Hypoxic pulmonary vasodilation: a paradigm shift with a hydrogen sulfide mechanism

Kenneth R. Olson, Nathan L. Whitfield, Shawn E. Bearden, Judy St. Leger, Erika Nilson, Yan Gao, and Jane A. Madden

Indiana University School of Medicine–South Bend, South Bend, Indiana; Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana; Department of Biological Sciences, Idaho State University, Pocatello, Idaho; SeaWorld Adventure Park, San Diego, California; and Department of Neurology, Medical College of Wisconsin, and Research Service, Zablocki Veterans Affairs Medical Center, Milwaukee, Wisconsin

Submitted 13 September 2009; accepted in final form 28 October 2009

Despite intense scrutiny and obvious clinical importance, the mechanism coupling the responses of pulmonary hypoxia to HVC, the so-called “O₂ sensor,” remains to be identified (1, 7, 27, 30, 33). Recent studies from our laboratory suggest that O₂-dependent metabolism of constitutively produced and vasoactive H₂S is the pivotal O₂ transducer (15). Support for this hypothesis has been obtained from studies on respiratory and systemic vessels from a variety of vertebrates and includes the following: 1) hypoxia and H₂S uniquely evoke the same response in isolated blood vessels from all vertebrate classes; 2) H₂S is enzymatically generated by vessels and adjacent tissue; 3) tissue H₂S concentration is inversely related to O₂ concentration; i.e., endogenous and exogenous H₂S is consumed in the presence of O₂, while endogenous H₂S rises in hypoxia; 4) hypoxia and H₂S appear to utilize a common activation process; and 5) hypoxia and H₂S depolarize bovine pulmonary vascular smooth muscle cells.

Diving mammals such as pinnipeds routinely experience profound hypoxia as a result of prolonged underwater activity, or terrestrial sleep apnea. Their arterial Po₂ often falls below 15–20 mmHg, which is well below the ∼25-mmHg level at which humans lose consciousness (11, 18, 21). Surprisingly, pulmonary arterial blood pressure does not increase in hypoxic pinnipeds, even during sleep apnea, when cardiac output remains constant (17). This finding is in marked contrast with the intense pulmonary hypertension that would be expected in terrestrial mammals under similar conditions (4, 13), and it suggests that the response of pinniped pulmonary arteries to hypoxia is different from that of their terrestrial counterparts.

In the present studies, we explored the possibility that pinniped pulmonary arteries were less sensitive to hypoxia by comparing the hypoxic response of isolated conductance pulmonary arteries (CPA) and resistance pulmonary arterioles (RPA) of the California sea lion (Zalophus californianus) with that of the cow (Bos taurus). The surprising observation that hypoxia diluted sea lion pulmonary arteries allowed us to further examine our hypothesis that O₂-dependent metabolism of H₂S is the vascular O₂ sensor.

MATERIALS AND METHODS

Animals

Holstein cow (Bos taurus, Mammalia) lungs were obtained from a nearby abattoir, placed in 4°C Krebs-Henseleit mammalian saline, and transported to the laboratory (South Bend or Milwaukee). The pulmonary arteries (4th–6th generation) were dissected out and stored in buffer at 4°C until use.

http://www.ajpregu.org
Lung tissue was obtained from euthanized (due to nonrepairable injuries or neurological problems associated with domoic acid toxicity) or fatally injured California sea lions (Zalophus californianus) that were delivered to the SeaWorld Adventure Park veterinary facility as part of the marine mammal rescue program of Southern California. The tissue was placed in a Kapak seal-a-meal bag with lactated Ringer solution and shipped overnight on ice to South Bend and Milwaukee. The pulmonary vessels were dissected free on the following morning and prepared for myography. Lungs with vessels that did not exhibit passive stress-relaxation or contract when exposed to 80 mM KCl were discarded. The suitability of this method of tissue procurement was verified in our laboratory by storage of cow lungs under similar conditions; no adverse effects were observed. All procedures were approved by the respective Institutional Animal Care and Use Committees.

Conductance Vessel Myography

Vessels were cut into 3- to 8-mm-long segments, mounted on 280-μm-diameter stainless steel wire hooks, and suspended in 5-ml water-jacketed smooth muscle baths filled with the appropriate buffer at 37°C. They were aerated with 95% air-5% CO2. One hook was stationary; the other was connected to a force-displacement transducer (model FT03C, Grass Instruments, West Warwick, RI). Tension was measured on a polygraph (model 7E or 7F, Grass). Polygraph sensitivity was set to detect changes as small as 5 mg. Data were archived on a personal computer at 1 Hz using Labtech Notebook software (Laboratory Technologies, Andover, MA) or SoftWire (Measurement Computing, Middleboro, MA). The chart recorders and software were calibrated before each experiment.

Length-tension relationships were derived from KCl-contrasted vessels and used to apply a reasonable baseline (resting) tension (~1.000 mg) for 0.5–1 h before experimentation. In a typical experiment, vessels were contracted twice with 80 mM KCl, and resting tension was reestablished over 30- to 45-min equilibration periods after the rinse and again before experimentation. Vessels were then precontracted with the thromboxane A2 mimetic U-46619 (10^-6 M) and, when stable, exposed to hypoxia (95% N2-5% CO2, <5 mmHg PO2).

To determine O2 sensitivity of conductance vessels, we exposed cow CPA to progressively lower PO2 by mixing 95% air-5% CO2 and 95% N2-5% CO2 with a Wösthoff gas-mixing pump. In preliminary experiments, we observed that hypoxic contractions were stronger in precontracted vessels. To determine whether the U-46619 precontraction affected vessel sensitivity to hypoxia, the PO2-response curves were obtained from otherwise unstimulated vessels and from vessels precontracted with 10^-5 or 10^-6 M U-46619.

To examine the contribution of potential sulfur donors to HVC, we exposed cow vessels to four consecutive periods of 20 min hypoxia-30 min of normoxia. Potential sulfur donors, cysteine (Cys), reduced glutathione (GSH), oxidized glutathione (GSSG), 3-mercaptopruvate (3-MP), or Cys + α-ketoglutarate (Cys + α-KG), all at 1 mM, were added after the second hypoxia exposure. Control vessels were exposed to four consecutive hypoxia treatments.

Resistance Vessel Myography

Isolated pulmonary arteries were cannulated with glass cannulas and placed in a water-jacketed plastic chamber. An artery segment was tied in place on the proximal cannula, and the lumen was flushed with Krebs-Henseleit-buffered (KHB) saline. The distal end of the artery was tied onto the distal cannula, and all side branches were tied off. The vessel was bathed in and superfused with KHB saline at 37°C and aerated with room air. A micrometer connected to the proximal cannula was used to remove slack from the artery. A KHB saline-filled syringe proximal to a pressure transducer on the inflow cannula was raised or lowered to set transmural pressure at the corresponding in vivo pressure (10 mmHg). A color video camera mounted on a stereomicroscope above the vessel chamber projected the artery image on a video monitor, and the external arterial diameter (±1.5 μm) was measured on a screen using a video scaler. The vessel diameter was always measured at the same point on the arterial wall using various distinguishing features, such as adhering connective tissue and side branches, located near the site. Diameters were measured immediately after the artery was mounted, after equilibration, and throughout the experimental protocols. Drugs were added to the superfusate, and hypoxia was achieved by gassing the superfusate with 95% N2-5% CO2, which consistently lowered the chamber PO2 to <50 mmHg.

One-Dimensional Western Blotting

Bovine pulmonary artery smooth muscle cells (PASMC) were grown to confluence, placed on ice, and lysed using 1% Triton X-100 lysis buffer. Sea lion pulmonary arteries were cut into small pieces. Cells and arteries were treated with a protease inhibitor cocktail and 100× phenylmethylsulfonyl fluoride and centrifuged at 22,600 g at 4°C for 15 min. The supernatant was collected and loaded onto a gel at uniform protein concentration. A Bis-Tris 4–12% 1.5-mm precast gel was run with a NuPAGE MES SDS running buffer and SeeBlue prestained molecular weight standard at 200 V for ~45 min, transferred to a 0.22-μm nitrocellulose membrane, and stained with Ponceau S for total protein load. The membrane was blocked for 30 min with BSA in TBS-Tween 20 buffer and probed overnight at 4°C with the appropriate primary antibody for the H2S-synthesizing enzymes, cystathionine β-synthase (CBS; Santa Cruz Biotechnology, Santa Cruz, CA), cystathionine γ-lyase (CSE; Novus Biologicals, Littleton, CO), or 3-mercaptopruvate sulfur transferase (3-MT; Sigma-Aldrich, St. Louis, MO; all at 1:2,000 dilution) and then for 1 h with the secondary antibody in 2% dry milk-TBS-Tween 20 solution. Enhanced chemiluminescence agent was applied, and the membrane was developed on CL-XX Posure film. The image was viewed on a Kodak IS2000 MMt Imaging Station.

Immunohistochemistry

Lung tissue was cut into small pieces and immersion fixed in fresh 4% paraformaldehyde overnight at 4°C. Tissues were then saturated with 30% sucrose in PBS and cryosectioned (Leica) at ~20°C. Sections (10 μm thick) were dried on glass slides, permeabilized, and blocked in PBS containing 0.2% Tween 20 and 1% BSA for 1 h. Sections were incubated overnight at 4°C in rabbit polyclonal anti-CBS (Santa Cruz Biotechnology), anti-CSE (Sigma GenoSys), or anti-3-MT (Sigma); all primary antibodies were diluted 1:1,000 in blocking/permeabilization buffer. Endogenous peroxidase was then quenched by 1 h of incubation in 0.3% H2O2, and slides were washed five times for 10 min each in PBS with 0.2% Tween. Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Vector Labs; 1:2,000 dilution) was incubated for 2 h at room temperature, and wash steps were repeated. Respective enzyme expression was then developed by 5 min of incubation in NOVARed horseradish peroxidase substrate (Vector Labs) to create a red/brown precipitate. Tissue sections were counterstained in hematoxylin for 1 min and ammonia for 30 s (to lighten and brighten the hematoxylin blue). Images were acquired with a Leica DMLFS microscope (objectives: ×10, NA 0.30 and ×40, NA 0.55), MicroPublisher 3.3 RTV camera, and QCapture software.

Tissue H2S Production

Cow or sea lion lung tissue (1.0 g) with no large vessels or airways was processed with a homogenizer (Ultraturrax model SDT, Ika-Werk) with an N-10 probe on ice in 4 ml of homogenization buffer and then centrifuged until the rotor speed reached 10,000 rpm. Homogenate (1.5 ml) was added to a water-jacketed glass metabolism chamber with ports for the H2S and O2 sensors and maintained at 37°C (see below). The homogenate was bubbled with N2 until O2 reached zero; then the stopper was lowered until no headspace remained and no
bubbles were visible. The homogenate was spiked to 100 μM pyridoxal-5-phosphate and allowed to incubate for 2 min, and Cys was added to a concentration of 1 mM. After 10 min, α-KG was added to a concentration of 1 mM, and the homogenate was allowed to incubate for 20 min. Air (1.5 μl) was injected, and the homogenate was allowed to consume all the O2 and resume production of H2S. Tissue was homogenized immediately before each run, and a sample was assayed for protein using the Lowry method.

**Effect of O2 on H2S Consumption**

The following studies were performed only on cow tissues because of the limited availability of tissues from sea lions.

**Homogenized cow lung.** Lung tissue (0.5 g) with no large vessels or airways was homogenized as described above on ice in 4 ml of homogenization buffer. In the metabolism chamber (37°C), 200 μl of supernatant were added to 1.3 ml of homogenization buffer and bubbled with air and N2, which were mixed with a Wösthoff gas-mixing pump to the desired PO2. The stopper of the chamber was lowered to remove all headspace, with care taken to ensure that no gas bubbles were trapped. The homogenate was spiked to 2 μM Na2S and allowed to incubate for ~4 min. Consumption rate was taken as a linear regression of the initial one-third of the consumption trace.

**Cow heart mitochondria.** Twenty grams of bovine heart ventricle were placed in 200 ml of mitochondria isolation buffer (MIB) with 850 μl of nargarse protease solution (Sigma) and minced on ice for 5 min. The minced tissue was processed on ice intermittently with an Ika Ultraturax T18 Basic homogenizer with an S18 N-19G probe until smooth. The homogenate was centrifuged at 1,300 g for 5 min, and the supernatant was vacuum filtered through four layers of cheesecloth and then centrifuged at 14,500 g for 10 min. The pellet was rinsed with MIB to remove the fluffy layer surrounding the mitochondrial pellet, resuspended in MIB, and centrifuged again at 14,500 g for 10 min. The pellet was rinsed and resuspended in a minimum volume of MIB. Mitochondrial respiratory control ratio buffer (1.5 ml) was placed in the diffusion chamber (37°C) and bubbled with air and N2, which were mixed using a Wösthoff gas-mixing pump until PO2 stabilized. The stopper was then lowered, with care taken to ensure that no gas bubbles were trapped. The buffer was then spiked to 2 μM Na2S, and 5 μl of ~20 mg/ml mitochondrial suspension were added. The mitochondria were allowed to consume the sulfide for ~4 min. Consumption rate was taken as a linear regression of the initial one-third of the consumption trace.

**Bovine PASMC.** Bovine PASMC were cultured in Cascade Biological Medium 231 with smooth muscle cell growth supplement at 37°C in an atmosphere of 5% CO2 in T-75 flasks. Cells were used between passages 6 and 8 and harvested with 0.25% trypsin-EDTA at near confluence. Cells were centrifuged at 1,000 rpm for 5 min and resuspended in ~3.5 ml of Medium 231 with supplement, counted, and kept on ice until use. Cell suspension (1.5 ml) was transferred to the metabolism chamber and equilibrated to 127 μM O2 (equivalent to systemic arterial PO2) or 40 μM O2 (equivalent to systemic venous PO2). Once the suspension was equilibrated, the stopper was lowered to remove all air, and the cells were spiked to 2 μM Na2S. H2S consumption was then measured in the same batch of cells at 7.5, 2, and 0 μM O2 in random order and determined at 127 or 40 μM O2 to ensure continued cell viability. There were no statistically significant differences between the initial and final H2S consumption rates at 127 or 40 μM O2.

**Metabolism chamber.** The metabolism chamber was constructed in-house and consisted of a lost-wax-cast soda lime glass inner member with O2 and H2S sensor ports on the side, orthogonal to each other and to the axis of the chamber and surrounded by an acrylic water jacket. A polyvinylidene difluoride (PVDF) stopper was machined to tightly fit into the opening and sealed with a Viton O-ring. A small hole drilled through the stopper permitted venting of the headspace air when the stopper was lowered into the chamber and provided an access port for injection of drugs or air bubbles with a Hamilton syringe. The chamber was placed on a magnetic stirrer and a PVDF-coated stir bar, constructed from the core of a typical laboratory micro stir bar that had been removed from the Teflon shell and inserted into a new shell of PVDF and heat-sealed, was used to minimize backdiffusion of O2, which can be a problem with Teflon-coated stir bars. Construction of the H2S sensor with a sensitivity of 14 nM H2S gas (~100 nM total sulfide) is described elsewhere (32). O2 was measured with a dissolved oxygen electrode (model 125/05D, Instech, Plymouth Meeting, PA) and was calibrated according to the manufacturer’s directions and verified with N2 equilibrated buffer and air-N2 gas mixtures of 20, 10, 5, 1, and 0% air-balance N2 obtained from a Wösthoff gas-mixing pump. On the basis of the signal-to-noise ratio, the O2 sensitivity was estimated to be ~<0.5 μM (~0.7 mmHg PO2). Both sensors were connected to a free radical analyzer (Apollo 4000, WPI, Sarasota, FL). Response time for the H2S and O2 sensors was 63% in 8 and 4 s, respectively.

** Buffers and Chemicals **

Mammalian KHB saline consisted of (in mM) 115 NaCl, 2.51 KCl, 2.46 MgSO4, 1.91 CaCl2, 5.56 glucose, 1.38 NaH2PO4, and 25 NaHCO3 (pH 7.4). Lung tissue homogenization buffer consisted of (in mM) 120 KCl, 2 NaH2PO4, 3 Na2HPO4, and 1 MgSO4 (pH 7.0). Respiratory control ratio buffer consisted of (in mM) 100 KCl, 50 MOPS, 5 K2HPO4, and 1 EGTA (pH 7.4). MIB consisted of (in mM) 220 mannitol, 70 sucrose, 5 MOPS, and 2 EGTA (pH 7.0). H2S was produced by dissolving Na2S into deoxygenated buffer under 100% N2 and titration to pH 7.4 with HCl. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Calculations**

Statistical comparisons were performed with the Student’s t-test or, when appropriate, with a paired t-test. Significance was assumed when P ≤ 0.05. The effective PO2 at which H2S consumption was half-maximal (P50, or EC50 when expressed as O2 concentration) was estimated from a curve fit by eye.

**RESULTS**

Hypoxia (<5 mmHg PO2) consistently contracted cow CPA (>500 μm diameter; Fig. 1, A and C). The response of sea lion CPA to hypoxia was variable: some vessels were relaxed (Fig. 1, B and C), whereas others contracted (not shown). Because CPA do not make a major contribution to pulmonary vascular resistance and may also exhibit hypoxic vasodilation (HVD) in terrestrial mammals (9), we examined the hypoxic responses of RPA (<400 μm diameter), where HVC is presumed pervasive. Here the results were more striking: moderate hypoxia (<50 mmHg PO2) constricted all cow CPA but dilated all sea lion RPA (Fig. 1D). The dichotomous hypoxic responses of RPA were not due to differences in the mechanical properties of the two vessels, inasmuch as vessels from both species were contracted by 80 mM KCl and the thromboxane A2 mimetic U-46619 (10−6 M, not shown).

The mechanical effects of H2S were identical to those produced by hypoxia in cow and sea lion pulmonary arteries. H2S (≥100 μM) contracted cow CPA (Fig. 1, A and C). In sea lion CPA, those vessels that were relaxed by hypoxia were also relaxed by H2S (Fig. 1, B and C) and those vessels that were contracted by hypoxia were also contracted by H2S (not shown). The response of RPA to H2S (300 μM) was also identical to the hypoxic response: H2S constricted all cow RPA and dilated all sea lion RPA (Fig. 1D).
Figure 2 shows the distribution of H$_2$S-synthesizing enzymes in sea lion RPA, cultured bovine PASMC, and cultured bovine pulmonary artery endothelial cells. CBS immunoreactivity was not evident in sea lion RPA or in bovine PASMC but was clearly present in bovine pulmonary artery endothelial cells. Immunohistochemical localization of CBS, CSE, and 3-MST antibodies in cow and sea lion lung tissue is shown in Fig. 3. In general, all three enzymes were abundantly distributed in the small airways and alveoli of both animals. There was noticeably less immunoreactivity in the adventitia and media of small vessels, whereas all three enzymes were often evident in the endothelium.

An amperometric (polarographic) H$_2$S sensor was used to examine H$_2$S production and O$_2$-dependent metabolism in real time in homogenized, but otherwise untreated, lung tissue from cow and sea lion (Fig. 4). H$_2$S production was observed in lung tissue from both animals in the presence of pyridoxal phosphate (a cofactor for CSE and CBS) and Cys and after addition of /$\mu$L$_2$51/$\mu$L$_2$4KG, but only under severely hypoxic conditions. Addition of air, theoretically sufficient to increase O$_2$ concentration to /$\mu$L$_2$11011/$\mu$L$_2$9262 8.5 /$\mu$L$_2$9262 M (in the absence of any concomitant O$_2$ consumption), immediately led to consumption of previously formed H$_2$S and prevented H$_2$S from increasing until nearly all the available O$_2$ was consumed. As the O$_2$ concentration fell, H$_2$S production resumed. These studies show that lung tissue from both animals produces H$_2$S and that O$_2$-dependent H$_2$S consumption inversely couples tissue H$_2$S concentration to ambient O$_2$ concentration.

To further examine the relationship between H$_2$S consumption and O$_2$ availability, we measured the rate of exogenous H$_2$S consumption as a function of PO$_2$ in bovine lung homogenate, cultured bovine PASMC, and mitochondria extracted from bovine hearts (Fig. 5). H$_2$S consumption in lung homogenate was maximal until PO$_2$ fell below 20 mmHg and decreased thereafter. The estimated PO$_2$ for half-maximal H$_2$S consumption (P$_{50}$) by lung homogenate was ~3.2 mmHg (EC$_{50}$, 4 $\mu$M O$_2$). H$_2$S consumption by bovine PASMC was maximal in the range of arterial and venous plasma PO$_2$, 100 and 32 mmHg, respectively (127 and 40 $\mu$M O$_2$) but fell to half-maximal at 6 mmHg (7.5 $\mu$M O$_2$). The rate of H$_2$S consumption by bovine PASMC was ~$\mu$L$_2$1021/10% of maximal at 1.5 mmHg (2 $\mu$M O$_2$). H$_2$S consumption by bovine heart mito-
chondria was sustained until $P_{O_2}$ fell below $5 \text{ mmHg}$, with an apparent $P_{50}$ of $0.8 \text{ mmHg}$ (EC$_{50}$ = $1 \mu \text{M O}_2$). These studies show a dose-dependent relationship between $O_2$ concentration and $H_2S$ consumption in a variety of tissue preparations.

The effects of prestimulation on force development and $O_2$ sensitivity of HVC were examined in cow CPA. Prestimulation with $10^{-6} \text{ M U-46619}$ significantly increased the strength of maximal HVC nearly three- to fivefold (Fig. 6A) but did not significantly increase the $O_2$ sensitivity (Fig. 6B). The $P_{50}$ values for $0$, $10^{-8}$, and $10^{-6} \text{ M U-46619}$ were $6$, $7$, and $10 \text{ mmHg}$, respectively. These studies show that although prestimulation affects the mechanical process of HVC, it has little, if any, effect on the $O_2$-sensing mechanism.

Potential sulfur donors, Cys, GSH, GSSG, 3-MP, and Cys + $\alpha$-KG, were added to prestimulated and unstimulated cow CPA to examine their ability to augment HVC. Cys and 3-MP slightly, but significantly, decreased the third HVC in prestimulated CPA (Fig. 7A), whereas HVC was significantly, and substantially, increased by all sulfur donors, except 3-MP and, at the fourth HVC, by GSSG in unstimulated vessels (Fig. 7B).

**DISCUSSION**

To our knowledge, our study is the first observation of HVD in a mammalian RPA. This is noteworthy in several aspects. 1) It challenges the paradigm that HVC is a universal attribute of the mammalian pulmonary vasculature (28). 2) HVD appears to be of physiological benefit during routine dives and sleep apnea. 3) It demonstrates the evolutionary plasticity of fundamental vascular responses that enables them to fit the needs of the organism. 4) The disparate responses to hypoxia in cow and sea lion pulmonary arteries provide a unique model system with which to dissect vascular $O_2$-sensing and signal transduction mechanism(s). This allowed us to provide additional evidence that $O_2$-dependent $H_2S$ metabolism is involved in vascular $O_2$ sensing.

**Hypoxic Pulmonary Vasodilation**

Given the pervasive thought that hypoxia constricts the mammalian pulmonary microcirculation, we initially hypothesized that HVC would have to be blunted in diving mammals such as pinnipeds to minimize an otherwise anticipated pulmonary hypertension. The HVD that we observed, although unanticipated, is clearly of greater homeostatic value, inasmuch as it will actually reduce pulmonary vascular resistance and potentially lower the metabolic load on the right ventricle. HVD may be even more beneficial during sleep apnea. It is well known that cardiac output decreases during a dive. The reduction in pulmonary blood flow could potentially offset HVC and minimize the potential for pulmonary hypertension. However, unlike a dive, cardiac output does not change during the entire apneic period when the animal is sleeping on land (17), even though the arterial $P_{O_2}$ may be as low as that encountered in a dive (11, 21). Under these circumstances,
even a modest HVC would produce pulmonary hypertension. HVD ensures that this does not occur.

**H2S and O2 Sensing**

We previously proposed that O2-dependent metabolism of H2S serves as a vascular and chemoreceptor O2 sensor (14–16). In this model, the constitutive cytosolic production of vasoactive H2S is offset by mitochondrial H2S oxidation. The oxidative capacity of tissues is such that tissue H2S concentration is nil during normoxia, but as O2 levels fall, the capacity to oxidize H2S progressively fails and tissue H2S increases. The existence, as well as sensitivity and efficacy, of this mechanism depends on a number of criteria. 1) The effects of H2S must mimic the hypoxic response, implying that these two stimuli utilize the same initial activation pathway. 2) Tissues must be capable of H2S synthesis. 3) The rate of H2S production or H2S metabolism must be intimately coupled to O2 availability, i.e., PO2. 4) O2–H2S coupling must occur at physiologically relevant levels of O2. We previously demonstrated various aspects of these criteria in a variety of vertebrates from hagfish and lamprey to rats and cows. The uniqueness of the present study is that it examines these criteria in the same organ of two mammals where the vascular responses to hypoxia are exactly the opposite.

The similarity of hypoxic and H2S responses in cow and sea lion pulmonary vessels, despite the opposite outcome (Fig. 1), is consistent with our previous observations that hypoxia and H2S produce identical responses in systemic and respiratory conductance vessels from vertebrates of every class, from hagfish to mammals, whether this response is contraction, relaxation, or multiphasic (15). Furthermore, the difference between cow and sea lion vessels is specific for hypoxia and H2S, inasmuch as other stimuli, such as 80 mM KCl and U-46619, consistently constricted both vessels. Collectively, these observations support criterion 1 and suggest that hypoxia and H2S regulate vascular diameter via a common pathway that
is upstream from the ultimate mechanical outcome, i.e., HVC or HVD.

Figures 2 and 3 show that pulmonary vessels and adjacent lung tissue have appropriate enzymes for H2S synthesis, and Fig. 4 demonstrates that H2S can be produced by these tissues under the appropriate conditions. H2S can be produced directly from Cys desulfuration via the enzymes CSE (also known as CGL; EC 4.4.1.1) and CBS (EC 4.2.1.22) or from Cys transamination to 3-mercaptopyruvate (with \( \alpha \)-KG, often serving as the amine acceptor) and subsequent desulfuration by the enzyme 3-MST (EC 2.8.1.2) (8, 19). CSE is the only desulfurating enzyme that has been identified in mammalian vessels (5, 25, 34–36). 3-MST appears to be the major pathway for H2S synthesis in the brain (19), but to our knowledge, our study is the first evidence of 3-MST in the vasculature.

The capacity for H2S synthesis in the airways is particularly noteworthy, inasmuch as it suggests that these tissues are also involved in O2 sensing. This is consistent with the well-known observation in terrestrial mammals that airway hypoxia is a more potent stimulus of HVC than mixed-venous (systemic) hypoxemia (13). Teleologically, it makes sense that the tissue that is the most immediately exposed to hypoxia (airways) or is the most intimately sensitive to it (systemic tissues) would be a leading contributor to the vasoactive response, be it constriction or dilation. This also explains why H2S is produced by so many extravascular tissues.

Addition of potential sulfur donors to otherwise unstimulated cow CPA greatly augmented HVC (Fig. 7A), suggesting that vascular smooth muscle can utilize sulfur via variety of biochemical pathways to increase H2S production. This effect was not noticed when the vessels were precontracted with U-46619. Although U-46619 may augment H2S production, it seems more probable that U-46619 prestimulation augments HVC independent of H2S production, and this masks the more subtle effects of the sulfur donors on HVC in otherwise unstimulated vessels. Precontraction is well known to enhance HVC, and in

![Fig. 6. Effect of prestimulation with the thromboxane A2 mimetic U-46619 on maximal (100% N2) hypoxic contraction (A) or O2 sensitivity (B) of cow CPA. Prestimulation significantly increased the magnitude of 2nd, 3rd, and 4th hypoxic contractions 3- to 4-fold (A) but did not affect O2 sensitivity (B). Values are means ± SE; n = 7 in each group in A and n = 5 for 10^-6 M U-46619, 4 for 10^-8 M U-46619, and 3 for no prestimulation (0 U-46619) in B. Curves were fit by eye.](http://ajpregu.physiology.org/)

![Fig. 7. Effects of potential sulfur donors, Cys, reduced glutathione (GSH), oxidized glutathione (GSSG), 3-mercaptopyruvate (3-MP), or Cys + \( \alpha \)-ketoglutarate (\( \alpha \)-KG), all at 1 mM, on the magnitude of hypoxic contraction of cow CPA that were prestimulated with 10^-6 M U-46619 (A) or unstimulated (B). Control vessels were exposed to 4 consecutive periods of hypoxia; sulfur donors were added after the 2nd hypoxic exposure. Values are means ± SE of number of vessels shown in parentheses below bars; values for the 3rd (gray bars) and 4th (black bars) hypoxic contractions were normalized relative to values for the second (white bars) hypoxic contraction. *Significantly different from respective control (Con).](http://ajpregu.physiology.org/)
many isolated preparations it is an absolute requirement for HVC (28). It has been suggested that prestimulation enhances mobilization and turnover of Ca2+ from intracellular stores, which then increases the availability of Ca2+ for contraction (2, 28). The fact that prestimulation increased the magnitude of HVC three- to fourfold in our experiments without affecting O2 sensitivity supports the suggestion that the effects of U-46619 are also indirect. Collectively, these studies plus the observations, in real time, of tissue H2S production (Fig. 4) support criterion 2 for H2S-mediated vasoactivity.

Figure 4 also shows that, in the presence of O2, any endogenously produced H2S is rapidly consumed and that H2S concentrations will remain low until O2 levels fall. This satisfies criterion 3, an inverse relationship between O2 and H2S.

Because it is not possible to measure intracellular H2S concentrations and there is still some uncertainty about subcellular O2 distribution, we can only indirectly address criterion 4, i.e., tissue H2S is inversely coupled to O2 at physiologically relevant P02 levels (or O2 concentrations). Figures 5 and 6 are, to our knowledge, the first attempt to do so. Unfortunately, there was not enough tissue from sea lions for a comparative analysis; nevertheless, we are confident that the cow samples are representative of a variety of O2-sensing tissues. The P50 for HVC in cow CPA was 6–11 mmHg for otherwise unstimulated vessels and vessels precontracted with U-46619 (Fig. 5B), even though the force of HVC was almost four times greater in the maximally precontracted vessels (Fig. 5A). Thus it is reasonable to assume that the P50 for the O2-sensing mechanism is relatively independent of the contractile process and must occur upstream from it. We can then compare this P50 with the P02, at which consumption is impaired: ~3 mmHg for homogenized lung tissue and ~6 mmHg for cultured PASMC (Fig. 5). The P50 values for H2S consumption and vascular activation are reasonably similar and may be even closer if one considers for the increased O2 diffusion distance that would be expected in an intact CPA. This argument is fortified by the even lower P50 of purified mitochondria (Fig. 5), where diffusion distances are even shorter. Although it is possible that the different O2 sensitivities of lung homogenate, cultured smooth muscle cells, and mitochondria are due to tissue-specific variations, it is clear that, in all preparations, at low O2 concentrations the ability to metabolize H2S progressively fails.

The efficacy of O2-dependent H2S metabolism can be put into the context of the effects of brief physiological hypoxia on vascular/tissue P02. In rats breathing room air (6), arterial, arteriolar, tissue (near venous capillaries), and venular P02 levels are 98, 52, 35, and 27 mmHg, respectively. These levels are typical of P02 in a variety of tissues (23), and all are sufficiently high to rapidly and completely consume H2S in our various preparations (Fig. 5). Moderate hypoxia produced by ventilation with 7% O2 for 1 min lowered these values to 32, 16, 11.4, and 9.6 mmHg, respectively. Under these conditions, venular and tissue P02 would be sufficiently low to begin to impair H2S consumption by PASMC and homogenized lung. There is considerably more uncertainty in the literature surrounding mitochondrial O2 concentrations, but because this is the metabolic O2 sink, it is most likely well below that of the interstitium and cytosol. Recently, using an invasive optical technique to estimate mitochondrial P02 in the rat heart in vivo, Mik et al. (12) found that, in normoxic rats, P02 was 10–20 mmHg in 26% of the mitochondria and 0–10 mmHg in only 10% of the mitochondria. Ventilating the rats with 10% O2 increased the fraction of mitochondria in the 0–10 mmHg P02 range to 46%. These results show that even moderate hypoxia produces a cascade of progressive hypoxia in all compartments, ultimately resulting in tissue and/or mitochondrial P02 levels that are not sufficient to sustain H2S consumption. Certainly, the ~15-mmHg P02 observed in arterial and venous blood of elephant seals during prolonged sleep apnea (21) would push intracellular and mitochondrial P02 of pulmonary and systemic tissues well into the range of impaired H2S consumption.

The efficacy of hypoxia-driven H2S liberation at physiologically relevant P02 across species, despite opposing mechanical responses of vessels, demonstrates a certain ubiquity of this mechanism in vascular O2 sensing. Ward (26) evaluated the most popular putative O2 sensors in the context of vascular and tissue P02 (Fig. 8). Of these, NADPH oxidase appeared to have the greatest dynamic range, progressing almost linearly from 15% activity at 2 mmHg to >80% activity at 120 mmHg. Other sensors, such as heme oxygenase-2 and mitochondrial cytochromes a and a3, were ~100% active over the entire range of P02 (0–120 mmHg), suggesting that they may not...
H2S and O2 have a long geochemical history of mutual exclusivity: the presence of one in the environment implies the absence of the other. Eukaryotic cells also have a long history with H2S, and, in fact, a number of the great extinctions appear to be associated with ambient hypoxia and elevated H2S. It seems reasonable to assume that the animals that survived these dramatic conditions developed methods to cope with abnormal levels of both gases. The present study supports the hypothesis that this reciprocity has been employed as a convergent O2-sensing/signaling mechanism in anatomically homologous blood vessels with dichotomous hypoxic responses. These studies are also the first to show hypooxic vasodilation in mammalian tissues. In: Signal Transduction and the Gasotransmitters, edited by Wang R. Totowa, NJ: Humana, 2004, p. 275–292.


REFERENCES


11. No conflicts of interest are declared by the author(s).

ACKNOWLEDGMENTS

The authors thank A. Lee for technical assistance.

GRANTS

This work was supported in part by National Science Foundation Grant IOS 0641436 (K. R. Olson) and National Center for Research Resources IDeA Networks of Biomedical Research Excellence Grant P20 RR-016454 (S. E. Bearden).

DISCLOSURES

No conflicts of interest are declared by the author(s).


