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Hydrogen Sulfide and Oxygen Sensing in the Cardiovascular System

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Abstract

Vertebrate cardiorespiratory homeostasis is inextricably dependent upon specialized cells that provide feedback on oxygen status in the tissues, blood, and on occasion, environment. These "oxygen sensing" cells include chemoreceptors and oxygen-sensitive chromaffin cells that initiate cardiorespiratory reflexes, vascular smooth muscle cells that adjust perfusion to metabolism or ventilation, and other cells that condition themselves in response to episodic hypoxia. Identification of how these cells sense oxygen and transduce this into the appropriate physiological response has enormous clinical applicability, but despite intense research there is no consensus regarding the initial hypoxia-effector coupling mechanism. This review examines an alternative mechanism of oxygen sensing using oxidation of endogenously produced hydrogen sulfide (H_2S) as the O_2-sensitive couple. Support for this hypothesis includes the similarity of effects of hypoxia and H_2S on a variety of tissues, augmentation of hypoxic responses by precursors of H_2S production and their inhibition by inhibitors of H_2S synthesis, and the rapid consumption of H_2S by O_2 in the range of intracellular/mitochondrial Po_2. These studies also indicate that, under normoxic conditions, it is doubtful that free H_2S has longer than a transient existence in tissue or extracellular fluid. *Antioxid. Redox Signal.* 12, 1219–1234.

Introduction

In coining the term "gasotransmitter," Rui Wang specified that these signaling molecules must be: (a) small molecules of gas, (b) freely membrane permeable, (c) endogenously and enzymatically generated and this must be regulated, (d) have defined functions at physiological concentrations, and (e) have specific molecular targets (68). With the exception of the first two, these are the same characteristics of neurotransmitters. One could add an additional criterion for gasotransmitters, or signaling systems in general: the optimal signaling mechanism should possess characteristics that either render it uniquely sensitive to the regulated parameter, or make it particularly efficacious in driving the effector response. Ideally it would have both. This review examines one gasotransmitter, hydrogen sulfide (H_2S), and its potential role in acute oxygen (O_2) sensing. Long-term (chronic) effectors of hypoxic responses that regulate genetic responses, such as the hypoxia inducible factor (HIF) transcription factor family, sustain and augment the initial responses but are not considered here.

In this review we will present evidence to support the hypothesis that O_2-driven H_2S metabolism is a physiologically significant O_2 sensor that responds to acute hypoxia in a variety of biological systems. In order to support this model, it must be demonstrated that H_2S can be produced and metabolized by O_2 sensing cells, H_2S is consumed by the cells in the presence of O_2, and H_2S initiates the appropriate effector response.

Variations of Hypoxia

While hyperoxia has deleterious consequences of its own, the most serious and pervasive threat to eukaryotic cells is hypoxia. This can be of either external or internal origin.

When terrestrial animals ascend to higher altitudes, ambient partial pressure of oxygen (Po_2) falls due to the decrease in barometric pressure according to the relationship: $Po_2 = fo_2 \cdot Pb$; where fo_2 is the altitude-independent mole fraction of oxygen in air (0.21) and Pb is the altitude-dependent barometric pressure. At 3,000 meters Pb and therefore Po_2 is 30% lower than at sea level. For climbers at the summit of Mount Everest (8,848 meters) the ambient (and arterial) Po_2 is ~43 mmHg, and for all practical purpose the lower limit for human survival (39). Burrowing animals may have little convective delivery of fresh air into their burrows and likewise experience very low (40–50 mmHg) Po_2s (55). Air-breathing animals that forage for food underwater (*i.e.*,

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whales, porpoises, seals, otters, penguins, and some ducks), may remain underwater for extended periods (up to 2 h) and since they are unable to obtain oxygen from the environment are subjected to an acute and abrupt environmental anoxia. Even the mammalian fetus experiences a form of external hypoxia relative to an adult as the arterial Po_2 is usually no more than 40 mmHg (69).

Aquatic hypoxia is even more problematic. Compared to air, water has a lower capacity for O_2 (1/30), diffusion through water is slower (Krogh's diffusion coefficients 1/200,000), and O_2 solubility decreases by $\sim$20% for every 10°C increase in temperature and another 20% in seawater compared to freshwater. Water's high viscosity (60 times that of air) increases ventilatory work and can decrease convective delivery of O_2 from O_2-rich surface water. Furthermore, ambient hypoxia occurs in aquatic environments seasonally, diurnally, or episodically, it is a common feature of both freshwater and saltwater environments, and it is often associated with increased [H_2S] (3).

Hypoxia of internal origin can be either global or regional. Global internal hypoxia can be produced by a decrease in the ventilation/perfusion ratio in the respiratory organs or impaired diffusion across the gas exchange surface. The former is most often the result of impaired ventilation [*i.e.*, a variety of hypoventilation syndromes (obesity, neuromuscular, skeletal, obstructive sleep apnea, chronic obstructive lung disease; COPD), and the latter from pulmonary edema (60)]. Often these clinical scenarios have multiple effects. Insufficient O_2 uptake produces hypoxemia (low blood O_2 content). Tissue hypoxia can result from delivery of hypoxemic blood (hypoxemic hypoxia) or insufficient blood flow relative to metabolism (ischemia or ischemic hypoxia).

O_2 Reporting, O_2 Responding, and O_2 Sensitive Cells

Cells can be divided into three main categories, depending on their response to changes in either oxygen content or Po_2: oxygen reporters, oxygen responders, or oxygen-sensitive cells. O_2 reporters monitor external or internal oxygen status and typically initiate distant cardioventilatory effector responses. O_2 responders monitor local or regional oxygen status and initiate effector responses in the same general area. O_2 sensitive cells respond metabolically to changes in oxygenation but typically they do not affect other cells.

O_2 reporters

Monitors of external hypoxia. Clusters of O_2-sensitive cells form neuroepithelial bodies (NEBs) that line the airways of mammalian lungs (26). NEBs are most prominent in neonatal lungs, especially near airway bifurcations. They are believed to monitor airway Po_2 and help initiate breathing and optimize ventilation/perfusion matching. NEBs act through both central reflexes and local (paracrine) stimulation of adjacent pulmonary vasculature (26). NEB cells are more responsive to airway hypoxia than hypoxemia. The role of NEB in adult animals is unclear and it remains to be identified if they function as a general monitor of environmental Po_2 in air-breathing vertebrates.

Aquatic vertebrates, such as fish and most larval and a few adult amphibians (*e.g.*, tadpoles, frogs, salamanders, and caecilians), are often subjected to environmental hypoxia and possess well-developed external O_2-reporting neuroepithelial (NECs) cells. NECs are scattered over the gill surface, and they are especially prevalent distally on the leading (inhalant) edge of the gill filament. In hypoxia-sensitive fish such as trout, they initiate a reflex bradycardia and increase ventilatory rate and amplitude when water Po_2 falls to 75–100 mmHg (38). In some fish (*e.g.*, trout), these NECs are predominantly on the first gill arch, the homolog of the mammalian carotid body; in others (bowfin, catfish) they are more evenly distributed over the gills and even orobranchial cavity (38).

Monitors of hypoxemia. Carotid bodies in mammals and other tetrapods are small paired organs located at the bifurcation of the internal and external carotid arteries and ideally positioned to monitor O_2 delivery to the brain. Because their blood flow and O_2 consumption per tissue weight is the highest of any organ, they are reporters of blood Po_2 rather than O_2 content. Carotid bodies are the primary, if not only, sensor of acute and chronic arterial hypoxemia in the adult mammal (38). Type I glomus cells, similar to the NEC of lower vertebrates, are the primary chemosensory cell. The Po_2 threshold for activation of isolated type I cells from the rabbit is $\sim$40 mmHg and half-maximal activation of many of these cells occurs $\sim$10 mmHg or less (5). Given their high metabolic rate, it is quite likely that the intracellular Po_2 at which the glomus cells are activated is considerably lower. Although many neurotransmitters have been identified in the carotid body, the main neurotransmitters appear to be acetylcholine and ATP (54). Discharge from the carotid bodies is integrated into complex cardiovascular reflexes. Hypoxemia initiates an increase in ventilation and peripheral vasoconstriction when breathing is unimpaired, while it produces a reflex bradycardia and peripheral vasoconstriction if breathing is not possible. Although fish lack carotid bodies, vascularly oriented NECs in the gills are homologous monitors of arterial Po_2. Unlike their mammalian counterparts, they appear more sensitive to blood oxygen content than blood Po_2 and they secrete serotonin in response to hypoxemia which also stimulates ventilation (38).

O_2-sensitive chromaffin cells are present in the adrenal medulla of fetal and newborn, but not adult, mammals and in the heart and vasculature (mainly systemic veins) of adults from the more primitive vertebrates (41). Hypoxemia stimulates the mammalian adrenomedullary chromaffin cells to release catecholamines into the bloodstream where they have stimulatory cardiorespiratory effects. Their location suggests that they provide information on the balance between O_2 delivery and tissue utilization although the low Po_2 threshold for secretion, $<$5 mmHg, suggests that they are more active during severe hypoxia. In fish, hypoxemia stimulates catecholamine secretion which initiates cardiorespiratory responses and increases O_2 transport by red blood cells.

O_2 responders

Blood vessels are perhaps the best example of local O_2 responders and this has been extensively studied in mammals where hypoxia contracts pulmonary vessels, relaxes systemic vessels, and in the fetus relaxes the ductus arteriosus. Hypoxic pulmonary vasoconstriction (HPV) decreases perfusion of poorly ventilated regions of the lung, and thus decreases the potential for partial unsaturation of pulmonary venous (systemic arterial) blood. Hypoxic systemic vasodilation (HSD)

increases tissue perfusion, thereby increasing O_2 delivery. Both HPV and HSD are intrinsic responses of pulmonary and systemic vascular smooth muscle cells (36). Although HPV is believed to be a unique attribute of the pulmonary circulation, this is not the case in nonmammalian vertebrates as hypoxia also constricts many systemic conductance (>500 μm dia) vessels in a range of vertebrates from hagfish and lamprey to birds (44). Furthermore, we have recently shown that hypoxia also dilates resistance (<400 μm dia) pulmonary arterioles in diving mammals (48) and birds (Olson and Madden unpublished). The effects of hypoxia on respiratory and systemic vessels from a wide range of vertebrates are summarized in Table 1 and several examples shown in Fig. 1.

O_2 sensitive cells

Essentially all cells are affected by hypoxia and the most pervasive responses to cellular hypoxia is hypometabolism and alterations in gene expression (12, 55). While metabolic depression has been induced by combined hypoxia and/or H_2S in small mammals, it is beyond the scope of this review. Cells from many tissues have also shown the ability to be 'conditioned' against injury resulting from reperfusion after prolonged ischemia by being pre-treated with several brief hypoxic episodes. These mechanisms will be considered in subsequent sections.

O_2 Sensing Mechanisms

O_2 reporting and O_2 responding cells

The search for O_2 sensors has heavily emphasized mammalian blood vessels and chemoreceptors. Initial studies suggested that depolarization due to closure of O_2-sensitive, voltage-gated potassium (Kv) channels in pulmonary arterial smooth muscle cells, or hyperpolarization after opening ATP-dependent potassium channels (KATP) in systemic vascular smooth muscle, would produce the appropriate change in intracellular calcium leading to vasoconstriction or dilation, respectively. Kv channels have been identified in most, if not all, O_2-sensing tissues including pulmonary arteries, carotid and neuroepithelial bodies, adrenal chromaffin cells (33, 41), and fish gill neuroepithelial cells (24). K^+ATP channels are also common in systemic vessels and these, along with the possible loss of the O_2-sensitive $K_v1.5$ and $K_v2.1$ channels, may account for HSD (65). It is now evident, however, that while O_2-sensitive K^+ channels may contribute to, or modulate, the hypoxic response, other factors "upstream" from these or other K^+ channels most likely couple hypoxia to K^+ channels (72).

Most studies seem to agree that HPV is ultimately achieved through an increase in intracellular Ca^{2+}, via voltage-gated, TRP channels, or store-operated channels and/or intracellular raynodine-sensitive stores, and by Ca^{2+} sensitization, via Rho kinase or other mechanisms (69). A number of intracellular signaling systems [*e.g.*, K^+ channels, protein kinase C (PKC)], are also activated downstream, but the initiating O_2 sensor/transducer that couples O_2 concentration or availability to the physiological response is unknown. Because mitochondria are the primary cellular O_2 consumers, it is reasonable to expect the sensing mechanism to reside in these organelles or to be closely associated with them, and this is the current consensus. How this is accomplished, however, remains hotly debated. There are currently three prominent theories for the "O_2 sensor", the redox theory, the ROS theory, and the AMP-activated kinase theory. The "redox theory" proposes that in normoxic conditions the proximal mitochondrial electron transport chain generates a diffusible reactive oxygen species (ROS), such as H_2O_2, that keeps Kv channels open and the resulting hyperpolarization ensures that the calcium channels remain closed, thereby maintaining relaxation. During hypoxia, mitochondrial ROS production falls and the cytosol becomes more reduced. Under these conditions, the Kv channels now close, which depolarizes the cell and ultimately produces HPV (72). The ROS hypothesis is based on an increase in ROS during hypoxia which then may inhibit Kv channels, open TRP channels via activation of phospholipase C, affect Ca^{2+} release by stimulating ryanodine channels in the sarcoplasmic reticulum, and increase Ca^{2+} sensitization (70). The redox and ROS theories depend on whether hypoxia decreases or increases ROS and this has not been resolved. This conundrum may be methodological, nevertheless, it has generated an interesting dialog (70). The AMP kinase hypothesis links energy state to HPV and is independent of redox statue or ROS. It is based on the increase in adenosine monophosphate (AMP)/ATP ratio that results from increased reliance on ATP synthesis from two ADP (forming 1 AMP and 1 ATP) when mitochondrial oxidative phosphorylation is compromised. The increased AMP/ATP ratio activates AMP kinase and the resulting downstream increase in cyclic adenosine diphosphate-ribose (cADPR) stimulates Ca^{2+} release from the ryanodine sensitive Ca^{2+} stores in the sarcoplasmic reticulum (11). The O_2-sensor has also been proposed to be metabolic products derived from O_2-dependent reactions catalyzed by enzymes such as NADPH oxidases, heme oxygenases, cytrochrome

Table 1. Phylogenetic Comparison of Hypoxia and H_2S Vasoactivity

	Systemic		*Pulmonary*		
	Hypoxia	*H_2S*	*Hypoxia*	*H_2S*	*Reference*
Resistance					
Cow			+	+	a
Seal			−	−	a
Penguin			−	−	b
Conductance					
Cow	−	−	(−)+	(−)+	c
Seal	− or +	− or +	− or +	− or +	c
Rat	−	−	+−+	+−+	c
Duck	+	+	−+	−+	c
Alligator	+	+	+−+	+−+	c
Toad	+	+	+	+	c
Trout	−+−	−+−	+	+	c
Shark	−	−			c
Lamprey	+	+	(+) −	(+) −	c
Hagfish	+	+	(+) −	(+) −	d,e
Perfused					
Rat (lung)			+	+	f
Trout (gill)			+	+	g

+, contraction; −, relaxation; multiple symbols (+−+) multiphasic response; symbols in parentheses are minor responses; symbols separated by 'or' indicate different responses in different vessels but same response to hypoxia and H_2S. Citations: a, 48; b, Olson and Madden, unpublished observations; c, 44; d, 47; e, 45; f, 35; g, Skovgaard and Olson, unpublished observations.

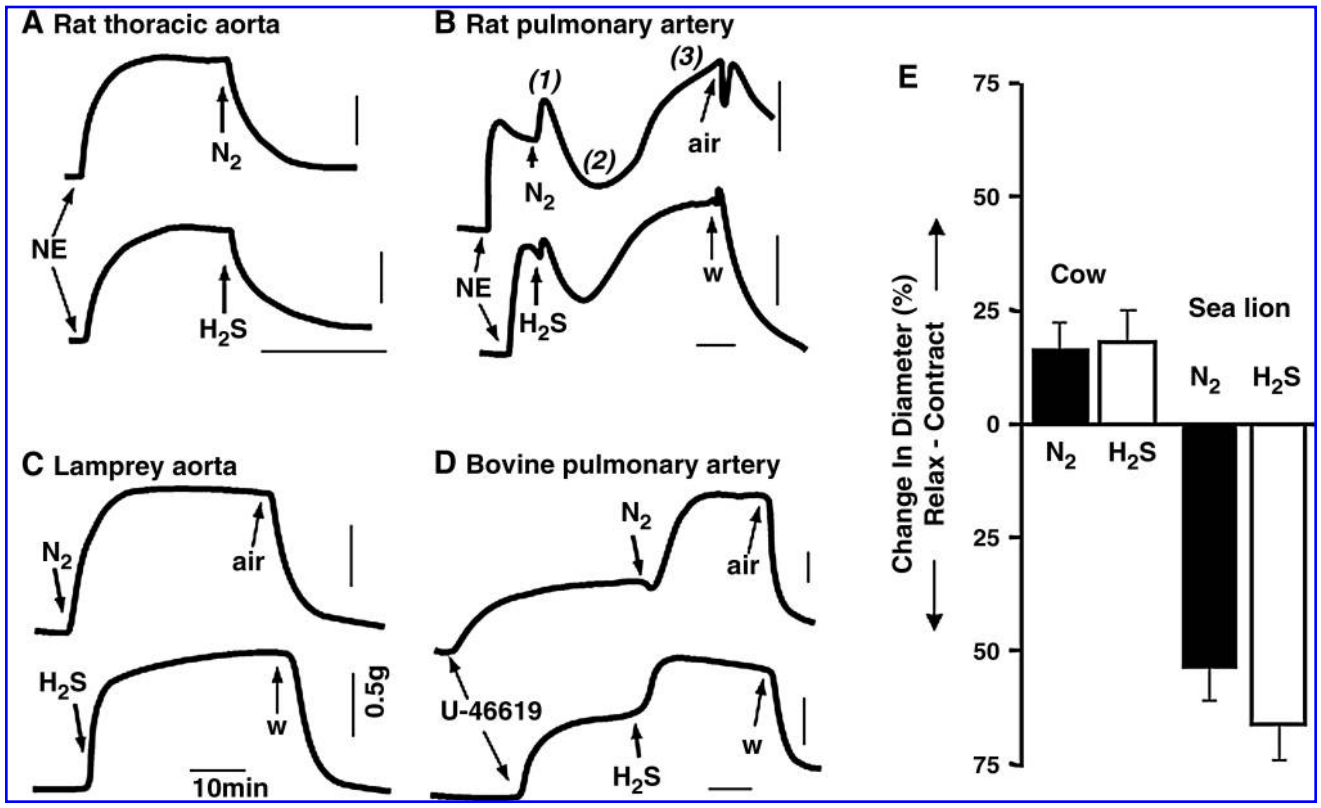

FIG. 1. Similarity of hypoxia and H_2S effects in blood vessels. Hypoxia (100% N_2) and H_2S (300 μmol l^{-1}; as total sulfide, $H_2S + HS^-$) produce identical responses in conductance (>500 μm diameter) vessels from (**A**) rat thoracic aorta, (**B**) rat pulmonary artery, (**C**) lamprey dorsal aorta, and (**D**) bovine pulmonary artery. Vessels pre-contracted with 10^{-6} mol l^{-1} norepinephrine (NE) or U-46619 (thromboxane A_2 mimetic; 10^{-7} mol l^{-1}); air, aeration with room air; w, wash; *1*, *2*, *3*, triphasic response. *Horizontal time bar* in A–D = 10 min, vertical tension scale = 0.5 g. (**E**) hypoxia (Po_2 ~50 mmHg) and H_2S (3×10^{-4} *M*) produce identical contractions of cow resistance (<500 μm diameter) pulmonary arteries while both stimuli relax sea lion resistance pulmonary arteries. Mean ± SE; *N* (animals, vessels) = cow (9, 9), sea lion (3, 5). A–D, adapted from ref. 44, with permission; E, adapted from ref. 48, with permission.

P-450 monooxygenases, and enzymes that degrade hypoxia-inducible factor-1 (69).

Hypoxic vasodilation may or may not be associated with a decrease in intracellular calcium, and has been proposed to be initiated by intracellular acidosis, redox control of cytosolic NADPH, and direct modulation of internal Ca^{2+} stores and Rho-kinase (65). Hypoxia dilates the ductus arteriosus *in utero*, whereas the normoxic vasoconstriction occurring after birth has been attributed to inhibition of the same Kv channels and Ca^{2+} entry through L-type channels as HPV, and also to an increase in Rho kinase (72).

In carotid body type I cells, hypoxia has been reported to activate NADPH oxidase and increase reactive oxygen species (ROS), activate AMP-activated protein kinase, inhibit mitochondrial metabolism, or stimulate heme oxygenase-2 (HO-2) production of carbon monoxide (54). In airway NEB, hypoxia has been proposed to inactivate NADPH oxidase thereby decreasing ROS (26, 41), and in adrenal chromaffin cells a mitochondrial mechanism decreases ROS (41).

Each of the above vascular and chemoreceptor O_2 sensing mechanisms has its proponents and opponents and arguments for and against these mechanisms have been recapitulated in numerous reviews (cf.; 33, 39, 60, 69, 70, 72). The most consistent observation is a lack of consensus.

O_2 sensitive cells— ischemic reperfusion injury and conditioning

Reperfusion injury (RI) is a progression of pathological events such as microvascular dysfunction, tissue necrosis, and apoptosis that follow restoration of blood flow to ischemic tissue. In the myocardium, where RI is most extensively studied, it may be expressed as myocardial stunning (a reversible mechanical dysfunction), no-flow phenomena (an inability to restore perfusion), arrhythmias, or cell death. While RI is detrimental, there is considerable evidence that many cells respond to brief episodic exposures to hypoxia by initiating a variety of mechanisms that protect against RI. This ischemic preconditioning (IPreC) is produced by several transient ischemic periods prior to the prolonged ischemia and can protect the heart from RI for 1–2 h (first window) and this is followed 24 h later by a second window of protection that lasts an additional 48–72 h. The early phase appears to be associated with modification of existing proteins, whereas the latter requires additional protein synthesis. Preconditioning is obviously of limited clinical value and it was not until the discovery that transient ischemic periods after the prolonged ischemia (ischemic postconditioning; IPostC) also conveyed protection that the therapeutic benefit of conditioning was

realized (16). The conditioning effect is also portable, remote ischemic preconditioning
(RIPreC) is produced by transient ischemia of one organ (often a limb) and it conveys a protective effect to a remote organ or tissue (63). Effects of ischemic conditioning have now been demonstrated in essentially all major organs including the heart, brain, liver, kidney, and skeletal muscle.

A number of factors have been proposed to mediate RI including oxidative stress, an abrupt increase in intracellular calcium, rapid restoration of pH following washout of lactic acid, and inflammatory responses (66). Any of these can open the mitochondrial permeability transition pore (MPTP) and initiate a cascade of cell death.

It is now clear that events during the early period of reperfusion are key to RI and that the benefits of both IPreC and IPostC address these events (16, 66). Activation of adenosine, bradykinin, and opioid G-protein coupled receptors appears to initiate cardioprotection. Adenosine directly activates PKC via phospholipases, whereas the others act through complex mechanisms that include phosphatidylinositol 3-kinase, Akt, nitric oxide synthase, guanylyl cyclase, PKG, opening of mitochondrial K_{ATP} channels, and redox activation of PKC. This involves the reperfusion injury survival kinases (RISK), P13 kinase, and MEK-ERK 1/2 pathways. While various isoforms of protein kinase C (PKC_α, PKC_ε, PKC_δ) are involved in IPreC and IpostC, they act downstream from ROS. The mitochondrial permeability transition pore may be the pivotal point of cell survival and Akt and ERK survival kinases are key in inhibiting pore formation until the ischemic injury is repaired (16, 66). Remote conditioning appears to be mediated by similar mechanisms but it is not known if the initial signal is neural or humoral. While ROS are generated upon reperfusion of the ischemic tissue, and the mitochondria appear to be the pivotal point through which RI and conditioning effects act, the molecular couple from hypoxia to the downstream effectors is unknown.

H_2S as an O_2 Sensor

Is there a universal O_2 sensor?

There is some opinion that the O_2 sensor should be a macromolecule, most likely a protein that would be drawn from a pool of proteins naturally undergoing oxido-reductive transitions (53), or an oxygen consuming or binding protein/organelle (60). Clearly there are a variety of proteins that are involved in O_2 sensing/signal transduction, but does this array of candidates suggest that O_2 sensing mechanisms evolved numerous times and now constitutes a highly redundant system, or that different sensing mechanisms may be uniquely adapted to specific tissues (69)? Alternatively, it may reflect the fact that these mechanisms are downstream effectors/modulators of a more fundamental and universal process.

The basic model of H_2S-mediated O_2 sensing

The proposed model of H_2S-mediated O_2 sensing (Fig. 2) does not require a complex or sophisticated O_2 "receptor". Instead, the signaling element is simply the balance between H_2S production and its destruction by available O_2. In other words, when intracellular O_2 falls, the rate of H_2S metabolism can no longer keep pace with production, intracellular H_2S concentration increases, and H_2S now activates the cell via a variety of cell-specific pathways. This model of H_2S-mediated O_2 sensing presumes that; (a) the effects of H_2S on O_2-sensitive tissues are the same as that of hypoxia and that H_2S activates the same intracellular processes, (b) H_2S is constitutively produced in or near the O_2 sensing cell, (c) H_2S is readily metabolized by these same tissues in an O_2-dependent manner. The following sections provide support for this model.

General aspects of H_2S signaling

As shown in this issue, and in numerous recent reviews, H_2S has an almost ubiquitous presence in tissues. Physiopharmacological effects of H_2S have been demonstrated in neurological, cardiovascular, gastrointestinal, genitourinary, endocrine, and immune systems and it has been proposed to be both pro- and anti-inflammatory (62, 68). H_2S has also been reported to induce suspended animation in small mammals and be of potential benefit in organ survival (2).

H_2S is synthesized in many, if not all, tissues (8). H_2S can be produced directly from cysteine desulfuration via the cytosolic pyridoxyl 5'phosphate-dependent enzymes, cystathionine λ-lyase (CSE, aka CGL; EC 4.4.1.1), cystathionine β-synthase (CBS; EC 4.2.1.22), or after cysteine transamination to 3-mercaptopyruvate (often with α-ketoglutarate, α-KG, as the amine acceptor) and desulfuration by the 3-mercaptopyruvate sulfurtransferase (3-MST; EC 2.8.1.2; refs. 34 and 58). There are numerous other potential metabolic pathways for H_2S generation that have been described for invertebrates (25) but they have not been systematically evaluated in mammalian tissues. It is also possible that H_2S may be liberated directly from sulfane sulfur or acid-labile sulfur by reducing or acidic conditions, respectively (58). CBS and CSE appear to be sensitive to Po_2 or intracellular redox state (34) and this may directly contribute O_2 sensitivity or may provide a long-term mechanism to bias the rate of constitutive H_2S production. Commonly used inhibitors of CSE include propargyl glycine (PPG) and β-cyanoalanine (BCA). Aminooxyacetate (AOA) is commonly used to inhibit CBS and hydroxylamine (HA) to inhibit both enzymes. Unfortunately, none of these inhibitors are specific for H_2S metabolism and often they are poorly absorbed by tissues (62).

H_2S oxidation has been demonstrated in vertebrate mitochondria. Sulfide-quinone oxidoreductase (SQR), a C-S-transhydrogenase in the glutathione reductase family, catalyzes electron transfer from H_2S to carbon centers in NAD(P)H-$NAD(P)^+$, and has been demonstrated in bacterial, invertebrate, and mammalian cells (17). SQR is a mitochondrial enzyme in eukaryotic cells and consumes one mole of O_2 for every mole of H_2S oxidized (17). Thus, O_2 consumption is obligatory during H_2S metabolism.

H_2S as a Vascular O_2 Sensor

Vascular effects of H_2S are similar to hypoxic responses

Mammalian systemic vessels. H_2S has long been known in mammals to dilate systemic vessels, decrease vascular resistance, and decrease arterial blood pressure (1, 14, 27, 28, 29, 31, 71, 76). The effects of H_2S on systemic vessels are identical to the effects of hypoxia (Fig. 1, Table 1).

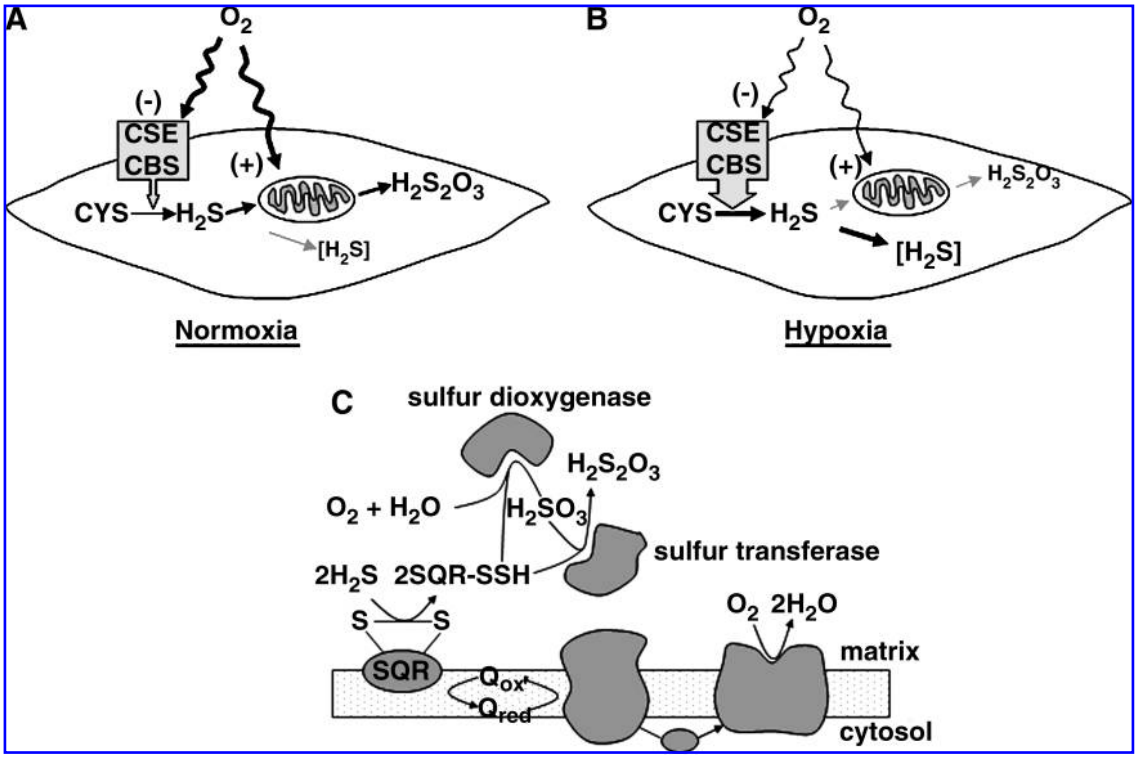

FIG. 2. Proposed mechanism using H_2S metabolism as an O_2 sensor (A, B) and suggested pathway for mitochondrial sulfide oxidation (C). H_2S is constitutively produced from cysteine (CYS) metabolism in the cytosol. During normoxia (**A**) H_2S is continuously oxidized to thiosulfite in the mitochondria, thereby maintaining low intracellular [H_2S]. A fall in oxygen availability (**B**) decreases mitochondrial H_2S oxidation resulting in an increase in biologically active [H_2S] and initiation of hypoxic responses. The enzymes generating H_2S from cysteine, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), also have the potential of O_2 sensitivity, thereby enabling either short-term regulation of [H_2S] or placing a long-term bias on the rate of H_2S metabolism. (**C**) A membrane-bound sulfide:quinone oxidoreductase (SQR) oxidizes sulfide to the level of elemental sulfur, simultaneously reducing a cysteine disulfide such that a persulfide group is formed at one of the cysteines (SQR-SSH). The electrons are fed into the respiratory chain via the quinone pool (Q_{ox}/Q_{red}), and finally transferred to oxygen by cytochrome oxidase (complex IV). A sulfur dioxygenase in the mitochondrial matrix oxidizes persulfides to sulfite (H_2SO_3), consuming molecular oxygen and water. The final reaction is catalyzed by a sulfur transferase, which produces thiosulfate ($H_2S_2O_3$) by transferring a second persulfide from the SQR to sulfite. A, B, adapted from ref. 42, with permission; C 17, with permission.

H_2S relaxes precontracted systemic vessels partly through activation of K_{ATP} channels and to a lesser extent through Ca^{2+}-dependent K^+ (K_{Ca}) channels and H_2S-mediated charybdotoxin/apimin-sensitive K^+ channels in the endothelium (27, 76, 77) and partly through a reduction in intracellular pH (31). H_2S-induced relaxation appears to depend on extracellular Ca^{2+} (76). Relaxation of rat aorta by exogenous H_2S does not depend on vascular prostaglandin synthesis, protein kinase C, or cAMP, nor does it involve superoxide or H_2O_2 production (27, 28). The relationship between H_2S and NO is far from resolved. H_2S relaxations have been reported to be independent of NO synthesis or cGMP activation (27, 28, 76) and Whiteman *et al.* (73) showed that H_2S combined with NO to produce a nitrosothiol that does not stimulate cGMP production in cultured RAW264.7 cells. NO production has been shown to be directly inhibited by H_2S (29) or indirectly stimulated by it through activating nuclear factor (NF-κB) which activated the extracellular regulated kinase 1/2 (ERK1/2) which in turn activated iNOS (23).

Reports of H_2S-mediated vasoconstrictory responses in mammalian systemic vessels are rare, and many of these show an endothelium-dependent effect that has been attributed to H_2S inactivation of NO. Low concentrations of H_2S ($<200\ \mu M$) produce endothelium-dependent contraction of human internal mammary arteries and rat and mouse aortas (1, 29, 71) and low-dose H_2S infusion increases blood pressure in the rat (1). These contractions have been proposed to result from H_2S inactivation of endothelial NO via production of an inactive nitrosothiol (1, 71), whereas Kubu *et al.* (29) showed that H_2S directly inhibited NO production. Conversely, H_2S and NO appear to combine to produce a vasoactive molecule that relaxes trout arteries (Dombkowski and Olson, unpublished). Other studies suggest that H_2S may have direct, albeit modest, constrictory effects on systemic vascular smooth muscle. Lim (32) observed 1 μM H_2S contractions of rat aortas that were partially independent of both the endothelium and K_{ATP} channels and due in part to down regulation of cAMP. This leaves the option open for a direct constrictory effect of H_2S.

Mammalian pulmonary vessels. H_2S produces the same effect in mammalian pulmonary arteries as hypoxia, that is,

contraction of bovine conductance (>500 μm diameter) pulmonary arteries (CPA) and triphasic contraction-relaxation-contraction of rat CPA (Fig. 1B and D, Table 1). The fact that H_2S was the only molecule found to mimic the signature triphasic response in rat CPA provided the first clue that H_2S may be involved in the hypoxic response (44). Perhaps the most convincing argument, however, comes from a recent examination of resistance (<500 μm diameter) pulmonary arteries (RPA) in the cow and sea lion where both H_2S and hypoxia contracted cow RPA, yet relaxed sea lion RPA (Fig. 1E; 48). This was not only the first demonstration of hypoxic relaxation in a mammalian RPA, but it provided additional evidence of the uniformity of H_2S and hypoxic responses in the vasculature. H_2S and hypoxia also increase vascular resistance in the perfused rat lung (35). In addition to these vasoactive responses, hypoxia and H_2S have other common effects. Both produce a similar depolarization of smooth muscle cells from bovine pulmonary arteries (44) and both cause phosphorylation of myosin light chain in cat pulmonary arteries (36; and unpublished).

Nonmammalian vessels. We have compared H_2S and hypoxic responses in respiratory and systemic conductance vessels from at least one species of each class of vertebrates (eight nonmammalian species total) and found that while both H_2S and hypoxia tend to contract respiratory vessels, their effects on systemic blood vessels are variable, some vessels contract, some relax, and in some the response is bi- or multiphasic; however, in every vessel the effect of H_2S and hypoxia are the same (Fig. 1C, Table 1) (44, 45, 47). H_2S and hypoxia also increase vascular resistance in the perfused trout gill (42; Skovgaard and Olson, unpublished observations). These disparate responses are not due to major differences in the contractile machinery as all vessels are similarly contracted by a variety of agonists such as elevated extracellular K^+ and thromboxane A_2 agonists and relaxed by NO donors or natriuretic peptides.

It is interesting to note that in support of the redox theory for hypoxic pulmonary vasoconstriction and ductus arteriosus vasodilation, Weir *et al.* (21) observe that only redox changes mimic oxygen by constricting pulmonary arteries and dilating the ductus arteriosus whereas other stimuli such as elevated extracellular K^+ contract both. H_2S appears to do the same in a considerable variety of vessels. One could argue that H_2S also reduces the cytosolic environment, however, antioxidants tend to suppress HPV, suggesting that the mechanism of H_2S vasoconstriction is not as a reductant.

H_2S metabolism in the vasculature

CBS and CSE are thought to be differentially expressed in tissues, CSE is prevalent in systemic and pulmonary arteries and the heart, but not found in brain, whereas only CBS is found in the brain (62). Recent studies have shown that 3-MST, not CBS, appears to be the major pathway for H_2S synthesis in the brain (58) but, other than our work (below) 3-MST has not been examined in the vasculature. In contrast to mammals, we have found both CSE and CBS mRNA in trout arteries, veins, heart, brain, liver, gut, gill, and skeletal muscle (46), suggesting a ubiquitous expression of these enzymes in fish. We have also found CBS mRNA and CBS immunoreactivity in the endothelium of sea lion and bovine pulmonary arteries (48).

Similarities between H_2S stimulation and activation of O_2-sensitive chemoreceptors

The involvement of H_2S in O_2-sensitive chemoreceptors in the carotid body is beginning to receive attention. In a preliminary study, Rong *et al.* (56) recorded afferent nerve activity from an isolated mouse carotid sinus nerve preparation and found that H_2S increased chemoreceptor afferent discharge in a concentration-dependent fashion through attenuation of large conductance, calcium-sensitive potassium (BKCa) channels. H_2S activity appeared specific for the glomus cells. They also found that O_2 sensitivity was enhanced in the presence of H_2S, whereas chemoreceptor response to hypoxia was abolished by the CBS inhibitor, AOA, but not by CSE inhibitors, PPG or BCA. Recently, Telezhkin *et al.* (64) identified CSE in glomus cells from the rat carotid body and found that H_2S inhibited BK channels in HEK 293 cells expressing the human BKCa channel alpha subunit. Thus the carotid body contains both the enzymatic machinery and effector targets for H_2S to function as a mediator of chemoreception.

There is also increasing evidence for H_2S mediation of oxygen-sensing NEC cells in the fish gill (46). Bolus injection of H_2S into the buccal (mouth) cavity produced a bradycardia similar to that produced by hypoxia (Fig. 3). Removal of the first pair of gill arches, the site of highest NEC density in trout and the homolog of the carotid body (38), greatly inhibited the H_2S bradycardia, whereas removal of the second pair did not affect the H_2S response. In addition, both hypoxia and H_2S depolarized isolated zebrafish NECs (46). Thus H_2S appears to be an active component of sensory transduction in a variety of vertebrate chemoreceptors.

H_2S and Myocardial O_2 Sensing

There is considerable, but at times somewhat conflicting, evidence that H_2S not only affects the heart but plays a role cardioprotection.

H_2S and cardiac function

H_2S has been reported to have negative inotropic effects in rats that appear to be mediated by opening of cardiac K_{ATP} channels (15). H_2S-mediated inhibition of adenylate cyclase also produced negative inotropic and chronotropic effects in adrenergically-stimulated rat hearts, although it was proposed that this may protect the heart during hypoxia-mediated adrenergic stimulation (75). Daily injections of H_2S reduced mortality in an adrenergically-stimulated myocardial ischemic injury model (14). In rats with a ligated left coronary artery H_2S decreased mortality and PPG inhibition of CSE, which was prevalent in the myocardium, increased mortality (78).

H_2S and ischemic conditioning

Preconditioning the heart with exogenous H_2S mimics IPreC and IPostC and has been shown to exert a positive cardioprotective effect against RI (Fig. 4) (4, 10, 18, 19, 49–51). Even the H_2S donor S-allylcysteine significantly lowered mortality and reduced infarct size in a rat model of acute

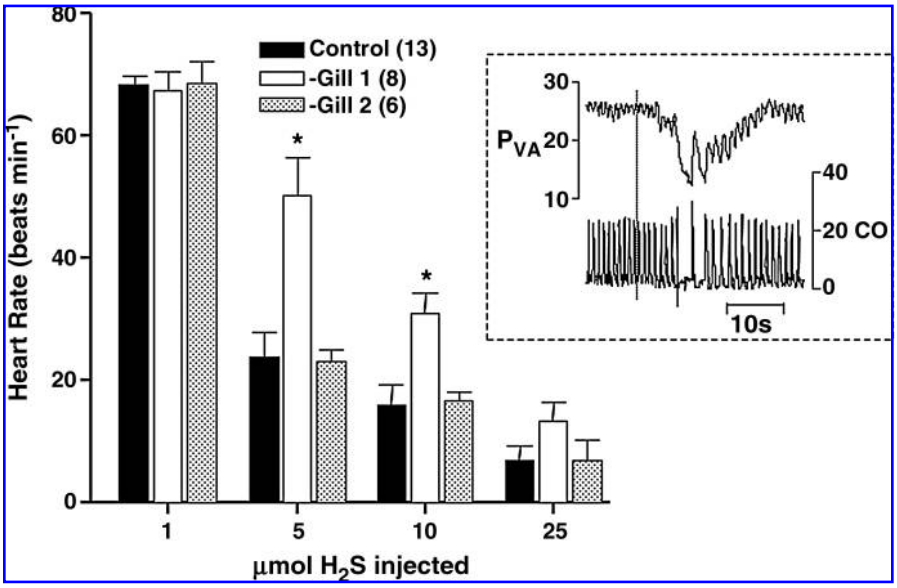

FIG. 3. Evidence for H_2S involvement in trout gill chemoreceptors. Bolus 1 ml injections of H_2S into the buccal cavity of an unanesthetized 600 g trout previously implanted with a pressure cannula in, and flow probe around, the ventral aorta produced bradycardia within 5–10 seconds that mimicked hypoxic bradycardia. Removal of the first pair of gills (-Gill 1), the primary location of chemoreceptors, decreased H_2S responses relative to control fish, and fish without the second arches (-Gill 2). Mean ± SE (N). *Inset* shows ventral aortic pressure (P_{VA}) and cardiac output (CO) responses to single 5 μmol H_2S injection (*dotted line*). Adapted from ref. 46, with permission.

myocardial infarction. These studies have also shown that H_2S-mediated cardioprotection utilizes similar activation pathways as IPreC, for example, activation of ERK and PI3K/Akt (19), activation of PKC_α, PKC_ε, and PKC_δ (49, 51), activation of sarcolemmal but not mitochondrial K_{ATP} channels (4, 50), stimulation of cyclooxygenase-2 (COX-2; 18), and inhibition of NO production (50).

Interaction Between Hypoxia and H_2S

Competition and enhancement

If H_2S mediates O_2 sensing then it would be expected that vessels maximally stimulated by one would be refractory to the other, whereas submaximal activation by one stimulus would enhance responsiveness to the other. This has been demonstrated in several studies of conductance vessels. We (44) found that the hypoxic relaxation of rat thoracic aorta or hypoxic contraction of bovine pulmonary arteries or lamprey aorta was greatly diminished or abrogated by high (300 μ*M*) H_2S and H_2S relaxation of rat thoracic aorta or hypoxic contraction of bovine pulmonary arteries or lamprey aorta was greatly diminished or abrogated by extensive bubbling with 95% N_2/5% CO_2 or 100% N_2 (mammal or lamprey respectively). We (45) also observed an increased H_2S sensitivity in lamprey aortas when ambient Po_2 was ~20–30 mmHg and in hagfish aortas when bubbled with 100% N_2 (Po_2 not measured but presumed to be above >1 mmHg based on previous studies). A similar increase in sensitivity to H_2S-mediated vasodilation of rat aortas aerated with 95% N_2/5% CO_2 was observed by Kiss *et al.* (27). Kiss *et al.*(27) also observed that in normoxia, but not hypoxia, low H_2S (50 μ*M*) slightly contracted rat aortas, whereas higher H_2S concentrations contracted both normoxic and hypoxic vessels. This is similar to the findings of Koenitzer *et al.* (28) in which low H_2S concentrations (10–100 μ*M*) contracted rat aorta equilibrated with room air (200 μ*M* O_2) and relaxed aortas when the Po_2 was ~30 mmHg (40 μ*M* O_2). They (28) attributed the contraction to an unidentified oxidation product of H_2S, rather than H_2S itself. It is doubtful that an oxidation product mediates the response of the lamprey or hagfish as H_2S-mediated contractions were observed at both high and low O_2 (45).

H_2S metabolism and hypoxic responses

If H_2S is to serve as an O_2 sensor, then alterations in H_2S metabolism would be expected to affect hypoxic responses. Inhibition of hypoxic responses with inhibitors of H_2S synthesis as well as augmentation of hypoxic responses with precursors of H_2S synthesis has been demonstrated in several O_2-sensing tissues.

In the rat aorta, the CSE inhibitor PPG completely inhibited hypoxic vasodilation (Fig. 5A),whereas the CBS inhibitor AOA, but not PPG, inhibited hypoxic vasoconstriction in bovine pulmonary arteries (Fig. 5B). Hydroxylamine, an inhibitor of both CSE and CBS, inhibited hypoxic vasoconstriction (44). Hydroxylamine also inhibited hypoxic vasoconstriction in the lamprey aorta (44). Hypoxic vasoconstriction in the hagfish dorsal aorta was inhibited by AOA but not by PPG or HA (45). In the mouse carotid body the CBS inhibitors AOA and HA abolished chemoreceptor afferent nerve discharge in response to hypoxia, whereas this was unaffected by the CSE inhibitor, PPG (56). Addition of compounds from which H_2S can be generated, such as cysteine, glutathione and cysteine in combination with α-ketoglutarate, have been shown to increase the magnitude of hypoxic vasoconstriction in the dorsal aorta of hagfish (45), lamprey (Fig. 5C; 44), bovine pulmonary artery (Fig. 5D; 44), and perfused rat lung (35).

Metabolic studies also suggest a role for endogenous H_2S in RI and conditioning. Although ischemia attenuated H_2S production, ischemic preconditioning (and exogenous H_2S) restored H_2S production during ischemia. CSE inhibitors PPG and BCA blocked the effect of IPreC and this blockade was reduced by coadministration of H_2S (4, 7, 50). RI is significantly reduced in mice with cardiac-specific overexpression of CSE (10). Other H_2S precursors also appear to mediate IPreC. The H_2S donor, S-allylcysteine (SAC), injected daily for 7 days significantly increased myocardial CSE activity, lowered mortality. and reduced infarct size in a rat model of acute myocardial infarction, whereas PPG significantly lowered

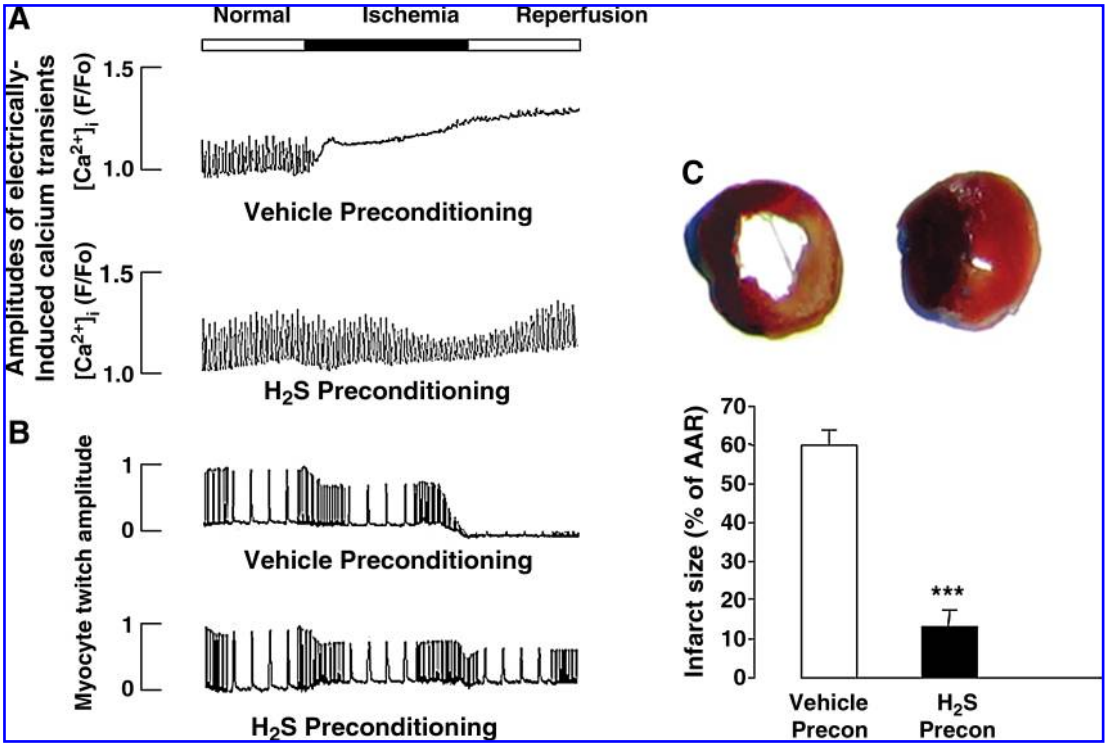

FIG. 4. Cardioprotection of H_2S preconditioning. (**A**) Immediate cardioprotective effects on electrically-induced $[Ca^{2+}]_i$ transients in single ventricular myocytes. Preconditioning with NaHS for three cycles (3 min each cycle, separated by 5 min of recovery) significantly increased the amplitude of $[Ca^{2+}]_i$ transients during ischemia/reperfusion. (**B**) Delayed cardioprotective effects on electrically-induced myocyte contraction in single ventricular myocytes. Isolated cardiac myocytes were treated with NaHS (100 μM) for 30 min and then cultured in normal DMEM solution for 20 h, followed by ischemia/reperfusion insults. Representative tracings showing that H_2S preconditioning increased myocyte twitch amplitude during ischemia/reperfusion. (**C**) *In vivo* study showing cardioprotection on myocardial infarct size in rats. A single bolus of NaHS (1 μmol/kg body weight) administered 1 day before myocardial infarction produced a strong infarct-limiting effect. Mean $\pm$ S.E.M. $^{***}p < 0.001$ *vs.* vehicle preconditioning; $n = 7$–8. A adapted from ref. 4, with permission; B adapted from ref. 18, with permission; C adapted from ref. 49, with permission. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

myocardial CSE activity and increased mortality and size of the myocardial infarct (7). S-propargyl-cysteine, a structural analogue of SAC, provided preconditioning effects in both adult rat hearts and neonatal cardiomyocytes (67).

Evidence for a direct inverse coupling between O_2 and H_2S

Until recently, measurement of tissue H_2S production, like that of circulating H_2S concentrations, were determined by indirect methods that usually entailed incubation of the sample for 30–90 min in a closed container, followed by terminating enzymatic reactions and either measurement of H_2S directly or after trapping on an alkaline or zinc acetate wick (43). While these may (or may not) be satisfactory for measuring long-term H_2S production, they do not appear suitable for measurement of H_2S concentration in blood or tissues. The advent of amperometric (a.k.a. polarographic) H_2S sensors permitted rapid, real-time measurement of H_2S in unadulterated biological samples (43).

Using the amperometric H_2S sensor, we examined the relationship between O_2 and H_2S with respect to tissue H_2S production and metabolism in O_2 sensing tissues and identification of the role of mitochondria in O_2-dependent H_2S metabolism. We observed three consistent findings in a variety of tissues; (a) hydrogen sulfide was only produced under relatively anoxic conditions, (b) addition of precursors of H_2S synthesis increased H_2S production, and (c) addition of O_2 resulted in net H_2S consumption. In minced trout heart (Fig. 6A and B) (74), cysteine produced a dose-dependent increase in the rate of H_2S production. H_2S production reverted to net consumption by addition of oxygen but resumed, presumably after the oxygen had been depleted. A similar cysteine-induced increase and O_2-mediated inhibition of H_2S production has been observed in the trout gill (46). Doeller *et al.* (8) also demonstrated that H_2S was produced by a variety of rat tissues, including aortas, in buffer containing $< 5\,\mu M$ O_2, whereas H_2S production was not observed in air-equilibrated ($\sim 200\,\mu M$ O_2) buffer.

The relationship between H_2S and O_2 is even more apparent when these gases are measured simultaneously. In homogenized rat lung (Fig. 6D), H_2S increases after addition of α-ketoglutarate, but production is converted to net consumption upon injection of small bubble of room air. Enough O_2 was present in this air bubble to theoretically increase O_2 concentration to $\sim 8.5\,\mu M$, however, the measured O_2 did not exceed $1\,\mu M$, presumably due to a slight lag in gas diffusion out of the air bubble and concomitant O_2 consumption by the tissue. H_2S

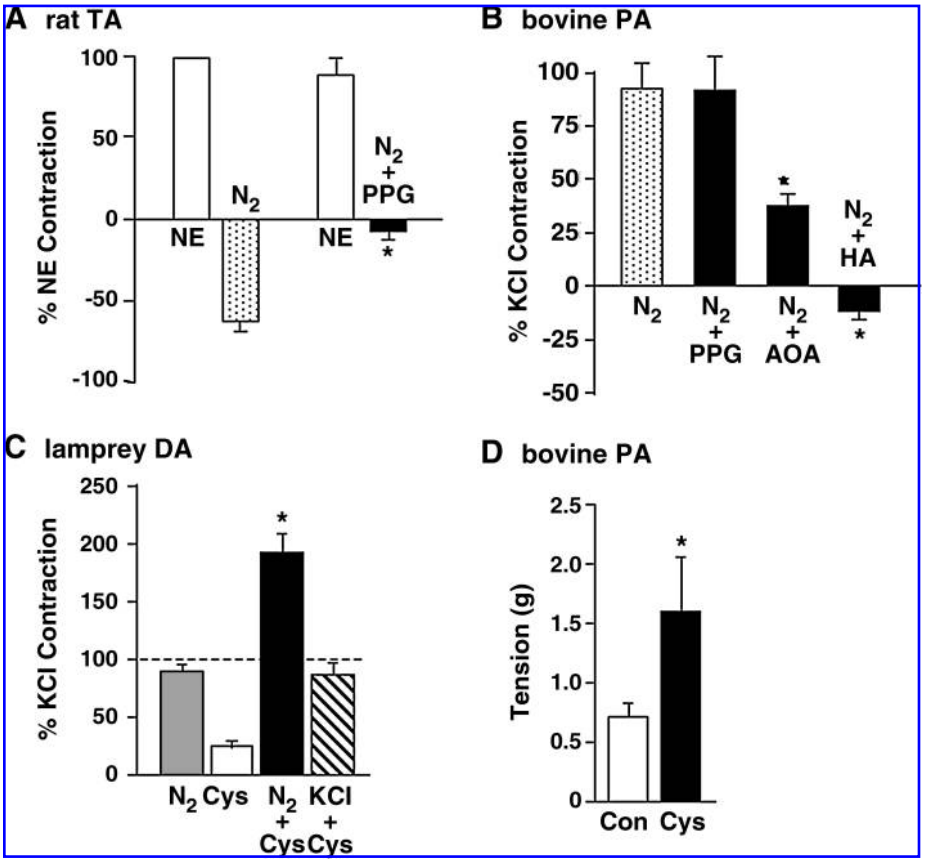

FIG. 5. Inhibition of hypoxic (N_2) responses by inhibitors of H_2S synthesis (A, B) and stimulation of hypoxic responses by H_2S precursors (C, D). (**A**) Hypoxic relaxation of a norepinephrine-contracted (NE; 10^{-6} moles l^{-1}) rat thoracic aorta (TA) is significantly (*) inhibited by the CSE inhibitor propargyl glycine (PPG). (**B**) Hypoxic contractions of bovine pulmonary arteries (PA) are not affected by propargyl glycine (P) but are increasingly inhibited by the CBS inhibitor aminooxyacetate (AOA) and the CBS + CSE inhibitor, hydroxylamine (H). (**C**) Hypoxic contraction of lamprey dorsal aorta (DA) is doubled by addition of 1 m*M* cysteine (N_2 + Cys), whereas Cys does not affect a 80 m*M* KCl contraction (KCl + Cys). (**D**) Cys increases hypoxic contraction of bovine pulmonary artery compared to control (Con). Adapted from ref. 44, with permission.

production did not resume until oxygen had been nearly completely depleted from the chamber. Figure 6E shows the average production and consumption from three rats. The O_2-mediated inhibition of H_2S production observed in the rat lung is essentially identical to our observations that H_2S production/consumption by homogenized lung tissue from the cow and sea lion is tightly coupled to oxygen at low Po_2 (48). The significance of these studies is that, while hypoxia has the opposite vasoactive effects on pulmonary arteries from terrestrial *versus* marine mammals, it has identical effects on H_2S metabolism. These studies also show that in normoxic conditions there is little, if any H_2S present.

The O_2 tension at which oxygen becomes rate limiting in H_2S consumption in homogenized bovine lung tissue is shown in Fig. 7A. H_2S consumption remains constant until Po_2 falls below 10 mmHg, and declines thereafter. The P_{50} for H_2S metabolism is ~4 mmHg. Although there is still considerable debate on mitochondrial Po_2, this value appears to be close to that encountered during hypoxia. In a recent study, Mik *et al.* (37) measured mitochondrial oxygen tension in rat hearts *in vivo* and observed that when arterial Po_2 (Pao_2) was normal, 10% of the mitochondria had a Po_2 of 5 mmHg or lower, 30% had a Po_2 of 5–15 mmHg, and the rest were >15 mmHg. When Pao_2 was reduced to 40 mmHg by inspiring 10% oxygen, over 45% of the mitochondria had a Po_2 of 5 mmHg or lower and 10% had a Po_2 of 5–15 mmHg. Thus this level of hypoxia places progressively more mitochondria in a O_2-deficient environment that is not sufficient for H_2S metabolism.

The percent activity of H_2S inactivation can be expressed as a function of Po_2 and compared to other potential O_2 sensors (Fig. 8) (69). It becomes evident that the metabolism of H_2S has two advantages over the other sensors, it is more efficient than most of the other proposed mechanisms in the range of cytosolic/mitochondrial Po_2, and it has a greater dynamic range going from complete H_2S consumption above 15 mmHg to almost no consumption at 2 mmHg. A similar comparison of the ability of purified bovine heart mitochondria to metabolize H_2S is also shown in Fig. 8 and shows that mitochondria are more efficient at lower Po_2. This curve looks to be very similar to, and appears to be a continuation of, the curve for mitochondrial cytochrome aa3 as described by Ward (69) and also depicted in Fig. 8. While these studies will need to be repeated with other tissues, they are, nevertheless, suggestive of an efficient sensing system operating at the level of the mitochondria.

H_2S Signaling

Autocrine, paracrine, or endocrine

Whether H_2S serves as a circulating molecular signal or is a local paracrine or autocrine messenger has been the subject of recent interest and controversy (43, 62) and is briefly summarized here. Nearly all studies published prior to 2000 reported plasma or blood H_2S concentrations <1 μmol l^{-1}. Since then, however, the number of studies showing plasma H_2S > 10 μmol l^{-1} has steadily increased and there are now a half dozen reports of plasma H_2S ~300 μmol l^{-1}, even though essentially all of these studies are on relatively few mammals, namely rats and mice (summarized in 43). However, there are a number of arguments against these elevated plasma titers. First, even 1 μmol l^{-1} H_2S in buffer is readily detected by the human nose (13) and >10 μmol l^{-1} should be obvious in plasma. Second, being a gas, H_2S will readily equilibrate

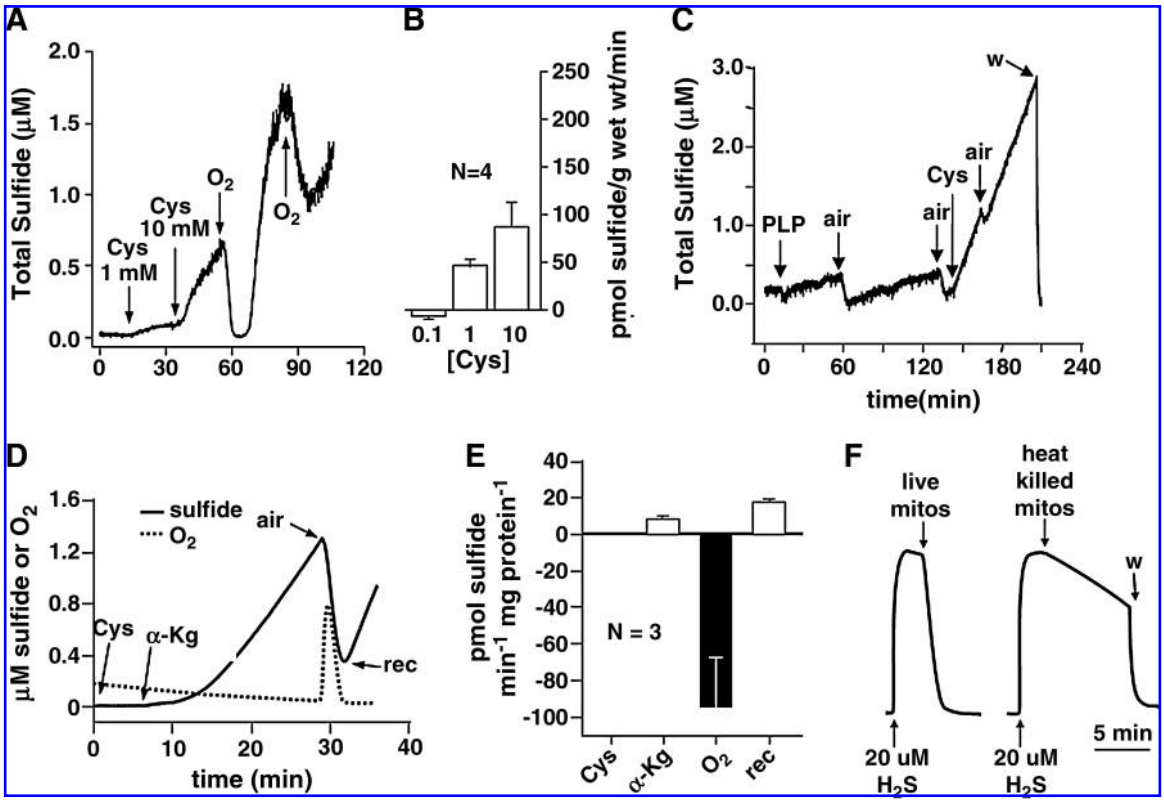

FIG. 6. Oxygen-dependent H_2S production and consumption in O_2-sensitive tissues measured in real-time with a amperometric H_2S sensor. (**A, B**) H_2S production (as total sulfide) by minced trout heart increases with increasing concentrations of cysteine (Cys; at $Po_2 \sim 0$). Injection of oxygen (O_2) sufficient to theoretically raise oxygen concentration to 100 μmol/l transiently results in net H_2S consumption. H_2S production resumes after the oxygen is consumed. (**A**) Single representative trace, (**B**) mean ± SE ($n = 4$). (**C**) H_2S production (as total sulfide) by homogenized trout gill occurs spontaneously after addition of pyridoxal 5′-phosphate (PLP, 1 m*M*); injection of air (theoretical O_2 concentration 10 μmol/l) produces net H_2S consumption. Addition of Cys increases H_2S production and a second injection of air produces net H_2S consumption; w, wash. (**D**, **E**) Relationship between O_2 concentration (*dotted line*) and H_2S production (as total sulfide; *solid line*) by homogenized rat lung. (**D**) In anoxia, H_2S production increases after addition of α-ketoglutarate (α-Kg, 1m*M*), in the presence of Cys (1 m*M*). Injection of oxygen (O_2) results in net H_2S consumption. When all O_2 is consumed, H_2S production is restored. (**E**) Net rate of H_2S production (positive values) or consumption (negative values) as a function of substrate and O_2. (**F**) H_2S consumption by purified bovine heart mitochondria; *left trace* shows rapid fall in H_2S concentration after addition of mitochondria (mitos). The rapid fall in H_2S concentration is prevented when the mitochondria are heat killed prior to addition (*right trace*); w, wash with H_2S-free buffer. A, B adapted from ref. 74, with permission; C adapted from ref. 46, with permission; D–F, Whitfield and Olson unpublished.

across the alveolar membranes (22) and should be readily detected in exhaled air, but it is not. Third, there is not enough cysteine in the body to sustain the predicted loss of H_2S in exhaled air for even a fraction of one day (13). Fourth, modern methods of H_2S analysis such as the polarographic (amperiometric) sensor (74) and HPLC separation (61) have failed to confirm these high levels and have reported plasma $H_2S < 1\ \mu\text{mol}\ l^{-1}$, and often undetectable. Fifth, measurements in real-time with the polarographic sensor of H_2S in fresh blood from mammals and fish, or in fish fitted with an extracorporeal loop have shown that exogenous H_2S rapidly disappears from the plasma (46, 74). Sixth, studies on tissue metabolism of H_2S (see section on Interaction between Hypoxia and H_2S, above) clearly show that in the presence of oxygen H_2S is readily consumed by tissues and under normoxic conditions there would be little if any favorable H_2S gradient from the tissue to blood. In fact, it has been shown that H_2S is essentially absent from tissues (13). Seventh, thus far, all attempts to free H_2S from a potential acid-labile or sulfate sulfur carrier in either plasma or red blood cells has failed to do so, even after adding exogenous H_2S (43). Sources of error that could potentially result in elevated H_2S values are beginning to be examined. The strongly alkaline antioxidant buffer mixed with plasma to drive all H_2S to S^{2-} for measurement with the sulfide ion selective electrode has been shown to generate free sulfide from plasma proteins and even from albumin solutions (74). The direct methylene blue method, which was originally developed for measuring H_2S in aqueous samples (59), is also commonly used for plasma H_2S measurements, even though it employs a strong acid and was never validated for measuring plasma or blood. It is evident that a standardized (and validated) approach to plasma H_2S measurements needs to be adopted. In the meantime, the most recent evidence with new methodologies supports the earlier (pre-2000) reports that H_2S, either free or bound to a carrier, does not circulate in the plasma. H_2S should be

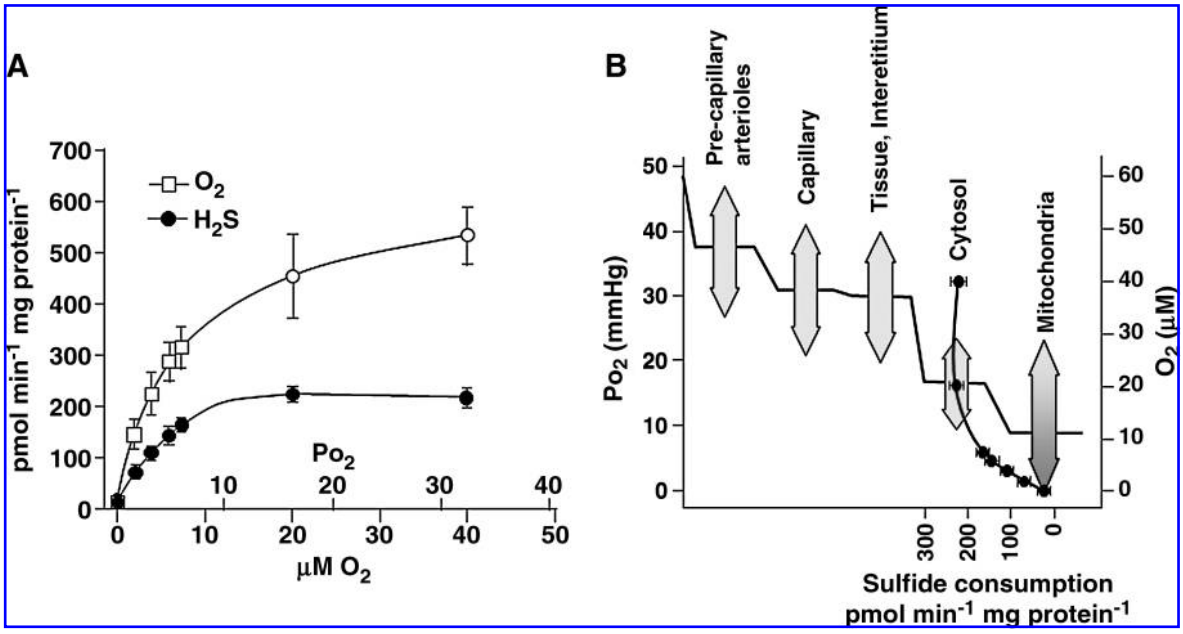

FIG. 7. (**A**) **Relationship between O_2 and H_2S consumption as a function of oxygen concentration in bovine lung homogenate.** O_2 and H_2S were continuously measured with amperometric sensors. The rate of H_2S consumption decreased when O_2 concentration fell below 10 μM. (**B**) O_2-dependent H_2S consumption by bovine lung homogenate from panel **A** plotted as a function of O_2 concentrations (*length of arrows* indicates range of reported [O_2]) in tissue and cells. There is sufficient oxygen from the vasculature to the cytosol and even normoxic mitochondria to effectively consume H_2S as rapidly as it is generated. During hypoxia, a fall mitochondrial O_2 will decrease H_2S consumption. A redrawn after ref. 48; B, O_2 concentrations from pre-capillary arterioles to cytosol redrawn from ref. 69 with mitochondrial O_2 concentration estimated to range from cytosolic to 0 (37).

considered an autocrine or paracrine signaling molecule in the cardiovascular system.

Because H_2S does not appear to circulate, perhaps the most important questions to be resolved are; what is the intracellular H_2S concentration, how is it is affected by various stimuli, and is there an intracellular compartmentalization of H_2S signaling? To date, there are no methods for measuring these parameters. It is even difficult to estimate intracellular H_2S concentration based on the concentration of exogenous H_2S necessary to elicit various effector responses. Most reports of the physiological/pharmacological effects of H_2S have recorded responses in the tens to hundreds of μmol l^{-1}. While it is possible that these concentrations could be achieved in very localized areas within the cell, there is no evidence to support or refute this. It is also possible that supraphysiological concentrations of exogenous H_2S are necessary to offset the very efficient intracellular mechanisms of H_2S oxidation. There is some support for this latter hypothesis in that both Koenitzer *et al.* (28) and Olson *et al.* (45) have shown that the apparent vascular sensitivity to H_2S is increased at low Po_2.

Mechanisms of action

At first glance, the myriad of effects that have been attributed to H_2S in a variety of biological systems that have been studied would suggest that the potential mechanisms of action of H_2S in the cardiovascular system are also seemingly limitless (62). Some of this no doubt is due to the concentrations of H_2S that have been used to elicit these responses (see above) and whether the observed effects are physiological or pharmacological. This question will largely remain unanswered until actual intracellular H_2S concentrations and sites of localized activity are known. At the risk of omission, some of the more prevalent mechanisms of H_2S action cited in Szabó, (62) and further examined in a recent paper by Muzaffar *et al*. (40) are shown in Fig. 9.The physiological importance of these remains to be defined.

Anecdotal Evidence for H_2S and O_2 Sensing

Geochemical evidence and evolution

Relationship between O_2 and H_2S in the environment. O_2 and H_2S are mutually exclusive in the environment, the presence of one generally precludes the existence of the other. This is evident on the geological time scale; sulfidic oceans prevalent in the reducing and O_2-deficient earth, disappeared as atmospheric O_2 increased, and then returned periodically as a result of upwelling of H_2S-rich deep ocean sediments during episodic declines in oceanic O_2 (30). This relationship is also evident in the present day, albeit in more restricted conditions, and appears as a mobile boundary layer between H_2S-rich anoxic and H_2S-deficient oxygenated water that changes with the level of oxygenation, receding when O_2 increases and growing when O_2 falls (3). By inference, the presence of one of these gases is indicative of the absence of the other.

Natural selection for hypoxia and H_2S tolerance. Periodic increases in ambient H_2S associated with a global decrease in

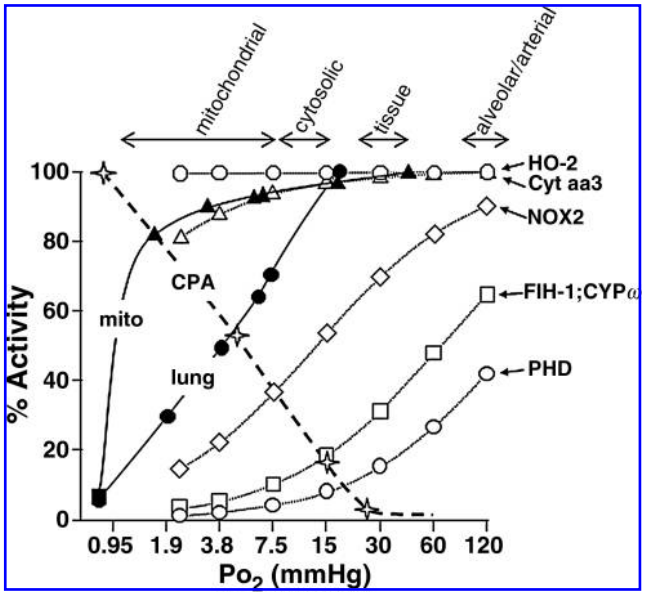

FIG. 8. Theoretical activity of potential O_2 sensors over physiologically encountered oxygen concentrations. H_2S fills a gap in sensitivity in the Po_2 range most likely encountered by the cytoplasmic/mitochondria environment during hypoxia. Abbreviations: Cyt aa3, mitochondrial cytochromes a and a3; CYPω, cytochrome P450 monooxygenase ω; FIH-1, factor inhibiting hypoxia inducible factor-1α, (HIF-1α); H_2S, hydrogen sulfide; HO-2, heme oxygenase 2; NOX2, NADPH oxidase; PHD, prolyl hydroxylase domain proteins involved in O_2-dependent hydroxylation of protein residues on HIF-1α. Redrawn and modified from ref. 69 with inclusion of H_2S data from Fig. 7A and the effect of O_2 on H_2S consumption by purified bovine mitochondria (mito; 48). Po_2 response curve of hypoxic contraction of isolated bovine conductance pulmonary arteries (CPA; *dashed line*) is shown for comparison (48).

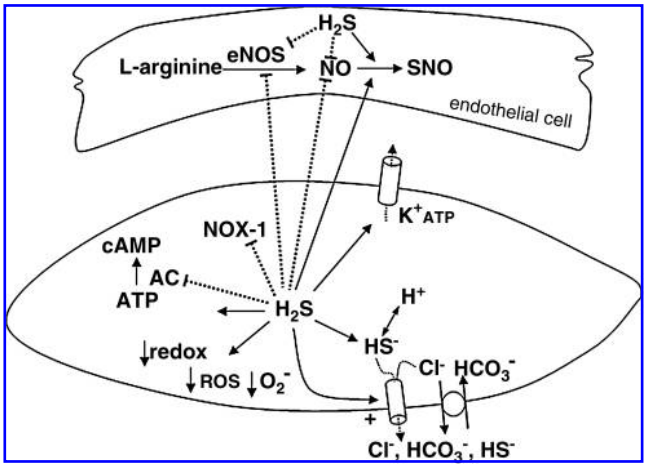

FIG. 9. Potential short-term effectors of H_2S activity in the cardiovascular system. The best characterized cardiovascular effector is an H_2S-mediated opening of ATP-sensitive potassium channels (KATP) which hyperpolarizes the cell and endothelial Ca^{2+}-dependent K^+ (K_{Ca}), and H_2S-mediated charybdotoxin/apimin-sensitive K^+ channels. H_2S may also inhibit NADPH oxidase (NOX-1), adenylyl cyclase (AC), and nitric oxide synthase (the endothelial form, eNOS is shown here). In addition, H_2S may directly bind, and therefore lower the concentration of, nitric oxide (NO) or combine with NO to form a nitrosothiol (SNO), it may lower the redox potential of the cell by combining with superoxide (O_2^-) or other reactive oxygen species (ROS). Because H_2S is a weak acid and is in equilibrium forming H^+ and HS^-, H_2S may lower intracellular pH and the HS^- may mimic or compete with Cl^- and HCO_3^- in a variety of ion channels and transporters.

O_2 have been proposed to cause several if not more of the great extinctions. Most notable among these was the mass extinction at the Permian–Triassic boundary that saw fall in atmospheric O_2 to as low as 13% (Po_2 ~100 mmHg) and destroyed ~70% of life on land and 90% of life in the water (20). Animals that passed through this gauntlet of natural selection, and those more recent, no doubt possess special adaptations to hypoxia (12, 20). It would seem that they would also have been selected for H_2S tolerance.

Origin of mitochondria—The key factor in H_2S-mediated O_2 sensing? Searcy (57) proposed that mitochondria originated from sulfide (H_2S)-oxidizing thermophilic bacteria and the nucleocytoplasm from sulfide-reducing Archaea. This supports the hypothesis that H_2S production and oxidation are compartmentalized intracellularly and that the mechanism for constitutive production of H_2S and its oxygen-dependent inactivation by mitochondria is a common feature of all eukaryotic cells.

Other H_2S–hypoxia interactions

A number of other studies have suggested the relationship between H_2S and hypoxia. Hypoxia increases H_2S production in the rat uterus (52), a tissue implicated in a variety of physiological and pathological responses to hypoxia (6). We have observed that hypoxia and H_2S have similar actions and appear to act through a common mechanism in the trout urinary bladder (9). Recent studies on the molecular mechanism of H_2S signaling suggest that H_2S may activate proteins by sulfhydration of cysteine residues and this process is far more extensive under hypoxic conditions (A.K.Mustafa, personal communication).

Concluding Comments

The excitement surrounding the myriad of potential physiological and pathophysiological actions of H_2S that are beginning to be examined is understandable. Potential therapies from H_2S-laden aspirin to suspended animation and perhaps pulmonary hypertension, myocardial infarction, and stroke are already garnering emotional if not financial support. But we also need to critically examine what is physiological and what is not. This review is a case in point. On one hand, it describes numerous studies of the effects of exogenous H_2S, and on the other it evaluates hypoxia-driven H_2S production as the signaling entity. But there is often 100-fold or greater H_2S concentration difference between the two. Is this because extra exogenous H_2S is required to overload endogenous metabolism, or are we really looking at pharmacological effects? These are questions we need to keep asking ourselves, and hopefully resolve with better methodology.

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Abbreviations Used

3-MST = 3-mercaptopyruvate sulfurtransferase
α-KG = α-ketoglutarate
Akt = genes coding for serine/threonine-specific protein kinases
AMP = adenosine monophosphate
AOA = aminooxyacetate
BCA = β-cyanoalanine
BKCa = large-conductance Ca^{2+}-dependent K^+ channels
cADPR = cyclic adenosine diphosphate-ribose
CBS = cystathionine β-synthase
COPD = chronic obstructive lung disease
COX-2 = cyclooxygenase-2
CPA = conductance pulmonary arteries
CSE = cystathionine λ-lyase
eNOS = endothelial nitric oxide synthase
ERK1/2 = extracellular regulated kinase ½
HA = hydroxylamine
HIF = hypoxia inducible factor
HO-2 = heme oxygenase-2
HPV = hypoxic pulmonary vasoconstriction
H_2S = hydrogen sulfide
HSD = hypoxic systemic vasodilation
iNOS = inducible nitric oxide synthase
IPostC = ischemic postconditioning
IPreC = ischemic preconditioning
K_{ATP} = ATP-dependent potassium channels
K_{Ca} = Ca^{2+}-dependent K^+ channels
Kv = voltage-gated potassium channels
MAPK = mitogen activated protein kinase
MEK–ERK = mitogen-activated protein kinase kinase–extracellular signal-related kinase
MPTP = mitochondrial permeability transition pore
NADPH = nicotinamide adenine dinucleotide phosphate
NEB = neuroepithelial body
NEC = neuroepithelial cells
NF-κB = nuclear factor-κB
NOS = nitric oxide synthase
Pao_2 = arterial partial pressure of oxygen
PKC = protein kinase C
PKG = protein kinase G
Po_2 = partial pressure of oxygen
PPG = propargyl glycine
RI = reperfusion injury
RIPreC = remote ischemic preconditioning
RISK = reperfusion injury survival kinases
ROS = reactive oxygen species
RPA = resistance pulmonary arteries
SAC = S-allylcysteine
SQR = sulfide-quinone oxidoreductase
TRP = transient receptor potential

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