Hydrogen sulfide mediates hypoxic vasoconstriction through a production of mitochondrial ROS in trout gills

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Running title: Effects of H$_2$S in perfused trout gills
Hypoxic pulmonary vasoconstriction (HPV) is an adaptive response that diverts pulmonary blood flow from poorly ventilated and hypoxic areas of the lung to more well-ventilated parts. This response is important for the local matching of blood perfusion to ventilation and improves pulmonary gas exchange efficiency. HPV is an ancient and highly conserved response, expressed in the respiratory organs of all vertebrates, including lungs of mammals, birds and reptiles, amphibian skin and fish gills. The mechanism underlying HPV and how cells sense low PO$_2$ remains elusive. In perfused trout gills (*Oncorhynchus mykiss*) acute hypoxia as well as H$_2$S caused an initial and transient constriction of the vasculature. Inhibition of the enzymes cystathionine-β-synthase and cystathionine-γ-lyase, which blocks H$_2$S production, abolished the hypoxic response. Individually blocking the four complexes in the electron transport chain abolished both the hypoxic and the H$_2$S mediated constriction. Glutathione, an antioxidant and scavenger of superoxide, attenuated the vasoconstriction in response to hypoxia and H$_2$S. Furthermore, diethyldithiocarbamate, an inhibitor of superoxide dismutase, attenuated the hypoxic and H$_2$S constriction. This strongly suggests that H$_2$S mediates the hypoxic vasoconstriction in trout gills. H$_2$S may stimulate the mitochondrial production of superoxide, which is then converted to H$_2$O$_2$. Thus, H$_2$O$_2$ may act as the ‘down stream’ signaling molecule in hypoxic vasoconstriction.

*Key words:* hydrogen sulfide, oxygen-sensing, hypoxia, mitochondria, reactive oxygen species, trout, gill resistance
INTRODUCTION

Hypoxic pulmonary vasoconstriction (HPV) is an adaptive response that diverts pulmonary blood flow from inadequately ventilated and hypoxic parts of the lung to better-ventilated areas (42). Thus, HPV is important for local matching of blood perfusion to ventilation; it improves pulmonary gas exchange efficiency and, consequently, maintains arterial oxygenation (4, 6, 42). The primary site of HPV is the precapillary pulmonary arterioles, where low alveolar oxygen causes a constriction of the vasculature that is reversible upon reoxygenation (47). Hypoxic vasoconstriction is an ancient and highly conserved response expressed in the respiratory organs of all vertebrates, including lungs of mammals, birds and reptiles, amphibian skin and fish gills (10, 17, 34, 35, 37, 42).

Numerous mechanisms have been proposed to explain how respiratory blood vessels sense low PO2 and transduce this signal into constriction, but none have received unequivocal support (44). Mitochondria are the main consumers of oxygen and, therefore, are considered a major candidate for the location of an oxygen-sensor. Several theories suggest that hypoxia alters redox state of the cell and mitochondrial production of reactive oxygen species (ROS), although it is not clear whether oxygen deprivation causes an increase or decrease in ROS production (3, 44, 45). The change in ROS may inhibit redox sensitive K+ channels resulting in a depolarisation of the pulmonary arterial smooth muscle cells (PASMC) causing contraction as intracellular Ca2+ concentration ([Ca2+]i) rises (1, 19, 48). Thus, the O2-sensor and signalling pathways for hypoxic vasoconstriction remain unresolved. Nevertheless, there is now evidence that a mechanism involving hydrogen sulfide (H2S) metabolism may explain oxygen-sensing and hypoxic vasoconstriction (20, 23, 25).

H2S has appeared as a gaseous signalling molecule of substantial biological potential involved in a vast number of physiological functions (40, 43). The gasotransmitter is produced in various tissues including endothelium and vasculature by two cytosolic
pyridoxyl-5’-phosphate dependent enzymes, cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE), as well as the pyridoxal-5’-phosphate independent enzyme 3-mercaptopopyruvate sulfurtransferase (3-MST) (32, 40). H_{2}S is an important regulator of blood pressure and infusion of H_{2}S donors elicits vascular responses that closely resemble those of hypoxia in vessels from all vertebrates studied so far (8, 25, 50). Recently, it was suggested that H_{2}S metabolism serves as an O_{2}-sensing mechanism that mediates hypoxic vasoconstriction or vasodilation in vascular smooth muscles (25). The model is based on the inverse relationship existing between \([O_{2}]\), which is not by itself vasoactive, and \([H_{2}S]\), which is. H_{2}S is constitutively synthesized in the cell cytoplasm and oxidized in the mitochondria, and the simple balance between H_{2}S production and the amount of O_{2} available for H_{2}S oxidation, i.e. tissue PO_{2}, therefore, determine the actual concentration of biologically active H_{2}S. However, the signalling pathway linking the increase in H_{2}S with vasoconstriction remains unknown.

Here we explore the hypothesis that H_{2}S is a vascular oxygen-sensor by identifying and comparing the mechanisms underlying H_{2}S and hypoxia mediated vasoconstriction. Although controversial, it seems that the HPV in mammals is dependent on the endothelium (16, 30, 33). In cyclostomes, a phylogenetically early group of vertebrates, however, hypoxia causes constriction of vascular smooth muscles independent of the endothelium (24). This indicates that hypoxic vasoconstriction is an ancient and fundamental response intrinsic to the vasculature that has acquired additional regulatory factors as vertebrates evolved to be more sensitive to hypoxia. Thus, HPV in mammals has evolved to be a complex process associated with several signalling pathways (24). The core mechanisms of the vascular O_{2}-sensor, therefore, are likely to be unravelled in phylogenetically ancient groups of vertebrates, such as fish, in which the secondary regulatory factors are absent. In the present study we show that
H$_2$S mediates hypoxic vasoconstriction in perfused trout gills through a stimulation of mitochondrial production of ROS.

**MATERIALS AND METHODS**

*Experimental animals*

Experiments were undertaken on rainbow trout, *Oncorhynchus mykiss*, of both sexes weighing between 300g and 700g. The animals were obtained from a local hatchery and kept in 2000L tanks with circulating aerated well water at 14°C and at a 12h:12h light:dark cycle. Trout were fed commercial trout pellets and were fasted no less than 48h prior to experimentation. Authorized investigators carried out experiments and animal protocols were approved by institutional IACUC review.

*Perfused gill preparation*

Trout were stunned by a blow to the head and the heart was exposed through a midventral incision. A 1ml injection of heparinized (1000 IU ml$^{-1}$) isotonic 0.9% wt/vol saline was given in the ventricle and allowed to circulate for 1 min. The fish was then decapitated and the first and second pair of gill arches were isolated and placed in ice cold physiological salt solution (PSS). The afferent branchial artery was cannulated using an 18G beveled and blunted needle connected to PE90 tubing and the catheter was connected to a Biopac SS13L pressure transducer (Biopac Systems, Inc., Goleta, CA). The gill arches were suspended in aerated tap water (300ml) and continuously perfused with filtered (0.2μM Metricel membrane filter, Gelman Science, MI) PSS (14°C) via a peristaltic pump (Buckler instruments, New York). A four-way stopcock on the aspiration end of the pump was used to switch between perfusates without interrupting flow. The pump speed was adjusted to produce a stable input pressure of
~ 40mmHg, which was equivalent to 35mmHg at the gill arch. After completed protocol, flow was measured by collecting perfusate at the out flow tubing for 1 min. Pressure transducers were calibrated daily against a static water column and signals from the pressure transducers were recorded with a Biopac MP35 data acquisition system (Biopac Systems, Inc., Goleta, CA) at 100Hz. Gill vascular resistance was calculated from flow and pressure. The gill arches were left 90-120min before the onset of experimental protocols to stabilize baseline. All experiments were carried out in a temperature-controlled room at 14°C.

Isolated efferent branchial arteries

Trout were killed by a blow to the head and the branchial basket was isolated. The efferent branchial arteries were dissected free and mounted on a wire myograph (Model 610M, Danish Myo Technology, Aarhus, Denmark) for recording of isometric tension (Mulvany and Halpern, 1977) using a Biopac MP100 data acquisition system (Biopac Systems, Inc., Goleta, CA) at 100 Hz. The vessels were immersed in 5ml PSS at 14°C and aerated with room air (pH ~ 7.8). The vessels were then left for 30min to stabilize and resting tension was normalized by adjusting the diameter of the vessel with a micrometer screw to a transmural pressure of 3.5kPa. All experiments were carried out in a temperature-controlled room at 14°C.

Chemicals and Solutions

All chemicals were purchased from Sigma-Aldrich unless otherwise stated: Aminooxyacetic acid hemihydrochloride (TCI America, MA), Antimycin A, L-cysteine hydrochloride, Diethylthiocarbamate, L-glutathione reduced, heparin sodium salt, hydrogen peroxide (Martin’s, IN), myxothiazol, DL-propargylglycine, rotenone, sodium azide (Acros, Belgium), sodium sulfide nonahydrate. Inhibitors were used in concentrations comparable to concentrations published elsewhere (e.g. 25, 31, 38, 45,46).
PSS (Cortland’s Ringer): (mM) 124 NaCl, 3 KCl, 0.57 MgSO₄, 2 CaCl₂, 5.5 Glucose, 12 NaHCO₃, 0.09 NaH₂PO₄, 1.8 Na₂HPO₄ (pH = 7.8). A PSS with high K⁺ concentration (KPSS, 80mM) was prepared by exchanging NaCl for KCl.

Hydrogen sulfide was administered as Na₂S, which in solution produces H₂S and HS⁻, collectively referred to in this study as H₂S. A new stock solution of Na₂S (100mM) was prepared daily in PSS and titrated to pH = 7.8. Hypoxic perfusate (PO₂ < 10 mmHg) was prepared by bubbling PSS with pure N₂ for 15 min, pH was then titrated back to 7.8 and the solution sealed. All solutions were prepared in PSS except the following ETC inhibitors: Rotenone was dissolved in acetone, which was diluted 10⁶ times in the final concentration; Antimycin A was dissolved in ethanol that was diluted 10⁴ times in the final concentration; myxothiazol was dissolved in ethanol, which was diluted 2x10³ times in the final concentration. The solvents did not have effects on gill resistance. All solutions were titrated to pH = 7.8.

Experimental protocols

Effects of increasing concentration of H₂S on gill resistance during normoxia and hypoxia. One group of gill arches received two consecutive exposures to increasing concentration of H₂S in the perfusate as follows: 0, 0.1, 1, 10, 100μM. The two exposures were separated by 1h. A second group of gill arches was subjected to a similar series of increasing H₂S concentration (0, 0.1, 1, 10, 100μM). Subsequently, this group was exposed to hypoxia (PO₂ < 10 mmHg) and the series of H₂S concentrations was repeated during the hypoxic conditions. Normoxic and hypoxic exposures to H₂S were separated by 1h. Each concentration was applied for 15min, which was adequate to reach a maximum response. Hypoxia was administered as hypoxic perfusate.

Hypoxia vs. H₂S. To evaluate the interactions between hypoxia and H₂S, gill arches were exposed to one stimulus while in the presence of the other stimulus. One group of gill arches
was exposed to hypoxia (PO$_2$ < 10 mmHg) and at the maximum hypoxic response in gill resistance H$_2$S was added in a final concentration of 100μM. Gill arches were then exposed to H$_2$S (100μM) and at the maximum response in gill resistance to H$_2$S hypoxia was introduced. A second group of gill arches was first exposed to H$_2$S then to hypoxia. Finally, all gill arches were contracted with a high K$^+$ solution (KPSS, 80 mM) at the maximum response to H$_2$S (100μM).

*Effects of inhibition of H$_2$S biosynthesis on the hypoxic and H$_2$S responses.* One group of gill arches were exposed to 100μM H$_2$S (45min) and two hypoxic exposures (PO$_2$ < 10 mmHg, 45min). The three exposures were separated by 1h and the sequence was alternated. A second group of gill arches were exposed to hypoxia (45min), then perfused with inhibitors of the H$_2$S synthesizing enzymes, AOA and PPG (5mM + 10mM, 30min), followed by hypoxia in the presence of AOA and PPG (45min). The two hypoxic exposures were separated by 1h.

*The effects of cysteine on the hypoxic response.* The effects of cysteine, the precursor for H$_2$S synthesis, on the hypoxia-mediated contraction were evaluated. Gill arches were exposed to three separate periods of hypoxia (PO$_2$ < 10 mmHg, 15min): hypoxia; hypoxia and cysteine after incubation with cysteine (1mM, 30min); hypoxia and cysteine after incubation with cysteine (10mM, 30min). The sequence of the exposures were alternated and separated by 30min.

*Effects of inhibition of the electron transport chain (ETC) on the hypoxic and H$_2$S responses.* Gill arches were exposed to hypoxia (PO$_2$ < 10 mmHg) and H$_2$S (100μM), then perfused (30min) with one of four inhibitors of the complexes in ETC followed by exposures to H$_2$S (100μM) and hypoxia in the presence of the ETC blocker. The sequence of H$_2$S and hypoxia was alternated and the two exposures were separated by 15min. The ETC inhibitors were the following: inhibitor of complex I, rotenone, 0.1μM; inhibitor of the proximal part of complex
III, myxothiazol, 2μM; inhibitor of the distal part of complex III, Antimycin A, 10nM; inhibitor of complex IV, Azide, 10mM.

Effects of the antioxidant glutathione on the hypoxic and H₂S responses. A group of gill arches was exposed to hypoxia (PO₂ < 10 mmHg, 15min), then perfused with glutathione (10mM, 30min) followed by a second hypoxic exposure in the presence of glutathione. A second group of gill arches was exposed to H₂S (100μM, 15min). The gill arches then received the following three treatments in alternating sequence: glutathione (10mM, 30min) followed by H₂S (100μM) in the presence of glutathione; glutathione (10mM, 30min) in hypoxic perfusate followed by H₂S (100μM) and glutathione in hypoxic perfusate; and finally glutathione (50mM, 30min) followed by H₂S (100μM) and glutathione.

The role of H₂O₂ in the hypoxic and H₂S response. One group of gill arches received an increasing concentration of H₂O₂ in the perfusate as follows: 0, 0.1, 1, 10, 100, 1000μM. A second group of gill arches was exposed to hypoxia (PO₂ < 10 mmHg) and H₂S (100μM) in alternate sequence, perfused for 30min with DDC (1mM), an inhibitor of the cytosolic superoxide dismutase, and then exposed to hypoxia and H₂S in the presence of DDC. Finally the effects of H₂S and hypoxia in the presence of H₂O₂ were evaluated. A group of gill arches was exposed to hypoxia (15min) on baseline or at the maximum response to H₂O₂ (100 or 1000μM). Similarly, a second group was exposed to H₂S (100μM, 15min) on baseline or at the maximum contraction with H₂O₂ (100 or 1000μM). The sequence of exposures in the two protocols was alternated. To conclude the protocol, gill arches were contracted with KPSS (80mM) at the maximum contraction with H₂O₂ (1000μM).

The effect of PEG-catalase on the hypoxic and H₂S response. These experiments were conducted in isolated efferent branchial arteries mounted in a myograph because this allowed for a long incubation time with catalase. Contractility of the vessels was evaluated by addition of KPSS 80mM to the bath. As a control, H₂O₂ (100μM) was applied (10min) on vessels
precontracted with noradrenaline (NA, $10^{-7}$M). Vessels were then washed three times with PSS and after 20min H$_2$S (100μM) was applied (10min) on precontracted vessels (NA, $10^{-7}$M). Vessels were again washed (3 x PSS) and after 20min hypoxia (2% O$_2$) delivered by a gas mixing pump (Wösthoff, Bochum, Germany) was applied (10min) on precontracted vessels (NA, $10^{-7}$M). Vessels were washed and then incubated with polyethylene glycol conjugated catalase (PEG-catalase, 2000 U ml$^{-1}$, 12h, 14°C). After incubation the vessels were washed three times with PSS and the protocol evaluating the effect of H$_2$O$_2$ (100μM), H$_2$S (100μM) and hypoxia (2% O$_2$) was repeated.

**Data analysis and statistics**

All data recordings were analyzed using AcqKnowledge data analysis software (version 3.7.1., Biopac, Goleta, CA). Data were evaluated using paired t-test or one-way and two-way ANOVA’s, for repeated measures when appropriate, followed by a Dunnett’s or Tukey *post hoc* test, respectively. Differences were considered statistically significant at a 95% level of confidence (P < 0.05). All data are presented as mean ± S.E.M.

**RESULTS**

The effects of increasing concentration of H$_2$S on gill resistance under normoxic and hypoxic conditions are shown in Figure 1. H$_2$S caused a concentration-dependent constriction of the gill vasculature indicated by an increase in gill resistance that was significant at 10μM (Fig. 1A). The concentration-response curve was unchanged in two consecutive exposures to H$_2$S under normoxic conditions. Because hypoxia caused a large drop in baseline resistance the effects of H$_2$S in normoxic and hypoxic conditions are compared as relative changes in gill resistance. Hypoxia caused a rightward shift in the concentration-response curves for H$_2$S (Fig. 1B).
The effects of \( \text{H}_2\text{S} \) (100\( \mu \text{M} \)) and hypoxia (\( \text{PO}_2 < 10 \text{ mmHg} \)) as a function of time are shown in Figure 2A. Both hypoxia and \( \text{H}_2\text{S} \) caused an initial (<5min) vasoconstriction of the gill vasculature that was followed by dilation in the hypoxic gills whereas the \( \text{H}_2\text{S} \) mediated constriction subsided and resistance returned to baseline values. Inhibition of CSE and CBS, the \( \text{H}_2\text{S} \) synthesizing enzymes, reduced the acute hypoxic vasoconstriction but had no significant effect on the hypoxic vasodilation (Fig. 2A and 2B). The responses of gill vascular resistance to \( \text{H}_2\text{S} \) during hypoxia and to hypoxia during exposure to \( \text{H}_2\text{S} \) are shown in Figure 2C. Individually, hypoxia and \( \text{H}_2\text{S} \) (100\( \mu \text{M} \)) produced the same magnitude of contraction, however, \( \text{H}_2\text{S} \) applied during hypoxia and hypoxia applied during \( \text{H}_2\text{S} \) exposure had virtually no effect. Conversely, potassium (KPSS) when applied during maximum contraction with \( \text{H}_2\text{S} \) further constricted the vasculature. Perfusion of the gills with cysteine, the precursor to \( \text{H}_2\text{S} \), prior and during the hypoxic exposure caused a concentration-dependent decrease in the hypoxic vasoconstriction (Fig. 3A). Also, cysteine decreased baseline gill resistance (Fig. 3B).

Inhibition of complex I, complex IV as well as the distal and proximal part of complex III in ETC significantly reduced or completely abolished the vascular effects of hypoxia and \( \text{H}_2\text{S} \) (Fig. 4). Perfusion with the antioxidant glutathione in a concentration of 10mM prior to and during hypoxic exposures reduced the hypoxic vasoconstriction (Fig. 5A). Glutathione (10mM) did not reduce the effect of \( \text{H}_2\text{S} \) significantly; however, glutathione (10mM) during hypoxic conditions and glutathione in a higher concentration (50mM) decreased the \( \text{H}_2\text{S} \) response (Fig. 5A). Moreover, glutathione decreased baseline resistance and did not mimic hypoxia (Fig. 5B).

DDC, an inhibitor of the superoxide dismutase, which catalyzes the conversion of the superoxide anion to hydrogen peroxide (\( \text{H}_2\text{O}_2 \)), attenuated the vascular response to hypoxia as well as \( \text{H}_2\text{S} \) (Fig. 6A). \( \text{H}_2\text{O}_2 \) caused a concentration-dependent constriction of the gill
vasculature similar to the concentration-response curve of H2S (Fig. 6B). Preconstricting the
gill vasculature with H2O2 reduced (100μM) or virtually abolished (1000μM) the effects of
hypoxia and H2S. However, potassium (KPSS) when applied during maximum contraction
with H2O2 (1000μM) further constricted the vasculature.

Isolated efferent branchial arteries were incubated for 12h with a high dose of the
enzyme PEG-catalase (2000 U ml⁻¹). Catalase catalyses the conversion of H2O2 to water and
oxygen, however, in the control experiment the effect of H2O2 was not changed after
incubation with catalase showing that the enzyme was not taken up by the arteries (data not
shown).

**DISCUSSION**

This study shows that acute hypoxic vasoconstriction in perfused trout gills is dependent on
the endogenous production of hydrogen sulfide. Furthermore, H2S and hypoxia work through
the same intracellular pathways involving an increase in the production of mitochondrial
ROS, which converted to H2O2 mediates the constriction. The present study provides
evidence that H2S metabolism is the initial step that links a fall in PO2 to a physiological
response and is the first to describe the mechanism underlying H2S mediated hypoxic
vasoconstriction.

*Evidence that H2S mediates the hypoxic vasoconstriction*

H2S induced a transient increase in gill resistance in perfused trout gills. In isolated
conducting efferent branchial arteries from trout (7), however, H2S produced a triphasic
response consisting of dilation, constriction and dilation. The difference in vessel type
(resistance vs. conductance) may explain the difference in responses. H2S caused a
concentration-dependent and reproducible increase in gill resistance in the micromolar range.
During hypoxic conditions the concentration response curve was right-shifted showing that the gill vasculature is less sensitive to H$_2$S during hypoxia. This is consistent with an *in vivo* study on submerged freshwater turtles exposed to anoxia for 14 days, where the H$_2$S mediated systemic vasoconstriction was abolished under anoxia (38). This suggests that the signal transduction pathway for H$_2$S-mediated vasoconstriction is oxygen-dependent. Indeed, Koenitzer *et al.* (13) argued that H$_2$S-mediated vasoconstriction of the rat aorta is highly oxygen-dependent and may be facilitated by one or more unidentified oxidation products of H$_2$S, rather than H$_2$S *per se*. However, Olson *et al.* (24) argue against H$_2$S-mediated contraction being dependent upon an oxidation product. They report hagfish and lamprey dorsal aorta to be more sensitive to H$_2$S in hypoxia.

Similar to H$_2$S, hypoxia caused a constriction of the gill vasculature, which developed into a vasodilation in prolonged hypoxia (> 5min). Hypoxic vasoconstriction is expressed in the respiratory organs of all vertebrates, including lungs of mammals, birds and reptiles, amphibian skin and fish gills (10, 17, 34, 35, 37, 42). The response is important for local matching of blood perfusion to ventilation; it improves pulmonary gas exchange efficiency and, consequently, maintains arterial oxygenation (4, 6, 42). In vertebrates from all classes, H$_2$S and hypoxia seems to have the same vascular effects whether this is a constriction, dilation or a multiphasic response (8, 25). Hypoxic pulmonary vasoconstrictions was assumed to be a ubiquitous response of mammalian pulmonary blood vessels, however, a recent study has shown that pulmonary arteries from California sea lions dilates in response to hypoxia and H$_2$S (28). Furthermore, systemic blood vessels typically dilate when exposed to hypoxia and H$_2$S, however, mesenteric arteries from a freshwater turtle constricts in response to anoxia as well as H$_2$S, strongly suggesting that H$_2$S may be involved in the hypoxic vascular responses (38).
Inhibition of endogenous H$_2$S production by PPG and AOA reduced the acute effect of hypoxia but had no significant effect on hypoxic vasodilation, suggesting that H$_2$S is only involved in the initial response to hypoxia. However, it is possible that the relaxation is mediated by H$_2$S generated through the CAT/3-MST pathway, which is not blocked by AOA or PPG. Another possibility is that the relaxation is produced by reduction of thiosulfate, which is independent of enzymes (21). The antagonists of endogenous H$_2$S production are notorious for their low potency, poor selectivity and limited cell-membrane permeability (40). Indeed, many in vivo studies report a lack of hemodynamic responses to pharmacological inhibitors of H$_2$S production, but hemodynamic responses to an exogenous H$_2$S donor (27, 38, 40). However, in isolated systemic and pulmonary vessels from many different vertebrates, the inhibitors of H$_2$S production have been successful in suppressing the vascular effect of hypoxia whether this is a constriction or dilation (25, 38).

H$_2$S (100uM) and hypoxia produced the same transient increase in gill resistance; however, when either stimuli was applied at the maximum response of the other stimuli, the effect of the second applied stimuli was reduced or completely abolished. High concentration of potassium, however, constricted the vasculature even in the presence of hypoxia or H$_2$S showing that the vasculature was not fully constricted. This suggests that H$_2$S and hypoxia exploit the same intracellular signaling pathways leading to a constriction of the gill vasculature. The same competitive interactions between H$_2$S and hypoxia were seen in lamprey and rat aortas as well as bovine pulmonary arteries (25).

The mechanism underlying H$_2$S mediated constriction at low H$_2$S concentrations is poorly understood, but mammalian studies suggest that it may involve an interaction between H$_2$S and NO (36). However, several studies have shown that fish lack vascular NO and an H$_2$S-NO interaction is unlikely (18, 22). H$_2$S may also mediate contraction through an
endothelium-independent mechanism involving inhibition of the cyclic AMP pathway (14) or through the release of arachidonic acid (5).

Is hypoxic vasoconstriction mediated through a change in ROS production?

Inhibition of complex I, the proximal and distal end of complex III and complex IV in the electron transport chain (ETC) strongly reduced or virtually abolished the H$_2$S and hypoxia mediated vasoconstriction, suggesting that both responses are mediated through the mitochondria. At several sites in the ETC, primarily from complex I and III, electrons can be lost to molecular oxygen to form ROS in the form of superoxide (41). There is some consensus that hypoxic vasoconstriction is mediated through a change in the production of ROS from the mitochondria although it is being debated whether it is through an increase or decrease in production of ROS (3, 44, 45). Interestingly, H$_2$S has been shown to both scavenge ROS as well as induce ROS production (9, 11) and it is tempting, therefore, to speculate that ROS could be a common link between hypoxic vasoconstriction and H$_2$S-mediated vasoconstriction. It may seem paradoxical that the low concentration of molecular O$_2$ present during hypoxia should cause an increase in the production of ROS; however, the production of ROS is proportional to the product of the concentration of oxygen and the concentration of a reductant ([O$_2$] x [electron doner]) (41). Thus, increasing the concentration of a reductant may increase the production of ROS although the concentration of [O$_2$] is low.

Perfusing the gills with glutathione, a naturally occurring antioxidant and scavenger of ROS, before and during the exposure to hypoxia strongly attenuated the hypoxic vasoconstriction, suggesting that the hypoxic vasoconstriction is mediated through an increase in ROS. Glutathione also reduced the effect of H$_2$S although a very high concentration (50mM) was needed. However, 10mM glutathione was able to significantly reduce the H$_2$S mediated vasoconstriction when applied during hypoxic conditions. This may be due to a
smaller production of ROS by H$_2$S in the hypoxic environment where [O$_2$] is low and, therefore, [O$_2$] x [electron donor] is decreased. The effect of glutathione on hypoxic vasoconstriction is consistent with studies in mammals showing that exogenous scavengers of ROS suppress HPV (15, 45, 49).

Surprisingly, there was a dose-dependent decrease in the hypoxic response when the gills were perfused with cysteine. L-cysteine is the main substrate for the synthesis of H$_2$S (32, 40) and we, therefore, expected cysteine to increase the hypoxic response in the gill vasculature if H$_2$S is mediating the hypoxic response. It has been shown that cysteine amplifies the vasoconstriction to prolonged hypoxia (15h) in lamprey dorsal aortas and bovine pulmonary arteries (25). The reduction in the hypoxic response may be explained in two ways. Loading the gill with cysteine prior to the hypoxic exposure could increase the production of H$_2$S and as such making the gill “hypoxic” and, therefore, it would be equivalent to exposing the gills to hypoxia at the maximum response to H$_2$S, which abolished the response to hypoxia (Fig. 2). In addition, glutathione is a tripeptide containing cysteine and the provision of cysteine is the rate-limiting factor in glutathione synthesis (12). Thus, loading the gills with cysteine may have increased the concentration of glutathione causing a reduction in hypoxic constriction through scavenging of ROS.

In mammals, the superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are important regulators of vascular tone and act as vasoconstrictors in intrapulmonary arteries (29). Pretreatment with diethyldithiocarbamate (DDC), a known inhibitor of superoxide dismutase, the enzyme that converts superoxide to H$_2$O$_2$, reduced the vascular effects of both H$_2$S and hypoxia. This indicates that H$_2$O$_2$ is the intracellular signal mediating the hypoxic vasoconstriction rather than superoxide. Also, we showed that H$_2$O$_2$ does indeed constrict the gill vasculature and with a concentration-response curve virtually identical to the concentration-response curve for H$_2$S. Furthermore, there was a dose-dependent suppression
in the responses to hypoxia and H$_2$S when the gill vasculature was exposed to either stimulant at a maximum constriction to H$_2$O$_2$. This would suggest that H$_2$S and hypoxia mediated vasoconstrictions share common intracellular pathways involving H$_2$O$_2$ and when the vasculature is activated by one stimulus, it cannot be further activated by the other. In the control experiment, high potassium still constricted the gill vasculature in the presence of the highest concentration of H$_2$O$_2$ showing that the reduction in the H$_2$S and hypoxia mediated constriction was not due to toxic effect of H$_2$O$_2$ on the cells. Nevertheless, the mixture of H$_2$S, hypoxia and H$_2$O$_2$ may have created a complicated chemistry with unforeseen effects and the reduction in the H$_2$S and hypoxia mediated constriction may be explained by the oxidation of H$_2$S by H$_2$O$_2$. To further establish the role of H$_2$O$_2$ in the H$_2$S and hypoxia mediated vasoconstriction, the gills were incubated with the enzyme catalase, which catalyses the conversion of H$_2$O$_2$ to H$_2$O and O$_2$. However, in the control experiment the H$_2$O$_2$ mediated constriction was not abolished after incubation with catalase showing that the enzyme was not taken up by the vasculature regardless of the higher concentration of catalase applied (2000 U ml$^{-1}$ vs. 200 U ml$^{-1}$; e.g. 15) and the longer incubation time (12h vs. 30-60min; e.g. 15) compared to mammalian studies. A study by Sundaresan et al. (39) showed a difference in the uptake of catalase between different cell types and that some cells do not take up catalase. Also, catalase was not taken up a 4°C, thus the low temperature (14°C) in the present study may impair the uptake of catalase. It is well known that H$_2$O$_2$ can inhibit redox sensitive K$^+$-channels and that the associated depolarization of pulmonary arterial smooth muscle cells leads to and activation of L-type calcium channels and constriction as [Ca$^{2+}$]$_i$ increases (2, 46). In fact, there is some evidence that the H$_2$S mediated contraction in trout vasculature involves calcium-dependent activation of myosin light-chain kinase and that calcium may enter through voltage-dependent L-type calcium channels (7).
In a comprehensive review, Ward (44) evaluated the most common putative oxygen-sensors in the light of physiologically relevant PO$_2$ values. He compared the P50 values for O$_2$-sensitive mechanisms including the cytochrome c oxidase (COX) in the mitochondria. The activity for COX remains close to a 100% as PO$_2$ falls well below estimated cytosolic values. Although mitochondrial PO$_2$ might be even lower then a reduction in PO$_2$ from 0.3 to 0.15 kPa only reduced the predicted COX activity by 15%, which does not correlate well with the sensitivity of tissues where inhibition of oxidative phosphorylation has been suggested to be the O$_2$-sensitive mechanism (44). Based on this, Ward (44) concluded that mitochondria in pulmonary arterial smooth muscle cells must either be specialized so that they are affected by higher levels of PO$_2$ than mitochondria in other tissues that are not O$_2$-sensitive, or that PASMC contain modulating influences that effectively increase the P50 (44). In a recent paper, Olson et al. (28) included the effect of O$_2$ on consumption of H$_2$S in bovine lung homogenate or cultured bovine PASMC in this analysis. This study revealed that H$_2$S consumption decreases from 100% to virtually zero between 20mmHg and 0mmHg, which is over the range of mitochondrial and intracellular PO$_2$ values expected to occur in normoxic and hypoxic tissues. In fact, the P50 for H$_2$S consumption is very close to the P50 for the hypoxic vasoconstriction in bovine pulmonary arteries (28). Seen in the light of the present study, it is seems very likely that the O$_2$-sensitive consumption of H$_2$S and the corresponding increase in intracellular [H$_2$S], which stimulates mitochondrial ROS production, is linking mitochondrial oxygen sensing with physiological relevant PO$_2$ values.

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REFERENCES


FIGURE LEGENDS

Figure 1. (A) Effects of increasing concentration of \( \text{H}_2\text{S} \) on gill resistance during two consecutive exposures. (B) Effects of increasing concentration of \( \text{H}_2\text{S} \) on the relative change in gill resistance during normoxia (black symbols) and hypoxia (open symbols). Gill resistance is expressed relative to control values (0\( \mu \)M \( \text{H}_2\text{S} \)) because of a large decrease in baseline from normoxic to hypoxic conditions. * indicates a significant difference from control values and † indicates a significant difference between normoxic and hypoxic values within one concentration (\( P < 0.05 \)). Data are mean ± S.E.M. (\( N = 6 \)).

Figure 2. (A) Changes in gill resistance during exposures (40min) to hypoxia (black symbols; \( N = 6 \)), hypoxia after inhibition of \( \text{H}_2\text{S} \) synthesis with AOA/PPG (light grey symbols; \( N = 6 \); AOA, aminooxyacetic acid hemihydrochloride, 5mM; PPG, DL-propargylglycine, 10mM) and 100\( \mu \)M \( \text{H}_2\text{S} \) (white symbols; \( N = 6 \)). * denotes a significant difference from baseline evaluated by a one-way or two-way ANOVA for repeated measures. (B) Effects of inhibition of \( \text{H}_2\text{S} \) production on the maximum change in gill resistance to hypoxia. The control group (\( N = 6 \)) received two consecutive exposures to hypoxia. The experimental group (\( N = 6 \)) received a first exposure to hypoxia and a second exposure to hypoxia after inhibition of \( \text{H}_2\text{S} \) synthesis (AOA, 5mM; PPG, 10mM). Grey bars indicate hypoxic exposures and checked bars indicate hypoxic exposures in the presence of inhibitors. * denotes a significant difference from the first hypoxic exposures (\( P<0.05 \)). (C) The effects of hypoxia (PO\(_2\) < 10 mmHg) and \( \text{H}_2\text{S} \) (100\( \mu \)M) are mutually competitive. Hypoxia (grey bars; \( N = 6 \)) and \( \text{H}_2\text{S} \) (light grey bars; \( N = 6 \)) produce similar contractions of trout gill vasculature, however, when added on top of the maximum \( \text{H}_2\text{S} \) (100\( \mu \)M) or hypoxic contraction, effects of hypoxia and \( \text{H}_2\text{S} \), respectively, are abolished. Potassium (dark grey bar, \( \text{K}^+ \), 80mM, \( N = 4 \)), when administered on top of a maximum \( \text{H}_2\text{S} \) (100\( \mu \)M) contraction, still contracts the gill vasculature (note different vertical
scale bars). * denotes a significant difference from hypoxic or H₂S contractions on baseline (P < 0.05). All data are mean ± S.E.M.

Figure 3. (A) Effects of cysteine (Cys1, 1mM; Cys10, 10mM) on the maximum change in gill resistance to hypoxia (PO₂ < 10 mmHg). (B) Effects of a 30min perfusion with cysteine on baseline. * denotes a significant difference from the maximum hypoxic contraction and † denotes a significant decrease in baseline (P < 0.05). All data are mean ± S.E.M., N = 6.

Figure 4. Effects of inhibition of the electron transport chain (ETC) on the maximum change in gill resistance to hypoxia or H₂S (100μM). (A) Rotenone, inhibitor of complex I in ETC (0.1μM, N = 6); (B) Myxothiazol, inhibitor of the proximal part of complex III in ETC (2μM, N = 7); (C) Antimycin A, inhibitor of the distal part of complex III in ETC (10nM, N = 6); (D) Azide, inhibitor of complex IV in ETC (10mM, N = 7). Grey bars indicate hypoxic responses, light grey bars indicate H₂S responses, and checked bars indicate the response to hypoxia or H₂S in the presence of ETC inhibitors. All data are mean ± S.E.M. * denotes a significant difference from the hypoxic or H₂S response (P < 0.05).

Figure 5. (A) Effects of glutathione (Glu10, 10mM; Glu50, 50mM) on the maximum change in gill resistance to hypoxia (grey bars) and 100μM H₂S (light grey bars). * denotes a significant difference from the hypoxic or H₂S response (P < 0.05). (B) Effects of a 30min perfusion with glutathione on baseline. † denotes a significant decrease in baseline (P < 0.05). All data are mean ± S.E.M., N = 7.
Figure 6. (A) Effects of DDC (diethyldithiocarbamate, 1mM, N = 7), blocking cytosolic superoxide dismutase, on hypoxic (PO$_2$ < 10 mmHg) and H$_2$S (100μM) mediated contractions of trout gill vasculature. Grey bars indicate hypoxic responses, light grey bars indicate H$_2$S responses and checked bars indicate hypoxic or H$_2$S responses in the presence of DDC. * denotes a significant difference from the hypoxic or H$_2$S contractions (P < 0.05). (B) Effects of increasing concentrations of H$_2$O$_2$ on gill resistance (N = 7). The concentration-response curve for H$_2$S from Figure 1A has been included for comparison (grey plot). † denotes a significant (P < 0.05) differences from control values (0μM). (C) The effects of H$_2$O$_2$ and hypoxia or H$_2$S are mutually competitive. Hypoxia (grey bars, N = 6) and 100μM H$_2$S (light grey bars, N = 7) produce similar contractions but when added on top of the maximum contraction to H$_2$O$_2$ (H$_2$O$_2$100, 100μM; H$_2$O$_2$1000, 1000μM) responses are reduced. Potassium (dark grey bar, K$^+$, 80mM, N = 6), when administered on top of the maximum 1000μM H$_2$O$_2$ contraction, still contracts the gill vasculature (note different vertical scale bars). * denotes a significant difference from the hypoxic or H$_2$S contraction (P<0.05). All data are mean ± S.E.M.
Figure 1. Skovgaard and Olson

A. Exposure to [H$_2$S] (μM)

B. Relative change in gill resistance (%)
Figure 2. Skovgaard and Olson
Figure 3. Skovgaard and Olson
Figure 4. Skovgaard and Olson
Figure 5. Skovgaard and Olson
Figure 6. Skovgaard and Olson