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Hydrogen sulfide as an oxygen sensor/transducer in vertebrate hypoxic vasoconstriction and hypoxic vasodilation

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Summary

How vertebrate blood vessels sense acute hypoxia and respond either by constricting (hypoxic vasoconstriction) or dilating (hypoxic vasodilation) has not been resolved. In the present study we compared the mechanical and electrical responses of select blood vessels to hypoxia and H_2S, measured vascular H_2S production, and evaluated the effects of inhibitors of H_2S synthesis and addition of the H_2S precursor, cysteine, on hypoxic vasoconstriction and hypoxic vasodilation. We found that: (1) in all vertebrate vessels examined to date, hypoxia and H_2S produce temporally and quantitatively identical responses even though the responses vary from constriction (lamprey dorsal aorta; lDA), to dilation (rat aorta; rA), to multi-phasic (rat and bovine pulmonary arteries; rPA and bPA, respectively). (2) The responses of lDA, rA and bPA to hypoxia and H_2S appear competitive; in the presence of one stimulus, the response to the other stimulus is substantially or completely eliminated. (3) Hypoxia and H_2S produce the same degree of cell depolarization in bPA. (4) H_2S is constitutively synthesized by lDA and bPA vascular smooth muscle. (5) Inhibition of H_2S synthesis inhibits the hypoxic response of lDA, rA, rPA and bPA. (6) Addition of the H_2S precursor, cysteine, doubles hypoxic contraction in lDA, prolongs contraction in bPA and alters the re-oxygenation response of rA. These studies suggest that H_2S may serve as an O_2 sensor/transducer in the vascular responses to hypoxia. In this model, the concentration of vasoactive H_2S in the vessel is governed by the balance between endogenous H_2S production and its oxidation by available O_2.

Key words: vascular smooth muscle, hydrogen sulfide metabolism, cysteine metabolism, redox signaling.

Introduction

Hydrogen sulfide (H_2S) has long been known as a noxious and toxic gas. However, recent studies have shown that vertebrate tissues synthesize H_2S and this endogenous gas is, in fact, a signaling molecule, thereby joining NO and CO as the third 'gasotransmitter' (Wang, 2002). H_2S has been shown to be a neuromodulator and neuroprotectant (Kimura et al., 2005), to exert effects in intestinal (Teague et al., 2002) and genito-urinary (Sidhu et al., 2001; Patacchini et al., 2005; Dombkowski, 2006) systems, and to have potent cardiovascular actions.

Until now, studies of the vasoactive effects of H_2S have been limited to systemic vessels. H_2S has been shown to be a vasodilator in mammalian vessels such as the rat thoracic aorta and portal vein (Hosoki et al., 1997; Zhao et al., 2001; Zhao and Wang, 2002; Zhang et al., 2003) and the perfused mesenteric bed (Cheng et al., 2004). In non-mammalian vertebrates H_2S may produce vasodilation, vasoconstriction or it may produce multi-phasic responses (Dombkowski et al., 2004; Dombkowski et al., 2005; Olson, 2005).

Information on the involvement of H_2S in the pulmonary vasculature is limited and indirect. H_2S toxicity is often associated with pulmonary edema (Roth, 2004), suggesting that either capillary permeability or pulmonary blood pressure is increased. In chronically hypoxic rats one of the enzymes responsible for H_2S synthesis is reduced in systemic vessels (Zhang et al., 2003). Plasma [H_2S] is also lower in these animals, suggesting that overall H_2S production is reduced. These data led the authors to infer that a hypoxia-induced loss

in tonic H_2S dilation contributed to chronic hypoxic pulmonary vasoconstriction, although they did not study the effects of H_2S on pulmonary vessels.

During the course of independent studies on the phylogeny of vascular responses to hypoxia (Smith et al., 2001; Olson et al., 2001; Russell et al., 2001) (M.J.R., R.A.D. and K.R.O., unpublished observation) and H_2S (Dombkowski et al., 2004; Dombkowski et al., 2005; Olson, 2005), we noticed that these two stimuli evoked similar, if not identical, responses in a variety of vessels, irrespective of whether this response was contraction, relaxation, or multi-phasic. Because hypoxia and H_2S also produced similar responses in trout urinary bladder (Dombkowski et al., 2006), this prompted us to investigate whether the relationship between these two stimuli in blood vessels was coincidental or if H_2S might be involved in the hypoxic response. In the present study, we compared the effects of hypoxia and H_2S (produced from dissolved NaHS or Na_2S) on mechanical responses of respiratory and systemic vessels from a variety of vertebrates. We then selected four vessels for further study based on their individual responses to these two stimuli: lamprey dorsal aorta (mono-phasic constriction), rat thoracic aorta (mono-phasic relaxation) and rat and bovine pulmonary arteries (multi-phasic responses). In select vessels, we examined the interaction between hypoxia and H_2S on mechanical responses of vessel rings, measured H_2S production by homogenized vessels, and evaluated the effects of inhibiting H_2S production or adding cysteine, the precursor for H_2S synthesis, on the hypoxic response. We also examined the effects of hypoxia and H_2S on smooth muscle transmembrane potential in bovine pulmonary arteries.

Table 1. *Response of vertebrate vessels in vitro to hypoxia (N_2) or hydrogen sulfide (H_2S)*

	Systemic		Respiratory	
	N_2	H_2S	N_2	H_2S
Rat	–	–	+/–/+	+/–/+
Cow	–	–	–/+	–/+
Duck	+	+	–/+	–/+
Alligator	+	+	+/–/+	+/–/+
Toad	+	+	+	+
Trout	–/+/–	–/+/–	+	+
Shark	–	–		
Lamprey	+	+		

White rat (*Rattus norvegicus*, Mammalia), Holstein cow (*Bos taurus*, Mammalia), Pekin duck (*Anas platyrhynchos domesticus*, Aves), American alligator (*Alligator mississippiensis*, Reptilia), marine toad (*Bufo marinus*, Amphibia), steelhead/rainbow trout (*Oncorhynchus mykiss*, Osteichthyes), sandbar shark (*Carcharhinus milberti*, Chondrichthyes), sea lamprey (*Petromyzon marinus*, Agnatha).

+, contraction; –, relaxation; symbols separated by / indicate multi-phasic responses. Modified in part from (Dombkowski et al., 2004; Dombkowski et al., 2005; Olson et al., 2001) and M.J.R., R.A.D. and K.R.O., unpublished observation. All responses represent vessels from at least four animals.

Materials and methods

Animals

Vessels from three animals, lamprey, rat, and bovine, were selected from the list compiled in Table 1 based on their distinct response to hypoxia and H_2S. All animal procedures received IACUC approval.

Sea lamprey (*Petromyzon marinus* L., 130–450 g) were trapped in streams feeding into the Great Lakes during the spring-summer spawning season and airlifted to the Indiana University School of Medicine-South Bend (IUSM-SB) where they were maintained in 500-liter rectangular tanks with aerated, flowing well water (15°C) and exposed to a 12 h:12 h L:D photoperiod. They were not fed. Lampreys were anesthetized in benzocaine (1:5000, w/v) and the dorsal aortas were dissected out and placed in lamprey buffered saline at 4°C until use.

White Sprague Dawley rats *Rattus norvegicus* (Berkenhour) (Mammalia) were anesthetized with 50 mg/animal pentobarbital and the viscera was removed and placed in 4°C Krebs–Henseleit mammalian saline. The thoracic aorta and pulmonary (first–third generation) arteries were dissected out and stored at 4°C until use.

Holstein cow *Bos taurus* L. (Mammalia) lungs were obtained from a nearby abattoir, placed in 4°C Krebs–Henseleit mammalian saline, and transported to IUSM-SB. The pulmonary arteries (fourth–sixth generation) were dissected out and stored at 4°C until use.

Vessel myography

Vessels were cut into 3–8 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 the animal's physiological temperature (lamprey, 14°C; rat and bovine, 37°C). Lamprey vessels were aerated with room air, mammalian vessels were aerated with 95% air, 5% CO_2. One hook was stationary; the other was connected to a Grass model FT03C force-displacement transducer (Grass Instruments, West Warwick, RI, USA). Tension was measured on a Grass Model 7E or 7F polygraph (Grass Instruments). Polygraph sensitivity was set to detect changes as small as 5 mg. Data was archived on a PC computer at 1 Hz using Labtech Notebook software (Laboratory Technologies Corp., Andover, MA, USA) or SoftWire (Measurement Computing, Middleboro, MA, USA). The chart recorders and software were calibrated prior to each experiment.

Length–tension relationships were derived from KCl-contracted vessels and used to apply an appropriate baseline (resting) tension (approximately 500–1500 mg) for 0.5–1 h prior to experimentation. In a typical experiment, vessels were contracted twice with 80 mmol l^{-1} KCl and resting tension was re-established between rinses and prior to experimentation (~30–45 min each). Hypoxia (P_{O_2} <5 mmHg) was achieved by aeration with 100% N_2 (lamprey) or 95% N_2/5% CO_2

(mammals). H_2S was produced by dissolving NaHS or Na_2S. In a number of experiments vessels were pre-contracted with KCl (lamprey aorta), norepinephrine (rat thoracic aorta and pulmonary artery) or the thromboxane A_2 mimetic, U-46619 (bovine pulmonary artery) agonist, prior to exposure to hypoxia or H_2S. Previous experience in our laboratory has shown that these agonists and doses (50–80% of maximal contraction) produce optimal and sustained force in the different vessels. Enzyme inhibitors were added 20–30 min prior to further treatment.

Hypoxia–H_2S interactions

To examine the interactions between hypoxia and H_2S, vessels were exposed to one stimulus while in the presence of the other. Lamprey aortas were contracted with 80 mmol l^{-1} KCl, washed, then exposed to hypoxia (N_2). When the hypoxic contraction had stabilized, H_2S was added to produce a final concentration of 3×10^{-4} mol l^{-1}. A second group of vessels was first exposed to H_2S, then to N_2. Rat thoracic aortas were treated with propranolol (10^{-5} mol l^{-1}) to block β-adrenoceptors then pre-contracted with norepinephrine (10^{-6} mol l^{-1}). When tension had stabilized they were exposed to either N_2 then H_2S (3×10^{-4} mol l^{-1}) after the N_2 relaxation stabilized, or *vice versa*. Bovine pulmonary arteries were pre-contracted with U-46619 (10^{-8} mol l^{-1}) then N_2 followed by 3×10^{-4} mol l^{-1} H_2S or *vice versa*. Both groups of bovine vessels were then re-oxygenated (air) and the hypoxia was repeated to evaluate recovery.

Transmembrane potential

The effects of hypoxia and H_2S on transmembrane potential (E_m) were measured in perfused bovine pulmonary arteries. Glass cannulae with matching tip diameters were inserted into each end of approximately 300 μm diameter, 15 mm long, vessels and secured with nylon suture. The vessels were immersed in Krebs–Henseleit buffer (37°C) in a water-jacketed chamber during cannulation and throughout the experiment. Side branches, if any, were tied off. A micrometer connected to the proximal cannula was used to take slack out of the artery and a pressure transducer connected close to this cannula allowed measurement of intravascular pressure. The inflow cannula reservoir was raised to produce an intravascular pressure of 10 mmHg and the vessels were continuously perfused and superfused with buffer aerated with 5% CO_2-balance air. A color video camera mounted on a stereomicroscope above the vessel was used to project an image of the artery on a video monitor and the vessel diameter (±1.5 μm) was measured on screen using a video scaler. Reference points such as adhering connective tissue, side branches, etc. located near the site of measurement insured that the diameter was always measured at the same point on the vessel wall. Vessel diameters were measured immediately after mounting the artery, after equilibration, and throughout the experimental protocols.

Membrane potentials were measured with glass microelectrodes filled with 3 mol l^{-1} KCl and having tip resistances between 50–80 MΩ. Impalements were made from the adventitial side of the vessel. Criteria for a successful impalement was an abrupt negative drop in voltage when the electrode entered the cell, an immediate return to baseline upon withdrawal of the electrode, and no change in electrode resistance. Because it was difficult to keep the electrode in the cell during an active response, E_m was measured in a number of cells before and after exposure to hypoxia or H_2S. Hypoxia was produced by perfusing and superfusing the vessels with buffer aerated with 5% CO_2 and 95% N_2. H_2S in the form of NaHS (1 mmol l^{-1}) was added directly to the perfusate and superfusate.

H_2S production

Lamprey dorsal aortas were pooled from six fish and bovine pulmonary arteries and veins were pooled from two cows. The vessels were homogenized on ice in 50 mmol l^{-1} phosphate buffer (pH 6.8; 1:9 tissue:buffer w/w). L-Cysteine (1 mmol l^{-1} bovine, 10 mmol l^{-1} lamprey) and pyridoxyl 5′-phosphate (2 mmol l^{-1}) were added (Zhao et al., 2003) and the mixtures were placed in sealed syringes along with a glass mixing bead, avoiding air bubbles, and gently agitated on a rotary mixer for 18–24 h at room temperature. The homogenate was briefly centrifuged to remove tissue debris and 0.5 ml of supernatant was added to an equal volume of antioxidant buffer to convert all H_2S gas and HS^- anion to sulfide (S^{2-}). Total sulfide anion was measured in triplicate samples with a sulfide electrode (Lazar Research Laboratories, Los Angeles, CA, USA) on a Fisher Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA, USA). Inhibitors (see Hosoki et al., 1997; Zhao et al., 2003) of cystathionine β-synthase (CBS), amino-oxyacetate (AOA; 1 mmol l^{-1}), cystathionine γ-lyase (CSE), D,L-propargylglycine (PPG; 10 mmol l^{-1}) and β-cyanoalanine (BCA; 5 mmol l^{-1}) and the general inhibitor of pyridoxyl 5′-phosphate-dependent enzymes, hydroxylamine (HA; 1 mmol l^{-1}) (Kery et al., 1999), were added to bovine vessels simultaneously with L-cysteine and pyridoxyl 5′-phosphate.

Contribution of the H_2S precursor, cysteine, to hypoxic responses

Lamprey aortas, bovine pulmonary arteries and rat thoracic aortas were prepared for myography as above, contracted twice with 80 mmol l^{-1} KCl and washed. Bovine pulmonary arteries and rat aortas were pre-contracted with U-46619 (10^{-7} mol l^{-1}) before each hypoxic exposure. The vessels were then exposed to hypoxia for 15–20 min and returned to air, washed 2 times and resting tension re-established. Cysteine (1 mmol l^{-1}) was added and 20 min later the procedure was repeated. As exogenous cysteine did not appear to be necessary for short-term hypoxic responses of bovine pulmonary arteries, paired vessels were exposed to hypoxia for over 15 h with or without cysteine, reoxygenated for 60 min and the U-46619 pre-contraction, hypoxia protocol repeated.

Physiological salines

Lamprey Hepes-buffered saline (in mmol l^{-1}): 145 NaCl, 3

KCl, 0.57 $MgSO_4$, 2 $CaCl_2$, 5 glucose, 3 Hepes acid, and 7 Hepes Na^+ salt, pH 7.8.

Mammalian Krebs–Henseleit bicarbonate-buffered saline (in mmol l^{-1}): 115 NaCl, 2.51 KCl, 2.46 $MgSO_4$, 1.91 $CaCl_2$, 5.56 glucose, 1.38 NaH_2PO_4, and 25 $NaHCO_3$, pH 7.4.

Chemicals

Stock solutions were prepared as follows: U-46619; 0.01 mol l^{-1}; and epinephrine, 0.01 mol l^{-1}. Propanolol (final concentration 10^{-5} mol l^{-1}) was added to the baths 15 min prior to epinephrine to block β-adrenoreceptor-mediated relaxation (Olson and Meisheri, 1989). All drugs were dissolved in distilled H_2O except U-46619, which was dissolved in 95% ethanol. Ethanol was not vasoactive at the concentrations used in these studies. Anti-oxidant buffer for the total H_2S assay was made of 15.6 mmol l^{-1} sodium salicylate, 3.7 mmol l^{-1} ascorbic acid and 21 mmol l^{-1} NaOH (pH >12). Unless otherwise stated all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

Data analysis

Dose–response curves were fit for individual vessels with Table Curve software (Jandel, Chicago, IL, USA). Statistical comparisons were made using Student's *t*-test or paired *t*-test where appropriate. One-way ANOVA followed by Student–Newman–Keul's test was used for multiple comparisons of means. Values are means ± s.e.m. Significance was assumed when $P \leqslant 0.05$.

Results

Myography

Table 1 compares the *in vitro* vascular response to hypoxia and H_2S in systemic and respiratory vessels in at least one animal from each vertebrate class. In some vessels both hypoxia and H_2S produced a mono-phasic contraction, in others a mono-phasic relaxation, and in others the response is multi-phasic. However, irrespective of the pattern of response, in each vessel the effects of hypoxia and H_2S were essentially identical. This is further illustrated in Fig. 1, where both stimuli produced a mono-phasic contraction in lamprey dorsal aorta (Fig. 1A), a mono-phasic relaxation in rat thoracic aorta (Fig. 1B), and a multi-phasic, contraction–relaxation–contraction, in rat pulmonary artery (Fig. 1C). In bovine pulmonary artery (Fig. 1D), hypoxia produced a slight relaxation followed by a sustained contraction, whereas the H_2S contraction appeared to be mono-phasic. However, as shown in Fig. 2, H_2S produced two different dose-dependent responses in bovine pulmonary arteries: relaxation at low concentrations and contraction at high concentrations. Between 10^{-5} mol l^{-1} and 10^{-3} mol l^{-1}, H_2S produced a bi-phasic relaxation followed by contraction. The response to 10^{-4} mol l^{-1} H_2S was similar to that produced by hypoxia in Fig. 1D.

The responses of lamprey aorta, rat thoracic aorta and bovine pulmonary arteries to H_2S during hypoxia and to hypoxia during exposure to H_2S are shown in Fig. 3. Individually, hypoxia and H_2S produced the same magnitude of contraction

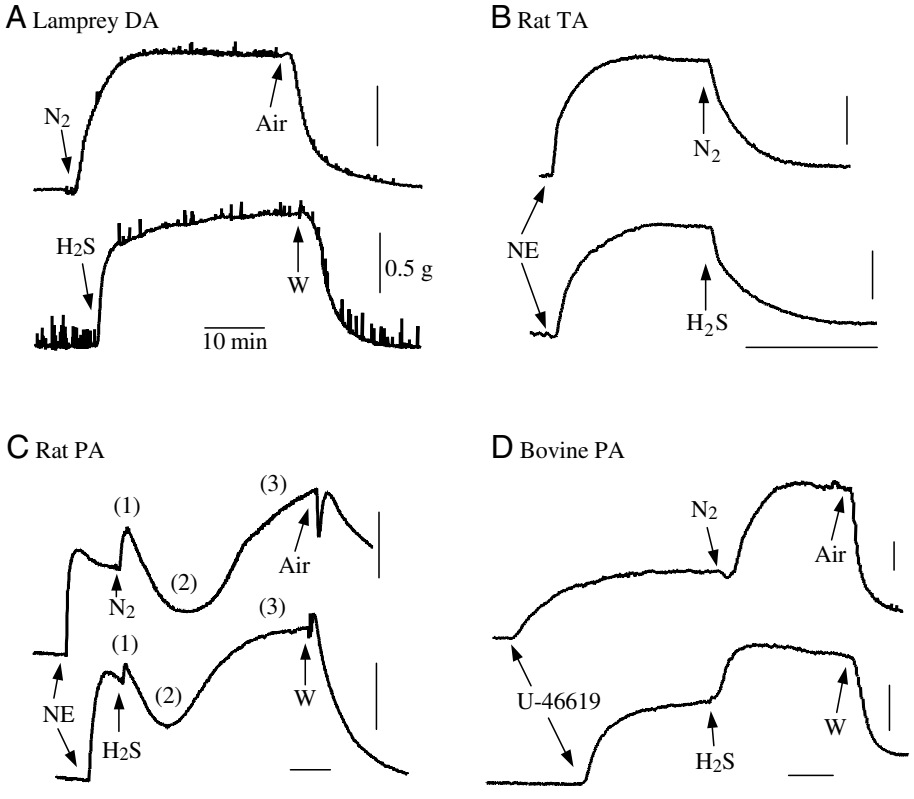

Fig. 1. The response of isolated vessels to hypoxia (N_2) or H_2S (1 mmol l^{-1}) is temporally and spatially similar, but vessel-specific. (A) Lamprey dorsal aorta (DA), (B) rat thoracic aorta (TA), (C) rat pulmonary artery (PA) pre-contracted with 10^{-6} mol l^{-1} norepinephrine (NE), (D) bovine pulmonary artery (PA) pre-contracted with 10^{-7} mol l^{-1} U-46619. A slight relaxation of bovine PA often precedes the hypoxic contraction; the H_2S contraction appears mono-phasic, but it also is multi-phasic (see Fig. 2). Horizontal lines=10 min; vertical lines=0.5 g tension; W=wash. (1), (2) and (3) in C, indicate stages in the multiphasic response. (A) N_2 trace adapted from (Olson et al., 2001), with permission; (C) H_2S trace adapted from Dombkowski et al. (Dombkowski et al., 2005), with permission.

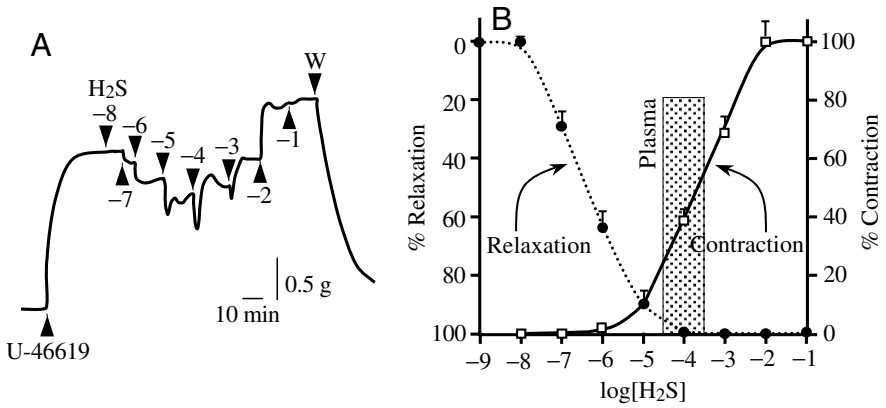

Fig. 2. H_2S has two dose-dependent effects on pre-contracted (10^{-7} mol l^{-1} U-46619) bovine pulmonary arteries. H_2S appears to produce a dose-dependent relaxation between 10^{-8} and 10^{-5} mol l^{-1}, whereas above 10^{-5} mol l^{-1} it produces a dose-dependent constriction. The EC_{50} for relaxation ($5.5\pm1.8\times10^{-7}$ mol l^{-1}) is significantly ($P\leqslant0.05$) different from the EC_{50} for contraction ($3.7\pm1.5\times10^{-4}$ mol l^{-1}). (A) Single trace of cumulative doses; arrowheads indicate log[H_2S] (in mol l^{-1}) and time of addition of U-46619; W=wash. (B) Relaxation (filled circles); contraction (open squares; values extrapolated where curves overlap ~10^{-5} mol l^{-1}). Values are means ± s.e.m. of 8 vessels. Stippled rectangle indicates range of H_2S reported in the rat plasma (see text for details).

in lamprey dorsal aorta (Fig. 3A), whereas H_2S applied during hypoxia produced a slight relaxation and a hypoxic contraction was significantly inhibited by prior application of H_2S. In rat thoracic aorta, H_2S produced a slight contraction when applied during hypoxia and hypoxic relaxation was inhibited by exposure to H_2S (Fig. 3B); similar results were obtained from seven other vessels. Hypoxia- and H_2S-mediated contractions of bovine pulmonary arteries were reversed to a relaxation when the vessels were previously treated with the other stimulus (Fig. 3C); after approximately 30 min aeration with air (95% air, 5% CO_2) both hypoxic and H_2S contractions were restored; similar results were obtained from seven other vessels.

Transmembrane potential

Table 2 shows the effect of hypoxia and H_2S on resting transmembrane potential (E_m) and diameter in bovine pulmonary arteries. Hypoxia and H_2S produced essentially identical depolarization and constriction.

H_2S production

Homogenates of lamprey aortas and bovine pulmonary arteries and veins produced H_2S (H_2S+HS^-) when incubated with cysteine and pyridoxyl 5′-phosphate (Fig. 4). H_2S production by lamprey vessels was twice that of bovine vessels. H_2S production by bovine pulmonary arteries was not significantly affected by the cystathionine γ-lyase (CSE)

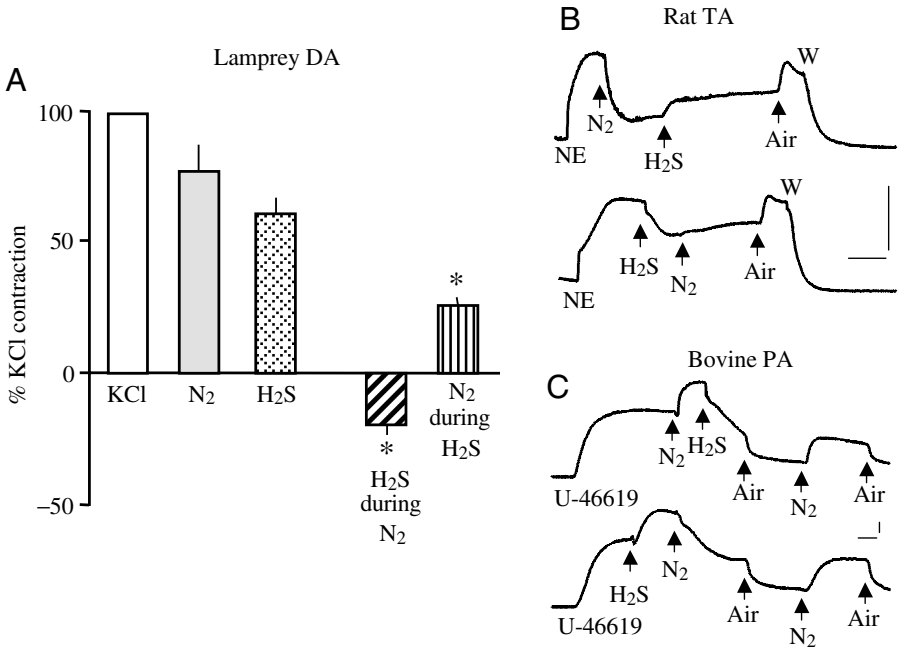

Fig. 3. The effects of hypoxia (N_2) and H_2S are mutually competitive. (A) Individually, N_2 and H_2S produce similar contraction of lamprey dorsal aorta (DA; normalized to 80 mmol l^{-1} KCl contraction=100%) while in the presence of N_2, H_2S (3×10^{-4} mol l^{-1}) relaxes and in the presence of H_2S, N_2 contractions are significantly ($P\leqslant0.05$; N=8 vessels) reduced. (B) In norepinephrine (NE; 10^{-6} mol l^{-1}) pre-contracted rat thoracic aortas initial exposure to hypoxia (N_2; top trace) or H_2S (3×10^{-4} mol l^{-1}; bottom trace) produces a typical relaxation, whereas subsequent application of either H_2S (top) or hypoxia (bottom) results in either a slight contraction or no response. (C) In U-46619 (10^{-6} mol l^{-1})-contracted bovine pulmonary arteries, 3×10^{-4} mol l^{-1} H_2S relaxes a pre-existing N_2 contraction and N_2 relaxes a pre-existing H_2S contraction. H_2S is lost from continuously aerated baths in C after which normal hypoxic contractions are restored. Values are means ± s.e.m., N=8 vessels; horizontal and vertical scale bars in B and C = 10 min and 0.5 g, respectively.

Table 2. *Effect of hypoxia and H_2S (1 mmol l^{-1}) on transmembrane potential (E_m) and percent reduction in diameter (contraction) in bovine pulmonary arteries (~300 μm diameter)*

	E_m	Contraction (%)
Control	–55±0.4 (18/8/4)	
Hypoxia	–45±0.7 (13/8/4)*	6.8±0.7 (10/5)
H_2S	–44±0.4 (14/7/4)*	7.9±0.8 (10/5)

Values are means ± s.e.m. (number of vessels impaled/number of arteries/number of cows, or number of arteries/number of cows). *Significantly different from control; effects of hypoxia and H_2S were not significantly different from each other.

inhibitor, D,L-propargylglycine (PPG), whereas it was significantly reduced by the cystathionine β-synthase (CBS) inhibitor, amino-oxyacetate (AOA). A combination of AOA and PPG was no more effective than AOA alone. H_2S production by pulmonary arteries and veins was inhibited by a mixture of AOA, PPG and the pyridoxyl 5′-phosphate-dependent enzyme inhibitor, hydroxylamine (HA). The three inhibitors combined were no more effective in pulmonary arteries than AOA alone.

Inhibition of H_2S synthesis and the hypoxic response

The effect of inhibitors of H_2S synthesis on the hypoxic response of lamprey and rat aortas and rat and bovine pulmonary arteries is shown in Fig. 5. In all vessels the response to hypoxia was partially or completely blocked by inhibitors of H_2S synthesis. The magnitude of hypoxic vasoconstriction in lamprey dorsal aorta was unchanged after three consecutive exposures to hypoxia (Fig. 5A, left bars), whereas in a second set of vessels, application of the pyridoxyl 5′-phosphate-dependent enzyme inhibitor, hydroxylamine (HA), after the initial hypoxia reduced the subsequent hypoxic vasoconstriction by over 80% (Fig. 5A, right bars). In otherwise untreated lamprey aortas, 1 mmol l^{-1} HA produced a contraction that was 31±3% (N=12) of a 80 mmol l^{-1} KCl contraction (not shown). In norepinephrine (NE; 10^{-5} mol l^{-1}) pre-contracted rat thoracic aorta, hypoxic vasodilation was nearly completely blocked by the CSE inhibitor D,L-propargylglycine (PPG; Fig. 5B) and in NE pre-contracted rat pulmonary arteries, both the hypoxic phase 1 contraction and phase 2 relaxation were partially inhibited by the CSE inhibitor, β-cyanoalanine (BCA; Fig. 5C). In rat pulmonary arteries, a combination of BCA, PPG and the CBS inhibitor amino-oxyacetate (AOA) was no more effective than BCA alone. Hypoxic vasoconstriction in U-46619 pre-contracted bovine pulmonary arteries was unaffected by the CSE inhibitor, PPG, but partly (~60%) blocked by AOA, converted to slight relaxation by HA and a strong relaxation by a combination of the three inhibitors (Fig. 5D). In U-46619 contracted bovine pulmonary arteries, the initial application of AOA produced a brief (<5 min) contraction that was only 3.5±1% (N=8) of a KCl (80 mmol l^{-1}) contraction (not shown). HA had no effect on un-stimulated bovine pulmonary arteries, but it completely relaxed U-46619 (10^{-7} mol l^{-1}) contracted arteries (not shown). Between 10^{-7} mol l^{-1} and 3×10^{-5} mol l^{-1}, HA produced a dose-dependent relaxation of KCl-contracted bovine pulmonary arteries that was equivalent to a 13±3% (N=8) reduction of a KCl contraction. HA between 3×10^{-5} mol l^{-1} and 3×10^{-3} mol l^{-1} produced a dose-dependent contraction of the same vessels (not shown). At 10^{-3} mol l^{-1}, the HA contraction was equivalent to 6±5% of a 80 mmol l^{-1} KCl contraction.

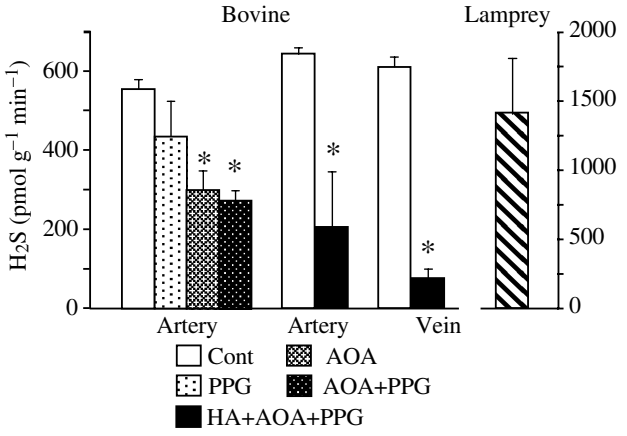

Fig. 4. H_2S is produced by homogenates of bovine pulmonary artery and vein and lamprey dorsal aorta. H_2S production by pulmonary arteries is inhibited by the CBS inhibitor, amino-oxyacetate (AOA; 1 mmol l^{-1}), the pyridoxyl 5′-phosphate-dependent enzyme inhibitor, hydroxylamine (HA; 1 mmol l^{-1}), but not by the CSE inhibitor D,L-propargylglycine (PPG; 10 mmol l^{-1}); HA also inhibits H_2S production in veins. Bovine vessels were pooled from two animals, lamprey aortas were pooled from six fish. Total sulfide (H_2S and HS^-) was measured in triplicate with ion-selective electrodes after alkaline conversion to S^{2-}. Values are means ± s.e.m.; *significantly different from respective control ($P\leqslant0.05$).

Contribution of the H_2S precursor, cysteine, to hypoxic responses

The contribution of cysteine to hypoxic vasoconstriction and dilation is shown in Fig. 6. Addition of 1 mmol l^{-1} cysteine to lamprey aortas produced a slight contraction, doubled the strength of the hypoxic contraction, but did not affect the strength of the KCl contraction (Fig. 6A). Glycine (1 mmol l^{-1}) also produced a slight contraction of lamprey aortas, but did not affect the strength of either hypoxic or KCl contractions (Fig. 6A). Exogenous cysteine did not appear to affect either the magnitude or rate of hypoxic relaxation of rat thoracic aortas (not shown). However, rat aortas incubated with cysteine contracted immediately upon re-oxygenation, whereas control vessels exhibited a transient further relaxation before contracting (not shown). The effects of cysteine incubation during long-term (15 h) hypoxia on subsequent hypoxic responses of rat aortas is shown in Fig. 6B. The magnitude of a U-46619 pre-contraction was increased by ~50% after 15 h

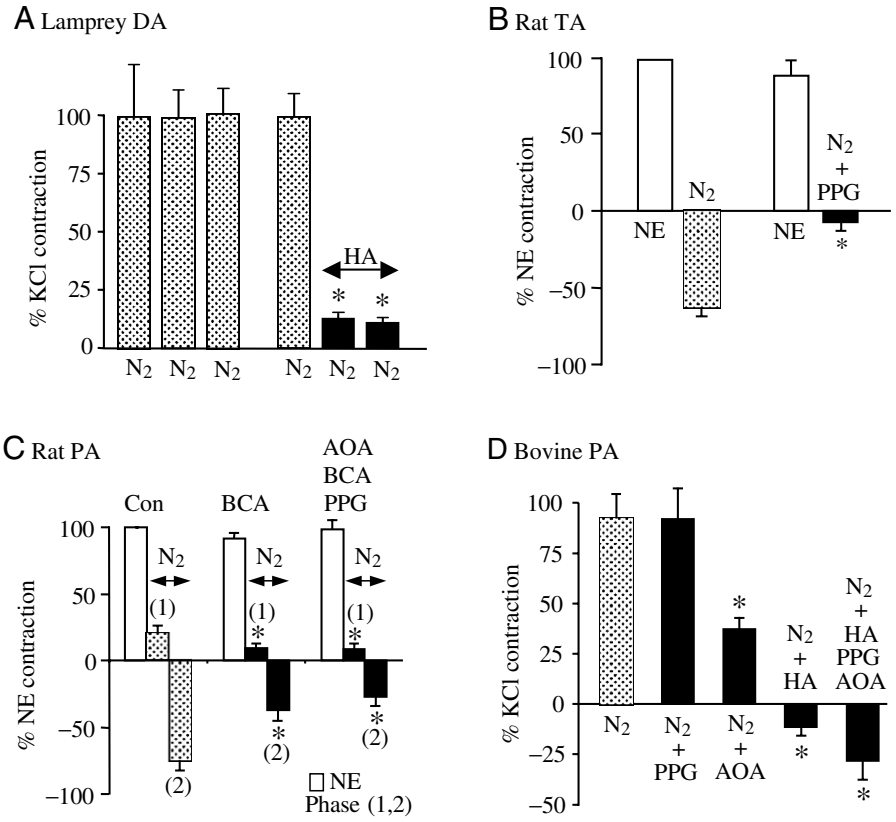

Fig. 5. Constrictory and dilatory responses of isolated vessels to hypoxia (stippled bars) is partially or completely prevented by inhibition of H_2S synthesis (black bars). (A) Hypoxic vasoconstriction in lamprey dorsal aorta (DA; *N*=8) is unaffected by three consecutive bouts of N_2 exposure (left bars), whereas 1 mmol l^{-1} of the pyridoxyl 5′-phosphate-dependent enzyme inhibitor, hydroxylamine (HA), reduces the N_2 response by over 80%. (B) Hypoxic vasodilation of norepinephrine (NE; 10^{-5} mol l^{-1}) pre-contracted rat thoracic aorta (TA) is nearly completely blocked by PPG (*N*=6). (C) In NE pre-contracted rat pulmonary arteries (PA), both the hypoxic phase 1 contraction (1) and phase 2 relaxation (2) are partially inhibited by the CSE inhibitor, β-cyanoalanine (BCA; 5 mmol l^{-1}) or a combination of 1 mmol l^{-1} AOA, 10 mmol l^{-1} BCA and 10 mmol l^{-1} PPG (*N*=8 each group). (D) Hypoxic vasoconstriction in 10^{-7} μmol l^{-1} U-46619 pre-contracted (contraction not shown) bovine pulmonary arteries is unaffected by the CSE inhibitor D,L-propargylglycine (PPG; 10 mmol l^{-1}), partly blocked by the CBS inhibitor amino-oxyacetate (AOA; 1 mmol l^{-1}) and converted to slight relaxation by HA and a strong relaxation by a combination of the three inhibitors (*N*=6 each group). Values are means ± s.e.m.; *significantly different from respective control ($P \leqslant 0.05$).

hypoxia ($P \leqslant 0.05$, *N*=16) and this was unaffected by the presence or absence of cysteine. Cysteine treatment significantly decreased the magnitude of the hypoxic relaxation (from 42±2% to 32±2% relaxation of the U-46619 contraction; control *vs* cysteine, respectively). Cysteine treatment did not affect the magnitude of the initial force recovered during re-oxygenation (262±138 mg *vs* 259±63 mg, control *vs* cysteine), but enabled the vessels to maintain tension during a 25 min recovery, whereas control vessels relaxed to baseline. A transient relaxation preceding re-oxygenation recovery was observed in control, but not cysteine-treated vessels; these responses were similar to those described above in vessels that were not exposed to 15 h hypoxia. Control vessels did not respond to a second hypoxic exposure, whereas hypoxic relaxation could be repeated in cysteine-treated vessels (Fig. 6B). Cysteine-treated vessels also relaxed when washed with U-46619-free buffer; control vessels did not (Fig. 6B). The apparent inability of control vessels to respond at the end of this experiment was not due to damage to the vessel as this protocol (U-46619 contraction, two consecutive hypoxia treatments and wash) could be repeated on both groups of vessels with identical results (not shown). Addition of cysteine to bovine pulmonary arteries did not affect the magnitude of a subsequent hypoxic contraction (not shown). However, after 15 h of hypoxia (Fig. 6C), hypoxic contraction of pulmonary arteries incubated with 1 mmol l^{-1} cysteine for 15 h was twice that of control vessels. Both cysteine-treated and control vessels relaxed upon re-oxygenation. Force developed during a second hypoxic exposure was not significantly different between the two groups, but the control vessels could not sustain the contraction (by 30 min they were completely relaxed) whereas cysteine-treated vessels were able to sustain the hypoxic vasoconstriction (at 30 min they retained 55% of their contractile force; Fig. 6C).

Discussion

Our experiments indicate that: (1) in at least one species from each vertebrate class, the vascular response to hypoxia and H_2S is similar, if not identical; (2) hypoxia and H_2S have essentially identical depolarizing effects on transmembrane potential; (3) the effects of hypoxia and H_2S seem to be competitive, in the presence of one stimulus, vascular responses to the second are greatly reduced or inhibited; (4) H_2S is vasoactive at physiologically relevant concentrations; (5) H_2S is produced by blood vessels *via* enzymatically mediated mechanisms; (6) hypoxic vasoconstriction and hypoxic vasodilation are partially or completely blocked by inhibitors of H_2S synthesis; (7) addition of cysteine, the precursor for H_2S production, enhances the hypoxic response. These results satisfy the criteria that H_2S is a biologically relevant gasotransmitter (Wang, 2002) in a variety of vertebrate blood vessels. Although

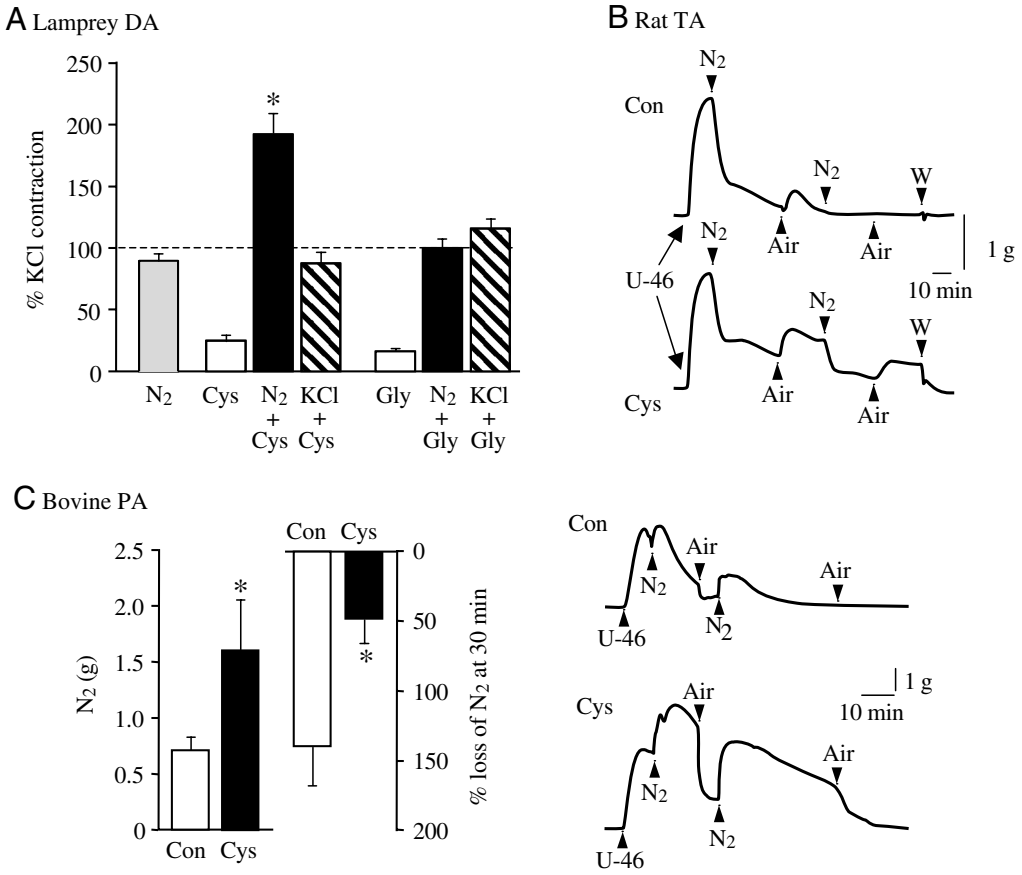

Fig. 6. Contribution of the H_2S precursor, cysteine, to hypoxic responses. (A) Addition of cysteine to lamprey dorsal aorta (DA) significantly and specifically increases the magnitude of a hypoxic contraction. In the absence of exogenous cysteine, a hypoxic contraction (N_2) develops as much force as a reference 80 mmol l^{-1} KCl contraction (broken line). Addition of cysteine (Cys; 1 mmol l^{-1}) produces a slight, transient contraction, doubles the strength of the hypoxic contraction (N_2+Cys; $P \leqslant 0.05$), but does not affect a second KCl contraction. Addition of glycine (Gly; 1 mmol l^{-1}) also produced a slight contraction but did not affect either hypoxic (N_2+GLY) or KCl (KCl+Gly) contractions. (B) Rat thoracic aortas (TA) were exposed to hypoxia for 15 h in the absence (Con) or presence of cysteine (Cys), returned to normoxia, pre-contracted with U-46619, and exposed twice to hypoxia. Incubation with cysteine significantly ($P \leqslant 0.05$) reduced the magnitude of the first hypoxic relaxation but enabled the vessels to respond to re-oxygenation and a second hypoxia. (C) Bovine pulmonary arteries (PA) were exposed to hypoxia for 15 h in the absence (Con) or presence of cysteine (Cys), returned to normoxia, pre-contracted with U-46619, and exposed twice to hypoxia. Incubation with cysteine increased the magnitude of the initial hypoxic contraction and the second hypoxic contraction was sustained longer.

considerable work remains to be done, our experiments also suggest that H_2S, or more likely the metabolism of H_2S, is an important component in the O_2 sensing/signal transduction cascade involved in the hypoxic response.

Vessel responses to hypoxia and H_2S are virtually identical

Vertebrate vessels from various species and from various vascular beds within the same species exhibit their own unique response to hypoxia, mono-phasic relaxations and mono or multi-phasic contraction. In every vessel we have examined thus far (Table 1, Fig. 1), as well as in studies by others (Olson et al., 2001; Zhao et al., 2001; Zhao and Wang, 2002; Dombkowski et al., 2005), the responses to H_2S are essentially identical to those produced by hypoxia. In lamprey dorsal aortas both hypoxia and H_2S produce a mono-phasic contraction (Fig. 1A) and both are dose-dependent (Olson et al., 2001; Dombkowski et al., 2005). In rat thoracic aorta these stimuli produce a mono-phasic, dose-dependent relaxation (Fig. 1B) (Zhao et al., 2001; Zhao and Wang, 2002). When hypoxia produces a multi-phasic response, so does H_2S. This similarity between hypoxic and H_2S responses is quite striking in rat pulmonary arteries where both stimuli produce an identical complex tri-phasic contraction–relaxation–contraction (Fig. 1C). To our knowledge only hypoxia and H_2S produce this characteristic tri-phasic response in rat pulmonary vessels. In bovine pulmonary arteries, hypoxia and H_2S (Fig. 1D and Fig. 3C, respectively) often produce a slight, transient dilation that precedes the sustained contraction.

The similarity between the effects of H_2S and hypoxia in both pulmonary and systemic vessels suggests that H_2S and hypoxia may share common activation pathways. Furthermore,

because we have observed identical effects of hypoxia and H_2S in vessels from at least one species in each vertebrate class (Table 1), we propose that H_2S mediation of a hypoxic response is common throughout vertebrate phylogeny and that it was a primordial feature of the earliest vertebrate vessels.

Vessel responses to hypoxia and H_2S are competitive

In addition to the similarity in the form of the response, in our experience in a variety of vessels, hypoxia and H_2S have been the only two stimuli whose effects are eliminated or reversed by pre-existing exposure to the other stimulus, whereas their response to other agonists is generally not affected (Fig. 3). These studies suggest that hypoxia and H_2S share a common and unique pathway in the excitation–contraction process that, when activated by one stimulus, cannot be further activated by the other.

In lamprey aorta (Fig. 3A), hypoxic and H_2S contractions are 76 and 60%, respectively, of an 80 mmol l^{-1} KCl contraction. Exposing the vessel to H_2S during a hypoxic contraction results in a small relaxation. Exposing the vessel to hypoxia during an H_2S contraction results in a contraction whose magnitude is less than 25% of the KCl contraction. By comparison, when lamprey aortas are pre-contracted with either 80 mmol l^{-1} KCl, or 10^{-6} mol l^{-1} epinephrine, the contractions produced by hypoxia (Olson et al., 2001) and H_2S (Dombkowski, 2006) are not diminished. In norepinephrine pre-contracted rat thoracic aortas, where initial exposure to either hypoxia or H_2S produces a characteristic relaxation, subsequent treatment with the other stimulus (H_2S or hypoxia, respectively) fails to relax, and in fact elicits a slight contraction (Fig. 3B). The inability of the second stimulus to produce additional relaxation is not due to a mechanical property of the vessel because the vessel is not completely relaxed by the first stimulus. In bovine pulmonary arteries, both hypoxia and H_2S produce a monophasic contraction that is additive to a pre-existing U-46619 contraction (Fig. 3C). However, secondary application of H_2S during hypoxia relaxes the hypoxic constriction and hypoxia relaxes the H_2S contraction (Fig. 3C). Thus the effects of hypoxia and H_2S appear to be uniquely competitive in both systemic and pulmonary vessels.

What is not yet clear is why in some instances the second stimulus not only inhibited, but reversed the effects of the first, i.e. H_2S reversed hypoxic contraction in the lamprey aorta (Fig. 3A) and bovine pulmonary artery (Fig. 3C) and hypoxic relaxation in the rat thoracic aorta (Fig. 3B) and hypoxia reversed the H_2S contraction in bovine pulmonary arteries (Fig. 3C). This effect may be due to different conditions produced by endogenous (hypoxia-mediated) and exogenous H_2S (NaHS or Na_2S), which could affect the magnitude and direction of the relative fluxes of H_2S gas and HS^- across the cell membrane. Similarly, when vessels are made hypoxic during H_2S treatment, actual [H_2S] may be greater than exogenous [H_2S] alone. This question may not be resolved until it is understood how exogenous and endogenous H_2S interact and how H_2S gas and HS^- move across cell membranes and mediate cellular responses.

Hypoxia and H_2S have similar effects on E_m

Hypoxia and H_2S have similar effects on vascular smooth muscle transmembrane potential (E_m); both hyperpolarize systemic vessels (Lombard et al., 1999; Frisbee et al., 2001; Zhao et al., 2001) and depolarize respiratory (bovine pulmonary) arteries (Table 2). Whether this means that both stimuli act through a common pathway is not known. While it is tempting to associate a change in E_m with intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) and relaxation or contraction, it is not clear if this is always the case. In a number of systemic vessels a substantial portion of hypoxic vasodilation is independent of [Ca^{2+}]$_i$ (Thorne et al., 2002) and vessels contracted with high ($\geqslant$80 mmol l^{-1}) KCl can still relax to hypoxia (Pearce et al., 1989; Shimizu et al., 2000). Similarly, the relaxant effect of H_2S in rat aortas has been shown to be only partially mediated by K^+ (K_{ATP}) channels and it requires extracellular Ca^{2+} (Zhao et al., 2001; Zhao and Wang, 2002). In rat pulmonary arteries cell depolarization can be uncoupled from the rise in [Ca^{2+}]$_i$ (Gelband and Gelband, 1997) and Ca^{2+} may enter the cell *via* voltage-independent (capacitative Ca^{2+}) pathways (Robertson et al., 2000). Both hypoxic pulmonary vasoconstriction in the rat (Robertson et al., 2000) and hypoxic vasoconstriction in the lamprey aorta (Olson et al., 2001) can occur in the presence of elevated extracellular [K^+]. Thus, the mechanism(s) responsible for the change in E_m during H_2S exposure remain to be elucidated.

Vessels produce H_2S enzymatically

H_2S production has now been demonstrated in vessels from lamprey (Fig. 4), rainbow trout (Dombkowski, 2006), rat (Hosoki et al., 1997; Zhao et al., 2001; Zhao et al., 2003; Wang et al., 2004) and cow (Fig. 4). This suggests that H_2S synthesis is a general property of vertebrate vascular smooth muscle.

Cysteine is the major source of H_2S production in mammals (Maclean and Kraus, 2004) and cysteine was added to our tissue homogenates to produce optimal enzymatic activity (S.K.H., N.L.W. and K.R.O., unpublished observation). A number of enzymes desulfurate cysteine. These include cystathionine β-synthase (CBS; EC 4.2.1.22), cystathionine (γ-lyase (CSE; EC 4.4.1.1), cysteine aminotransferase (EC 2.6.1.3), mercaptopyruvate sulfurtransferase (MST; EC 2.8.1.2), rhodanase (thiosulfate cyanide sulfurtransferase; EC 2.8.1.1) and cysteine lyase (EC 4.2.1.10) (Maclean and Kraus, 2004; Stipanuk, 2004). Most vascular studies have focused on CSE and CBS as the potential H_2S-generating enzymes, although other enzymes may also be involved (Maclean and Kraus, 2004).

CSE is thought to be the primary enzyme for H_2S synthesis in mammalian vessels. CSE mRNA has been identified in human systemic vessels and rat pulmonary arteries; in rat systemic vessels, both CSE mRNA and the 43 kDA CSE protein have been identified (Hosoki et al., 1997; Zhao et al., 2003; Cheng et al., 2004; Wang et al., 2004). We have also found CSE mRNA in systemic arteries, veins and respiratory vessels of trout (R. Wang, R.A.D. and K.R.O., unpublished observation). Unlike nitric oxide synthase and hemeoxygenase,

CSE mRNA in rat vessels is confined to vascular smooth muscle and is absent from the endothelium (Wang et al., 2004). The CSE inhibitor, propargyl glycine (PPG), inhibits H_2S production in rat vessels (Hosoki et al., 1997; Zhao et al., 2003), but in bovine pulmonary arteries PPG did not significantly affect either H_2S production (Fig. 4), or hypoxic contraction (Fig. 5). This suggests that enzymes other than CSE also contribute to vascular H_2S production.

Previous studies have indicated that CBS is not involved in H_2S synthesis in mammalian vessels. CBS mRNA has not been detected in mammalian (rat and human) vessels and the CBS inhibitor, aminooxyacetic acid (AOA) did not block H_2S production by rat vessel homogenates (Hosoki et al., 1997; Zhao et al., 2001; Zhao et al., 2003; Wang et al., 2004). However, our findings suggest that in a number of vertebrates, CBS and perhaps other enzymes are important in vascular H_2S production. AOA partially inhibits H_2S production in homogenized bovine pulmonary arteries and veins (Fig. 4), and trout arteries and veins (Dombkowski, 2006), and it partially inhibits the hypoxic contraction of bovine pulmonary arteries (Fig. 5). We have also found that CBS mRNA is ubiquitously expressed in trout systemic arteries, veins and respiratory vessels (R. Wang, R.A.D. and K.R.O., unpublished observation). Collectively, these studies suggest that CBS is also involved in vascular H_2S production.

Although we did not systematically examine all enzyme inhibitors in all vessels, hydroxylamine appeared to have the greatest inhibitory effect on H_2S production and hypoxic responses. CBS, CSE and cysteine lyase all depend on the co-factor, pyridoxal 5′-phosphate (PLP) for enzymatic activity (Maclean and Kraus, 2004, and as hydroxylamine is a general inhibitor of PLP-dependent enzymes (Kery et al., 1999), it is possible that multiple enzymes contribute to H_2S production in some vessels.

Inhibition of H_2S production inhibits hypoxic vasoconstriction and hypoxic vasodilation

The inhibition of both hypoxic vasoconstriction and hypoxic vasodilation by inhibitors of H_2S synthesis (Fig. 5) further supports the hypothesis that the vascular response to hypoxia and H_2S are interrelated. These studies also suggest that H_2S synthesis may depend on different, even vessel-specific, enzymes and that other enzymes, in addition to CBS and CSE, may be involved in H_2S production. PPG, which inhibits CSE and H_2S production by rat aorta (Zhao et al., 2003), essentially abolished hypoxic vasodilation in rat thoracic aortas (Fig. 5B) and the CSE inhibitor β-cyano-L-alanine (BCA), partially inhibited hypoxic phase 1 contraction and phase 2 relaxation in rat pulmonary arteries and was no less effective than AOA, BCA and PPG combined. However, CBS appears to account for at least part of the hypoxic generation of H_2S by bovine pulmonary arteries as the CBS inhibitor, AOA, reduced hypoxic vasoconstriction by 50%, whereas the CSE inhibitor, PPG, was ineffective (Fig. 5D). This is consistent with the predominance of CBS in H_2S synthesis by these vessels (Fig. 4). Although CBS mRNA has not been identified in mammalian vessels, we have found it in trout vessels (R. Wang, R.A.D. and K.R.O., unpublished observation) suggesting a potential function.

The inhibitors used in our, and essentially all other studies on blood vessels, are somewhat non-specific, e.g. AOA inhibits the malate–aspartate shuttle (Bunger et al., 1980), PPG inhibits L-alanine transaminase (Burnett et al., 1980), BCA interacts with NMDA receptors (Roy et al., 1996) and hydroxylamine is a NO donor in rat aorta (Beranova et al., 2005). Thus it is possible that some of the effects of these inhibitors on hypoxic vasodilation and hypoxic vasoconstriction may not involve H_2S. While it was beyond the scope of this study to characterize the non-specific effects of these inhibitors on the mechanical properties of blood vessels, several points are worth noting. First, we (Fig. 4) and others (Hosoki et al., 1997; Zhao et al., 2003) have clearly demonstrated that H_2S synthesis by blood vessels is sensitive to specific enzyme inhibitors. Second, inhibition of hypoxic vasodilation and hypoxic vasoconstriction also appears somewhat dependent on specific enzyme inhibitors, i.e. PPG is a better inhibitor of hypoxic relaxation of the rat aorta than AOA, whereas AOA is more effective than PPG in inhibiting hypoxic vasoconstriction in the bovine pulmonary artery. Third, the direct effects of inhibitors on vessel tension do not necessarily mimic their effect on hypoxic vasodilation and hypoxic vasoconstriction; HA contracts lamprey aortas, but inhibits hypoxic vasoconstriction, and AOA has essentially no effect on U-46619 pre-contracted bovine pulmonary arteries, but also inhibits hypoxic vasoconstriction. The effects of HA on bovine pulmonary arteries, however, could be non-specific through the release of NO (Beranova et al., 2005). To our knowledge, there are no specific inhibitors of H_2S synthesis and clearly they are needed to resolve this issue.

Addition of H_2S precursor cysteine affects hypoxic responses

Cysteine, which is the precursor for H_2S production, doubled the force of a hypoxic contraction in both lamprey aortas and bovine pulmonary arteries (Fig. 6A,B), suggesting that under these circumstances H_2S production was also increased. The cysteine effect was not apparent in bovine pulmonary arteries unless they had been exposed to prolonged hypoxia whereas pre-hypoxic exposure was not needed in the lamprey. The difference between lamprey and bovine vessels may be due to the amount of endogenous cysteine stored in the vessel or to differences in metabolism. Incubation with cysteine also changed the hypoxic responses of rat aortas, but it unexpectedly augmented the recovery during re-oxygenation rather than the hypoxic relaxation. It is not clear how this occurred.

H_2S as a mediator of the hypoxic response

Collectively, the above experiments indicate that H_2S participates in vascular responses to hypoxia. We propose that it is the metabolism of H_2S that serves as the 'O_2 sensor/transducer' in vascular smooth muscle. In this model the concentration of vasoactive H_2S is regulated by the simple balance between endogenous vascular H_2S production and its

oxidation by available O_2. As described below, the stoichiometric relationship between [O_2] and [H_2S] in vascular smooth muscle and the primordial precedent for H_2S production in the cytosol and oxidation in the mitochondria add anecdotal support to our model.

Recent studies have shown that P_{O_2} in the walls of systemic arterioles in many vascular beds is around 50 mmHg, partly because they supply much of the O_2 to tissues, and partly because the rate of O_2 consumption by the vessel wall is very high, up to 500 times that of resting skeletal muscle (Tsai et al., 2002; Shibata et al., 2005). With an oxygen solubility around 10^{-6} mol l^{-1}mmHg^{-1} (see Shibata et al., 2005), the O_2 concentration in arteriolar smooth muscle would be $5–6\times10^{-5}$ mol l^{-1}. This is strikingly similar to most reports showing plasma [H_2S] in rats at around $4–6\times10^{-5}$ mol l^{-1} (Zhao et al., 2001; Geng et al., 2004; Yan et al., 2004; Yusuf et al., 2005). Unfortunately, intracellular [H_2S] is unknown. It may equal or exceed plasma [H_2S], but it may also be lower due to compartmentalization within the cell; it is well known that 10^{-6} mol l^{-1} H_2S is toxic to isolated mitochondrial cytochrome *c* oxidase, but not to intact cells (see Dombkowski et al., 2005). Thus if there is excess dissolved O_2 relative to H_2S, as would be expected in normoxia, continual oxidation, and therefore inactivation, of H_2S would be expected. However, even moderate hypoxia would lower arteriolar [O_2], which would then decrease the rate of H_2S oxidation and allow intracellular [H_2S] to increase. This scenario is feasible. A 1 min reduction in inspired P_{O_2} in rats, which is similar to that experienced in humans during sleep apnea, lowers P_{O_2} in cremaster muscle arterioles to 15.8 mmHg (Johnson et al., 2005). This is essentially half that of plasma [H_2S]. Furthermore, Doeller et al. observed (Doeller et al., 2005) that intact segments of rat aortas produced significant amounts of H_2S when the incubation medium [O_2] was 4 μmol l^{-1}, but they could not detect H_2S production when the medium [O_2] was raised to 200 μmol l^{-1}, i.e. normoxia. We predict that the resultant rise in [H_2S] due to decreased oxidation during hypoxia will then initiate the appropriate vascular responses. The continual oxidation of H_2S during normoxia may also contribute to the high O_2 demand of vascular smooth muscle.

Intracellular compartmentalization of H_2S production and oxidation appears to be a common property of all eucaryotic cells and we propose that smooth muscle cells employ this to regulate [H_2S]. There is accumulating evidence that mitochondria originated from sulfide (H_2S)-oxidizing bacteria and the nucleocytoplasm from sulfide-reducing (H_2S generating) Archaea (Searcy, 2003). Where ancient (and some modern) eucaryotic cells shuttled sulfur to generate ATP, we propose that smooth muscle uses this mechanism to regulate the level of H_2S-mediated vasoactivity. In fact, H_2S oxidation has been demonstrated in chicken liver mitochondria (Yong and Searcy, 2001) and H_2S production has been observed in cells lacking mitochondria such as human erythrocytes (Searcy and Lee, 1998). The observations that H_2S production is essentially the same in normoxic and hypoxic erythrocytes (Searcy and Lee, 1998), whereas pieces of rat thoracic aorta produce H_2S when hypoxic, but consume H_2S when normoxic (Doeller et al., 2005), are also consistent with our model.

It is also possible that vascular H_2S production is actively regulated as CBS appears to have a number of O_2-sensitive regulatory sites (Maclean and Kraus, 2004; Stipanuk, 2004; Banerjee and Zou, 2005). This needs further investigation. Even if the short-term H_2S response to hypoxia is not enzymatically regulated in vascular smooth muscle, tonic regulation of H_2S production could contribute to resting (normoxic) tone and bias the pattern and magnitude of the hypoxic response.

Function of multi-phasic H_2S effects

The distinct phases of the H_2S response in respiratory vessels (Table 1) may have specific physiological functions at different P_{O_2} values. As shown in Fig. 2, low [H_2S] relaxes bovine pulmonary arteries and higher [H_2S] contracts them. According to our hypothesis, much of the H_2S produced by the vascular smooth muscle cells during normoxia will be oxidized and the resultant low [H_2S] will dilate the vessels and minimize pulmonary vascular resistance. However, during hypoxia, [H_2S] will increase and result in the characteristic constriction. Although H_2S levels have not been measured in bovine plasma or in smooth muscle intracellular fluid, [H_2S] in (putatively normoxic) rat plasma is usually around 4×10^{-5} mol l^{-1} (Zhao et al., 2001; Geng et al., 2004; Yan et al., 2004; Yusuf et al., 2005). In fact, it has been estimated that vascular H_2S may approach 10^{-4} mol l^{-1} in some vessels (Zhao and Wang, 2002), although intracellular [H_2S] may be somewhat lower due to compartmentalization. As shown in Fig. 2, maximum dilation to H_2S in bovine pulmonary artery occurs around 10^{-5} mol l^{-1} H_2S and this is also the threshold for constriction. A tenfold increase in [H_2S] to 10^{-4} mol l^{-1} will shift the vessel from nearly complete relaxation to 40% of a maximal H_2S contraction.

Distinct dose-dependent dilatory and constrictory effects of H_2S are not unique to bovine pulmonary arteries; we have also characterized them in rainbow trout efferent branchial (systemic) arteries where they overlap with plasma titers (Dombkowski et al., 2004), and in Pekin duck pulmonary arteries (Dombkowski et al., 2005). This suggests that the P_{O_2}–H_2S system is a versatile bipolar effector of vascular responses to ambient O_2 in many vertebrates.

Acute vs chronic hypoxia

Previous investigators (Zhang et al., 2003; Zhang et al., 2004) have suggested that H_2S is inversely related to hypoxic pulmonary hypertension. They (Zhang et al., 2003; Zhang et al., 2004) reported that hypoxia decreased H_2S production in rat pulmonary arteries and they concluded that the resultant loss in H_2S-mediated vasodilation contributed to the observed increase in pulmonary vascular resistance. While this conclusion appears to contradict ours, it likely represents a fundamentally different mechanism. First, they (Zhang et al., 2003; Zhang et al., 2004) examined the effects of chronic (3 weeks, 6 h per day, 10% O_2) hypoxia *in vivo*, whereas our study focuses on the immediate

effects of acute hypoxia in isolated vessels. Second, they found that hypoxia produced vascular hypertrophy, but because they did not directly examine the response of pulmonary vessels to H_2S, they could only assume that it would be a relaxation similar to that observed in the aorta (Zhao et al., 2001; Zhao and Wang, 2002). Our present and prior studies (Dombkowski et al., 2004) show that H_2S constricts many respiratory vessels. Although it is likely that many of the effects of chronic hypoxia are mediated by genomic factors (as shown by vascular remodeling), it is doubtful that these would contribute to the acute hypoxic responses we observed.

Overview

Hypoxic vasoconstriction and dilation have been observed in blood vessels from all classes of vertebrates and although numerous factors, endothelial and otherwise, can modify these responses, it would seem that the basic mechanisms intrinsic to vascular smooth muscle have a conservative evolutionary history. Perhaps this is best exemplified in cyclostomes where the mono-phasic hypoxic vasoconstriction is unencumbered by endothelial and other evolutionary embellishments. H_2S production and vasoactivity have a similar phylogenetic profile and, based on the present study, H_2S appears to be interwoven with the hypoxic responses. To date, little is known regarding the mechanism(s) of H_2S vasoactivity other than its demonstrated ability to open K_{ATP} channels and initiate vasodilation. However, H_2S may prove to be the most versatile gasotransmitter because of its unique ability to participate in redox reactions, form S-nitrosothiols, buffer at physiological pH, and exert biological effects either as a gas or anion. Undoubtedly this versatility is appropriately utilized by vascular smooth muscle.

List of abbreviations

AOA	amino-oxyacetate
BCA	β-cyanoalanine
bPA	bovine pulmonary artery
CBS	cystathionine β-synthase
CSE	cystathionine (γ-lyase)
HA	hydroxylamine
lDA	lamprey dorsal aorta
MST	mercaptopyruvate sulfurtransferase
NE	norepinephrine
PLP	pyridoxal 5′-phosphate
PPG	D,L-propargylglycine
rA	rat aorta
rPA	rat pulmonary artery

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