_2S to thiosulfate, thereby increasing H_2S concentration. In addition, previously formed thiosulfate can no longer be oxidized to sulfate and in the increased reducing environment in the mitochondrial matrix, this thiosulfate is reduced to H_2S and sulfite (not shown). Production of H_2S from thiosulfate is likely to be faster than H_2S production from RSH and may be the initial event in hypoxic signaling.

problems with signaling *via* inhibition of complex IV. First, it puts the cart before the horse. It is unclear what factor(s) would cause H_2S concentration to increase to this level. In addition, H_2S concentrations $>1\ \mu M$ would be expected to produce an obvious odor. Second, if H_2S is from an endogenous source, this should result in a fatal positive feedback. If initial, low-level H_2S concentrations partially inhibited CCO, this would further reduce the rate of H_2S oxidation and H_2S concentrations would continue to increase to the point where CCO is completely and irreversibly inhibited. While partial CCO inhibition could be considered a general mechanism for shutting down metabolism and conserving resources, it would not explain how O_2-sensing cells are activated by hypoxia. Third, arguments can be made against H_2S inhibition of O_2 consumption based on mass balance of sulfur and O_2 metabolism. A 70 kg adult male ingests $\sim$26 mmol of sulfur amino acids per day, and total sulfur intake is probably $\sim$40 mmol per day (62, 118). O_2 consumption is $\sim$250 ml/min (179), or 360 L/day, which is equivalent to 15 mol of O_2 per day. Assuming all ingested sulfur is ultimately excreted as sulfate (SO_4); the rate of O_2 utilization to sulfur excretion (4O/S) is 250:1. It is difficult to conceive how sulfide could increase to the point where it has a significant impact on O_2 consumption and, therefore, ATP production. Fourth, inhibition of CCO by exogenous H_2S (15) is unlikely to reflect the *in vivo* situation where H_2S production and metabolism is most likely spatially restricted (122).

H_2S as a downstream effector of CO-mediated O_2 sensing

The relationship between hypoxia and H_2S signaling in the carotid body, adrenal medulla, and cerebral vasculature has been suggested to be a secondary event downstream of the initial O_2 sensor, CO (Fig. 2B) (60, 139). In the carotid body, it has been proposed that hypoxia inhibits constitutive CO production by hemeoxygenase 2 (HO-2), which then relieves the heretofore tonic inhibitory effect of CO on cystathionine γ-lyase (CSE) and H_2S levels increase, closing large-conductance Ca^{2+}-dependent K^+ channels (BK_{Ca}) (87, 162, 163) or TWIK-related acid-sensitive potassium (TASK) (15) channels. This depolarizes glomus cells and releases neurotransmitter. In this sense, H_2S is a mediator of the actual O_2 sensor, CO (139). The role of H_2S in these studies is supported by observations that CSE knockout mice or the CSE inhibitor, propargyl glycine (PPG), inhibits hypoxic responses in both the carotid body and adrenal medulla (97, 136). However, it is unclear how or whether CO actually inhibits CSE (112). In addition, HO-2 null mice have an intact chemoreceptor response to hypoxia (131).

The inhibitory effects of CO on CBS have also been proposed to mediate hypoxic vasodilation in the cerebral cortical circulation (111). Here, HO-2 that is present in neurons and endothelia constitutively generates substantial quantities of CO which inhibits CBS located in the endfeet of astrocytes in contact with the vessel wall. In normoxia, CO inhibition of CBS inhibits release of vasodilator H_2S; whereas the inhibition is lost in hypoxia, resulting in vasodilation.

The anatomical arrangement of CBS and HO-2 is likely unique to the cerebral vasculature, as it does not appear to be found in other systemic vessels. Since CBS controls sulfur flux into the trans-sulfuration pathway and away from the remethylation cycle (60), is unclear what impact CO in the brain has on overall sulfur metabolism or remethylation, both of which appear to be highly susceptible to O_2 availability in this model. In addition, inhibitors of CSE, but not CBS inhibit hypoxic vasodilation in other systemic vessels such as the rat aorta (125). Obviously, additional studies are required to

elucidate the relationship between CO and H_2S in the carotid and other systemic vessels as well as in the pulmonary circulation.

O_2 sensing by O_2-dependent H_2S inactivation

We proposed that there was a direct metabolic coupling between H_2S and O_2 (125). In this mechanism, H_2S is constitutively generated through sulfur metabolism but intracellular H_2S concentration is maintained at low levels through concomitant oxidation (Fig. 2C). H_2S signaling is achieved through the inability of H_2S oxidation to keep pace with H_2S production during hypoxia. Since we initially proposed this mechanism, a considerable amount of evidence has accumulated that not only supports this hypothesis but also expands the scope of H_2S-mediated O_2 sensing. This includes evidence that (i) H_2S production is O_2 independent, whereas there are numerous effectors of O_2-dependent H_2S metabolism; (ii) O_2-dependent H_2S metabolism is regulated at physiologically relevant Po_2s; (iii) the effects of exogenous H_2S mimic hypoxia and compounds that inhibit or augment H_2S production inhibit or augment hypoxic responses, respectively; (iv) H_2S acts on effector mechanisms which are known to mediate hypoxic responses; and, (v) as stated in the introduction, the reciprocal relationship between O_2 and H_2S has been inexorably intertwined throughout evolution, albeit with the ironic twist that the molecule that was once used as the primary energy source is now the reporter for the molecule that replaced it. Points i–iv are considered in the next sections.

Metabolic Relationships Between H_2S and O_2

The next few paragraphs and Figure 3 illustrate the basic pathways of H_2S production and metabolism. Not surprisingly, essentially all H_2S production is independent of O_2. Conversely, O_2 is directly involved in nearly all aspects of H_2S degradation and indirectly involved in other aspects of H_2S metabolism.

H_2S production

H_2S can be generated from the classical trans-sulfuration pathways or through reduction of sulfur in persulfides (R-S_n, where $n = 2$–8). Trans-sulfuration mechanisms have been (and still are) examined in considerable detail, whereas persulfide reduction is only beginning to be understood in the context of H_2S production.

There are four enzymes: cystathionine β-synthase (CBS), CSE (also known as CGL) and tandem catalysis by cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST). CAT initially transfers the sulfur from cysteine to α-ketoglutarate, forming 3-mercaptopyruvate. The sulfur is then transferred to 3-MST, forming a persulfide, which is subsequently released as H_2S by a reducing agent such as thioredoxin (Trx) or dihydrolipoic acid.

It is evident that L-cysteine and L-homocysteine account for most H_2S production. CBS, CSE, and CAT/3-MST are cytosolic enzymes, whereas CAT and 3-MST are also present in mitochondria (71). 3-MST is especially abundant in the mitochondrial matrix (103), presumably enabling access to a threefold higher cysteine concentration here than in the cytosol (42). Hypoxia appears to increase CBS in the mitochondria (164) and stress-related stimuli can translocate CSE to the mitochondria, although this may take hours (42); additional details are provided in sections "CBS Translocation to Mitochondria" and "CSE Translocation to Mitochondria." Initially, it was believed that CBS was predominantly found in the brain and CSE was found in the cardiovascular system [reviewed in Kimura (73)], although a broader distribution is now becoming apparent (6, 73, 129). H_2S can also be derived from D-cysteine; however, this appears limited to the brain and kidney, where it protects the former from oxidative stress and the latter from re-perfusion injury (147). CBS, CSE, and CAT are pyridoxal 5′ phosphate-dependent, enzymes. *S*-adenosylmethionine allosterically activates CBS (152) and although CBS contains a heme group that can be inhibited by CO, neither nitric oxide (NO) nor O_2 appears effective at physiological concentrations (4). CSE and both cytosolic and mitochondrial CAT activity are inhibited by calcium, independent of calmodulin (104, 105).

H_2S metabolism (inactivation)

Theoretically, mitochondrial metabolism is a far more efficient and controllable means of regulating H_2S than simple diffusion out of the cell (122). In fact, there is considerable evidence showing that mitochondria efficiently oxidize H_2S [reviewed in Olson (121)]. Sulfide:quinone oxidoreductase (SQR), 3-MST, rhodanase (Rde), thiosulfate reductase (TR), sulfur dioxygenase (ETHE1), and sulfite oxidase (SO) are mitochondrial enzymes that oxidize H_2S to sulfate (SO_4^{2-}) for subsequent excretion. Sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$) are intermediates. SQR is bound to the inner mitochondrial membrane and intimately associated with the respiratory chain "supercomplex" (58). This provides a close association with an O_2-sensing process, as the latter also appears to be a mitochondrial event (155). Not surprisingly, 3-MST, Rde, and TR are abundant in the mitochondrial matrix and, to a lesser extent, the intermembrane space (78).

H_2S oxidation begins with H_2S binding to the highly conserved Cys-Cys disulfide bridge of SQR. The sulfide is oxidized to elemental sulfur forming SQR persulfide (sulfane sulfur) with the now-reduced SQR cysteine (SQR-SSH). Two H_2S and two SQR are involved: One H_2S sulfur is transferred to the mitochondrial sulfur dioxygenase, ETHE1, where it is oxidized to sulfite and the second sulfur is transferred from the SQR to sulfite by sulfur transferase producing thiosulfate. One electron from each of the two H_2S are fed *via* the quinone pool (Q) into the respiratory chain (98) and they ultimately reduce O_2 at complex IV. SQR is bound to the inner mitochondrial membrane, and it is believed that sulfur is shuttled from SQR by an as-yet unidentified mobile carrier, possibly glutathione (GSH), dihydrolipoate, Trx, or sulfite (59, 63, 165). SQR is found in all tissues except the brain (58, 82, 89) (but see "2. ETHE1" section), which may account for the pronounced sensitivity of the nervous system to H_2S toxicity. The capacity of cells to oxidize sulfide appears to be greater than the estimated rate of sulfide production (11, 44) and in *Caenorhabditis elegans* sqrd-1, the gene encoding SQR is increased eightfold after exposure to H_2S (108). Thus, it is expected that intracellular H_2S concentrations are very low under normoxic conditions.

FIG. 3. Pathways for H_2S production and degradation. H_2S is synthesized from homocysteine or cysteine by the cytosolic enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), or the tandem action of cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST) in both the cytosol and mitochondria. Other potential mechanisms for H_2S biosynthesis have been described in invertebrates (68) (dotted enclosure) but have yet to be confirmed in mammals. 3-mercaptopyruvate can also be produced from D-cysteine in the peroxisome by D-amino acid oxidase (DAO). During hypoxia, CBS migrates from the cytosol to the mitochondrial matrix and in the absence of Lon protease degradation, generates H_2S from the abundance of cysteine in the matrix. Oxidation of H_2S in the mitochondria is initiated by an 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 and forms 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 (Trx) reductase (TRD) in combination with glutathione (GSH) and sulfite 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 O_2 consumption. An alternative oxidase (AOX) that oxidizes H_2S without concomitant ATP production has also been observed in invertebrates. H_2S can also be regenerated from thiosulfate by 3-MST or Rde in the presence of other reducing disulfides such as Trx or dihydrolipoic acid (DHLA). Circled numbers indicate actual or potential hypoxia-sensitive sites (see sections "Metabolic Relationships Between H_2S and O_2" and "Effectors of H_2S Metabolism" for details). Modified from Olson (121).

In normoxia, most thiosulfate is further metabolized to sulfate by TR and SO. Although sulfur excretion as thiosulfate would conserve O_2, apparently there is little need for this when O_2 is plentiful and thiosulfate excretion in vertebrates is generally low. In fact, it is not clear why most vertebrates, especially terrestrial ones, would be bothered with this pathway at all, except that it may be an important avenue for regeneration of H_2S during hypoxia (124). A number of reviews (69, 73, 121, 152, 154) provide additional details of H_2S metabolism.

Effectors of H_2S Metabolism

It is now evident that there are a number of mechanisms with which O_2 can potentially influence H_2S concentration, thereby contributing to O_2 sensing. Some act over a relatively short timescale and contribute to acute hypoxic responses while others, operating on a longer timescale, may bias the background levels of H_2S. A number of examples are presented where H_2S or its metabolites are increased due to deficiencies in enzyme activity. Since O_2 is also involved in

these pathways, it is presumed that hypoxia will have a qualitatively similar effect, although this has rarely been examined. Additional factors may also indirectly contribute to the O_2-sensing process. These mechanisms are briefly described next; the reader is referred to corresponding numbers in Figure 3.

Rapid effectors of H_2S concentration

①. Electron transport: The absence, or reduced availability, of the terminal electron acceptor (O_2) prevents electron flow down the respiratory chain, enabling H_2S that was derived from trans-sulfuration to accumulate. This was our initial hypothesis of H_2S-mediated O_2 sensing (125).

②. ETHE1: The mitochondrial dioxygenase, ETHE1, uses molecular O_2 and water to oxidize the mobile persulfide from SQR to form sulfite. Inhibition of this pathway will prevent H_2S binding to SQR and enable H_2S and thiosulfate to accumulate. This has been shown in human and animal models of ETHE1 deficiencies (28, 36, 46, 166).

③. Sulfite oxidase: SO in the mitochondrial intermembrane space catalyzes the oxidation of sulfite to sulfate by transferring an atom of O_2 from water to sulfite and in the process, the enzyme undergoes a two-electron reduction (140). These electrons are then transferred from SO to cytochrome C and then into the electron transport chain, effectively inversely coupling sulfite concentration to O_2 availability. Humans with SO deficiency present with elevated urinary thiosulfate (113). Interestingly, SO is expressed in lung alveolar cells but not vessels (107).

④. Thiosulfate reduction: Thiosulfate is a simple persulfide and any factor that promotes its accumulation, for example, inhibition of electron transport, ETHE1, or SO can potentially produce H_2S. Thiosulfate is easily reduced to SO_2^{2-} and H_2S by endogenous reductants in the mitochondria, and this is catalyzed by 3-MST or TR (also known as Rde) (103, 178). This has been demonstrated in a variety of mammalian and non-mammalian tissues (124) and is likely favored during hypoxia when thiosulfate can accumulate in the mitochondria and the matrix becomes selectively reduced (183). This mechanism is predicted to be relatively rapid and may be considered the initial event in O_2 sensing; it would also conserve biologically relevant thiols by recycling sulfur.

Medium- to long-term effectors of H_2S concentration

⑤. CBS translocation to mitochondria: The mitochondrial heat shock protein (mtHsp 70) transports CBS from the cytosol to the mitochondrial matrix, where under normoxic conditions it is degraded by Lon protease. Oxygenation of the prosthetic heme group in the CBS appears to be key for Lon protease degradation (164). Loss of this O_2 in hypoxia prevents CBS degradation, enabling CBS to accumulate. Mitochondrial CBS concentration doubles within 10 min of hypoxia and increases sixfold within 1 h. Normoxia rapidly restores CBS to control levels within 5 min of normoxic reperfusion. This correlates with a hypoxic increase in mitochondrial, but not cytosolic H_2S production, which could be blocked by aminooxyacetate (AOA). H_2S generated by mitochondrial CBS prevents Ca^{2+}-mediated cytochrome C release from mitochondria, thereby preventing mitochondrial swelling, and decreases generation of reactive oxygen species (ROS). This has been proposed to explain the effects of exogenous H_2S on myocardial and hepatic ischemia/reperfusion injury (164).

⑥. CSE translocation to mitochondria: Hypoxia stimulates CSE translocation from the cytosol to the mitochondria in vascular smooth muscle cells to take advantage of a threefold increase in cysteine concentration and presumably generate ATP from H_2S, supposedly providing some protection from hypoxia (42). However, there are several problems with this theory: First, anaerobic metabolism is sufficient for energy production in vascular smooth muscle, even during hypoxia (35), and second, electron transport cannot continue in the absence of O_2. However, the H_2S formed by CSE translocation could obviously contribute to O_2 sensing and hypoxic vasodilation.

⑦. Cysteine dioxygenase: Cytosolic cysteine dioxygenase (CDO) irreversibly catalyzes the oxidation of cysteine to cysteinesulfinate, effectively eliminating sulfur from entering the H_2S pool (154). CDO activity is dynamically regulated by cysteine (as much as 450-fold), and this is important in the detoxification of excess dietary or metabolic cysteine. Conversely, the H_2S-forming trans-sulfuration pathway is not similarly regulated (154). In the absence of CDO, sulfur is redirected through the desulfuration pathway, which then increases thiosulfate and H_2S production (142, 173). Since O_2 is the only other substrate in CDO-mediated cysteine oxidation, it is likely that hypoxia will also impair cysteine oxidation and favor H_2S production. While this is not likely a rapid response, it could place a long-term bias on chronic O_2 sensing. The striking similarities between CDO and ETHE1 pathologies support sulfide/H_2S as a common factor (173). Apart from this, the inability of CDO to handle a large transient cysteine load may partially explain how hypoxic responses are augmented by exogenous cysteine (see ''Physiological Evidence for H_2S-Mediated O_2 Sensing'' section).

Indirect O_2 effects

⑧. 3-MST and Trx catalytic-site cysteines: Many enzymes contain cysteine in the catalytic site and since they generally have a low pKa, they are redox active (116). One example is the catalytic cysteine in 3-MST (Cys^{247}; rat), which, being exposed, is readily oxidized to a sulfenyl (R-SO) by O_2, peroxide (H_2O_2), or other oxidants. This inactivates the enzyme. The sulfenyl can be reduced by reduced Trx (117), and reactivated. Monomeric Rat 3-MST can also dimerize by mild oxidation of two other exposed cysteines, Cys^{154} and Cys^{263}, which also inactivates the enzyme. A defect in 3-MST activity, presented clinically as mercaptolactate-cysteine disulfidia, is believed to be associated with deficient H_2S production (117). It has been proposed that these three cysteines enable 3-MST to serve as an effective antioxidant (117). However, this could also be considered a key component of the O_2-sensing mechanism. Since hypoxia increases ROS in the cytosol while decreasing ROS in the mitochondrial matrix of both pulmonary and systemic arterial smooth muscle cells (183), this would be expected to augment mitochondrial H_2S production while inhibiting production in the cytosol. Parenthetically, the now-oxidized Trx can be reduced by Trx reductase using nicotinamide adenine dinucleotide phosphate (NADPH), which may be an overlooked but key explanation for why NADPH has been central in many O_2-sensing theories (52).

⑨. System X_c^-: System X_c^- is a cystine/glutamate antiporter that transports L-cystine into cells in exchange for glutamate (12). While system X_c^- is generally regarded as a mechanism to ultimately increase intracellular L-cysteine for subsequent glutathione synthesis and antioxidant protection, it could also provide a substrate for H_2S formation. In this regard, system X_c^- is upregulated in murine neural stem cells by hypoxic preconditioning, although the preconditioning periods were quite long, 45 min and 4 h (149). H_2S also increases X_c^- transporter activity in primary cultures of rat cortical neurons, which increases cystine uptake and intracellular cysteine (74). This could potentially provide a positive-feedback enhancement of H_2S production in hypoxia as well. It is also interesting that the highest activity of system X_c^- in the central nervous system is found in the hippocampus (149), which was one of the first areas in the central nervous system where the physiological effects of H_2S (selective enhancement of NMDA receptor-mediated responses and facilitation of hippocampal long-term potentiation) were demonstrated (1).

Inverse Relationship Between O_2 and H_2S

The fact that O_2 and H_2S do not typically coexist in tissues is pretty much a universal finding. H_2S is rapidly consumed in gills and gill mitochondria from sulfide-adapted mussel, *Geukensia demissa*, in normoxic conditions but reduced 50-fold by anoxia (79). In rat tissues, O_2 either inhibits H_2S production or it may be associated with H_2S consumption and both are reversed by hypoxia (30, 44). Similar observations have been made in a variety of tissues from numerous vertebrates (32, 89, 94, 126, 128, 186). A compelling argument for H_2S-mediated O_2 sensing can be made by comparing bovine and sea lion lungs. While both tissues exhibit an identical reciprocal relationship between H_2S production/consumption and O_2, both hypoxia and H_2S constrict bovine pulmonary arteries but these stimuli dilate pulmonary arteries from the sea lion (Fig. 4) (129).

O_2-Dependent H_2S Metabolism Occurs at Physiologically Relevant P_{O_2}

There is relatively little information on the specific P_{O_2}s at which H_2S oxidation becomes impaired. We used polarographic H_2S sensors to monitor the rate of tissue and mitochondrial H_2S consumption at carefully controlled P_{O_2} (129). This can then be compared with the P_{O_2} that produces hypoxic vasoconstriction or the O_2 sensitivity of other putative O_2-sensing mechanisms. As shown in Figure 5A, the efficiency of H_2S oxidation by bovine lung homogenate, bovine pulmonary arterial smooth muscle cells, or purified bovine heart mitochondria begins to fail when P_{O_2} falls below 30 mmHg; and half-maximal inhibition (P_{50}) of H_2S oxidation occurs around 4–7 mmHg for tissue and <1 mmHg for isolated mitochondria. These P_{50}s are physiologically relevant, as the corresponding P_{O_2}s are routinely encountered during hypoxia (182). Not surprisingly, the P_{50} for H_2S oxidation of bovine lung or pulmonary arterial smooth muscle is essentially identical to the P_{50} of hypoxic pulmonary vasoconstriction of bovine pulmonary arteries and also similar to that of lamprey and New Zealand hagfish dorsal aortas. Since mitochondria function at a P_{O_2} well below cytosolic P_{O_2}s, their P_{50} is also correspondingly lower as is the P_{50} for hypoxic vasoconstriction of the hypoxia-tolerant hagfish dorsal aorta. A slightly higher P_{50} (7.5 mmHg) has been observed for H_2S oxidation in mitochondria of *G. demissa* (79), which may reflect the ability of these animals to live in sulfidic environments.

The carotid body has an unusually high metabolic rate and O_2 sensitivity (16). Although O_2-dependent H_2S metabolism has not been measured in the carotid body, its corollary, O_2-dependent H_2S production has been measured (136) and it also correlates exceptionally well with O_2 sensitivity (Fig. 5B).

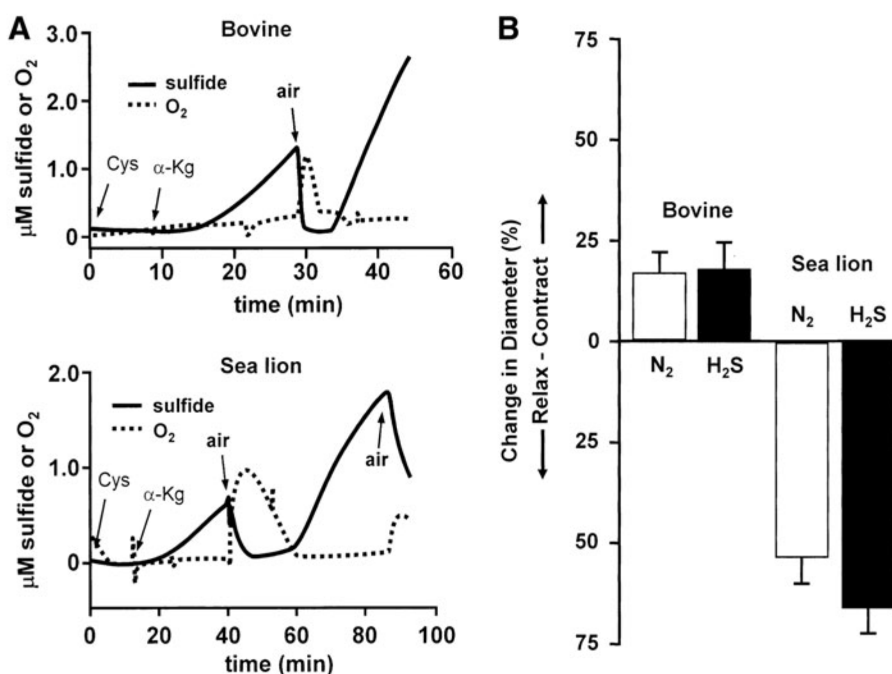


FIG. 4. Comparison of the effects of O_2 on tissue H_2 production (A) and vessel response (B) in bovine and sea lion tissues. **(A)** Continuous recording of O_2-dependent H_2S production by homogenized bovine and sea lion lungs measured in real time with amperometric electrodes. Cysteine (Cys, 1 m*M*) and α-ketoglutarate (α-Kg, 1 m*M*) were added at *arrows*. H_2S production steadily increased until samples were exposed to O_2 (air), at which time the H_2S was consumed. H_2S production resumed when the O_2 was consumed. **(B)** Effects of hypoxia (N_2) and H_2S (3×10^{-4} *M*) on U-46619 (10^{-6} *M*) precontracted bovine and sea lion resistance pulmonary arteries (RPA; <400 *μM* dia). Both stimuli contracted bovine but relaxed sea lion RPA. Redrawn from Olson *et al.* (129).

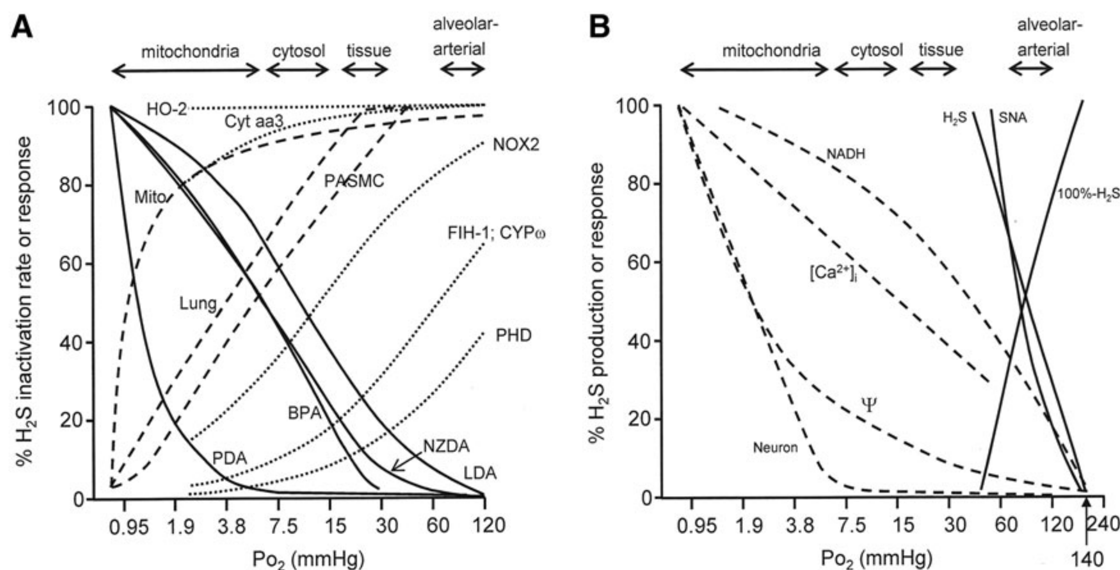


FIG. 5. Effect of O_2 on H_2S metabolism and tissue response. **(A)** Comparison of O_2 sensitivity of H_2S consumption by homogenized bovine lung (Lung), pulmonary arterial smooth muscle cells (PASMC), and bovine heart mitochondria (Mito) to hypoxic vasoconstriction in bovine pulmonary arteries (BPA), lamprey dorsal aorta (LDA), and dorsal aortas from New Zealand and Pacific hagfish (NZDA and PDA, respectively). O_2 sensitivity of tissue H_2S consumption is similar to O_2 sensitivity in vessels from O_2-sensitive vertebrates (bovine, lamprey, and New Zealand hagfish), whereas O_2 sensitivity in Pacific hagfish aortas is considerably lower commensurate with their tolerance to hypoxia. *Double-headed arrows* above figure show approximate ranges of O_2 tension in different tissue and cellular compartments. The partial pressure of oxygen (Po_2) values at which H_2S metabolism is impaired are at the low end of cytosolic and mitochondrial Po_2 and would be expected during hypoxia. *Thin lines* indicate efficacy of other putative O_2-sensing mechanisms, HO-2, mitochondrial cytochromes a and a3 (Cyt aa3), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2), factor inhibiting hypoxia-inducible factor-1α (FIH-1), cytochrome P450 monooxygenase ω (CYPω), and prolyl hydroxylase domain proteins involved in O_2-dependent hydroxylation of protein residues on hypoxia-inducible factor (HIF)-1α (PHD). **(B)** Comparison of O_2 sensitivity of afferent sinus nerve activity from the carotid body (SNA) to H_2S production (H_2S; or its inverse, 100%-H_2S) and components of intracellular signaling in the carotid body. The P_{50} for carotid activation is essentially identical to the P_{50} for H_2S production, which is more evident when the latter is expressed as the inverse (100%-H_2S). The P_{50} for intracellular excitation events, mitochondrial NADH, intracellular calcium ($[Ca^{2+}]_I$), mitochondrial transmembrane potential (Ψ), or activation of sympathetic neurons (Neuron). Carotid sinus nerve activation and H_2S production are well below the P_{50}s of the intact carotid body or H_2S production and more in line with other tissues in **(A)**. **(A)** Adapted from Refs. (102, 127, 129, 182). **(B)** Adapted from Refs. (16, 136).

Mechanism of H_2S Signaling

Sulfur is a highly reactive molecule that is able to bind with its own species as well as with nitrogen and O_2 moieties. This has led to a wide variety of potential sulfide signaling mechanisms, some of which are well established while others are only speculative. These are briefly described in the next few paragraphs; detailed reviews can be found in Refs. (75, 86, 134, 135, 167, 168, 181, 196). The downstream consequences of these initial signaling events on ion channel permeability, transport processes, enzyme activity, or structural proteins are not considered.

Reactions with sulfur

Cysteine is important in protein structure as well as an active component in many enzyme reactive centers; cysteine sulfur is also the most reactive nucleophile in the cell. Thus, it is susceptible to modification by a variety of molecules, including sulfide. Low-molecular-weight sulfides, namely H_2S and polysulfides (H_2S_n, where $n = 2$–8), can modify cysteine sulfur by either creating polysulfides or breaking disulfide bonds. Sulfide can also reverse the effects of cysteine nitrosylation and sulfenylation/sulfinylation by displacing nitrogen and O_2. These sulfur modifications are described next and shown in Figure 6. It should be noted that many of these reactions also generate other signaling molecules (H_2O_2, $HO_2^{\bullet}$, HNO, and H_2S_2) that may have additional (and perhaps greater) stimulatory effects.

Sulfhydration. The term ''sulfhydration'' was initially proposed by Mustafa *et al.* (114) to describe the mechanism of H_2S signaling by which H_2S combined with a protein thiol to form a persulfide (RSSH). Unlike nitroylsation, this was proposed to activate enzymes such as GAPDH and the authors reported that as many as 30% of protein thiols were sulfhydrated. The term ''sulfhydration'' is probably more correctly denoted as ''sulfuration'' (169). Sulfurated proteins and a variety of potential metabolic pathways for their production were previously described by Toohey (167), who designated the reactive sulfur as ''sulfane'' sulfur [see also Toohey (168)]. This further confounds the nomenclature, as ''sulfane'' is now the IUPAC name for H_2S (86). More importantly, it has recently been suggested that sulfuration of protein cysteine by H_2S is not possible, because the H_2S sulfur is in its most reduced form and, therefore, incapable of oxidizing cysteine sulfur (50, 75, 169, 193, 196). However,

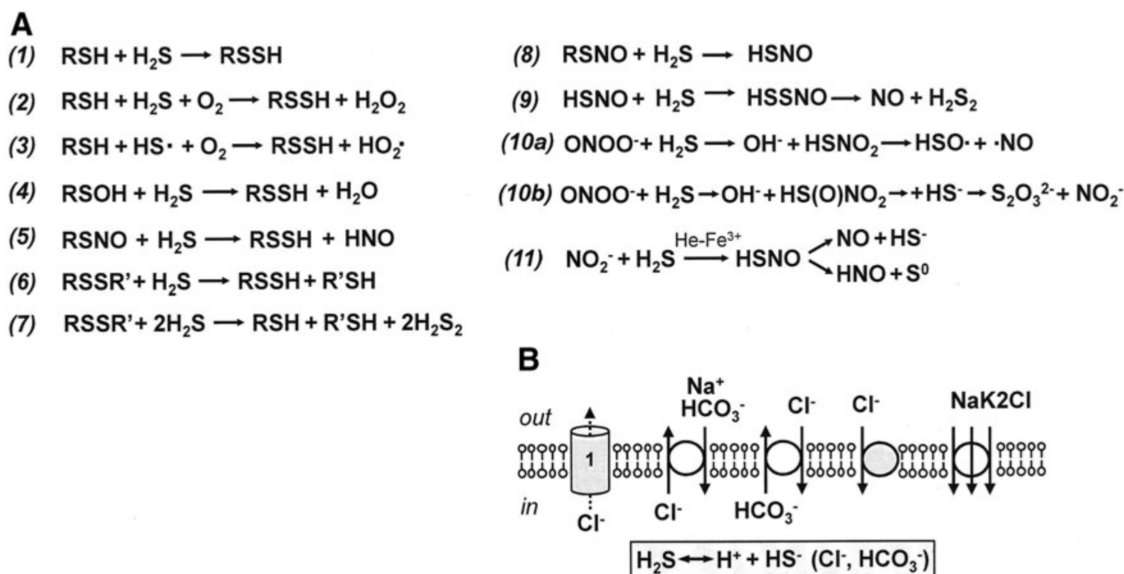
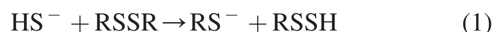


FIG. 6. Mechanisms of sulfide signaling. (A) Sulfhydration of a protein thiol. The generic mechanism proposed by Mustafa *et al.* (115); (1) is generally believed to be not possible, as the reduced sulfide is incapable of oxidizing cysteine sulfur. However, there are a number of other possible mechanisms, including spontaneous oxidation of H_2S (2), metal-catalyzed oxidation of H_2S with a radical intermediate (3), reaction with sulfenic acids (4), reaction with S-Nitrosothiols (5), or reduction of the disulfide bond (the latter may not be thermodynamically favorable) (193). It should be noted that reactions (4) and (5) can also proceed without sulfhydryl formation, thereby restoring the original thiol. Two-step reduction of a disulfide bond involving two H_2S (7). Reactions with nitrogenous compounds include an H_2S reaction with S-nitrosols to produce thionitrous acid (HSNO) (8), an HSNO reaction with excess H_2S to form HSSNO ($SSNO^-$), which slowly decomposes to nitric oxide (NO), and H_2S_2 (possibly *via* a disulfide radical, $SS^{\bullet -}$, intermediate, not shown) (9), scavenging peroxynitrite to form thionitrite ($HSNO_2$), which can produce $HSO^\bullet$ and $^\bullet NO$ under anerobic conditions (10a) or produce sulfinyl nitrite [HS(O)NO], which under aerobic conditions can react with additional H_2S and form $S_2O_3{}^{2-}$ and $NO_2{}^-$ (10b), or iron-catalyzed reduction of nitrite ($NO_2{}^-$) to form NO or nitroxyl (HNO) (11). Many of the reactions in **(A)** also generate other signaling molecules (H_2O_2, $HO_2{}^\bullet$, and HNO) that may have additional and perhaps greater stimulatory effects. **(B)** At physiological pH, from half to two-thirds of dissolved H_2S exists as the anion, HS^- in intracellular and extracellular fluid, respectively. This HS^- can compete with a variety of chloride (Cl^-) and bicarbonate ($HCO_3{}^-$) anion channels and transporters, thereby affecting transmembrane potential.

sulfhydration can be achieved through previous oxidation of H_2S or H_2S reduction of S-nitrosols or sulfenyl sulfur (193).

Disulfide bond cleavage. Certain protein disulfide bonds may serve as specific ''receptors'' for H_2S. Two H_2S molecules can reduce disulfide bonds in a two-step reaction; although a sulfhydrated intermediate is formed, it does not appear to be stable (161, 196). H_2S is proposed to initiate a nucleophilic attack in which 2 HS^- are involved in a two-step reaction:



$$HS^- + RSSR \rightarrow RS^- + RSSH \quad (1)$$

$$HS^- + RSSH \rightarrow RSH + HSSH \quad (2)$$

This reaction appears specific for H_2S as opposed to other thiols (cysteine, glutathione, *etc.*), because H_2S can penetrate deeply into the protein and H_2S may have some property in excess of its reducing ability to interact with these bonds (161). Unlike sulfhydration, disulfide bonds readily reform when H_2S is removed (196). H_2S appears to use this mechanism to close K_{ATP} channels by breaking disulfide bonds in the SUR subunit (65). In reaction 2 (above), the disulfide product, HSSH can also act as an oxidant and interact with other proteins (50).

Reduction of sulfenyl sulfur and S-nitrosothiols. Similar to the mechanism of sulfhydration (above), H_2S could signal by reducing sulfenyl or S-nitroso groups without formation of a persulfide. This would restore the original thiol. H_2S can also release NO from nitrosothiols, thereby initiating the NO signaling cascade (130).

Reactions with NO and related compounds

With NO. A number of *in vitro* studies in a marine echiuran worm and fish (67), mammalian heart (191, 192), and RAW246.7 cells or liver homogenate (185) have shown that addition of H_2S (as a sulfide salt or slow-releasing donor) to an NO donor (often sodium nitroprusside [SNP]) elicits a response in the tissue that is either greater than or different from the parent compounds. It has been proposed that this new signaling molecule is nitroxyl (HNO/NO^-), a one-electron reduced and protonated form of NO (191, 192), and/or a S-nitrosothiol (thionitrous acid [HSNO]) (40, 185). The manner in which H_2S and NO react has not yet been resolved, and a number of possibilities have been proposed (75). Neither HNO nor HSNO can be produced by the direct

interaction of H_2S/HS^- and NO as the former is a diamagnetic reductant or nucleophile, whereas NO is a paramagnetic free radical (43, 75, 86). However, one-electron reduction of H_2S/HS^- to produce the thiyl radical ($HS^•$) or a one-electron oxidized species of NO, such as nitrite (NO_2^-), can react to produce $HSNO/SNO^-$ (40). $HSNO/SNO^-$ is reported to be further metabolized to NO^+, NO, and NO^- with a variety of distinct stimulatory functions, and since it can diffuse freely across membranes, it may facilitate transnitrosation of proteins (40). SNP appears to be a poor choice of an NO donor, as it directly reacts with H_2S and produces HNO and although this is a source of HNO, the chemistry does not appear to be similar to H_2S/NO interactions in biological systems (38). $HSNO/SNO^-$ can further react with additional H_2S and form H_2S_2 (40). Recently, Cortese-Krott *et al.* (23) showed that excess H_2S (HS^-; from Na_2S or Gyy4137) interacted with S-nitroso-N-acetyl-DL-penicillamine (SNAP) and formed nitropersulfide anion ($SSNO^-$). This was more stable than SNO ($t_{1/2} >> 30$ min *vs.* <2 min), prolonged the release of NO from SSNO, and sustained vasodilator activity. The remaining hydropersulfide (HSS^-) can also mediate a variety of biological functions as described earlier, but again, the physiological relevancy of this is questionable.

With peroxynitrite. H_2S can scavenge peroxynitrite and form thionitrite ($HSNO_2$), which can then produce $HSO^•$ and $^•NO$ under anerobic conditions or sulfinyl nitrite [HS(O)NO] under aerobic conditions (39). The latter can react with additional H_2S and form $S_2O_3^{2-}$ and NO_2^-.

Reduction of nitrite. Nitrite (NO_2^-) has been proposed to be a regulator of hypoxic signaling *via* a heme-iron catalyzed reduction to NO, with the latter then mediating hypoxic vasodilation and protection against ischemia/reperfusion injury. However, the biochemistry has been difficult to resolve (176). Myoglobin (Mb) heme appears to be key to hypoxic vasodilation, as it is present in rat aortic smooth muscle and in Mb null rats ($Mb^{-\backslash-}$) hypoxic vasodilation is impaired (170). Recently, Miljkovic *et al.* (106) showed that H_2S is a rapid mediator of nitrite reduction by providing reducing equivalents to Fe^{3+}, thereby generating both NO and its reduced congener, nitroxyl (HNO). Vasodilation produced by acute hypoxia in mice *in vivo* appears to be independent of NO production, and it is associated with reduced plasma nitrite concentration and increased RSNOs (174). This is consistent with H_2S, as the initial hypoxic signal that mediates downstream increases in both H_2S_2 and HNO as shown in Figure 7. It also explains, parenthetically, why nitrite is an antidote for H_2S poisoning.

As an anion

Chloride is accumulated against an electrochemical gradient by a variety of cells, including vascular smooth muscle, and it is an important regulator of vasoactivity (21, 99). Metabolically generated bicarbonate (HCO_3^-) can interact with Cl^- antiporters and produce either vasodilation or vasoconstriction (95, 96). With the pK_{a1} of ~ 6.9 for the reaction $H_2S \leftrightarrow HS^- + H^+$, half of intracellular sulfide and more than two-thirds of extracellular sulfide is also an anion. Although H_2S freely diffuses across lipid membranes (100), it is conceivable that HS^- can also substitute for, or compete with, the main regulatory anions, Cl^- and HCO_3^- (Fig. 6B). This is supported by observations that HS^- is transported into mammalian red blood by the AE1 antiporter (64), and H_2S-mediated vasodilation appears to involve chloride/bicarbonate channels (76, 84).

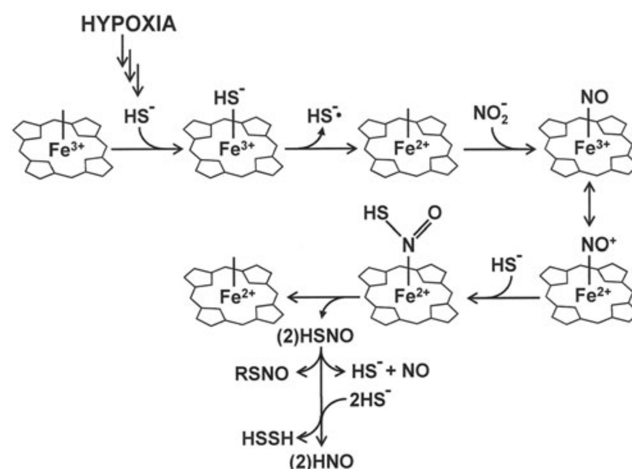


FIG. 7. Proposed mechanism of H_2S signaling in myoglobin-mediated formation of vasoactive molecules. H_2S produced during hypoxia reduces ferric (Fe^{3+}) myoglobin to ferrous (Fe^{2+}) myoglobin, liberating a thiyl radical ($HS^{-•}$). Ferrous myoglobin then binds NO_2^- and the myoglobin is re-oxidized, forming NO. Reduction of the iron forms a nitrosyl-Fe^{2+} that then binds an additional H_2S which results in the liberation of thionitrus acid (HSNO) and this can also give rise to H_2S, polysulfide (HSSH), NO and its reduced congener, nitroxyl (HNO), and protein S-nitrosothiol (RSNO), all of which could contribute to vascular signaling. Modified from Miljkovic *et al.* (106).

Physiological Evidence for H_2S-Mediated O_2 Sensing

Cardiovascular system

Identical responses to hypoxia and H_2S, be it constriction, dilation, or multi-phasic, have been observed in more than 30 studies on isolated vessels, perfused organs, and intact animals encompassing all classes of vertebrates (27, 94, 123, 125, 128, 129, 150, 151). Not surprisingly, H_2S also dilates the mouse ductus arteriosus (5), which would be expected to keep this vessel patent in the relative hypoxic intrauterine environment. Perhaps the strongest argument for the commonality of H_2S and hypoxia is the observation that both constrict bovine pulmonary arteries, whereas they dilate pulmonary arteries from the sea lion (129) (Fig. 4B).

Compounds that augment or inhibit H_2S production augment or inhibit hypoxic responses. Sulfur donors, especially cysteine, are well known to augment hypoxic responses (Fig. 8). Cysteine increases the magnitude of hypoxic vasoconstriction of isolated lamprey aortas (125), bovine pulmonary arteries (125, 129), and the perfused rat lung (94). Both reduced and oxidized glutathione augment hypoxic vasoconstriction in pulmonary arteries and the perfused rat lung and cysteine plus α-ketoglutarate (presumably utilizing the CAT/3-MST pathway) increases hypoxic vasoconstriction in bovine pulmonary arteries (94, 129). The presence of exogenous cysteine also sustains hypoxic vasoconstriction

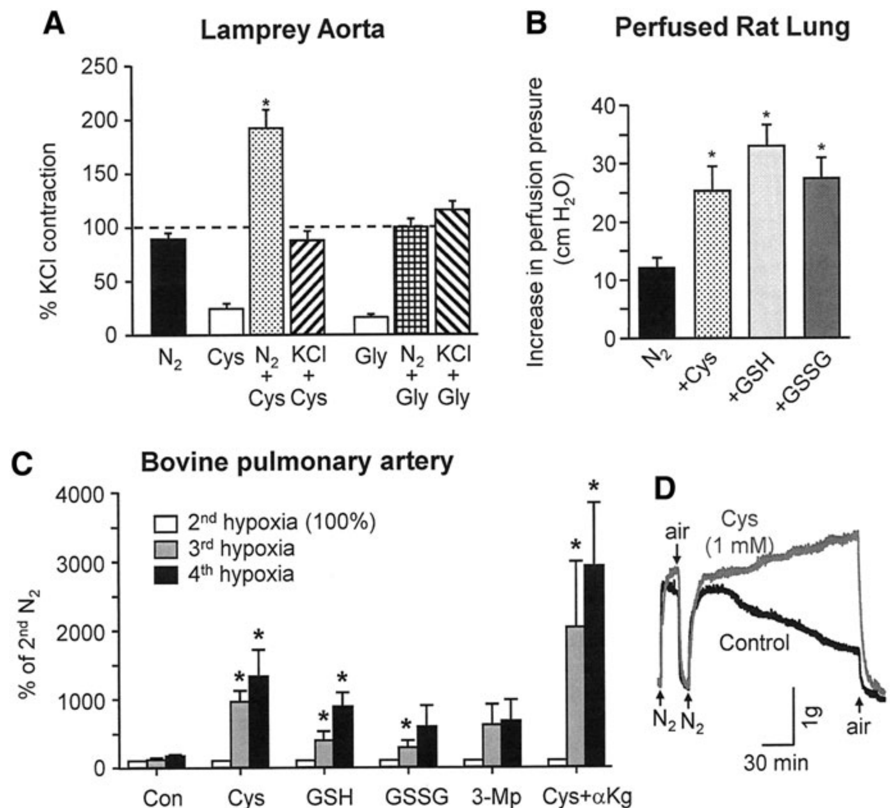


FIG. 8. Potential sulfur-donating molecules augment hypoxic vasoconstriction in vertebrates. (A) Cysteine (Cys, 1 m*M*) nearly doubles a hypoxic contraction but does not affect a KCl (80 m*M*) contraction in the lamprey aorta. Glycine (Gly, 1 m*M*) does not affect the hypoxic response. **(B)** Cys (1 m*M*) and reduced or oxidized glutathione (GSH, GSSG, 1 m*M*) increase perfusion pressure in the perfused rat lung. **(C)** 1 m*M* Cys, GSH, GSSG, and Cys + α-Kg enhance consecutive hypoxic contractions of bovine pulmonary arteries (BPA). **(D)** Representative myograph traces demonstrating the ability of Cys (1 m*M*) to prolong a hypoxic contraction in an isolated BPA. From Olson (120).

(Fig. 8D), enhances hypoxic relaxation of rat aortas (14) and perfused trout gills (150).

Although inhibitors of H_2S biosynthesis have a variety of inherent problems (3, 156), they have been shown to inhibit hypoxic responses of lamprey aorta, bovine pulmonary arteries, rat aorta, and perfused trout gills and rat lungs (94, 125, 150) (Fig. 9). With these caveats, CSE appears to be the major pathway for H_2S production by systemic vessels, and CBS plus perhaps CAT/3-MST contribute to H_2S production in bovine pulmonary vessels, whereas CSE may be important in the rat lung (94).

Respiration

General effects on respiration

Although high levels of H_2S inhibit respiration, an intravascular injection or inhalation of lower concentrations of H_2S mimics hypoxic hyperventilation in fish (126), birds (77), and mammals (7, 53, 55–57, 141, 175). These effects appear to be mediated through both central and peripheral mechanisms.

H_2S mediation of central respiratory centers

Injection of H_2S into the cerebral ventricles produces a K_{ATP} channel-mediated dose-dependent bradycardia and hypotension (90) mimicking the hypoxic diving reflex in mammals. H_2S increases discharge frequency from the pre-Bötzinger (pB) dorsal inspiratory respiratory group, and it may initially produce transient inhibition of the pB by stimulating the nearby parafacial respiratory group (pFRG) (19, 61). Discharge frequency of hypoglossal rootlets in medullary slices of female neonatal Sprague–Dawley rats is transiently decreased and then increased as H_2S is increased from 100 to 300 μM and then decreased at 400 μM H_2S (61). The effect of 100 μM H_2S was mimicked by 200 μM cysteine and the CBS activator S-adenosyl-L-methionine (AdoMet) and inhibited by the non-specific inhibitor, hydroxylamine. In a later study, Chen *et al.* (19) found that H_2S increased burst frequency in the pB complex and produced biphasic (initial decrease followed by an increase) responses in slices that contained the pFRG. Lesioning the pFRG removed the initial inhibitory effect of H_2S. A selective micro-injection into the preBötC increases discharge frequency whereas it decreases discharge frequency when injected into the pFRG, thus identifying the effect of H_2S in the two different respiratory centers. In other studies using a similar preparation (132, 133), the authors found that H_2S prevented the inhibitory effect of hypoxia on burst activity and these effects were suppressed by pretreatment with the K_{ATP} channel inhibitor glibenclamide and enhanced by cysteine. Hydroxylamine postponed recovery from hypoxic inhibition and significantly enhanced hypoxia-induced increase in malondialdehyde content in the slices. Hypoxia-induced upregulation of *c-fos* mRNA could be antagonized by SAM but was increased by hydroxylamine. These studies suggest that H_2S helps protect the medullary respiratory centers from hypoxic injury *via* an antioxidation effect and by downregulation of *c-fos*.

Although removal of the first pair of gill arches, the site of peripheral chemoreceptors in the rainbow trout, inhibited hypoxic bradycardia, it did not affect hypoxic hyperventilation, nor did addition of the CBS inhibitor, AOA, or the CSE inhibitor, PPG (126). This suggests that fish have central chemoreceptors as well; however, it remains to be determined whether H_2S signaling is involved.

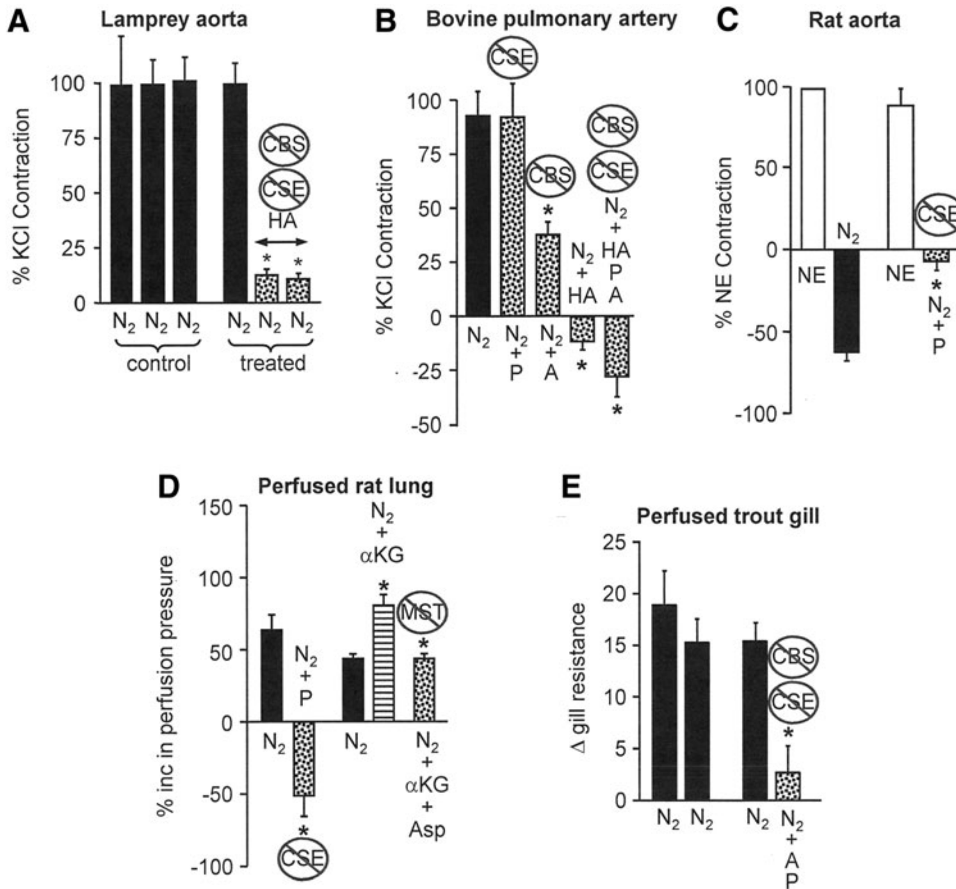


FIG. 9. Inhibiting H_2S production inhibits hypoxic vasoconstriction and vasodilation. Inhibitors of H_2S biosynthesis inhibit hypoxic vasoconstriction in the lamprey aorta **(A)**, and BPA **(B)** and hypoxic vasodilation of the norepinephrine (NE, 1 μM) precontracted rat aorta **(C)**. Inhibition of H_2S biosynthesis also inhibits hypoxia vasoconstriction in the perfused rat lung **(D)** and perfused trout gill **(E)**. A, aminooxyacetate a CBS inhibitor (1 m*M*); Asp, aspartic acid, an inhibitor of MST (10 m*M*)); HA, CBS, and CSE inhibitor hydroxylamine (1 m*M*); P, propargyl glycine a CSE inhibitor (10 m*M*; α-Kg, α-ketoglutarate a substrate for MST. From Olson (120).

H_2S mediation of peripheral chemoreceptors, NEC, and carotid body

H_2S stimulates peripheral chemoreceptors (NEC) in the trout gill, and this mediates hypoxic bradycardia (126). H_2S also depolarizes NEC isolated from zebrafish gills, as does hypoxia. Although both CBS and CSE transcripts have been identified in the gills of rainbow trout, the specific location of these enzymes has not been resolved (126). Since NEC are the antecedents of carotid glomus cells (66), it is likely that NEC signaling is homologous to that in their mammalian counterparts.

A number of studies have shown that exogenous H_2S depolarizes the mammalian carotid glomus cells, increases afferent nerve activity, and *in vivo* mimics hypoxic hyperventilation (15, 26, 87, 97, 136, 145). As described in section ''H_2S as a Downstream Effector of CO-Mediated O_2 Sensing,'' this presumably entails closing BK_{Ca} and/or TASK channels. Low doses of exogenous H_2S have a synergistic effect on the hypoxic response and accelerate the sinus nerve response to hypoxia, whereas maximal stimulatory concentrations prevent hypoxic responses (87, 97). This is similar to that observed in blood vessels and suggests that hypoxia and H_2S operate through a common effector mechanism. However, it should be noted that H_2S has been reported to inhibit release of ATP and acetylcholine in the carotid body (41). Since the latter results seem to be inconsistent with all other studies on the carotid body, they need to be confirmed.

CBS and CSE immunoreactivity has been identified in glomus cells in the cat, rat, and wild-type (WT; $CSE^{+/+}$) mouse but CSE immunoreactivity is absent in CSE null ($CSE^{-/-}$) mice (41, 87, 97, 136). This has afforded a means of approximating the sources of endogenous production, which can be complicated by the nonspecificity of enzyme inhibitors. Li *et al.* (87) inhibited hypoxia-stimulated afferent nerve activity in the mouse carotid body *in vitro* and blunted hypoxic hyperventilation *in vivo* with either AOA or hydroxylamine, suggesting that H_2S is derived from CBS as PPG and β-cyanoanaline were ineffective. Other studies have suggested that the main source of glomus cell H_2S is CSE, as hypoxic responses were not demonstrated in the presence of CSE inhibitors or in CSE null mice and CBS inhibitors were generally ineffective (26, 97, 136, 145). However, lower inhibitor concentrations of AOA and PPG, when combined, had a greater inhibitory effect than AOA alone, suggesting some degree of CBS activity as well. Perhaps related is the observation that PPG enhances the hyperventilatory response to hypercapnia (26, 136). This suggests that a CBS- or MST-mediated CO_2-sensing pathway was unmasked. If this turns out to be the case, it will add another dimension to H_2S signaling in chemoreceptor cells. Recent studies have shown that the commonly employed CBS inhibitor, AOA, is even a more potent inhibitor of CSE (3) and the role of CBS-mediated H_2S production in chemoreceptor cells needs to be re-evaluated. Nevertheless, is evident that H_2S is intimately involved in peripheral chemoreceptor function.

Hypoxia (Po_2 ~30 mmHg) increases H_2S production in rat carotid bodies (97, 136) (Fig. 5B) and this, but not baseline production, is inhibited by PPG. H_2S production was also lower in $CSE^{-/-}$ than in WT mice, and hypoxia or the CO donor ($[Ru(CO_3)Cl_2]_2$) increased H_2S production in WT, but not $CSE^{-/-}$ animals. (Conversely, breathing 100% O_2 suppresses H_2S-induced hyperventilation, suggesting that enhanced O_2 increases H_2S metabolism by the glomus cells) (175). However, published rates of H_2S production by glomus cells should be viewed with caution. Assuming a cell with 15% protein and 70% cytosolic water by weight, 1 nmol H_2S/mg cell protein is equivalent to 214 μmol H_2S/L in the cytosol. Using this conversion, the basal tissue production rate of 55 nmol H_2S/h/mg protein reported in mouse glomus cells by Peng *et al.* (136) would increase cytosolic H_2S to12 m*M* in 1 h. Doing so would essentially consume all of the glutathione sulfur in a typical cell in 1 h. Peak production of 1300 nmol H_2S/h/mg protein reported by Peng *et al.* (136) in rat carotid bodies would increase cytosolic H_2S to 278 m*M* in 1 h. This would consume all of the cell's sulfur in less than 15 min.

The effects of H_2S on glomus cells have been attributed to BK_{Ca} channels (87, 162, 163) or TASK channels (15). Li *et al.* (87) showed that in whole cell recordings of mouse glomus cells, both hypoxia and H_2S inhibited BK_{Ca} channels and AOA could inhibit the hypoxic affect. CO reversed the H_2S inhibitory effect on the channels. K_{ATP} channels do not seem to be involved (136).

Calcium influx is a requirement for hypoxic and H_2S activation of glomus cells (15, 87, 97, 136). Hypoxia-, but not H_2S-mediated increases in intracellular calcium are inhibited by PPG and do not occur in $CSE^{-/-}$ mice.

Glomus cells exhibit an unusually high sensitivity to hypoxia (Fig. 5B), and an increase in afferent nerve activity is often observed when Po_2 falls below 100 mmHg (16, 97, 136). Glomus activation is often regarded as a sequential process by which the O_2 sensor (CO, H_2S, inhibition of oxidative phosphorylation, i.e., the metabolic hypothesis, or others) initiates potassium channel closure and the resulting cellular depolarization increases calcium entry. This increases intracellular calcium, initiates neurotransmitter release, and activates neuronal afferents. However, recent studies have suggested that this may be a two-step process in which the O_2 sensor initiates an increase in intracellular calcium and a second regulatory step involving either a downstream effector or the activated sensor (or a mechanism coupled to it) acts through protein kinase C (PKC) to modulate the magnitude of responsiveness (34). Although the exact mediators of this process are unknown, it is interesting that H_2S can directly affect both potassium (and even calcium) channels and PKCε (122).

Other studies suggest that H_2S mediates chemoreflex activity under pathological conditions. Heart failure, hypertension, and renal failure activate the carotid body, which contributes to breathing instability and autonomic dysfunction (145). In a rat model of congestive heart failure, PPG inhibition of CSE decreased the hypoxic chemoreflex response and afferent sinus nerve activity, as it did in normal rats. PPG also led to breathing stability, decreased sympathetic nervous system activity, substantially reduced apnea index and breath rate variability, and partially reversed heart rate and systolic blood pressure variability (26).

Role of CO

It has thus far been difficult to determine whether CO is the actual O_2 sensor and H_2S modulates this response or vice versa. As described earlier, a number of studies have suggested that CO is the O_2 sensor and the H_2S effects are modulatory. The CO donor $[Ru(CO_3)Cl_2]_2$ reverses the stimulatory effect of H_2S on the sinus nerve and the inhibitory effect of H_2S on BK_{Ca} channels and inhibits H_2S production by glomus cells in WT but not $CSE^{-/-}$ mice (87, 136). Conversely, hypoxic hyperventilation is not affected in HO-2 null mice (131). H_2S appears to have more of a direct effect on BK_{Ca} channels, whereas CO appears modulatory (162, 163) and HO inhibitors such as zinc protoporphyrin IX directly bind to H_2S (33). Obviously, this field needs an additional study.

H_2S mediation of peripheral chemoreceptors, adrenal medulla

Chromaffin cells, containing both CBS and CSE, line the posterior cardinal vein and anterior kidney in trout and are homologous to adrenal medullary cells in mammals. They respond to hypoxia by a CBS-mediated release of H_2S and epinephrine into the systemic circulation (137). As with type I glomus cells, catecholamines are released by exogenous H_2S and this requires extracellular calcium, suggesting that H_2S is involved in chromaffin cell depolarization.

Mammalian adrenal medullary chromaffin cells are considered to provide an O_2-sensing role during neonatal development of the carotid bodies (66). CSE immunoreactivity has been found in rat and mice neonatal chromaffin cells and hypoxic stimulation of catecholamine secretion appears to be mediated in mice and rats by H_2S, likely through CSE (136, 195). Exogenous H_2S directly induces catecholamine release from adrenal cells cultured from rats by inhibiting BK_{Ca} channels.

Airway receptors

In spontaneously breathing chickens, low concentrations (0.2%) of inhaled H_2S stimulate ventilation, an effect that may be mediated in part by airway receptors (77, 172). Vagal and sensory neurons in mammalian airways may also be activated by H_2S (158).

Mechanical effects on airway smooth muscle

Hypoxia and H_2S relax tracheal and bronchiolar airway smooth muscle, although there is some species specificity regarding the efficacy of H_2S (20, 80). Hypoxic relaxation is accompanied by a decrease in intracellular calcium and may or may not be mediated by K_{ATP} channels (88, 177). H_2S-mediated relaxation of airways appears to be independent of K_{ATP} channels, prostaglandins, and NO (80). In an elegant study, Castro-Piedras and Perez-Zoghbi (18) showed that H_2S dilated small (~200 μM) airways in lung slices and they observed a considerably lower EC_{50} (36 μM) than the 300 μM reported by Kubo *et al.* (80). These differences could be due to increased small airway sensitivity to H_2S and/or due to the closed conditions in the study by Castro-Piedras and Perez-Zoghbi (18), which prevented H_2S volatilization. Conversely, much higher, H_2S concentrations (EC_{50} = 1.3 m*M*) produce a dose-dependent contraction in guinea pig main

bronchae and distal trachea that appears to be mediated by activation of vanilloid neurons (171). We (Olson, unpublished) have also observed H_2S relaxation of bovine bronchioles in the range reported by Kubo *et al.* (80).

Other Tissues

Hypoxia and H_2S also relax nonvascular smooth muscle of fish urinary bladder and the gastrointestinal tract, both of these stimuli produce a unique and transient increase in spontaneous contraction frequency and amplitude before the onset of the inhibitory effects (31, 32). H_2S also relaxes human corpus cavernosum and urinary bladder smooth muscle (24, 45). Addition of exogenous cysteine, which presumably enhances or sustains H_2S production, augments hypoxic relaxation of trout urinary bladder (31) and salmon intestine (32). Consistent with findings in other tissues, inhibitors of H_2S biosynthesis inhibit hypoxic relaxation of rainbow trout urinary bladder (31) and rainbow trout and Coho salmon intestine (32).

General Metabolism

In addition to cells and tissues which serve as homeostatic O_2 sensors, there is some evidence that H_2S-mediated O_2 sensing is involved in general cellular metabolism and function. As described earlier, cells can use H_2S as a substrate for ATP production and this can serve as an endogenous stimulator of cellular bioenergetics (49, 157). These possibilities are currently being explored, albeit with limited success thus far, in protecting tissue from ischemia/reperfusion injury, organ preservation before transplantation, protective metabolic depression during bypass surgery or after severe trauma associated with shock, sepsis, and acute lung injury.

H_2S can also produce metabolic depression (akin to ''suspended animation'') in small mammals (9, 10), and it has been implicated in torpor and hibernation (159, 160), anapyrexia (81), and hypoxia-induced radiation resistance (194). H_2S has also been shown to protect cells from both hypoxia- and hyperoxia-induced redox imbalance and apoptosis (13, 83, 85, 92, 101, 189, 190). The beneficial effects of H_2S in treating ischemia/reperfusion injury and in pre- and post-conditioning have been well documented, and it has been suggested that the beneficial effects of exogenous H_2S are due to mimicking transient hypoxia, which is also used in conditioning (128).

It is also evident that H_2S is involved in long-term O_2 sensing through its inhibitory effects on hypoxia inducible factor (HIF)-1α expression (70, 148, 149, 188), although opposite effects have also been reported (17, 91, 93). In an elegant study, Ma *et al.* (93) identified a novel genetic pathway where an increase in H_2S produced by prolonged (24 h) hypoxia increases HIF-1 expression in *C. elegans*. They showed that H_2S promotes the interaction of CYSL-1 with EGL-9, thereby removing the inhibitory effect of EGL-9 on HIF-1. This mechanism is independent of EGL-9 hydroxylation of HIF-1 and does not utilize the von Hipple–Lindau pathway for HIF-1 degradation. Interestingly, they (93) also showed that CYSL-1 is in the family of CBS proteins, albeit with insignificant enzymatic activity, and they propose that a mammalian CYSL-1-like CBS protein is involved in protection from reperfusion injury.

Conclusions

O_2-dependent inactivation of H_2S is an effective, highly accurate, and tightly coupled mechanism that is used by a variety of O_2-sensing tissues and cells to detect O_2 availability and to coordinate supply and demand. A variety of observations support this hypothesis. H_2S metabolism appears ubiquitous, even predating oxidative phosphorylation. H_2S is produced by cells independent of O_2, whereas numerous O_2-dependent biochemical pathways affect its destruction and this occurs at physiologically relevant O_2 tensions. Protein cysteines, which are often key regulatory components of protein structure and enzymatic activity, readily react with H_2S and initiate downstream signaling processes. Vertebrate cardiovascular and respiratory systems, the primary effectors of O_2 supply, are replete with examples of H_2S-mediated O_2 sensing and their functions can be enhanced or inhibited by factors that promote or prevent H_2S metabolism. However, challenges remain. Additional information is needed on H_2S metabolism and interaction with sulfides (including H_2S itself), other putative O_2-sensing mechanisms, and effector processes. Better inhibitors of H_2S metabolism are needed as are more specifically targeted and controlled H_2S ''donors.'' Finally, this field needs to develop into the realm of therapeutic application, which, of course, is one of the goals of scientific investigation.

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Address correspondence to:
Dr. Kenneth R. Olson
Department of Physiology
Indiana University School of Medicine—South Bend
1234 Notre Dame Ave.
South Bend, IN 46617

E-mail: kolson@nd.edu

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Abbreviations Used

α-Kg = α-ketoglutarate
Ψ = mitochondrial transmembrane potential
3-MST = 3-mercaptopyruvate sulfurtransferase
AdoMet = S-adenosyl-L-methionine
AMP = adenosine monophosphate
AOA = aminooxyacetate
AOX = alternative oxidase
BK_{Ca} = large-conductance Ca^{2+}-dependent K^+ channels
BPA = bovine pulmonary arteries
CAT = cysteine aminotransferase
CBS = cystathionine β-synthase
CCO = cytochrome C oxidase
CDO = cytosolic cysteine dioxygenase
c-fos = proto-oncogene
CO = carbon monoxide
CPA = conductance pulmonary arteries
CSE = cystathionine γ-lyase
CYPω = cytochrome P450 monooxygenase ω
Cyt aa3 = mitochondrial cytochromes a and a3
DAO = D-amino acid oxidase
DHLA = dihydrolipoic acid
ETHE1 = mitochondrial sulfur dioxygenase
FIH-1 = factor inhibiting hypoxia inducible factor-1α
GSH = reduced glutathione
GSSG = oxidized glutathione
H_2S = hydrogen sulfide
HA = hydroxylamine
HIF = hypoxia inducible factor
HNO = nitroxyl
HO-2 = hemeoxygenase-2
HS(O)NO = sulfinyl nitrite
HSNO = thionitrous acid
$HSNO_2$ = thionitrite
$HS^{-\bullet}$ = thiyl radical
K_{ATP} = ATP-dependent potassium channels
K_{Ca} = Ca^{2+}-dependent K^+ channels
Kv = voltage-gated potassium channels
LDA = lamprey dorsal aorta
Mito = mitochondria
mtHsp 70 = mitochondrial heat shock protein
NADPH = nicotinamide adenine dinucleotide phosphate
NE = norepinephrine
NEB = neuroepithelial body
NEC = neuroepithelial cell
NO = nitric oxide
NO_2^- = nitrite
NOX2 = NADPH oxidase
NZDA = New Zealand hagfish dorsal aorta
O_2 = oxygen
PASMC = pulmonary arterial smooth muscle cells
pB = pre-Bötzinger
PDA = Pacific hagfish dorsal aorta
pFRG = parafacial respiratory group
PHD = prolyl hydroxylase domain proteins on HIF-1α
PKC = protein kinase C
P_{O_2} = partial pressure of oxygen
PPG = propargyl glycine
Rde = rhodanase
ROS = reactive oxygen species
RPA = resistance pulmonary arteries
SNA = sinus nerve activity
SNP = sodium nitroprusside
SO = sulfite oxidase
SQR = sulfide:quinone oxidoreductase
ST = sulfur transferase
TASK = TWIK-related acid-sensitive potassium
TR = thiosulfate reductase
TRD = Trx reductase
Trx = thioredoxin
WT = wild-type