Hydrogen Sulfide as an Oxygen Sensor

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Abstract

Eukaryotic cells depend upon oxygen (O_2) for their survival and elaborate mechanisms have evolved in multicellular animals, especially vertebrates, to monitor the availability of environmental O_2, the efficiency of O_2 extraction from the environment, ensure adequate O_2 delivery to tissues and even to regulate cellular metabolism when O_2 availability is compromised. In vertebrates, specialized O_2 "sensing" cells have developed to carry out many of these processes. Although all O_2 sensing cells ultimately couple low P_{O_2} (hypoxia) to physiological responses, how these cells actually detect hypoxia, i.e., the "O_2 sensor" remains controversial. We have recently proposed that hydrogen sulfide (H_2S) through its O_2-dependent metabolism is a universal and phylogenetically ancient O_2 sensing mechanism. This hypothesis is based on a variety of experimental evidence including; (1) the effects of exogenous H_2S mimic hypoxia, (2) H_2S production and/or metabolism is biochemically coupled to O_2, (3) tissue H_2S concentration is inversely related to P_{O_2} at physiologically relevant $P_{O_2}s$, (4) compounds that inhibit or augment H_2S production inhibit and augment hypoxic responses, (5) H_2S acts upon effector mechanisms known to mediate hypoxic responses, (6) H_2S was key to the origin of life and the advent of eukaryotic cells and the reciprocal relationship between O_2 and H_2S has been inexorably intertwined throughout evolution. The evidence for H_2S-mediated O_2 sensing is critically examined in this review.

Keywords

Hypoxia • Cardiovascular • Chemoreceptors • Mitochondria

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Abbreviations

3MP	3-Mercaptopyruvate
3-MST	3-Mercaptopyruvate sulfur transferase
AMPK	AMP-activated protein kinase
AOA	Amino-oxyacetate
AOX	Alternative oxidase
Asp	Aspartic acid
CAT	Cysteine aminotransferase
CBS	Cystathionine β-synthase
CDO	Cysteine dioxygenase
CO	Carbon monoxide
CSE	Cystathionine γ-lyase
Cys	Cysteine
DAO	D-amino acid oxidase
DHLA	Dihydrolipoic acid
EC_{50}	Effective concentration for half-maximal activity
ETHE1	Mitochondrial sulfur dioxygenase
Gly	Glycine
GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulfide
HA	Hydroxylamine
HIF	Hypoxia-inducible factor
HPC	Hypoxic pulmonary vasoconstriction
HSD	Hypoxic systemic vasodilation
IK_{Ca}	Intermediate conductance potassium channel
K_{ATP}	Adenosine triphosphate sensitive potassium channel
KCl	Potassium chloride
K_i	Inhibition constant
K_v	Voltage-gated potassium channels
NEB	Neuroepithelial bodies
NEC	Neuroepithelial cells
NO	Nitric oxide
O_2^-	Superoxide
PASMC	Pulmonary artery smooth muscle cells
pB	Pre-Bötzinger respiratory group
PKCε	Protein kinase C epsilon
PLP	Pyridoxal 5′phosphate
P_{O_2}	Partial pressure of oxygen
PPG	Propargyl glycine

Rde	Rhodanase
RI	Ischemia reperfusion injury
ROS	Reactive oxygen species
R-SO	Sulfenyl
$S_2O_3{}^{2-}$	Thiosulfate
SO	Sulfur oxidase
$SO_3{}^{2-}$	Sulfite
$SO_4{}^{2-}$	Sulfate
SQR	Sulfide:quinone oxidoreductase
ST	Sulfur transferase
TASK	Acid-sensitive potassium channel
TR	Thiosulfate reductase, a.k.a. rhodanase
TRD	Thioredoxin reductase
Trx	Thioredoxin
α-Kg	α-ketoglutarate

2.1 Introduction

Specialized O_2 sensing tissues in vertebrates are strategically placed to monitor ambient O_2, O_2 transport in blood, and to match blood flow with ventilation or tissue demand. Neuroepithelial cells (NEC) are present on the external surfaces of fish gills and monitor water Po_2 (Milsom and Burleson 2007). This is especially important for these aquatic vertebrates because, compared to air, water has considerably less O_2 (1/30), slower rates of O_2 diffusion (Krogh's diffusion coefficients 1/200,000) and higher viscosity (60 times). Even more problematic, aquatic O_2 levels can vary both temporally (minutes to seasons) and spatially within meters (Bickler and Buck 2007). In lungs of newborn mammals, cells similar to neuroepithelial-like cells are found in clusters (called neuroepithelial bodies, NEB) near airway bifurcations and here they may be important in the transition from the relatively hypoxic uterine environment during and shortly after birth (Kemp et al. 2002). External O_2 sensors other than NEB are relatively uncommon in terrestrial vertebrates. Presumably this is because atmospheric O_2 is relatively constant (21 %) and internal O_2 sensors appear to be able to accommodate changes in O_2 availability (such as in borrows or with increasing altitude) if needed. Arterial O_2 sensors that monitor blood Po_2 and O_2 delivery are found in fish as vascular-facing NEC and in higher vertebrates as the type I glomus cells of carotid and aortic bodies. (It is perhaps no coincidence that the first gill arch of fish is the homolog of the mammalian carotid body.) Mammalian adrenal medullary cells and homologous chromaffin cells that line systemic veins in fish secrete catecholamines in response to hypoxemia (Nurse et al. 2006; Perry et al. 2000) and may monitor tissue O_2 extraction. Perhaps the best characterized O_2 sensing tissues are the blood vessels themselves. It has generally been accepted that hypoxia relaxes systemic vessels thereby matching tissue perfusion to metabolic demand, whereas hypoxia

contracts pulmonary vessels to match ventilation to perfusion (Sylvester et al. 2012). However, recent studies have shown that many systemic vessels in non-mammalian vertebrates are contracted by hypoxia (Russell et al. 2008) and hypoxia relaxes pulmonary vessels in diving mammals to prevent pulmonary hypertension that would otherwise occur during a prolonged dive (Olson et al. 2010). Although the vascular response to hypoxia is intrinsic to vascular smooth muscle (Madden et al. 1992) there appears to be considerable plasticity in the functional organization of this response. These atypical responses have been key to evaluating H_2S-mediated mechanisms for acute O_2 sensing as described in this chapter.

There is considerable controversy concerning the actual mechanism with which these cells detect O_2 levels or availability and then transduce this into physiologically relevant signals and of the numerous proposed O_2 sensing mechanisms none have received unanimous support. Our work has suggested that the O_2 dependent metabolism of hydrogen sulfide (H_2S) is an effective and efficient mechanism of H_2S sensing. This review presents the evidence supporting our hypothesis.

2.2   Mechanism(s) of O_2 Sensing

Despite the apparent ubiquity of O_2 sensing tissues in vertebrates there has been little consensus on the mechanism that specifically measures P_{O_2} or O_2 concentration. Potassium channels have long been a likely candidate (Weir and Archer 1995) although it is now believed that they operate downstream of the actual sensing mechanism. The various theories of O_2 sensing mechanisms have been extensive reviewed Sylvester et al. (2012). Because mitochondria account for most of a cell's O_2 consumption they are central to most O_2 sensing theories and implicit in the three most prevalent theories, the redox hypothesis, the reactive oxygen species (ROS) hypothesis and the energy state/AMPK (AMP-activated protein kinase) hypothesis. In the redox hypothesis, hypoxia suppresses mitochondrial oxidative phosphorylation which further reduces the cytosol and decreases ROS production. Voltage-gated potassium (K_v) channels that were tonically kept open during normoxia by ROS now close and the resulting cell depolarization opens voltage-gated calcium channels and the influx of calcium produces contraction. Essentially the opposite occurs in the ROS hypothesis where hypoxia increases mitochondrial production of ROS, namely superoxide (O_2^-) and probably more importantly hydrogen peroxide (H_2O_2). The ROS thus produced activate a variety of intracellular signaling cascades that also increase intracellular calcium concentration. In the energy state/AMPK hypothesis, hypoxia decreases mitochondrial ATP production which increases the AMP to ATP ratio and activates AMP kinase. The resulting production of cyclic ADP ribose then brings about an increase in intracellular calcium and contraction. It should be noted, however, that vascular smooth muscle and endothelial cells can and do derive their energy from glycolysis and even though they respond to hypoxia, their ATP levels do not appear to be affected (Dromparis and Michelakis 2013). Other O_2 sensing mechanisms such as heme oxygenase (which generates the gasotransmitter carbon monoxide, CO), plasma

membrane bound NADPH oxidase or a yet identified hemoprotein or mitochondrial complex III and nitric oxide (NO) have also been described for various tissues (Evans et al. 2011; Gonzalez et al. 2010; Haldar and Stamler 2013; Waypa and Schumacker 2010; Wolin et al. 2010).

2.3   H₂S Oxidation as an O₂ Sensing Mechanism

We (Olson et al. 2006) originally proposed that the O_2-dependent metabolism of endogenously generated, and biologically active H_2S functioned as an efficient O_2 sensing mechanism that initiated hypoxic pulmonary vasoconstriction (HPC) and hypoxic systemic vasodilation (HSD). This hypothesis appears to fulfill the criteria for an O_2 sensing mechanism (Olson 2011) in that; (1) the effects of exogenous H_2S mimic hypoxia, (2) H_2S production and/or metabolism is biochemically coupled to O_2, (3) tissue H_2S concentration is inversely related to P_{O_2} at physiologically relevant P_{O_2}s, (4) compounds that inhibit or augment H_2S production inhibit and augment hypoxic responses, (5) H_2S acts upon effector mechanisms known to mediate hypoxic responses, (6) H_2S-mediated O_2 sensing has an evolutionary precedent and a phylogenetic history. The following sections describe how these criteria have been met.

2.3.1   The Effects of Exogenous H₂S Mimic Hypoxia

2.3.1.1 Cardiovascular System

Mechanical responses to hypoxia are identical to those produced by H_2S in all vessels (Fig. 2.1). Relaxation of rat thoracic aorta and portal vein was one of the first "physiological" effects of H_2S identified (Hosoki et al. 1997) and this mimics the well-known hypoxic vasodilation of mammalian systemic vessels. Similar hypoxic and H_2S vasodilations have been observed in all classes of vertebrates from the most primitive hagfish and lamprey to mammals (cf Russell et al. 2008; Dombkowski et al. 2005). This response has now been observed in over 20 mammalian studies. H_2S also dilates the mouse ductus arteriosus (Baragatti et al. 2013) which would be expected to keep this vessel patent in the relative hypoxic intrauterine environment. H_2S vasodilation of mammalian systemic vessels has also been observed in perfused organs (Cheng et al. 2004) and in vivo (Derwall et al. 2011; Leffler et al. 2010; Mustafa et al. 2009; Yan et al. 2004; Yang et al. 2008). Consistent with hypoxic pulmonary vasoconstriction, H_2S constricts isolated bovine pulmonary arteries (Olson et al. 2006, 2010), increases vascular resistance in perfused fish gills (Skovgaard and Olson 2012) and perfused rat lungs (Madden et al. 2012) and increases pulmonary arterial blood pressure in vivo (Derwall et al. 2011; Sowmya et al. 2010).

Hypoxic responses of non-mammalian vertebrates are considerably more variable in that systemic vessels can be either dilated or contracted by hypoxia and often these effects are multiphasic. Nevertheless, in over 30 animals from all
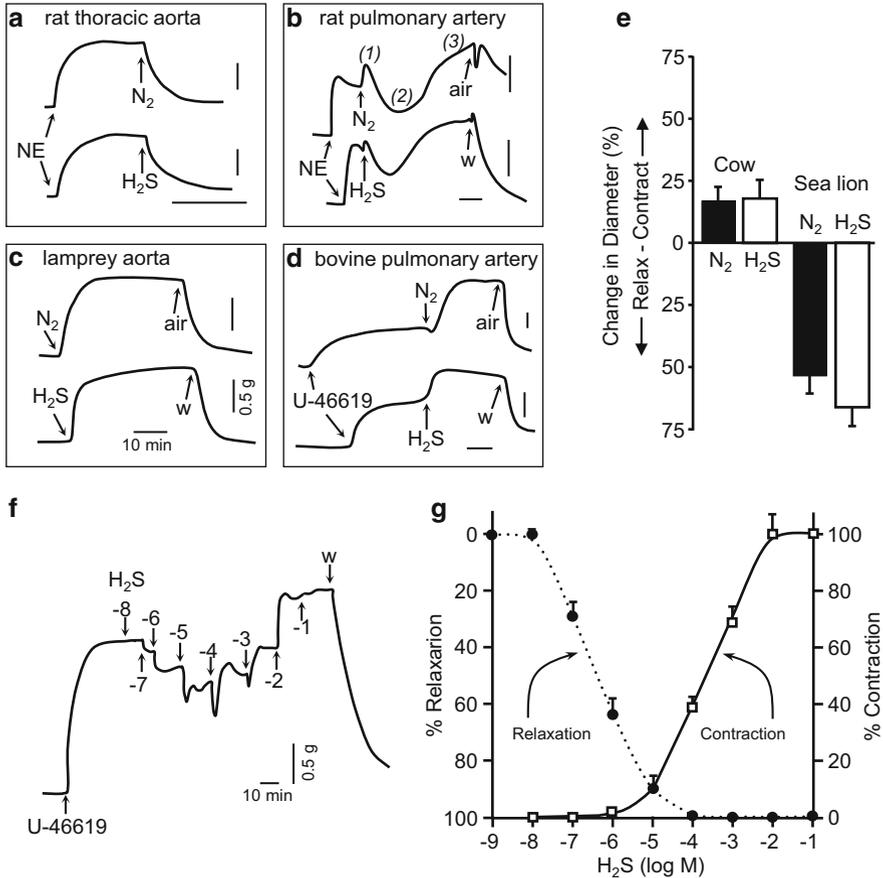
Fig. 2.1 Hypoxia (95 % N_2/5 % CO_2 **a**, **b**, **d**, **e**; or 100 % N_2, **c**) and H_2S (300 µmol/l) produce identical responses in conductance (>500 µm diameter) vessels from rat thoracic aorta (**a**), rat pulmonary artery (**b**), lamprey dorsal aorta (**c**) and bovine pulmonary artery (**d**). Vessels pre-contracted with 1 µmol/l norepinephrine (NE) or U-46619 (thromboxane A_2 mimetic; 0.1 µmol/l); *air* aeration with room air; *w* wash; *1, 2, 3*, tri-phasic response. *Horizontal time bar* in **a–d** = 10 min, vertical tension scale = 0.5 g. (**e**), hypoxia ($P_{O_2} \sim 50$ 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). (**f**, **g**), H_2S has two dose-dependent effects on pre-contracted (0.1 µmol/l U-46619) bovine pulmonary arteries. H_2S appears to produce a dose-dependent relaxation between 10^{-8} and 10^{-5} mol/l, whereas above 10^{-5} mol/l it produces a dose-dependent constriction. The EC_{50} for relaxation ($5.5 \pm 1.8 \times 10^{-7}$ M) is significantly ($p < 0.05$) different from the EC_{50} for contraction ($3.7 \pm 1.5 \times 10^{-4}$ M). (**f**) single trace of cumulative doses, *arrows* indicate log M [H_2S]; *w* wash; (**g**) average (+SE) of eight vessels, *filled circles* denote relaxation and *open squares* contraction (values extrapolated where curves overlap ~ 10^{-5} M) (**a–d**, **f**, **g** Adapted from Olson et al. 2006, with permission; **e** adapted from Olson et al. 2010, with permission)

vertebrate classes, the hypoxic responses of both systemic and respiratory vessels are consistently mimicked by H_2S (summarized in Olson and Whitfield 2010). This includes the signature multiphasic contraction-relaxation-contraction of rat pulmonary arteries (Fig. 2.1b) and the unique hypoxic and H_2S-mediated dilation of sea lion pulmonary resistance vessels (Fig. 2.1e). It is also evident that many of these multiphasic responses such as the initial relaxation followed by constriction in bovine pulmonary arteries are the results of separate, but dose-dependent effects of H_2S (Fig. 2.1f), a hint of which can also be seen during the onset of hypoxia (Fig. 2.1d). Hypoxia and H_2S also relax non-vascular smooth muscle of fish urinary bladder and the gastrointestinal tract and in the gastrointestinal tract both of these stimuli produce a unique and transient increase in spontaneous contraction frequency and amplitude prior to the onset of the inhibitory effects (Dombkowski et al. 2006, 2011). H_2S also relaxes human corpus cavernosum and urinary bladder smooth muscle (d'Emmanuele di Villa Bianca et al. 2009; Fusco et al. 2012).

Involvement of H_2S has also been shown in other hypoxia-related responses. In the cardiovascular system this includes angiogenesis, ischemia reperfusion injury (RI) and pre- and post-conditioning against RI (Bian et al. 2006; Cai et al. 2007; Calvert et al. 2010; Lavu et al. 2010; Liu et al. 2010; Pan et al. 2006; Papapetropoulos et al. 2009; Szabo and Papapetropoulos 2011; Wang et al. 2010a, b; Yong et al. 2008). H_2S also contributes to hypoxia-induced radiation resistance (Zhang et al. 2011) and it is a cryogenic mediator of hypoxia-induced anapyrexia (Kwiatkoski et al. 2012).

2.3.1.2 Respiration

Intravascular injection or inhalation of H_2S at low concentrations has long been known to mimic hypoxemia by stimulating respiration in many mammals (Beauchamp et al. 1984; Reiffenstein et al. 1992; Haggard and Henderson 1922; Haouzi et al. 2009 [in mice but not rats], 2011; Haouzi 2012; Van de Louw and Haouzi 2012) and birds (Klentz and Fedde 1978). This may have both a central and peripheral component mediated through the carotid bodies. Intracerebroventricular injection of H_2S produces a K_{ATP} channel-mediated dose dependent bradycardia and hypotension (Liu et al. 2011) akin to the hypoxic diving reflex. In more specific studies it was observed that 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 (Chen et al. 2013; Hu et al. 2008). H_2S stimulates peripheral chemoreceptors (neuroepithelial cells) in the fish gill (Olson et al. 2008) and mammalian carotid body (Li et al. 2010; Makarenko et al. 2012; Peng et al. 2010) and it stimulates the mammalian adrenal medulla (Peng et al. 2010; Zhu et al. 2012) and homologous fish chromaffin cells (Perry et al. 2009). H_2S may also contribute to the sequela of events in which heart failure, hypertension and renal failure activate the carotid body leading to breathing instability and increased sympathetic nerve activity (Schultz et al. 2012).

2.3.2 H_2S Production and/or Metabolism is Biochemically Coupled to O_2

2.3.2.1 Biosynthesis

Pathways for H_2S synthesis and metabolism are shown in Fig. 2.2. L-cysteine and L-homocysteine account for most H_2S production through the activity of four enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE aka CGL) and sequential catalysis by cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3-MST). H_2S may be synthesized in the cytosol as CBS and CSE and CAT/3-MST are cytosolic enzymes, or it may be synthesized in the mitochondria as CAT/3-MST are present there as well (Kamoun 2004). 3-MST is especially abundant in the mitochondrial matrix (Mikami et al. 2011a) where it can take advantage of threefold higher cysteine concentration than in the cytosol (Fu et al. 2012). CSE can also be translocated to the mitochondria by a variety of stress-related stimuli (Fu et al. 2012). Initially it was believed that CBS was predominantly found in the brain and CSE in the cardiovascular system (reviewed in Kimura 2010), although a broader distribution is becoming evident, i.e., CBS has been identified in vascular endothelium, CAT and 3-MST in vascular endothelium and brain and MST, but not CAT, in vascular smooth muscle (Kimura 2010; Olson et al. 2010). CBS and CSE have also been identified in human plasma (Bearden et al. 2010). H_2S can be generated from D-cysteine, however, this pathway appears limited to the brain and kidney where it protects the former from oxidative stress and the latter from re-perfusion injury; it may be of limited function elsewhere (Shibuya et al. 2013). CBS, CSE and CAT are pyridoxal 5′phosphate (PLP)-dependent, enzymes. S-adenosylmethionine allosterically activates CBS (Stipanuk 2004). CBS contains a heme group that can be inhibited by presumably physiological levels of carbon monoxide (CO; inhibition constant, $K_i = 5.6$ μM) and although it is also inhibited by nitric oxide (NO), the K_i is so high (320 μM) that it is of questionable physiological significance; O_2 does not affect CBS activity (Banerjee and Zou 2005). CSE and both cytosolic and mitochondrial CAT activity are inhibited by calcium, independent of calmodulin (Mikami et al. 2011b, 2013).

2.3.2.2 Metabolism (Inactivation)

Mathematical models of H_2S diffusion out of cells versus intracellular metabolism suggest that most H_2S is inactivated intracellularly (Olson 2013), and this is supported by the considerable body of evidence that mitochondria efficiently oxidize H_2S (reviewed in Olson 2012c). Mitochondrial enzymes, sulfide:quinone oxidoreductase (SQR), 3-MST, rhodanase (Rde), thiosulfate reductase (TR), sulfur dioxygenase (ETHE1) and sulfite oxidase (SO) ultimately oxidize H_2S to sulfate (SO_4^{2-}) which is then excreted. Sulfite (SO_3^{2-}) and thiosulfate ($S_2O_3^{2-}$) are intermediates. SQR is bound to the inner mitochondrial membrane and it is closely associated with the respiratory chain "supercomplex"(Hildebrandt 2011) which provides a hint at its O_2 sensing function as the mitochondrion is a lead candidate for the site of O_2 sensing (Sylvester et al. 2012). Not surprisingly, 3-MST,
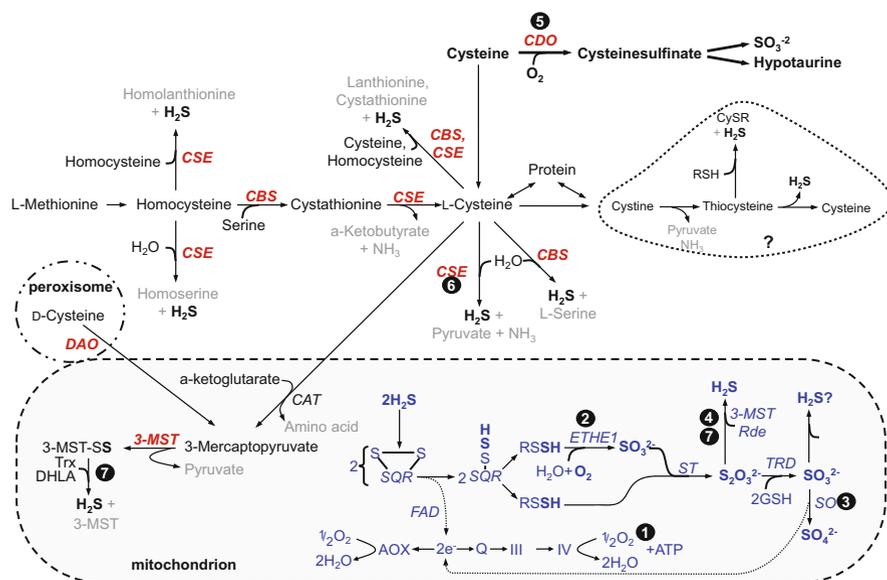
Fig. 2.2 Pathways for H₂S production and degradation. H₂S is synthesized from homocysteine or cysteine by the cytosolic enzymes cystathionine β-synthase (*CBS*), cystathionine γ-lyase, or the tandem action of cysteine aminotransferase (*CAT*) and 3-mercaptopyruvate sulfur transferase (3-*MST*) in both the cytosol and mitochondria. Other potential mechanisms for H₂S biosynthesis have been described in invertebrates (*dotted enclosure*; Julian et al. 2002) 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*). Oxidation of H₂S in the mitochondria (*blue letters*) is initiated by interaction with the enzyme sulfide:quinone oxidoreductase (*SQR*) producing two SQR persulfides that are then transferred to a mobile sulfide carrier (RS**SH**), one of which is oxidized to sulfite (SO₃²⁻) by mitochondrial sulfur dioxygenase (*ETHE1*). Sulfur transferase (*ST*) transfers the other persulfide to form thiosulfate (S₂O₃²⁻). H₂S may be regenerated from thiosulfate by 3-MST or rhodanase (*Rde*), or the thiosulfate may be oxidized to sulfate (SO₄²⁻) by the sequential actions of thioredoxin reductase (*TRD*) in combination with glutathione (*GSH*) and sulfur oxidase (*SO*). Oxidation of H₂S donates electrons to the respiratory chain (Q, III, IV) that has been shown in invertebrates and mammals to result in ATP production and O₂ consumption. An alternative oxidase (*AOX*) that oxidizes H₂S without concomitant ATP production has also been observed in invertebrates. H₂S can also be regenerated from thiosulfate by 3-mercaptopyruvate sulfur transferase (3-*MST*) or rhodanase (*Rde*) in the presence of other reducing disulfides such as thioredoxin (*Trx*) or dihydrolipoic acid (*DHLA*). *Circled numbers* indicate actual or potential hypoxia-sensitive sites (see text for details) (Modified from Olson et al. (2012c), with permission)

rhodanase and thiosulfate reductase are abundant in the mitochondrial matrix and to a lesser extent the intermembrane space (Koj et al. 1975). H₂S oxidation begins with H₂S 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 H₂S and two SQR are involved, one persulfide sulfur is transferred to sulfur dioxygenase (SDO) where it is oxidized to sulfite while sulfur from the second persulfide is transferred from the SQR to sulfite

by sulfur transferase (ST) producing thiosulfate. One electron from each of the two H_2S are fed via the quinone pool (Q) into the respiratory chain (Marcia et al. 2010) 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 a mobile carrier such as glutathione (GSH), dihydrolipoate, thioredoxin or even sulfite (Hildebrandt and Grieshaber 2008; Jackson et al. 2012; Theissen and Martin 2008). One of the mobile persulfides is oxidized to sulfite by ETHE1 which consumes molecular O_2 and water in the process. Metabolism of H_2S through SQR appears ubiquitous in tissues although the brain may be an exception (Hildebrandt 2011; Lagoutte et al. 2010; Linden et al. 2011; but see Ackermann et al. 2011). The capacity of cells to oxidize sulfide appears to be greater than the estimated rate of sulfide production (Bouillaud and Blachier 2011; Furne et al. 2008). Thus it is expected that intracellular H_2S concentrations are very low under normoxic conditions.

Under normoxic conditions, most thiosulfate is further metabolized to sulfate by thiosulfate reductase (TR) and sulfite oxidase (SO). Elimination of one sulfur atom as sulfate is accompanied by four atoms of O_2. Even though sulfur excretion as thiosulfate would conserve O_2, apparently there is little need for this when is O_2 plentiful and thiosulfate excretion in vertebrates is generally low. In fact, it is not clear why most vertebrates, especially terrestrial ones, would bother with this pathway at all. However, as described below, it may be an important avenue for regeneration of H_2S during hypoxia. Details of H_2S biosynthesis and metabolism can be found in (Kabil and Banerjee 2010; Kimura 2010; Olson 2012c; Stipanuk 2004; Stipanuk et al. 2009).

2.3.2.3 H_2S Oxidation via Electron Transport

In our original mechanism of H_2S-mediated O_2 sensing (Olson et al. 2006) we proposed that H_2S was constitutively produced in the cytosol through transsulfurration and oxidized in the mitochondria. As the amount of O_2 available to the mitochondria determined the rate of H_2S oxidation, and hence H_2S concentration, this was the "O_2 sensor." Although this is still likely an integral mechanism, it is now evident that there are a number of other mechanisms with which O_2 can influence H_2S concentration and thereby contribute to O_2 sensing. These mechanisms, some of which are expected to rapidly respond to O_2 while others may provide a longer bias of H_2S concentration, are described in the following paragraphs and shown numerically in Fig. 2.2. While many of these pathways remain to be verified in the context of O_2 sensing, there is accumulating evidence, largely through studies on enzyme deficiencies, that their dysfunction will impact sulfide metabolism and either directly or indirectly increase H_2S concentration.

Rapid Effectors of H_2S Concentration

Electron transport: Disruption of electron flow down the respiratory chain by insufficient O_2 delivery to the mitochondrion at complex IV still remains a very likely and highly effective mechanism to regulate H_2S as it directly couples O_2 availability to H_2S inactivation. This will prevent any further oxidation of H_2S that was derived from

transsulfurration. This pathway of H_2S oxidation has been well-established in the context of ATP synthesis (Goubern et al. 2007; Lagoutte et al. 2010; Modis et al. 2013).

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. Although the effects of O_2 on this pathway have not been examined in detail, they would presumably be similar to the well-characterized ETHE1 deficiencies in experimental animals (ETHE1$^{-/-}$ mice) and humans, the pathology of which is characterized by greatly elevated tissue H_2S and thiosulfate (Di Meo et al. 2011; Drousiotou et al. 2011; Giordano et al. 2011; Tiranti et al. 2009).

Sulfite oxidase: Sulfite oxidase (SO) in the mitochondrial innermembrane 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 2-electron reduction (Rajapakshe et al. 2012). These electrons are then transferred from SO to cytochrome c and shuttled into the electron transport chain with molecular oxygen as the terminal acceptor. This couples sulfite concentration to O_2 availability and suggests that a hypoxia-induced increase in sulfite would increase thiosulfate concentration and ultimately H_2S production. SO deficiency in a human was first demonstrated in 1967 and, as might be expected, this patient presented with elevated urinary thiosulfate (Mudd et al. 1967).

Thiosulfate reduction: As described above, inhibition of either ETHE1 or SO will prevent further oxidation of thiosulfate and this thiosulfate can now directly produce H_2S under appropriate conditions by reduction. The sulfur atoms on thiosulfate have different oxidation states, the inner sulfur is +5 and the outer (persulfide) sulfur is -1. In the presence of a reducing reagent such as the endogenous mitochondrial reductant, dihydrolipoic acid (DHLA), 3-MST or thiosulfate reductase (TR; aka rhodanase, Rde) can catalyze the removal of the persulfide and generate H_2S (Mikami et al. 2011a; Villarejo and Westley 1963). H_2S generation from thiosulfate under these conditions has been demonstrated in a variety of mammalian and non-mammalian tissues and it is enhanced by hypoxia (Olson et al. 2013). Thiosulfate can also be generated from D-cysteine by D-amino acid oxidase through formation of 3-mercaptopyruvate (Huang et al. 1998) although the biological significance of this pathway is unknown. H_2S generation from thiosulfate will be greatest where thiosulfate concentrations are the highest and when the immediate environment becomes more reduced and the relevant reducing molecules become more available. Thiosulfate concentrations are probably highest in or near the inner mitochondrial membrane, the site of SQR. This is also likely to experience the greatest increase in hypoxia-induced reducing equivalents such as DHLA or thioredoxin because as cells become hypoxic, reactive oxygen species (ROS) increase in both the cytosol and mitochondrial intermembrane space, whereas ROS decrease in the mitochondrial matrix (Waypa et al. 2010). Thus, thiosulfate reduction could significantly contribute to the initial increase in H_2S concentration that activates the O_2 sensing cascade and by recycling sulfur it conserves biologically relevant thiols.

Long-Term Effectors of H$_2$S Concentration

Cysteine dioxygenase: Cysteine dioxygenase (CDO), a cytosolic enzyme, effectively eliminates sulfur from entering the H$_2$S pool by irreversibly catalyzing the oxidation of cysteine to cysteinesulfinate which can be further metabolized to hypotaurine, then taurine or sulfite and then sulfate for excretion (Stipanuk et al. 2009). One of the primary functions of CDO is believed to be the detoxification of excess dietary or metabolic cysteine as CDO activity is dynamically regulated by cysteine (as much as 450-fold) whereas cysteine desulfuration, the H$_2$S-forming transsulfuration pathway is not regulated (Stipanuk et al. 2009). CDO also contributes to degradation of methionine and homocysteine after their conversion to cysteine (Stipanuk and Ueki 2011). Impairment of this pathway, which has been demonstrated in CDO knockout (CDO$^{-/-}$) mice, redirects sulfur through the desulfuration pathway and increases thiosulfate and H$_2$S production (Ueki et al. 2011; Roman et al. 2013). As molecular O$_2$ is the only other substrate in CDO-mediated cysteine oxidation it is likely that hypoxia will impair cysteine oxidation and favor H$_2$S production. It is not likely that this would contribute to the rapid on/off signaling observed acute hypoxia, but it could place a long-term bias on chronic H$_2$S-mediated H$_2$S sensing. An inability of this pathway to handle a large transient cysteine load may partially explain how hypoxic responses are augmented by exogenous cysteine (see below). Ueki et al. (2011) also noted the striking similarities between CDO$^{-/-}$ and ETHE1$^{-/-}$ pathologies and suggested that sulfide/H$_2$S was the common factor.

CSE translocation to mitochondria: In vascular smooth muscle cells, hypoxia stimulates CSE translocation from the cytosol to the mitochondria where it utilizes the threefold increase in cysteine concentration to generate H$_2$S which can be subsequently used in ATP synthesis (Fu et al. 2012). It has been proposed that this H$_2$S improves mitochondrial ATP production and it is protective during hypoxia (Fu et al. 2012). There are, however, several problems with this hypothesis. First, vascular smooth muscle can derive sufficient energy from anaerobic metabolism and does not need oxidative phosphorylation to supply energy, even during hypoxia (Dromparis and Michelakis 2013). Second, because ATP generated from H$_2$S is ultimately dependent on O$_2$, it is unclear what the electron acceptor will be in the absence of O$_2$. However, the H$_2$S formed by CSE translocation could clearly contribute to O$_2$ sensing and hypoxic vasodilation.

Indirect O$_2$ Effects

3-MST and thioredoxin catalytic-site cysteines: Many enzymes contain cysteine in the catalytic site and because these cysteines generally have a low pKa, they are redox active (Nagahara 2011). The catalytic cysteine in 3-MST (Cys^{247}; rat) is one example. The exposed, Cys^{247} sulfur is readily oxidized to a sulfenyl (R-SO) by O$_2$, peroxide (H$_2$O$_2$) or other oxidants under mild oxidizing conditions; the sulfenyl is also reduced (to R-SH) by reduced thioredoxin (Trx) but not GSH (Nagahara 2012). Monomeric Rat 3-MST can also dimerize by mild oxidation of two other exposed cysteines, Cys^{154} and Cys^{263}; both oxidization of Cys^{247} and dimerization inactivate the enzyme. A defect in 3-MST activity, presented clinically

as mercaptolactate-cysteine disulfidia, is believed to be associated with deficient H_2S production (Nagahara 2012). While the three external cysteines of 3-MST may well allow it to serve as an effective antioxidant (Nagahara 2012) it could also be a key component of the H2S-mediated O_2 sensing mechanism. Furthermore, as 3-MST is found in both the cytosol and mitochondrial matrix and during hypoxia ROS in the cytosol increase while ROS in the matrix decrease, in both pulmonary and systemic arterial smooth muscle cells (Waypa et al. 2010), this will favor mitochondrial H_2S production and inhibit it in the cytosol. Parenthetically, the now-oxidized Trx can be reduced by thioredoxin reductase (TRD) using NADPH; which may be an overlooked, but key explanation for why NADPH has been central in many O_2-sensing theories (Gupte and Wolin 2008).

2.3.3 Tissue H_2S Concentration Is Inversely Related to Po_2 at Physiologically Relevant Po_2s

If H_2S and O_2 coexist in the environment or in cells it is only transient. In fact, hypoxia, or more often anoxia, is generally (although rarely appreciated) a requisite for all measurements of tissue H_2S production, other than those using polarographic H_2S sensors (Olson 2012a). Using the polarographic sulfide sensor, originally developed by Jeroschewski et al. (1996), Kraus and Doeller (2004) observed that excised gills and gill mitochondria from sulfide-adapted mussel, *Geukensia demissa*, rapidly consumed H_2S in the presence of O_2 and that the rate of H_2S consumption was reduced 50-fold in anoxia and 75 % inhibited by cyanide. The authors estimated the P_{50} (the partial pressure of O_2 at half maximal rate) for H_2S oxidation in mitochondria of approximately 7.5 mmHg. Both Furne et al. (2008) and Doeller et al. (2005) measured the relationship between O_2 and H_2S production in rat tissues and observed that H_2S production was suppressed at normoxic Po_2 and Furne et al. (2008) observed a switch from H_2S production in hypoxia to H_2S consumption in normoxia in mouse liver and brain. Similar observations have been made in a variety of other tissues (Fig. 2.3; Dombkowski et al. 2011; Linden et al. 2011; Madden et al. 2012; Olson et al. 2008, 2010; Olson and Whitfield 2010; Whitfield et al. 2008). A compelling argument for H_2S-mediated O_2 sensing can be made by comparing bovine and sea lion lungs (Olson et al. 2010); while both tissues clearly demonstrate an identical and reciprocal relationship between H_2S production/ consumption and O_2 (Fig. 2.3a, b), both hypoxia and H_2S constrict the former and dilate the latter (Fig. 2.1e).

    To date, there has only been one study in which the rate of H_2S consumption by tissue has been measured at carefully controlled Po_2 (Olson et al. 2010). As shown in Fig. 2.3d, the efficiency of H_2S oxidation by bovine lung homogenate, bovine pulmonary arterial smooth muscle cells, or purified bovine heart mitochondria begins to fail at physiologically relevant Po_2s and at Po_2s routinely encountered during hypoxia H_2S metabolism becomes highly sensitive to O_2 availability. Further demonstration of the physiological relevancy of this process is the observation that the Po_2 at which the ability of pulmonary arterial smooth muscle cells to
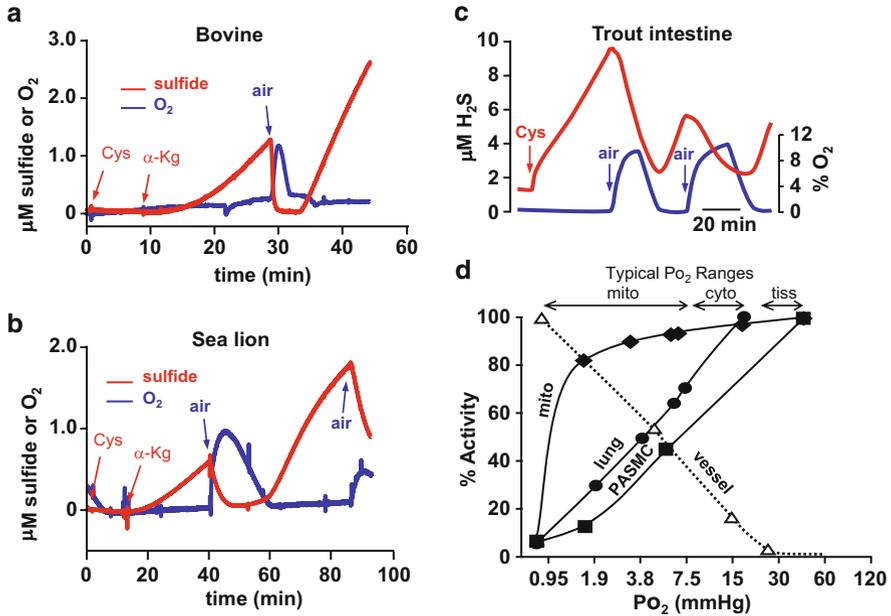
Fig. 2.3 Reciprocal relationship between O_2 and H_2S in tissues. (**a–c**) Realtime measurements of O_2 and H_2S in homogenized cow lung (**a**), homogenized sea lion lung (**b**) and homogenized trout intestine (**c**) using gas-specific polarographic electrodes. H_2S production increases after addition of 1 mM cysteine (Cys) and α-ketoglutarate (α-Kg) or Cys alone but only in the absence of O_2. Injection of a small air bubble (air) which increases O_2 concentration immediately reduces H_2S concentration all tissues. When the O_2 is consumed, H_2S concentration again increases. These results show that H_2S production is inversely related to O_2 in a variety of tissues, even though bovine pulmonary arteries are constricted by hypoxia whereas sea lion pulmonary arteries and trout intestines are relaxed. (**d**) H_2S consumption as a function of O_2 availability (P_{O_2}) by bovine mitochondria (mito), homogenized bovine lung tissue (bovine lung) and bovine pulmonary artery smooth muscle cells (*PASMC*). H_2S is rapidly metabolized (100 % inactivation rate) until P_{O_2} falls to ~ 15 mmHg (1–2 mmHg for mito); H_2S metabolism rapidly fails as P_{O_2} continues to fall. The P_{O_2} values at which H_2S metabolism is impaired are at the low end of cytosolic and mitochondrial P_{O_2} and would be expected during hypoxia. The P_{O_2} at which H_2S metabolism is reduced to half (P_{50}) in lung tissue and PASMC is essentially the same P_{O_2} that produces half-maximal hypoxic vasoconstriction of bovine pulmonary arteries (vessel). (**a**, **b**, **d**, Modified from Olson et al. 2010, **c** modified from Dombkowski et al. 2011, with permission)

oxidize H_2S is halved (P_{50}) is identical to the P_{50} of hypoxic pulmonary vasoconstriction. As might be expected, mitochondria function at a P_{O_2} below the cytosolic P_{O_2} and it is evident in Fig. 2.3d that the H_2S oxidation curve is left-shifted accordingly. These studies clearly show that the metabolism of H_2S is O_2 dependent, that the ability of tissues to metabolize H_2S fails at physiologically relevant P_{O_2}s, and this provides a sensitive and efficient mechanism for O_2 sensing.

2.3.4 Compounds that Augment or Inhibit H₂S Production Augment or Inhibit Hypoxic Responses

The ability of sulfur donors especially cysteine to augment hypoxic responses has been well documented (Fig. 2.4). Cysteine increases the magnitude of hypoxic vasoconstriction of isolated lamprey aortas (Olson et al. 2006), bovine pulmonary arteries (Olson et al. 2006, 2010) and it increases vascular resistance in the perfused rat lung (Madden et al. 2012). 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 (Madden et al. 2012; Olson et al. 2010). Continuous utilization of cysteine to sustain a hypoxic vasoconstriction is evident in Fig. 2.4d where it clearly sustains the hypoxic response. Exogenous cysteine also augments hypoxic relaxation of rat aortas (Bucci et al. 2010), the relaxation component of the perfused trout gill (Skovgaard and Olson 2012) as well as hypoxic relaxation of trout urinary bladder (Dombkowski et al. 2006) and salmon intestine (Dombkowski et al. 2011).

Hypoxic responses of lamprey aorta, bovine pulmonary arteries, rat aorta and the perfused trout gill and rat lung are also inhibited by inhibitors of H₂S synthesis (Fig. 2.5; Olson et al. 2006; Madden et al. 2012; Skovgaard and Olson 2012). Although inhibitors of H₂S are notoriously nonspecific and often poorly absorbed by tissues (Szabó 2007), their application can provide some information on the biosynthetic pathways that are being used to produce H₂S. Not surprisingly, CSE appears to be the major pathway for H₂S production by systemic vessels (Fig. 2.5c). However, in bovine pulmonary arteries inhibition of CBS, but not CSE, reduces the hypoxic response, whereas hydroxyl amine, which inhibits all pyridoxal phosphate dependent enzymes, including CBS, CSE and CAT, completely inhibits hypoxic vasoconstriction (Fig. 2.5b). This suggests that both the CBS and CAT/3-MST contribute to H₂S production in bovine pulmonary vessels. The CAT/3-MST pathway can also be utilized in the rat lung as the competitive inhibitor, aspartate, prevents the augmented hypoxic response produced by exogenous α-ketoglutarate (Fig. 2.5c). There appears to be some species variation in the enzymatic pathways employed as CSE may be a major component of H₂S production mediating hypoxic responses of the perfused rat lung (Madden et al. 2012). Inhibitors of H₂S biosynthesis have also been shown to inhibit hypoxic relaxation of rainbow trout urinary bladder (Dombkowski et al. 2006) and rainbow trout and Coho salmon intestine (Dombkowski et al. 2011).

Inhibiting CBS in the mouse carotid body decreases hypoxia-stimulated afferent nerve activity in vitro and blunts the hypoxic hyperventilation in vivo (Li et al. 2010). Conversely, Peng et al. (2010) observed an inverse P_{O_2}-dependent increase in H₂S production by rat carotid bodies and both H₂S production and sinus nerve activity could be blocked by inhibiting CSE; in a mouse CSE knockout ($CSE^{-/-}$) hypoxic responses of glomus cells were significantly reduced. Hypoxia-evoked catecholamine
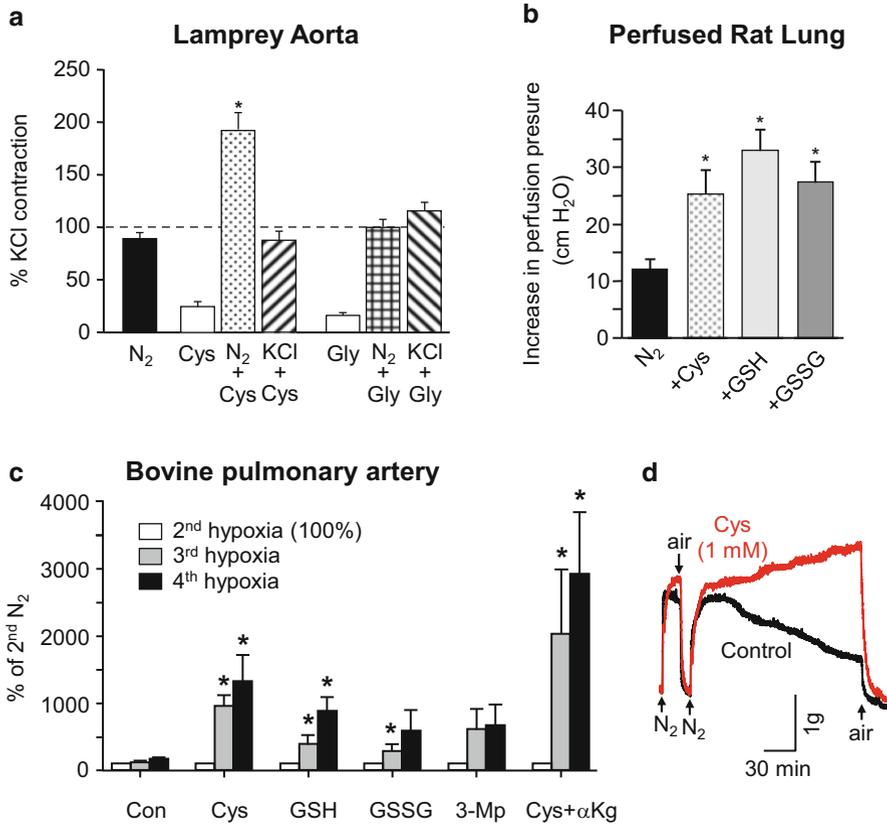
Fig. 2.4 Substrates for H$_2$S biosynthesis augment hypoxic responses. (**a**) In the lamprey aorta cysteine (Cys, 1 mM) nearly doubles a hypoxic contraction but does not affect a KCl (80 mM) contraction. Both Cys and glycine (Gly, 1 mM) had a slight effect but Gly does not affect the hypoxic response. (**b**) Cys (1 mM), reduced glutathione (GSH, 1 mM) and oxidized glutathione (GSSG, 1 mM) all significantly increase perfusion pressure in the perfused rat lung. (**c**) Cys (1 mM), GSH (1 mM), GSSG (1 mM) and Cys plus α-ketoglutarate (Cys + αKg, 1 mM) enhance consecutive hypoxic contractions of bovine pulmonary arteries. (**d**) Representative myograph traces illustrating the ability of Cys (1 mM) to prolong a hypoxic contraction in an isolated bovine pulmonary artery (Modified from Olson 2012b, with permission)

secretion from adrenal glands was also inhibited in CSE$^{-/-}$ mice or by inhibiting CSE in rats Peng et al. (2010). Clearly additional studies are needed so sort out the specific metabolic pathways for H$_2$S production in the glomus cells, but nevertheless, a strong case can be made for the involvement of H$_2$S in hypoxic signal transduction.
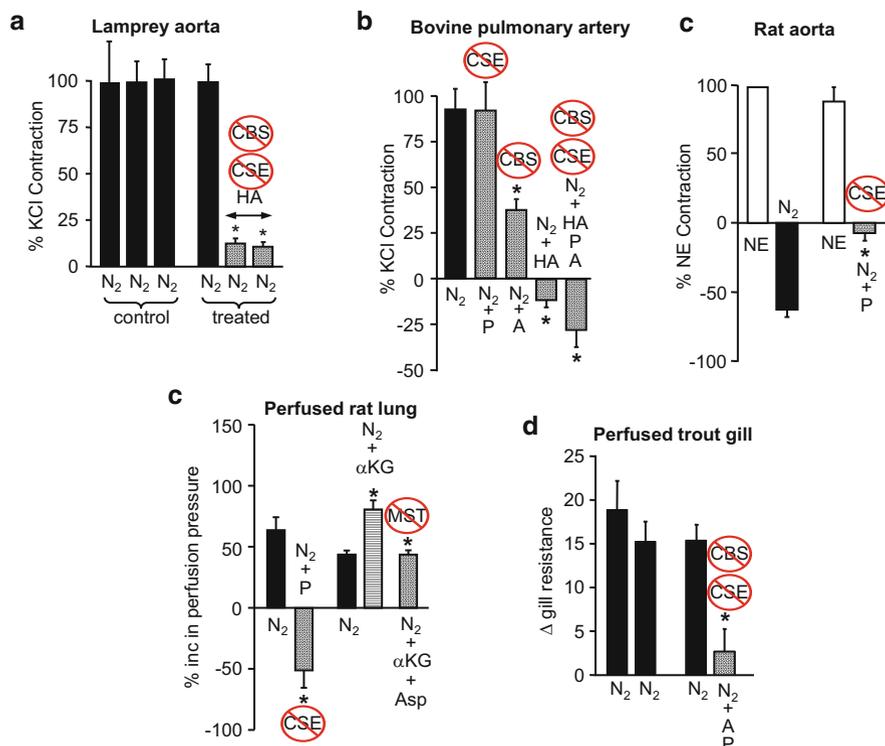
Fig. 2.5 Inhibitors of H_2S biosynthesis inhibit hypoxic vasoconstriction in the lamprey aorta (**a**), and bovine pulmonary artery (**b**) and hypoxic vasodilation of the norepinephrine (NE, 1 μM) precontracted rat aorta. Inhibiting H_2S biosynthesis also inhibits hypoxic vasoconstriction in the perfused rat lung (**c**) and perfused trout gill (**d**). *CBS* cystathionine β-synthase, *CSE* cystathionine γ-lyase, *A* amino-oxyacetate a CBS inhibitor (1 mM), *P* propargyl glycine a CSE inhibitor (10 mM), *HA*, *CBS* and *CSE* inhibitor hydroxylamine (1 mM), *α-Kg* α-ketoglutarate a substrate for mercaptopyruvate sulfur transferase (*MST*), *Asp* aspartic acid, an inhibitor of MST (10 mM) (From Olson 2012b, with permission)

2.3.5 H₂S Acts Upon Effector Mechanisms Known to Mediate Hypoxic Responses

The recent identification of H_2S signaling through sulfhydration of protein cysteine molecules (Mustafa et al. 2009) has not only contributed to our overall understanding of H_2S signaling pathways, but it has shed some light on the mechanisms of H_2S signaling in hypoxia. Because many proteins such as enzymes and structural proteins are regulated through active-site cysteines (Nagahara 2011) is also evident that H_2S signaling is most likely an autocrine activity and that even within the cell it must be highly spatially regulated. This is supported by the models predicting that hypoxic signaling proceeds in the immediate mitochondrial environment (Olson 2013).

As would be expected, the mechanisms with which inhibits or activates H_2S cells is commensurate with the intended outcome of H_2S signaling.

It is well-known that hypoxic vasodilation is mediated in part by K_{ATP} channels (Weir and Archer 1995) and these channels were one of the first targets identified for H_2S signaling (Zhao et al. 2001). Since then a variety of channels in vascular smooth muscle and endothelial cells have been shown to be affected by H_2S leading to vasodilation. These include K_{ATP}, intermediate conductance (IK_{Ca}) and K_v7 potassium channels (Jiang et al. 2010; Liang et al. 2011; Martelli et al. 2013; Mustafa et al. 2011). H_2S also relaxes newborn piglet cerebral arteries by increasing $[Ca^{2+}]$ in the sarcoplasmic reticulum. This stimulates Ca^{2+} sparks, increases current through K_{Ca} channels and hyperpolarizes the cells thereby lowering global intracellular $[Ca^{2+}]$ (Liang et al. 2012). H_2S did not directly affect K_{Ca} channels in these studies. H_2S activates the Kir 6.1 subunit of K_{ATP} channel through sulfhydration of specific cysteine residues, especially Cys-34, this decreases the inhibitory effect of ATP on these channels while increasing binding of the activator phosphatidylinositol (4,5)-bisphosphate (PIP2)28 to Kir 6.1; other channels such as the endothelial intermediate conductance (IK_{Ca}) channel and other cysteine residues such as Cys^6 and Cys^{26} may also be sulfhydrated and contribute to H_2S relaxation (Jiang et al. 2010; Mustafa et al. 2011). Intermittent hypoxia down regulates CSE and increases vascular tone via the loss of H_2S activation of BK_{Ca} channels (Jackson-Weaver et al. 2011) suggesting a longer time-scale of vascular regulation.

H_2S can also activate cells. We (Sudhahar et al. 2013) have recently shown that H_2S induces membrane trafficking of protein kinase Cε (PKCε) through specific sulfhydration of Cys-13 and Cys-74 in the C2 domain. This is consistent with the well known role of PKCε activation in hypoxic pulmonary vasoconstriction (Sylvester et al. 2012). H_2S also activates the carotid body through inhibition of large-conductance calcium activated potassium (BK_{Ca}) channels (Li et al. 2010; Telezhkin et al. 2009, 2010) and/or inhibition of background (TASK) potassium channels (Buckler 2012); both lead to membrane depolarization and voltage-gated Ca^{2+} entry. Although specific channels were not identified, inhibition of potassium channels is consistent with hypoxia- and H_2S-mediated depolarization of zebrafish neuroepithelial cells (Olson et al. 2008), bovine pulmonary arteries (Olson et al. 2006) and lamprey aortas (Madden and Olson, unpublished). H_2S stimulates catecholamine release from rat adrenal cells via H_2S inhibition of $I_{K(Ca)}$ current (Zhu et al. 2012). In other cells, H_2S directly increases BK channel activity in rat GH(3) pituitary tumor cells through its reducing action on sulfhydryl groups of the channel protein (Sitdikova et al. 2010). A direct link between H_2S signaling and hypoxia has been shown by Tao et al. (2012) where the Cys1045-Cys1024 disulfide bond of VEGFR2 is targeted by H_2S and serves as a specific molecular switch for hypoxia mediated migration of vascular endothelial cells.

It is not too surprising that the acute hypoxia signal, H_2S, interacts with the long-term hypoxia signaling hypoxia-inducible factors (HIFs). In general, H_2S decreases HIF-1α expression in a variety of mammalian tissues (Kai et al. 2012; Si et al. 2013; Wu et al. 2012). H_2S inhibits HIF-1α protein accumulation during hypoxia (1 % O_2) or hypoxia-mimetic conditions by enhancing HIF2α phosphorylation independent of

protein synthesis or ubiquitin-proteasomal degradation (Kai et al. 2012; Wu et al. 2012). Hypoxic pre- (and post-)conditioning, which appears to involve H_2S signaling (Bian et al. 2006; Lavu et al. 2010; Pan et al. 2006; Yong et al. 2008) is also associated with a decrease in HIF1-α expression (Sims et al. 2012). Interestingly, H_2S has the opposite effect on the nematode, *Caenorhabditis elegans*, where it increases HIF-1 activity, although this effect appears to be independent of hypoxia-mediated HIF-1 expression (Budde and Roth 2010). Other effectors of H_2S-mediated cellular protection from hypoxia include heat shock protein 90 (Meng et al. 2011), inhibition of ROS-activated pathways such as NF-κB/COX-2 (Yang et al. 2011) and ERK1/2 and p38MAPK (Lan et al. 2011).

Direct competition between hypoxia and H_2S for the same effector response, seen as the inability of one stimulus to produce a response when the tissue is activated by the other, has been demonstrated in a variety of blood vessels (Olson et al. 2006; Skovgaard and Olson 2012). This provides additional, albeit indirect, evidence that H_2S is involved in hypoxic signaling.

2.3.6 H₂S-Mediated O₂ Sensing Has an Evolutionary Precedent and a Phylogenetic History

H_2S was likely an important energy source and structural entity in the origin of life and for the first 500 million years after the origin of eukaryotes, the latter occurring in sulfidic and anoxic environments (reviewed in Olson 2012c). Thus, there is a long evolutionary history of H_2S in intracellular energy trafficking and signaling. Some of these facets are retained today as SQR, the initial enzyme in H_2S oxidation, is an integral component of the mitochondrial electron transport chain, as is SO (Hildebrandt 2011). It is evident in the present day, however, that H_2S and O_2 are not mutually compatible either in the environment, or in cells. Thus, as the Earth's O_2 levels began to rise some 800 million years ago, environmental H_2S fell and H_2S was no longer available as a substrate for energy production. However, it is clear that cells retained much of their metabolic capabilities and with an ironic, but well established metabolic twist, they now use H_2S as a reporter of O_2 availability.

Conclusions

There is now considerable evidence that the reciprocal relationship between H_2S and O_2 provides cells with an effective and accurate mechanism with which to couple O_2 availability to a variety of effector responses in O_2 sensing and perhaps all cells. This hypothesis is supported by the ubiquitous similarity between the effects of hypoxia and H_2S on a variety of tissues, that net tissue H_2S is exquisitely controlled by O_2 availability at physiologically relevant O_2 levels, that factors that augment or inhibit H_2S production have a similar effect on hypoxic responses and that the downstream effectors for hypoxia and H_2S appear to be identical. This mechanism appears to have originated early on in

evolution and it is likely widespread in the animal kingdom. The next step in evaluating this signaling mechanism will be to determine how, and where, this mechanism operates at the sub-cellular level.

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