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Oxygen dependency of hydrogen sulfide-mediated vasoconstriction in cyclostome aortas

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SUMMARY

Hydrogen sulfide (H_2S) has been proposed to mediate hypoxic vasoconstriction (HVC), however, other studies suggest the vasoconstrictory effect indirectly results from an oxidation product of H_2S. Here we examined the relationship between H_2S and O_2 in isolated hagfish and lamprey vessels that exhibit profound hypoxic vasoconstriction. In myographic studies, H_2S (Na_2S) dose-dependently constricted dorsal aortas (DA) and efferent branchial arteries (EBA) but did not affect ventral aortas or afferent branchial arteries; effects similar to those produced by hypoxia. Sensitivity of H_2S-mediated contraction in hagfish and lamprey DA was enhanced by hypoxia. HVC in hagfish DA was enhanced by the H_2S precursor cysteine and inhibited by amino-oxyacetate, an inhibitor of the H_2S-synthesizing enzyme, cystathionine β-synthase. HVC was unaffected by propargyl glycine, an inhibitor of cystathionine λ-lyase. Oxygen consumption ($\dot{M}_{O_2}$) of hagfish DA was constant between 15 and 115 mmHg P_{O_2} (1 mmHg=0.133 kPa), decreased when P_{O_2} <15 mmHg, and increased after P_{O_2} exceeded 115 mmHg. 10 μmol l^{-1} H_2S increased and ⩾100 μmol l^{-1} H_2S decreased $\dot{M}_{O_2}$. Consistent with the effects on HVC, cysteine increased and amino-oxyacetate decreased $\dot{M}_{O_2}$. These results show that H_2S is a monophasic vasoconstrictor of specific cyclostome vessels and because hagfish lack vascular NO, and vascular sensitivity to H_2S was enhanced at low P_{O_2}, it is unlikely that H_2S contractions are mediated by either H_2S–NO interaction or an oxidation product of H_2S. These experiments also provide additional support for the hypothesis that the metabolism of H_2S is involved in oxygen sensing/signal transduction in vertebrate vascular smooth muscle.

Key words: hypoxic vasoconstriction, oxygen sensing, vascular smooth muscle.

INTRODUCTION

Hypoxic vasoconstriction (HVC) was first observed in the mammalian pulmonary vasculature by von Euler and Liljestrand (von Euler and Liljestrand, 1946) and it is now generally accepted that in mammals this response is unique to the pulmonary circulation, whereas hypoxic vasodilation (HVD) is the prominent response of systemic vessels (Weir and Archer, 1995). Although HVC and HVD may be modulated by endothelial-derived and/or circulating substances (Félétou et al., 1995; Jacobs and Zeldin, 2001; Kerkhof et al., 2001; Liu et al., 2001; Aaronson et al., 2002; Deussen et al., 2006), the basic responses are intrinsic to the vascular smooth muscle cell (Madden et al., 1992). In non-mammalian vertebrates, HVC has been observed in both systemic and respiratory conductance vessels (Olson et al., 2001; Russell et al., 2001; Smith et al., 2001; Russell et al., 2007) and HVC appears to be an intrinsic response of vascular smooth muscle cells in the cyclostome dorsal aorta as well (Olson et al., 2001). HVD has only recently been systematically examined in non-mammalian vertebrates (Russell et al., 2007) and while it is common in systemic vessels it is not necessarily the predominant response.

How vascular smooth muscle cells 'sense' hypoxia and transduce this into a mechanical response, either HVC or HVD, is unknown. We recently proposed that the metabolism of H_2S is involved in the O_2-sensing signal transduction process. Our model is based on the balance between constitutive cellular production of vasoactive hydrogen sulfide (H_2S) and its oxidation to inactive products by available O_2 (Olson et al., 2006). Furthermore, this model appears to be applicable to both HVC and HVD and evidence for a H_2S-mediated hypoxic relaxation has even been observed in the trout urinary bladder (Dombkowski et al., 2006).

There is also relatively little information on the mechanism through which H_2S elicits mechanical responses in the vasculature. H_2S-mediated vasodilation has been demonstrated in mammalian systemic vessels and at least part of this response is due to H_2S opening of ATP sensitive potassium (K_{ATP}) channels on the vascular smooth muscle cell and to release of nitric oxide (NO) from the endothelium (Zhao et al., 2001; Zhao and Wang, 2002; Wang et al., 2004). H_2S-mediated vasoconstriction has been demonstrated in mammalian pulmonary vessels (Olson et al., 2006) and in a variety of both pulmonary and systemic vessels from non-mammalian vertebrates (Dombkowski et al., 2005). Although it is unlikely that H_2S contractions are mediated through either K_{ATP} channels or endothelial-derived vasoconstrictor substances, the mechanism(s) of H_2S-mediated vasoconstriction is unknown.

Recently, Koenitzer et al. (Koenitzer et al., 2007) examined the effects of H_2S on rat thoracic aortas at high (200 μmol l^{-1}, ~150 mmHg) and low (40 μmol l^{-1}, ~30 mmHg) partial pressures of O_2 and showed that vascular relaxation was more sensitive to H_2S at low oxygen concentration ($[O_2]$) and that H_2S-mediated contractions were present at high, but not low $[O_2]$. They postulated that the decreased sensitivity of the H_2S-mediated vasorelaxation at high $[O_2]$ was due to the combined effect of rapid oxidation (and therefore inactivation) of vasodilatory H_2S plus the generation of a

vasoconstrictor oxidation product of H_2S that would compete with the H_2S relaxation. Thus H_2S does not directly produce vasoconstriction. The identity of this oxidation product was not determined.

There are other possible explanations for the results of Koenitzer et al. (Koenitzer et al., 2007) that seem equally or more plausible. First, Koenitzer et al. (Koenitzer et al., 2007) only examined rat aortas and these vessels relax when exposed to either hypoxia or lower (and perhaps more physiological?) concentrations of H_2S and thus a contraction would not normally be expected. Second, our theory of H_2S metabolism in vascular O_2 sensing predicts that as $[O_2]$ falls, endogenous $[H_2S]$ increases. Thus at low P_{O_2} we would expect greater sensitivity to exogenous H_2S when applied against a background of elevated endogenous H_2S, consistent with the observations of Koenitzer et al. (Koenitzer et al., 2007). Furthermore, we also think that H_2S directly produces vasoconstriction in vessels that exhibit hypoxic vasoconstriction (e.g. hagfish and lamprey aortas) because it seems unlikely to us that cellular concentrations of an oxidation product of H_2S would be increasing when P_{O_2} is falling.

In the present study we examined the interaction between $[O_2]$ and $[H_2S]$ in the dorsal aorta of the most ancient extant craniate, the hagfish. This vessel was chosen because it has a mono-phasic, $[O_2]$-dependent HVC that is endothelium independent, and does not involve K_{ATP} channels, products of lipoxygenase, cyclooxygenase, cytochrome P_{450} enzyme activity, or α-adrenergic, muscarinic, nicotinic, purinergic or serotoninergic receptors (Olson et al., 2001). If our hypotheses that H_2S directly produces vasoconstriction and that endogenous H_2S increases when P_{O_2} falls is correct, we expect to see an increased sensitivity of H_2S-constriction at low $[O_2]$, not an unmasking of H_2S relaxation. We also provide additional evidence for H_2S metabolism in the O_2 sensing mechanism by examining the contribution of its precursor, cysteine, and the effects of inhibitors of H_2S synthesis on HVC. The effects of H_2S, cysteine and enzyme inhibitors on vessel O_2 consumption were measured to determine whether H_2S exposure increased $\dot{M}_{O_2}$ and inhibitors decreased it. For comparison, we examined the O_2 sensitivity of H_2S contraction in lamprey aortas. These vessels are identical to hagfish aortas in their response to hypoxia (Olson et al., 2001) and evidence for the role of H_2S in O_2 sensing has been described (Olson et al., 2006).

MATERIALS AND METHODS

Animals

New Zealand hagfish, *Eptatretus cirrhatus* Forster (1122±165 g, *N*=26) were collected in Akaroa Harbour, New Zealand, and held in 14°C seawater aquaria at the University of Canterbury, Christchurch, NZ for at least 1 week prior to use. They were anesthetized in a combination of AQUI-S^{TM} (200 p.p.m.; Lower Hutt, New Zealand), benzocaine (400 p.p.m.) and MS-222 (400 p.p.m.). The ventral and dorsal aortas (VA and DA) and afferent and efferent branchial arteries (ABA and EBA) were removed cleaned of excess fat and blood and placed in 4°C hagfish Hepes saline until use (within 1–2 days). The saline was changed daily prior to use.

Sea lamprey (*Petromyzon marinus* L.; 130–450 g) were captured by the US Geological Survey, Biological Resources Division, in Michigan during the spring-summer spawning migration and airlifted to Indiana University School of Medicine–South Bend (IUSM-SB). They were housed in 500 l rectangular tanks in aerated, flowing well water (15°C), and exposed to a 12 h:12 h light:dark photoperiod. They were not fed. Lamprey were anesthetized in benzocaine (1:5000, wt:vol), and the vessels were dissected out and placed in Cortland buffered saline at 4°C until use.

Salmon (*Oncorhynchus tshawytscha* Walbaum) were obtained from a nearby hatchery, anaesthetized with 22 p.p.m. AQUI-S^{TM} in their holding tanks and then killed by pithing the brain and proximal spinal cord. The ventral aorta and afferent branchial arteries were rapidly excised and stored at 4°C in freshwater salmon Ringer's solution; the dorsal aorta is firmly attached to the vertebral column and cannot be removed intact. Storage and preparation of the salmon vessels for respirometry was identical to that described below for hagfish.

Experimental procedures were approved by the University of Canterbury's Animal Ethics Committee and the IUSM-SB IACUC.

Myography

Vessels were cut transaxially into 3–4 mm long segments, mounted on 280 μm diameter stainless-steel hooks and suspended in 20 ml, water-jacketed (12°C) smooth muscle chambers and bubbled with room air. Tension was measured with Grass FT03C force-displacement transducers (Grass Instruments, West Warwick, RI, USA) and collected electronically using Biopac model MP35 (Biopac Systems Inc., Goleta, CA, USA), or measured with MLT0210 isometric force transducers (ADInstruments, Castle Hill, Waverley, NSW, Australia) using Powerlab® systems with bridge amplifiers (ADInstruments). Data were archived at 2 Hz on notebook computers. A resting tension of 500±50 mg (Olson et al., 2001) was applied to the vessels for 45–60 min prior to experimentation. Vessels were maximally contracted with the acetylcholine analog carbamylcholine chloride (carbachol, 10 μmol l^{-1}; Fig. 1) until tension plateaued (30–45 min), then rinsed four times with buffer and resting tension re-established over the ensuing 60 min. They were then contracted a second time with 10 μmol l^{-1} carbachol, the rinse repeated, and the vessels were allowed to stabilize and resting tension re-established for the next 1–2 h. The tension produced by the second application of carbachol was used as the reference contraction for subsequent experiments.

Protocols

Carbachol dose response

Cumulative carbachol dose–response curves were initially obtained for efferent branchial arteries (Fig. 1) and dorsal aortas (not shown). Carbachol at 10 μmol l^{-1} produced maximum contraction in both

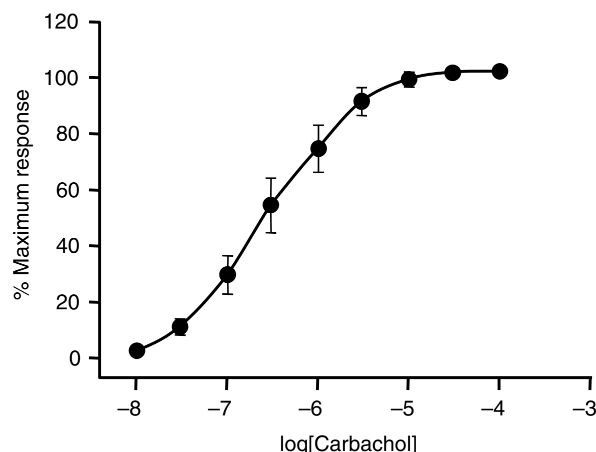

Fig. 1. Cumulative carbachol dose–response curve for efferent branchial arteries (mean ± s.e.m.; *N*=4).

vessels. Therefore, carbachol at this concentration was used twice at the beginning of experiments, first for initial activation and second for a reference contraction. A third carbachol (10 μmol l^{-1}) was also applied at the end of many experiments to determine if other treatments had non-specific effects on vascular reactivity.

Effect of H_2S on buffer pH

The dissolution of Na_2S in water produces H_2S and HS^- (collectively referred to in this study as H_2S) and increases pH. Because extracellular alkalinity can contract vascular smooth muscle independently of other exogenous stimuli (Smith et al., 2006), the buffering capacity of Hepes samples over the range of 1 μmol l^{-1} to 10 mmol l^{-1} H_2S was measured in triplicate using an Orion 911600 semi-micro pH electrode (Beverly, MA USA) and a PHM 84 pH meter (Radiometer, Copenhagen, Denmark).

H_2S dose-dependent responses

Cumulative H_2S dose–response curves (1 μmol l^{-1} to 1 mmol l^{-1}) were obtained for otherwise unstimulated, normoxic (bubbled with room air; 21% O_2) dorsal aortas and efferent branchial arteries. To determine if H_2S relaxed vessels, a second series of experiments were conducted with the vessels pre-contracted with 150 mmol l^{-1} KCl or 0.3 μmol l^{-1} carbachol prior to the H_2S doses. In pilot studies, H_2S had no effect on ventral aortas or afferent branchial arteries (N=4) and these vessels were not examined further.

Effect of P_{O_2} on H_2S responses

The effect of graded hypoxia on the H_2S dose response of hagfish dorsal aortas was examined by initially aerating groups of vessels with either 100% room air (P_{O_2}=157 mmHg), 6% air/94% N_2 (P_{O_2}=10 mmHg), or 100% N_2 (P_{O_2}<1 mmHg) for 20–30 min prior to and during the H_2S treatments (1 mmHg=0.133 kPa). The air/N_2 mixture was controlled with a Wöstoff type 1M 300/a-F gas mixing pump (H. Wöstoff, Bochum, Germany). The P_{O_2} was measured in one myograph chamber using a Microelectrodes MI-730 oxygen electrode and meter (Bedford, NH).

The effect of moderate hypoxia on the H_2S dose response of lamprey dorsal aortas was examined using the following protocol. Vessels were contracted twice with 80 mmol l^{-1} KCl, washed twice after each contraction, and then vigorously bubbled with 100% N_2 to produce a maximal HVC. After recovery (normoxia) the flow of N_2 was reduced to produce HVC that was 20±6% of the maximal HVC. This is equivalent to a bath P_{O_2} of 20–30 mmHg (Olson et al., 2001). Cumulative doses of H_2S (10 nmol l^{-1}–1 mmol l^{-1}) were applied during this moderate hypoxia.

Involvement of H_2S mechanisms in hagfish hypoxic vasoconstriction

The involvement of H_2S in hagfish HVC was examined by measuring the response of hypoxia-contracted (100% N_2) aortas to serial additions of the substrate for H_2S synthesis: L-cysteine (0.1, 1 and 10 mmol l^{-1}), amino-oxyacetate (AOA; 0.1, 1 and 4 mmol l^{-1}), an inhibitor of cystathionine β-synthase (CBS), D,L-propargylglycine (PPG; 0.1, 1 and 4 mmol l^{-1}), an inhibitor of cystathionine λ-lyase, or hydroxylamine (0.01, 0.1 and 1 mmol l^{-1}), a general inhibitor of pyridoxyl 5′-phosphate-dependent enzymes. Following a standard carbachol (10 μmol l^{-1}) contraction the vessels were thoroughly washed and gassed with 100% N_2 for 20–30 min until the hypoxic contraction stabilized. Cumulative doses of cysteine or inhibitors were applied during the hypoxic contraction followed by a final application of 10 μmol l^{-1} carbachol. Vessels were not washed prior to the final carbachol. The effects of hypoxia, cysteine, inhibitors and final carbachol were normalized relative to the reference carbachol contraction.

Oxygen consumption by hagfish dorsal aortas

Hagfish dorsal aortas used in the oxygen consumption ($\dot{M}_{O_2}$) experiments were stored and maintained in hagfish Hepes buffer containing gentamicin sulfate (200 μg ml^{-1}) to reduce the potential for micro-organisms to contribute to $\dot{M}_{O_2}$ (Rudin et al., 1970). Four to six aortas, 3–8 mm long, were freed of any remaining connective tissue and fat and threaded onto a 280 μm diameter stainless steel wire frame. They were then placed into 1 ml of either air-saturated or reduced [O_2] (P_{O_2} ~60 mmHg) Hepes buffer in model RC300 respirometers (Strathkelvin Instruments, Glasgow, Scotland) fitted with IL 1302 oxygen electrodes and maintained at 12°C. The electrode signal was fed into a Strathkelvin model 781 O_2 meter and then *via* a Powerlab 4SP to a notebook running Chart 5 software (both ADInstruments). Treatments were introduced into the respirometer *via* a small slot in the electrode holder with a 50 μl Hamilton syringe (Hamilton Co., Reno, Nevada, USA) fitted with a length of polythene tubing (Portex Ltd, Hythe, Kent, England; o.d. 0.61 mm, i.d. 0.28 mm). Oxygen consumption was determined from the equation:

$$\dot{M}_{O_2} = \Delta P_{O_2} \frac{\times 60 \times \alpha O_2 \times V}{t \times M} ,$$

where $\dot{M}_{O_2}$ is the rate of oxygen consumption (mmol O_2 mg^{-1} min^{-1}), ΔP_{O_2} is the change in P_{O_2} during the treatment period (in mmHg), αO_2 is the solubility of O_2 in seawater (in mmol O_2 l^{-1} $mmHg^{-1}$; hagfish plasma has very similar ionic composition), V is the volume of the respirometer (in l), t is the time (in s) between P_{O_2} measurements and M is the mass of the vessels in the respirometer (in mg).

The relationship between P_{O_2} and $\dot{M}_{O_2}$ was measured in vessels that were allowed to deplete the oxygen content from air saturation (P_{O_2} ~155 mmHg) down to zero. These experiments showed that the vessels could efficiently regulate their $\dot{M}_{O_2}$ between a P_{O_2} of 15 and 115 mmHg (see Results). Subsequent experiments on the effects of H_2S, cysteine and inhibitors of H_2S production were performed between 40 and 60 mmHg P_{O_2} where $\dot{M}_{O_2}$ was otherwise independent of P_{O_2}.

A cumulative H_2S concentration–$\dot{M}_{O_2}$ response was established for 1 μmol l^{-1}–1 mmol l^{-1} H_2S in both un-contracted and carbachol (100 μmol l^{-1})-contracted vessels. The effects of the H_2S precursor, L-cysteine (1 and 1 mmol l^{-1}) and inhibitors of H_2S production, AOA and HA (both 10 μmol l^{-1}–1 mmol l^{-1}), on $\dot{M}_{O_2}$ were also determined.

Oxygen consumption by salmon vessels

In the initial studies on hagfish vessels it became evident that contracting the vessels with carbachol had no effect on oxygen consumption. This was unexpected as it has been shown that contracting mammalian vessels increases oxygen consumption (Koenitzer et al., 2007). To determine whether this was an actual physiological difference or an experimental artifact, we repeated the studies with ventral aortas and afferent branchial arteries isolated from chinook salmon using the general protocol described above for hagfish vessels.

Chemicals

The composition of hagfish Hepes-buffered saline was (in mmol l^{-1}): 497.95 NaCl, 8.05 KCl, 5.10 $CaCl_2$, 9.00 $MgCl_2$, 3.04 $MgSO_4$, 3.00 Hepes [N-(2-hydroxyethyl)piperazine-N=–(2-ethane-sulfonic acid)]

acid form, 6.99 Hepes sodium salt, 5.55 glucose, pH 7.8. The composition of lamprey Cortland saline was (in mmol l^{-1}): 124 NaCl, 3 KCl, 2 $CaCl_2$, 0.57 $MgSO_4$, 12 $NaHCO_3$, 0.09 NaH_2PO_4, 1.8 Na_2HPO_4, 5.5 glucose, pH 7.8. The composition of salmon Ringer was (in mmol l^{-1}; 136.89 NaCl; 2.11 KCl; 0.99 $MgCl_2$; 1.30 $CaCl_2$; 3.00 Hepes acid form; 6.99 Hepes sodium salt; 0.30 sodium glutamate; 0.40 L-glutamine; 0.02 sodium aspartate; 0.05 DL-carnitine; 10.00 glucose, pH 7.6. AOA was purchased from ACROS Organics (Morris Plains, NJ, USA) all other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Calculations

Concentration response curves were expresses as a percentage of the maximal response. Vessel responses were normalized to the second carbachol contraction produced prior to experimentation. At the end of an experiment the vessel was blotted on paper toweling, weighed and vessel tension was normalized to wet mass, i.e. mg tension g^{-1} wet mass. Because the hypoxic responses of individual vessels were reproducible (Olson et al., 2001), each vessel served as its own control and treatment effects were statistically examined by paired *t*-test or repeated measures tests. Results are presented as mean ± s.e.m. Student's *t*-test and analysis of variance (ANOVA) were used for comparisons between vessels. Significance was assumed when $P \leqslant 0.05$.

Significant differences in rates of $\dot{M}_{O_2}$ and responses to drugs were determined using a repeated measures ANOVA. Where significant differences were calculated between means, Tukey's *post-hoc* tests showed which means were significantly different from each other. Paired Student's *t*-tests were used to detect differences between carbachol-treated and -untreated vessels in the $\dot{M}_{O_2}$ data (controls and at each concentration of H_2S). Significance was assumed when $P \leqslant 0.05$. All analyses were performed in Prism 4.00 (Graphpad software, San Diego, CA, USA).

RESULTS

Carbachol dose response of hagfish efferent branchial arteries

Carbachol produced a dose-dependent contraction of efferent branchial arteries (Fig. 1) with an EC_{50} of 0.275±0.148 μmol l^{-1} (*N*=4). Peak force was achieved at a concentration of 10 μmol l^{-1} carbachol.

Effects of H_2S on pH

The effects of increasing concentrations of H_2S on pH of hagfish Hepes buffer is shown in Fig. 2. Buffering was very efficient between 1 μmol l^{-1} and 1 mmol l^{-1} H_2S and increased less than 0.3 pH unit between 1 mmol l^{-1} and 3 mmol l^{-1} H_2S. However, pH increased nearly 2.5 units between 3 and 10 mmol l^{-1} H_2S. H_2S contractions appeared to be independent of pH between 1 μmol l^{-1} and 1 mmol l^{-1} H_2S, but concentrations above 1 mmol l^{-1} alkalinized the medium and this appeared to greatly augment H_2S contractions. H_2S concentrations were limited in subsequent experiments to 1 mmol l^{-1} in order to minimize the possibility of pH interference and to avoid complications that might result from changes in ionic strength or composition due to increasing the buffering capacity, or titration.

Vascular effects of H_2S

H_2S produced dose-dependent contractions in otherwise unstimulated dorsal aortas and efferent branchial arteries of hagfish (Fig. 3) but had no affect on ventral aortas or afferent branchial arteries (not shown). H_2S produced essentially identical dose-dependent contractions in KCl (150 mmol l^{-1}, *N*=4) and carbachol (0.3 μmol l^{-1}, *N*=4) pre-contracted dorsal aortas and in carbachol (0.3 μmol l^{-1}, *N*=4) pre-contracted efferent branchial arteries (data not shown for pre-contracted vessels). There was no obvious H_2S-mediated relaxation in either pre-contracted or otherwise un-stimulated vessels.

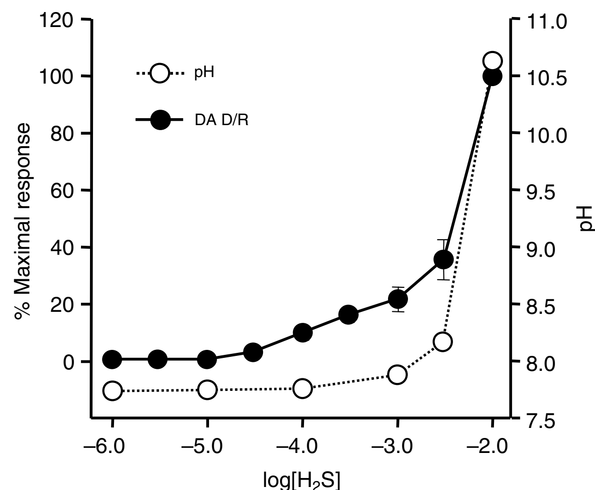

Fig. 2. Effects of H_2S (administered as Na_2S) on buffer pH (open circles, *N*=2) and contraction of dorsal aortas (filled circles, *N*=4). pH is relatively stable at an [H_2S] of ≤1 mmol l^{-1}, but higher concentrations produce increasing alkalinity and appear to augment aortic contraction.

Effect of P_{O_2} on H_2S responses of hagfish and lamprey dorsal aortas

Hagfish dorsal aortas bubbled with 100% N_2 were significantly more sensitive to low H_2S concentrations than aortas bubbled with room air and the H_2S dose–response curve of hypoxic (anoxic) vessels appeared to have two components (Fig. 3). H_2S-mediated contractions of hagfish aortas bubbled with 6% air/94% N_2 (data not shown) were not significantly different from aortas bubbled with room air.

H_2S produced dose-dependent contractions of lamprey dorsal aortas (Fig. 3). Moderate hypoxia increased H_2S sensitivity between 10 and 300 μmol l^{-1} H_2S (*P* value at 30 μmol l^{-1} H_2S was 0.053). Hypoxia did not affect the magnitude of the 1 mmol l^{-1} H_2S contraction which in hypoxia was 41±5% and in normoxia 43±4% of a 80 mmol l^{-1} KCl contraction. H_2S at concentrations between 10 nmol l^{-1} to 1 μmol l^{-1} did not affect either normoxic or hypoxic vessels (not shown).

Involvement of H_2S mechanisms in hagfish hypoxic vasoconstriction

In these experiments vessels were contracted with 10 μmol l^{-1} carbachol, washed, then continuously contracted with 100% N_2 aeration. During the hypoxic contraction, the vessels were given cumulative additions of cysteine or inhibitors and this was followed, without washing the vessels, by 10 μmol l^{-1} carbachol.

The effect of L-cysteine, a substrate for H_2S synthesis, on hypoxic contractions of hagfish dorsal aortas is shown in Fig. 4, top left panel. 100 μmol l^{-1} cysteine produced a consistent, but statistically insignificant increase in the force of the N_2 contraction. Increasing cysteine to 1 mmol l^{-1} significantly ($P<0.05$) contracted the vessels to approximately double that in the original N_2

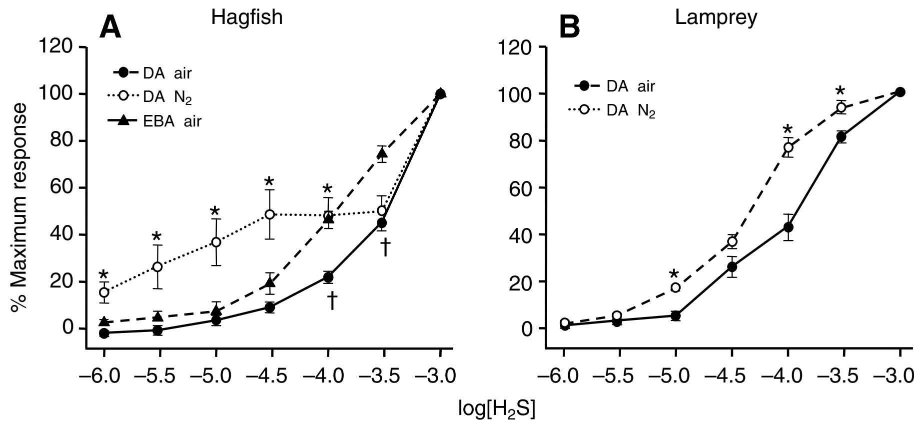

Fig. 3. (A) H_2S dose–response curves for hagfish dorsal aortas (filled circles; *N*=22) and efferent branchial arteries (filled triangles; *N*=8) bubbled with room air, and dorsal aortas bubbled with 100% nitrogen (open circles; *N*=10). Aortas bubbled with nitrogen were significantly (*) more sensitive to low concentrations of H_2S than air-bubbled aortas and appear to have a two-phase response to H_2S. H_2S at 100 and 300 μmol l^{-1} produces a significantly greater response in normoxic efferent branchial arteries than normoxic dorsal aortas (†). (B) H_2S dose–response curves for lamprey dorsal aortas bubbled with room air (filled circles; *N*=8) or during moderate hypoxia (open circles; *N*=8). Hypoxic vessels were significantly (*) more sensitive to H_2S. Values are means ± s.e.m.

contraction. Raising cysteine to 10 mmol l^{-1} produced an immediate relaxation back to the pre-cysteine (N_2) level ($P<0.05$). The carbachol (10 μmol l^{-1}) contraction at the end of the experiment, in the presence of N_2 and 10 mmol l^{-1} cysteine, was not significantly different from the reference carbachol contraction (90±14% of reference, *N*=7).

The effect of the cystathionine β-synthase (CBS) inhibitor, amino-oxyacetate (AOA), on hypoxic vasoconstriction of the dorsal aorta is shown in Fig. 4 top right panel. Hypoxic contractions were unaffected by 100 μmol l^{-1} and 1 mmol l^{-1} AOA. 4 mmol l^{-1} AOA completely inhibited the hypoxic contraction ($P<0.05$). The carbachol (10 μmol l^{-1}) contraction at the end of the experiment, in the presence of 4 mmol l^{-1} AOA, was not significantly different from the reference carbachol contraction (111±12% of reference, *N*=8).

As shown in Fig. 4, lower left panel, the cystathionine λ-lyase (CSE) inhibitor, propargyl glycine (PPG; between 100 μmol l^{-1} and 4 mmol l^{-1}) had no effect on the hypoxic contraction. A carbachol (10 μmol l^{-1}) contraction at the end of the experiment, in the presence of PPG, was similarly unaffected (104±7% of reference, *N*=8).

Hydroxylamine (HA), an uncoupler of pyridoxyl 5′-phosphate-dependent enzymes including CBS and CSE, at 10 μmol l^{-1} significantly increased the force of the N_2 contraction (Fig. 4, lower right panel). Increasing HA to 100 μmol l^{-1} and 1 mmol l^{-1} produced slight, but statistically insignificant, further increases in tension. The carbachol (10 μmol l^{-1}) contraction at the end of the experiment, in the presence of HA, was not significantly different from the reference carbachol contraction (103±12% of reference, *N*=8).

Vessel O_2 consumption

The relationship between P_{O_2} and oxygen consumption ($\dot{M}_{O_2}$) in uncontracted and carbachol pre-contracted hagfish dorsal aortas is shown in Fig. 5A. $\dot{M}_{O_2}$ was well maintained around 2.4 pmol mg^{-1} min^{-1} between 15 and 115 mmHg P_{O_2} but doubled between 115 and 155 mmHg and fell to zero as P_{O_2} approached zero. $\dot{M}_{O_2}$ fell to 90% of the regulated rate at a P_{O_2} of 12 mmHg and the P_{O_2} at which the regulated $\dot{M}_{O_2}$ fell to half (P_{50}) was 3 mmHg. Pre-treating hagfish aortas with 100 μmol l^{-1} carbachol did not significantly affect $\dot{M}_{O_2}$. $\dot{M}_{O_2}$ was also well-regulated in unstimulated salmon vessels between 15 and 115 mmHg P_{O_2} but the rate of oxygen consumption per unit tissue mass was five times that of hagfish aortas (Fig. 5B). Pre-treating salmon vessels with 100 μmol l^{-1} carbachol nearly doubled $\dot{M}_{O_2}$ at all but the lowest P_{O_2}.

The effects of H_2S, cysteine, AOA and HA on $\dot{M}_{O_2}$ are summarized in Fig. 6. 10 μmol l^{-1} H_2S significantly stimulated $\dot{M}_{O_2}$ whereas 100 μmol l^{-1} and 1 mmol l^{-1} significantly inhibited $\dot{M}_{O_2}$. A 12-fold increase in $\dot{M}_{O_2}$ was produced by 10 mmol l^{-1} cysteine; in many experiments 1 mmol l^{-1} cysteine often appeared to increase $\dot{M}_{O_2}$ as well, although this was not statistically significant. $\dot{M}_{O_2}$ was inhibited by either 10 mmol l^{-1} AOA or 10 mmol l^{-1} HA. Other concentrations of AOA (10 μmol l^{-1}–1 mmol l^{-1}) and HA (10 μmol l^{-1}–1 mmol l^{-1}) did not significantly affect $\dot{M}_{O_2}$. Pre-contraction with 100 μmol l^{-1} carbacol did not significantly affect $\dot{M}_{O_2}$ in vessels treated with 10 μmol l^{-1}, 100 μmol l^{-1} or 1 mmol l^{-1} H_2S (Fig. 6), 10 mmol l^{-1} cysteine, 10 mmol l^{-1} AOA or 10 mmol l^{-1} HA (*N*=5 for all; data not shown), although $\dot{M}_{O_2}$ of vessels in 10 μmol l^{-1} H_2S was significantly greater than $\dot{M}_{O_2}$ of vessels in 100 μmol l^{-1} H_2S (Fig. 6).

DISCUSSION

The present studies support our hypotheses that, (1) H_2S directly produces vasoconstriction, and (2) the metabolism of H_2S is involved in the oxygen sensing and/or signal transduction cascade in hypoxic vasoconstriction of the hagfish aorta.

H_2S as a vasoconstrictor

As shown in Fig. 3, the H_2S sensitivity in both hagfish and lamprey aortas increased when P_{O_2} was decreased. Hagfish vessels bubbled with 100% N_2 responded to 1 μmol l^{-1} H_2S, whereas 100 μmol l^{-1} H_2S was required to contract vessels bubbled with room air (Fig. 3). Similarly, the apparent H_2S thresholds for hypoxic (P_{O_2} ~20–30 mmHg) and normoxic lamprey vessels were 10 and 30 μmol l^{-1} H_2S, respectively (Fig. 3). Furthermore, the H_2S dose–response curves for both animals were left-shifted in low P_{O_2}. Thus although the effect of hypoxia on the H_2S response of lamprey vessels was less dramatic than that of hagfish vessels (probably because the hypoxia was less severe), the basic responses were, nevertheless, quite similar. As described below, these results support the hypothesis that H_2S has direct vasoconstrictory activity in specific vessels.

Koenitzer et al. (Koenitzer et al., 2007) observed a bi-phasic effect of H_2S on rat aortas; low H_2S concentrations produced dilation and high concentrations (1 mmol l^{-1}) produced contraction. They also found that H_2S-mediated dilation of rat aortas became more sensitive to H_2S at low P_{O_2}. These authors (Koenitzer et al., 2007) suggested that the H_2S-mediated vasoconstriction of aortas bubbled with room air was due to an oxidation product of H_2S, not H_2S itself, and that the reason rat aortas became more sensitive to H_2S during hypoxia

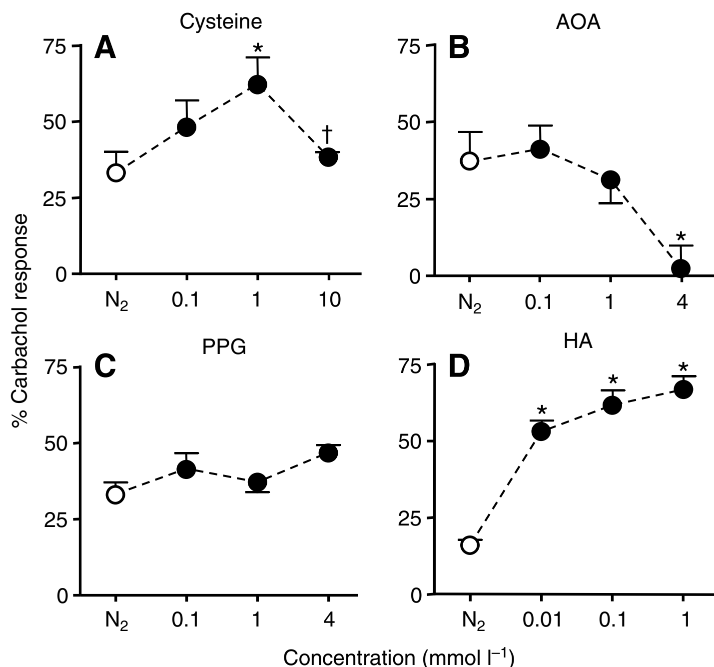

Fig. 4. Effects of (A) L-cysteine, the substrate for H_2S synthesis, (B) the cystathionine β-synthase inhibitor, amino-oxyacetate (AOA), (C) the cystathionine λ-lyase inhibitor, propargyl glycine (PPG) and (D) an uncoupler of pyridoxyl 5′-phosphate-dependent enzymes, hydroxylamine (HA) on hypoxic (100% N_2) vasoconstriction of hagfish dorsal aorta. Vessels were continuously contracted with N_2 then given cumulative additions of cysteine or inhibitors followed by 10 μmol l^{-1} carbachol (CBC). Values are expressed as the average tension (mean ± s.e.m.) as the percentage of the reference carbachol contraction (100%; dashed line). Hypoxic contractions (N_2) were generally ~30% of the reference carbachol contraction. Cysteine at 1 mmol l^{-1} approximately doubled the force of the original N_2 contraction, whereas raising cysteine to 10 mmol l^{-1} relaxed the vessels back to the original N_2 level (* significant increase from N_2; † significant decrease from 1 mmol l^{-1} cysteine; *N*=7). Hypoxic contractions were unaffected by 100 μmol l^{-1} and 1 mmol l^{-1} AOA. At 4 mmol l^{-1} AOA, the hypoxic contraction was completely inhibited (*; *N*=8). PPG did not significantly affect the N_2 contraction (*N*=8). HA, 10 μmol l^{-1} HA significantly (*) increased the force of the N_2 contraction and tension was maintained at 100 μmol l^{-1} and 1 mmol l^{-1} (*N*=8).

was because in the absence of this putative oxidation product there was no offsetting constrictory stimulus to compete with the direct H_2S dilation. Our findings argue against these hypotheses. First, H_2S only constricted hagfish and lamprey dorsal aortas and therefore the increased sensitivity observed at low P_{O_2} could not be due to removal of a competing (in this case dilatory) process. Second, it seems unlikely that production of this hypothetical vasoconstrictory oxidation product of H_2S would increase when the vessels are bubbled with 100% N_2. An alternative, and we think more plausible, explanation for the increased H_2S sensitivity, and one that is consistent with our (Olson et al., 2006) hypothesis of H_2S involvement in HVC (see below), is that when P_{O_2} falls endogenous H_2S increases. Therefore, less exogenous H_2S is required for vasoconstriction.

Ali et al. (Ali et al., 2006) and Kubo et al. (Kubo et al., 2007) observed the opposite effects of Koenitzer et al. (Koenitzer et al., 2007), i.e. low H_2S concentrations (<200 μmol l^{-1}) contracted, and elevated H_2S concentrations (200–1600 μmol l^{-1}) relaxed rat aortic rings. They attributed the low-dose H_2S contraction to H_2S combining with NO and thereby removing the tonic NO-mediated vasodilation (Ali et al., 2006), or directly inhibiting endothelial nitric oxide synthase (Kubo et al., 2007). This also is unlikely to occur in either hagfish or lamprey dorsal aortas because, (1) there is at present no evidence for endothelial NO production by either of these vessels (Olson et al., 2001), (2) exogenous NO produces a modest contraction in the ventral aorta of the hagfish, *Myxine glutinosa* (Evans and Harrie, 2001), and (3) NO synthesis from L-arginine and O_2 would be expected to be reduced during prolonged hypoxia. Our studies suggest that H_2S may directly constrict specific vessels and that this response is an intrinsic property of the smooth muscle cells. Clearly, however, variations in this response can be achieved through H_2S interactions with other vasoregulatory mechanisms.

H_2S metabolism in O_2 sensing

Our model of the role of H_2S metabolism in oxygen sensing and/or signal transduction appears to accommodate both hypoxic vasoconstriction (HVC) and hypoxic vasodilation (HVD) in vertebrate smooth muscle (Olson et al., 2007). This model is based on the balance between H_2S production by vascular tissue and its inactivation through oxidation, and it provides a simple and rapid mechanism that couples the concentration of a vasoactive molecule directly to P_{O_2}. The model is supported by observations that the responses of a wide variety of vessels (either constriction, dilation or multi-phasic) to hypoxia and H_2S are identical, H_2S is constitutively produced by blood vessels, cysteine the metabolic precursor of H_2S, augments HVC and inhibitors of H_2S production inhibit HVC and HVD. The present study provides additional support for the involvement of H_2S in HVC in hagfish vessels.

Similarity of vascular responses to H_2S and hypoxia

The responses of New Zealand hagfish vessels to H_2S are in many respects similar to those produced by hypoxia. H_2S and hypoxia (Olson et al., 2001), appear to be exclusively vasoconstrictory in hagfish dorsal aortas and efferent branchial arteries because they consistently contracted both un-stimulated and pre-contracted vessels. Conversely, neither H_2S nor hypoxia produced a sustained response in ventral aortas or afferent branchial arteries. H_2S and hypoxic (Olson et al., 2001) contractions of aortas and efferent branchial arteries were also unaffected by pre-contraction with KCl. Furthermore, because KCl pre-contraction presumably depolarizes smooth muscle cells it is likely that the mechanism of H_2S excitation is independent of cell depolarization; evidence for depolarization-independent HVC in these vessels has also been presented previously (Olson et al., 2001). This is in contrast to the H_2S-mediated relaxation of rat aorta (Zhao et al., 2001; Zhao and Wang, 2002) and trout efferent branchial arteries (Dombkowski et al., 2004) where elevated KCl partially inhibits the response. Collectively, these findings suggest that H_2S contraction and HVC have a common, or at least similar, excitation pathway in hagfish vessels. This is consistent with other studies (Olson et al., 2001; Dombkowski et al., 2004; Dombkowski et al., 2005; Olson et al., 2006; Russell et al., 2007) that have shown that the vascular response to hypoxia is identical to that of H_2S irrespective of whether this response is contraction, relaxation, multi-phasic, or, as in the case of hagfish ventral aorta and afferent branchial arteries, no response at all. We have also observed identical hypoxic and H_2S responses in trout

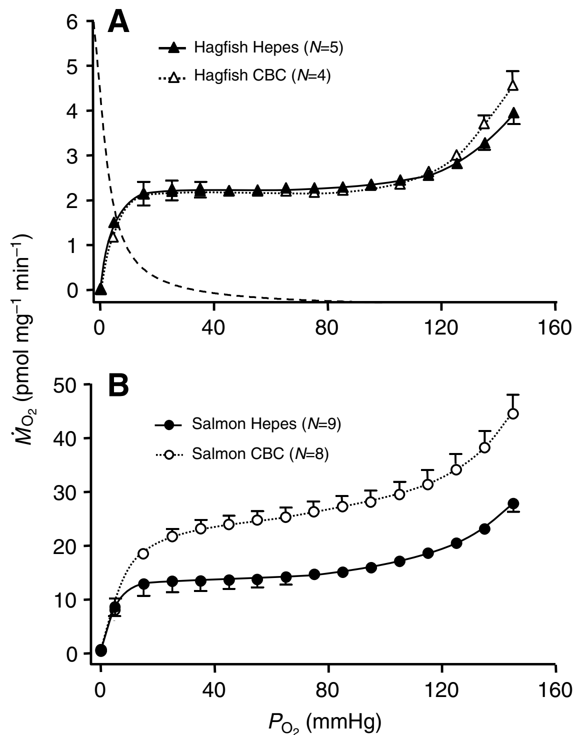

Fig. 5. Effect of P_{O_2} on oxygen consumption ($\dot{M}_{O_2}$) by hagfish dorsal aortas (A) and salmon vessels (B). $\dot{M}_{O_2}$ is tightly regulated in unstimulated hagfish aortas (filled triangles) between a P_{O_2} of 15 and 115 mmHg (1 mmHg=0.133 kPa), but varies with P_{O_2} at either extreme. At a P_{O_2} of 12 mmHg $\dot{M}_{O_2}$ falls to 90% of the regulated level and $\dot{M}_{O_2}$ is halved at 3 mmHg (P_{50}). $\dot{M}_{O_2}$ was not affected by pre-contracting hagfish aortas with 100 μmol l^{-1} carbachol (CBC; open triangles). $\dot{M}_{O_2}$ was also regulated in unstimulated salmon vessels (B; filled circles) between a P_{O_2} of 15 and 115 mmHg; however, per unit tissue mass it was five times greater than that of hagfish aortas. Pre-treatment of salmon vessels with 100 μmol l^{-1} carbachol nearly doubled $\dot{M}_{O_2}$ at all P_{O_2} (open circles). Mean ± s.e.m.; *N* indicates the number of groups of 4–6 vessels per group in each experiment (standard error not shown when within the symbol). Broken line shows the P_{O_2} dependency of hypoxic contraction in hagfish dorsal aortas [redrawn from Olson et al. (Olson et al., 2001)].

urinary bladder (Dombkowski et al., 2006) and thus hypoxia and H_2S appear to have a common, or at least similar excitation pathway in vertebrate smooth muscle in general.

Metabolic coupling of HVC to H_2S production

Cysteine, which is presumed to be the precursor of H_2S production in animals (Julian et al., 2002) increases the magnitude of HVC at lower concentrations (Fig. 4) suggesting that it increases tissue production of H_2S. This is consistent with a cysteine-enhanced HVC in lamprey dorsal aortas and bovine pulmonary arteries and enhanced HVD observed in rat thoracic aortas (Olson et al., 2006). Further elevation of the cysteine concentration (10 mmol l^{-1}) inexplicably reduced the HVC in hagfish dorsal aorta. This may be due to a feedback-type inhibitory effect of cysteine on H_2S production, as we (R.D., S. Head, N. Whitfield and K.O., unpublished observation) have also observed elevated cysteine (10 mmol l^{-1} or 100 mmol l^{-1}) inhibition of H_2S production in homogenized bovine heart or trout vessels, respectively, which is consistent with feedback inhibition. Alternatively, cysteine at a concentration of 10 mmol l^{-1} may be toxic to smooth muscle cells. However, the fact that carbachol contractions on top of 10 mmol l^{-1} cysteine were not significantly different from the reference contractions, suggests that cytotoxicity of cysteine at 10 mmol l^{-1} is unlikely, and the reason for this inhibition remains to be identified.

The effects of inhibitors of H_2S production provide additional evidence for H_2S signaling in hypoxic responses. As shown in Fig. 4, amino-oxyacetate (AOA), an inhibitor of cystathionine β-synthase (CBS) completely inhibited HVC in hagfish aortas, whereas the cystathionine λ-lyase (CSE) inhibitor, propargyl glycine (PPG) was ineffective (Fig. 4). This suggests that HVC in the hagfish dorsal aorta is dependent upon H_2S synthesis *via* CBS. This is in contrast to mammalian systemic vessels where CSE, but not CBS, catalyzes H_2S production (Hosoki et al., 1997; Zhao et al., 2003). We (Olson et al., 2006) have also shown that inhibition of CSE, but not CBS, blocked HVD in rat aortas, whereas inhibition of CBS, but not CSE, blocked HVC in bovine pulmonary arteries. Interestingly, in trout, H_2S produces a tri-phasic relaxation-contraction-relaxation (Dombkowski et al., 2004) and these vessels appear to possess both CBS and CSE (G. Yang, R. Wang and K.O., unpublished observation). These studies not only support the hypothesis of H_2S as a vascular O_2 sensor but they also provide additional evidence that different enzymes for H_2S production, CBS and CSE, may mediate HVC and HVD, respectively in different vessels.

Contractions produced by carbachol while hagfish dorsal aortas were exposed to AOA or PPG were not significantly different from the reference contraction produced by carbachol in the absence of inhibitors. Thus the inhibitory effect of AOA on HVC could not be due to general inhibition of the contractile apparatus. Separate H_2S and ligand-mediated responses have also been observed in other vessels (Dombkowski et al., 2004; Olson et al., 2006), indicating that the pathway for H_2S activation is not shared with some of the more common ligand-mediated mechanisms.

It is not clear why hydroxylamine potentiated HVC in hagfish aortas, although a non-specific effect seems likely. Hydroxylamine inhibits at least 100 enzymes that use pyridoxyl 5′-phosphate as a co-enzyme (Kery et al., 1999; Tang et al., 2005) including CBS and CSE. Although we expected it to act in a manner similar to AOA and inhibit vasoconstriction, it was the most potent constrictor tested, on a molar basis. Interestingly, despite turning the vasa vasorum brown, probably as a result of an action on heme groups (Canty and Driedzic, 1987; Nichols and Weber, 1989), and contracting the vessels, the vessels remained viable and the response to the final carbachol exposure was not diminished.

Effect of P_{O_2} on O_2 consumption

As shown in Fig. 5, hagfish aortas display a remarkable ability to maintain O_2 consumption ($\dot{M}_{O_2}$) over a wide range of ambient P_{O_2} (~15–115 mmHg). It is not clear why the vessels lose their regulatory ability when P_{O_2} exceeds ~115 mmHg, but this may be near the maximum P_{O_2} these vessels encounter in the wild, i.e. in air-saturated seawater (inspired P_{O_2} of 156 mmHg), arterial P_{O_2} was 109 mmHg (Forster et al., 1992). When P_{O_2} falls below ~15 mmHg, $\dot{M}_{O_2}$ also falls. The P_{O_2} over which $\dot{M}_{O_2}$ decreases is quite similar to the P_{O_2} at which HVC increases (dashed line in Fig. 5).

The P_{O_2} at which $\dot{M}_{O_2}$ begins to decrease in hagfish dorsal aortas (15 mmHg) is somewhat less than the 20 mmHg critical P_{O_2} (P_{O_2} at which $\dot{M}_{O_2}$ was reduced by 5%) in isolated rat aortas (Koenitzer et al., 2007). However, the $\dot{M}_{O_2}$ for rat aortas [78 pmol mg^{-1} min^{-1} (Koenitzer et al., 2007)] is 32.5 times greater than the $\dot{M}_{O_2}$ for hagfish aortas (2.4 pmol mg^{-1} min^{-1}). Even assuming a Q_{10} of 2.4, the 25°C temperature difference between our study and that of Koenitzer et al. (Koenitzer et al., 2007) would only account for a sixfold difference in $\dot{M}_{O_2}$. In fact, these differences would probably be even

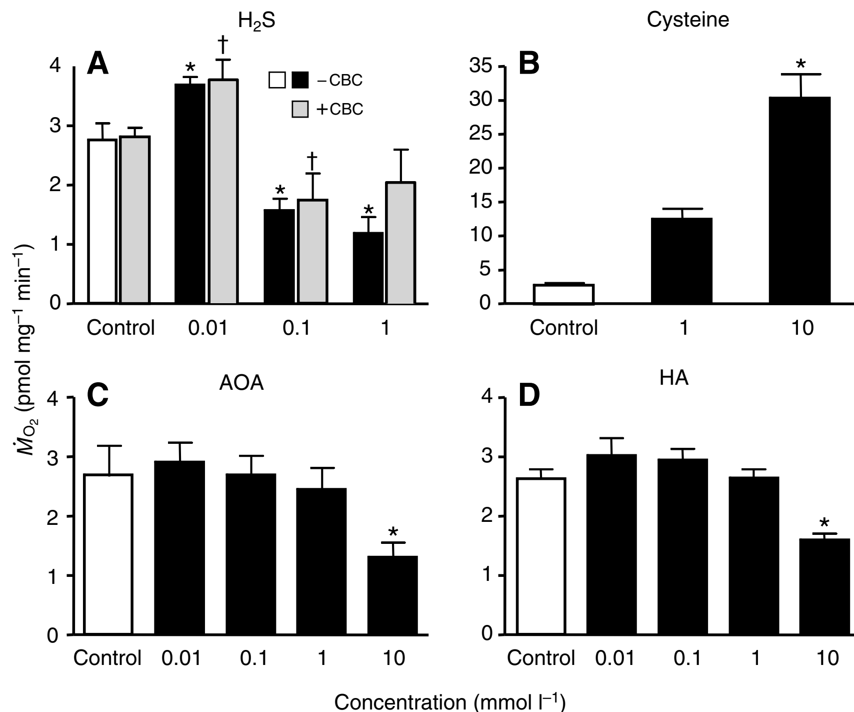

Fig. 6. Effect of (A) H_2S (as Na_2S), (B) the substrate for H_2S synthesis, L-cysteine (cysteine), and inhibitors of H_2S production, (C) amino-oxyacetate (AOA) and (D) hydroxylamine (HA), on oxygen consumption ($\dot{M}_{O_2}$) by hagfish dorsal aortas. $\dot{M}_{O_2}$ was stimulated by 10 μmol l^{-1} H_2S and 10 mmol l^{-1} cysteine and inhibited by 100 μmol l^{-1} and 1 mmol l^{-1} H_2S and by 10 mmol l^{-1} AOA and 10 mmol l^{-1} HA. Carbachol (100 μmol l^{-1}) pre-treatment (+CBC) did not affect $\dot{M}_{O_2}$ at any [H_2S] compared to untreated (–CBC) vessels, although $\dot{M}_{O_2}$ was significantly different between 10 μmol l^{-1} and 100 μmol l^{-1} H_2S in carbachol pretreated vessels. Mean + s.e.m.; N=7 (H_2S), 5 (cysteine), 4 (AOA), 4 (HA) groups of 4–6 vessels per group; *significantly different from respective control; †significantly different from +CBC control.

greater if the O_2 solubility coefficients were accounted for; mammalian (human) plasma at 37°C is 1.26 μmol l^{-1} $mmHg^{-1}$ and seawater (with the same osmolarity of hagfish plasma) at 12°C is 1.72 μmol l^{-1} $mmHg^{-1}$ (Boutilier et al., 1984).

Koenitzer et al. (Koenitzer et al., 2007) also showed that $\dot{M}_{O_2}$ more than doubled when rat aortas were contracted with phenylephrine. We did not find any difference in $\dot{M}_{O_2}$ between un-contracted and contracted hagfish aortas, perhaps because $\dot{M}_{O_2}$ was so low to begin with, or, more likely, because once hagfish aortas are contracted they are able to maintain tension with little additional energy expenditure. The latter point may be related to the hypoxia tolerance of hagfish vessels where hypoxic contractions can be sustained for 8 h of continuous aeration with 100% N_2 (Olson et al., 2001). Many non-mammalian vertebrates, especially the more 'primitive' ones are considerably more hypoxia tolerant than mammals because of their ability to downregulate cellular metabolism and balance ATP demand with ATP supply (Boutilier, 2001). Hypoxia tolerance varies across hagfish species and interestingly, *E. cirrhatus* does not voluntarily tolerate an ambient P_{O_2} of less than 45 mmHg (82 μmol l^{-1}) at 11°C (Forster, 1992). Clearly, the lack of an increase in $\dot{M}_{O_2}$ was not due to the technique used as carbachol nearly doubled $\dot{M}_{O_2}$ in salmon.

Despite the elevated metabolic rate of rat aortas, the tension (in mg tension mg^{-1} wet mass) produced by KCl contraction of rat aortas, which varies from 240 (Olson et al., 2001) to 720 (Resende et al., 2004) is only 2.5–7.5 times greater than a KCl contraction of hagfish dorsal aorta (94±12, N=4; data from this study). Thus is appears in rat vessels that either more oxygen is consumed for non-contractile-related activities, or that force development is energetically less efficient.

Relationship between O_2 consumption and H_2S production

In many organisms, O_2 consumption is affected by H_2S. At low [H_2S], O_2 often increases because of the use of H_2S in mitochondrial ATP synthesis or for H_2S detoxification; at elevated [H_2S], O_2 consumption often decreases because of H_2S inhibition of mitochondrial cytochrome *c* oxidase (Grieshaber and Völkel, 1998), or perhaps even a general metabolic depression (Blackstone et al., 2005). H_2S also affects $\dot{M}_{O_2}$ in un-contracted hagfish aortas (Fig. 6) in a manner consistent with that described by Grieshaber and Völkel (Grieshaber and Völkel, 1998). An increase in $\dot{M}_{O_2}$ is also predicted by our (Olson et al., 2006) model of H_2S oxidation by blood vessels as a mechanism to inactivate H_2S during normoxia. Higher (100 μmol l^{-1} and 1 mmol l^{-1}) [H_2S] inhibits $\dot{M}_{O_2}$ (Fig. 3) but not tension development (Figs 2, 3). This likely reflects the inherently low energy cost of force development, consistent with our observation that $\dot{M}_{O_2}$ does not change even during maximal carbachol contraction and it also provides a mechanism for sustaining HVC even when mitochondrial energy production is compromised.

The increase in $\dot{M}_{O_2}$ produced by cysteine and the decrease in $\dot{M}_{O_2}$ produced by AOA (Fig. 6) are also consistent with a positive and negative effect on H_2S production by hagfish dorsal aortas. It is not clear why 10 mmol l^{-1} cysteine appeared to decrease tension, yet increase $\dot{M}_{O_2}$. This suggests that H_2S oxidation continues, although the mechanism that causes contraction is subject to feedback inhibition. However, other explanations are also plausible, i.e. the experimental conditions were different (anoxia in myograph studies, P_{O_2} ~40-50 mmHg in H_2S studies), this cysteine concentration is near the threshold for both processes, or there are temporal differences in responses. The effects of hydroxylamine on $\dot{M}_{O_2}$ do not correlate with its effects on tension and may also be nonspecific as it inhibits many other enzymes (Zollner, 1989).

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