Hydrogen sulfide as an endogenous regulator of vascular smooth muscle tone in trout

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Hydrogen sulfide as an endogenous regulator of vascular smooth muscle tone in trout. Am J Physiol Regul Integr Comp Physiol 286: R678–R685, 2004; 10.1152/ajpregu.00419.2003.—Hydrogen sulfide (H2S) is an endogenous vasodilator in mammals, but its presence and function in other vertebrates is unknown. We generated H2S from NaHS and examined the effects on isolated efferent branchial arteries from steelhead (stEBA) or rainbow (rtEBA) trout. H2S concentration was measured colorimetrically (CM) and with ion-selective electrodes (ISE) in rainbow trout plasma. NaHS produced a triphasic response consisting of a relaxation (phase 1), constriction (phase 2), and relaxation (phase 3) in both unstimulated vessels and in stEBA precontracted with carbachol (Carb). Phase 1 and phase 3 in stEBA were decreased and phase 2 increased in unstimulated vessels by K+ and phase 3 in stEBA were decreased and phase 2 increased in vessels and in stEBA precontracted with carbachol (Carb). Phase 1

Second, H2S can be generated by mammalian tissue from two pathways: (1) from the cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) and it is produced directly in arterial and venous tissue by CSE (6, 24). Third, plasma titers of H2S have been reported to be between 45 (24) and 300 (22) μM, which are well within the range of the effective concentration for half-maximal response (EC50) of vessels in vitro (125 μM; 24). Fourth, this system responds to perturbation of the cardiovascular system; pulmonary hypoxia decreases both lung tissue CSE and plasma H2S concentration ([H2S]; 22). Fifth, there is compelling evidence that H2S acts on a specific smooth muscle target, the ATP-sensitive potassium (KATP) channel (24).

H2S has been proposed (21) to be an endogenous “gaso transmitter” similar to two other vasoactive gases, nitric oxide (NO) and carbon monoxide (CO). In fact, in some instances H2S appears to synergize with NO, augmenting relaxation in rat thoracic aorta and portal veins (6). It is likely that the relationship between these two gases affords an additional level of cardiovascular integration.

To our knowledge, the physiological function(s) of H2S, or the presence of H2S in the plasma, have not been investigated in nonmammalian vertebrates, and it is unclear if there is an evolutionary precedent for H2S as a gasotransmitter. The existence of H2S in fish is perhaps most intriguing. First, they are the most ancient of vertebrates. Second, many studies have been unable to show that fish vessels produce NO or release NO-like relaxing factors (8, 10, 13, 18), although this is still somewhat controversial (11). Thus H2S may be similarly lacking, or conversely, if present it may be a predominant vasoregulator. Third, aquatic vertebrates may be subjected to substantially elevated ambient H2S both through natural production and from a variety of industrial and agricultural processes (3, 16), and vasoregulatory capability may therefore be compromised by exogenous H2S.

In the present study we examined the actions of H2S derived from NaHS on isolated blood vessels and measured plasma H2S concentration ([H2S]) in trout. Our findings provide strong support for H2S as a physiologically important endogenous vasoactive “gasotransmitter” in trout, and they suggest that the cardiovascular system may be a potential focal point of H2S toxicity.

MATERIALS AND METHODS

Animals. Steelhead trout (Oncorhynchus mykiss, Skamania strain, 3–7 kg) of either sex were used for most isolated vessel studies. They were captured by the Indiana Department of Natural Resources (DNR) during the fall migration and kept at the Richard Clay Bodine State Fish Hatchery until the spawning season (January–March). The

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Rainbow trout (O. mykiss, kamloops strain, 0.3–0.8 kg) of either sex were used for measurement of plasma [H2S] and several studies on isolated vessels. They were purchased from a local hatchery (Homestead Trout Farm, Harrietta, MI), kept in circulating 2,000-liter tanks containing well water at 12–15°C, aerated with filtered room air, and exposed to appropriate seasonal light/dark cycles. The fish were fed a maintenance diet of commercial trout pellets (Purina, St. Louis, MO). Isolated vessels were obtained from trout stunned by a blow to the head. All procedures followed National Institutes of Health guidelines and were approved by the local Institutional Animal Care and Use Committee.

**Smooth muscle preparation.** One-half- to one-centimeter-long segments of third and fourth gill arch efferent branchial arteries from the steelhead trout (stEBA) were used for most experiments. Third and fourth arch EBA from rainbow trout (rEBA; 3–5 mm long) were used for several myography experiments, and first through fourth arch rEBA (3–6 mm long) were used to measure cGMP production (see below). The effects of a single dose of NaHS (3.7 × 10⁻⁴ M) were also examined on precontracted bulbus arteriosus (BA), celiacomesenteric arteries (CMA), and anterior cardinal veins (ACV) from steelhead trout. The vessels were isolated and mounted on 280-μM-diameter stainless steel wire hooks and suspended in 5-mL water-jacketed smooth muscle baths filled with 14°C HEPES buffer and aerated with room air. The bottom hooks were stationary; the upper ones were connected to Grass model FT03C force-displacement transducers (Grass Instruments, West Warwick, RI). Tension was measured on a Gould Series 8000S (Gould Instrument Systems, Valley View, OH) or a Grass model 7F polygraph (Grass Instruments). Data were archived on a personal computer at 1 Hz using Labtech Notebook software (Laboratory Technologies, Andover, MA). The chart recorders and software were calibrated before each experiment. With this system, a change in tension equivalent to 5 mg could be detected.

Baseline (resting) tension of 500 mg was applied to the EBA (BA, 750 mg; CMA, 500 mg; ACV, 350 mg) for 0.5–1 h before experimentation. The vessels were then contracted with 80 mM KCl, washed twice, and resting tension was reestablished. This process was repeated, and resting tension was reestablished for a minimum of 30 min before further experimentation. These two “pretreatment” contractions were found to be necessary to produce optimal responses thereafter.

The cumulative dose-response characteristics of NaHS, which forms H2S in solution (23), were examined in otherwise unstimulated vessels. A consistent and repeatable triphasic, relaxation-contraction-relaxation response was produced by 3.7 × 10⁻⁴ M NaHS, and this dose was used for subsequent inhibitor studies in stEBA with each vessel serving as its own control. The properties of the triphasic responses were examined by addition of a cyclooxygenase inhibitor, indomethacin (Indo, 10⁻⁵ M), a K_ATP channel inhibitor, glibenclamide (Gly, 10⁻⁵ M), a tripartite cocktail consisting of the lipoxygenase inhibitor esculetin (Escu, 10⁻³ M), the cytochrome P-450 and K_Ca channel inhibitor clotrimazole (Clo, 10⁻⁵ M), and Indo (10⁻⁵ M), or the soluble guanylate cyclase inhibitors 1H-[1,2,4]oxadiazolo-[4,3-a]quinazolin-1-one (ODQ; 10⁻⁵ M) and NS-2028 (10⁻⁶ M). After resting tension was established, the vessels were treated with NaHS in the absence of inhibitor, washed twice, and, after resting tension was reestablished, the inhibitor was added and 10–15 min later the vessels were again treated with NaHS. Only one study was performed per vessel.

Three groups of stEBA were used to examine the calcium dependency of the phase 2 contraction. The first group was stimulated with NaHS, as described above, washed twice, then treated with either L-type Ca²⁺ channel inhibitor, methoxyverapamil (D600, 10⁻⁴ M), or the myosin light-chain kinase inhibitor ML-9 (10⁻⁴ M), and after 10–15 min treated again with NaHS. In the other two reference groups, either KCl (80 mM) or norepinephrine (NE, 3 × 10⁻⁶ M) was substituted for the two NaHS treatments. The NE-contracted vessels were incubated for 10 min with 10⁻⁵ M propranolol before NE addition to prevent β-adrenoceptor stimulation (12). A second L-type Ca²⁺ channel inhibitor was examined using rEBA; stEBA were unavailable. In these experiments, third and fourth arch rEBA were equilibrated at 500 mg resting tension and contracted twice with KCl as above. They were then contracted with 3.7 × 10⁻³ M NaHS, washed three times, treated with 10⁻⁵ M nifedipine for 15 min and contracted a second time with 3.7 × 10⁻³ M NaHS. The higher NaHS dose was chosen to produce a strong contraction in these small vessels; at 3.7 × 10⁻³ M NaHS the typical triphasic response was observed; however, the magnitude of contraction was not sufficient to allow statistical examination of the nifedipine effects. The role of extracellular calcium in phase 2 contraction was examined in an additional group of rEBA after removal of extracellular calcium and addition of 0.1 M EGTA to chelate any residual Ca²⁺ for 30 min before 3.7 × 10⁻³ M NaHS treatment. As with stEBA, consecutive NaHS treatments produced identical responses in rEBA, and each vessel was used as its own control.

Due to the prominent vasodilatory effect of 3.7 × 10⁻⁴ M NaHS on unstimulated stEBA (phase 3), this response was examined further on stEBA precontracted with other agonists. A cumulative dose-response relationship was determined for carbamylcholine chloride [carbachol (Carb); Fig. 1]. The cumulative dose-response relationship for both KCl contractions indicated that 30 mM produced ~80% of the maximal contraction (EC₅₀); the EC₅₀ for Carb was 4.0 ± 0.6 × 10⁻⁶ M (n = 7).

The phase 3 relaxation produced by 1.1 × 10⁻³ M NaHS (approximate EC₅₀) was also examined on stEBA precontracted with voltage-mediated (30 mM KCl or 15 mM K₂SO₄), or ligand-mediated (3 × 10⁻⁶ M NE or 10⁻⁵ M Carb) stimuli. The dose-response characteristics for KCl contractions indicated that 30 mM produced ~100% contraction (Dombkowski, unpublished), and this lower concentration was used in these experiments. All agonist doses were selected to produce 80–100% of a maximal contraction. NaHS was added after...


tension plateaued (10–15 min) and only one agonist was used per vessel. Propranolol (10⁻⁵ M) was added 10 min before NE.

cGMP production. The effects of NaHS on cGMP production were examined in rTEBA; stTEBA were unavailable. First through fourth arch rTEBA were removed from 12 trout and placed in cold (4°C) HEPES buffer. To obtain enough tissue, EBA from eight fish (2 from each arch 1, 2 from arch 2, etc.) were pooled for each sample; three samples were assayed for each treatment. Each group of eight vessels was mounted in series in the myograph chamber, between the force transducer and the fixed hook, by inserting hair-pin wires in the lumens of adjacent vessels. In this way all vessels could be equilibrated with the same resting tension and the time course of the NaHS response could be monitored and recorded. Optimal resting tension (1.5 g) for this arrangement was derived from the length-tension response to 80 mM KCl in a pilot study. The vessels were contracted twice with KCl (with appropriate rinses) and resting tension was reestablished after each treatment. Thirty to sixty minutes later the vessels were treated with 3.7 × 10⁻⁴ or 3.7 × 10⁻³ M NaHS until the response was maximal (~30 min). The vessels were then removed, frozen in liquid nitrogen, and stored at ~80°C until analysis. Control vessels were contracted twice with KCl as above and equilibrated in the myographs for the same duration; other vessels were treated with 10⁻³ M sodium nitroprusside (SNP) for comparison. The cGMP assay was performed according to the manufacturer’s directions (Correlate-EIA, Assay Designs, Ann Arbor, MI) without overnight acetylation.

Plasma H₂S concentration, colorimetric assay. Trout were removed from the tank, manually restrained, and blood (2–4 ml) was removed from the hemal arch via a 21-gauge stainless steel needle into an unheparinized syringe and immediately centrifuged. Two aliquots of plasma were used to perform duplicate measurements for each sample. A colorimetric technique (15; modified slightly by 22) was used to determine H₂S concentration in duplicate plasma samples from four fish. Briefly, 0.1 ml of plasma was added to a test tube containing 0.5 ml of 1% zinc acetate and 2.5 ml of distilled water. Then, 0.5 ml of 20 mM N,N-diethyl-p-phenylenediamine dihydrochloride in 7.2 M HCl and 0.4 ml of 30 mM FeCl₃ in 1.2 M HCl were added and the mixture was incubated for 20 min at room temperature. After incubation, the protein in the plasma was precipitated with 1 ml of 10% trichloroacetic acid followed by centrifugation. Absorbance was measured at 670 nm with a Spectro UV-Vis Auto PC Scanning spectrophotometer (Labomed, Culver City, CA) and compared with NaHS standards (at the pH of the incubation mixture essentially all the hydrosulfide anion, H₂S⁻, in plasma should exist as H₂S, which then is stoichiometrically equivalent to converting H₂S⁻ from the NaHS standard to H₂S).

Plasma H₂S concentration, ion-selective electrode. Blood was withdrawn and plasma isolated as described above. H₂S was measured on duplicate plasma samples with a sulfide electrode (Lazar Research Laboratories, Los Angeles, CA) on a Fisher Accumet AR50 pH meter (Fisher Scientific, Pittsburgh, PA) following the manufacturer’s directions. Standards were prepared from NaHS.

Effect of NaHS on bath pH. To determine if NaHS affected bath pH, a myograph chamber was filled with 5 ml HEPES buffer at 14°C and aerated with room air while three consecutive doses of NaHS (each dose raised bath NaHS by 1 mM) were added. Bath pH was measured every 5 min for 30 min with a Fisher Accumet Selective Ion Analyzer (model 750, Fisher Scientific) and electrode (SensoreX model S200C combination pH electrode, SensoreX, Garden Grove, CA). The electrode was calibrated in the same myograph chamber with pH 4 and 10 standards under the same conditions. This experiment was done in triplicate. Although bath pH rose with each NaHS addition, reaching a plateau in ~15 min, the average rise in bath pH per millimole NaHS was only 0.048 ± 0.011 (n = 3) pH units, and this was not sufficient to account for any of the NaHS effects on the vessels.

Data analysis. Dose-response curves were fit for each vessel using Table Curve (Jandel, Chicago, IL). Student’s t-tests were used for comparisons between groups of vessels (different preluminal experiment) and paired t-tests and Wilcoxon signed rank tests were used for within-vessel comparisons (SigmaStat, Jandel). Results are provided as means ± SE. Significance was assumed at P ≤ 0.05.

Chemicals. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). ODQ was purchased from Calbiochem-Novabiochem (San Diego, CA). Concentrations for the HEPES-buffered trout saline (pH 7.8) were as follows (in mM): 145 NaCl, 3 KCl, 0.57 MgSO₄·7H₂O, 2 CaCl₂·2H₂O, 5 glucose, 3 HEPES acid, and 7 HEPES Na⁺ salt. HEPES buffer and NaHS stock solutions were used within 72 h of preparation.

RESULTS

NaHS effects on resting tone. Addition of 3.7 × 10⁻⁴ M NaHS to otherwise unstimulated rTEBA produced a triphasic response consisting of an initial relaxation, then contraction, followed by another relaxation (Fig. 2). Phase 1 (relaxation) was of variable magnitude (Fig. 2, A and B) and on occasion absent altogether. When present, it lasted 3–5 min. Phase 2 (contraction) began within 3–5 min after the onset of phase 1. If phase 1 was present, then phase 2 returned tension to or slightly above the original resting tension (Fig. 2A). If phase 1 was minimal, phase 2 increased tension above resting tension (Fig. 2B). Phase 3 (relaxation) predominated in both magnitude and duration at NaHS concentrations of 3.7 × 10⁻³ M or below. During this phase, the vessels often relaxed below resting tension (Fig. 2A), and this relaxation was sustained until the H₂S gas evolved from the bath or the bath was replaced. At, or above, 10⁻³ M NaHS, the phase 2 contractions dominated and there was essentially no phase 1 or phase 3 relaxation for the duration of the NaHS exposure (>40 min). Phase 2 contractions were dose dependent, and it appeared as though there were two components, the first with high sensitivity and low efficacy and the second with lower sensitivity, but more efficacious (Fig. 3). However, phase 3 relaxations were most pronounced at NaHS concentrations between the two components of phase 2 and it made separating the components
difficult. The overall EC$_{50}$ for phase 2 was 1.79 ± 0.03 × 10$^{-3}$ M (n = 7). Phase 2 contractions were sustained at the highest dose tested (0.1 M NaHS) for over 40 min without any apparent adverse effect on the vessel; on removal of the NaHS, the vessel rapidly relaxed and a subsequent 80 mM KCl application had no apparent effect on the vessel; on removal of the NaHS, the vessel rapidly relaxed and a subsequent 80 mM KCl application had no apparent adverse effect on the vessel; on removal of the NaHS, the vessel rapidly relaxed and a subsequent 80 mM KCl application had no apparent adverse effect on the vessel. The soluble guanylate cyclase inhibitor ODQ (Fig. 5) significantly increased the phase 2 contraction to 176 ± 16% of control (n = 7), but did not significantly affect phase 3. For each phase, the effects of ODQ alone were not significantly different from Escu-Clot-Indo effects, suggesting that the predominant mechanism is Indo sensitive. The soluble guanylate cyclase inhibitor ODQ (Fig. 5) potentiated the phase 1 relaxation (348 ± 94%; n = 7) as well as the phase 2 contraction (250 ± 55%), but inhibited the phase 3 relaxation. The soluble guanylate cyclase inhibitor NS-2028 also inhibited phase 3, but it did not affect either phase 1 or phase 2 (Fig. 5).

Role of calcium in phase 2 contraction. The effects of the L-type calcium channel inhibitor, methoxyverapamil (D600), and the myosin light chain kinase inhibitor, ML-9, on contractions produced by 3.7 × 10$^{-4}$ M NaHS (phase 2), 80 mM KCl, and 3 × 10$^{-6}$ NE in stEBA are shown in Fig. 6. D600 had no effect on phase 2 NaHS contractions (97 ± 30% of control; n = 8), whereas KCl and NE contractions were significantly reduced to 37 ± 4% (n = 7) and 26 ± 7% (n = 7) of control. ML-9 significantly reduced both NaHS (38 ± 9%, n = 7)- and KCl (42 ± 8%, n = 7)-induced contractions; NE contractions in the presence of ML-9 were not examined. D600 did not affect resting tension or either phase 1 or phase 3. ML-9 did not affect resting tension or phase 1 relaxation. Phase 3 was reduced by ML-9, although this was probably due to the prior inhibitory effect of ML-9 on phase 2. Conversely in stEBA (Fig. 6), the L-type calcium channel blocker nifedipine significantly reduced a 3.7 × 10$^{-3}$ M NaHS phase 2 contraction to 68.6 ± 5.5% of control (n = 6), and removal of extracellular Ca$^{2+}$ significantly reduced the phase 2 contraction to 42.5 ± 13.7% of control (n = 6). There was no significant difference between the nifedipine and zero extracellular Ca$^{2+}$ treatments, nor was the tension produced by a second 3.7 × 10$^{-3}$ M NaHS treatment significantly different.
consistent phase 1 relaxation, a predominant phase 3 relaxation at 10^{-10} M NaHS (n = 7; D600; n = 7; ML-9) or contractions produced by 80 mM KCl (n = 7; D600; n = 7; ML-9) or 3 \times 10^{-6} M norepinephrine (NE; n = 7) in steelhead trout and the effects of 10^{-5} M nifedipine (NF) or zero external calcium (0 Ca^{2+}) on phase 2 contraction produced by 3.7 \times 10^{-3} M NaHS in rainbow trout. *Significantly different from respective controls (P < 0.05).

from the first NaHS treatment (302.5 \pm 41.4 vs. 243.5 \pm 35.8 mg/mg vessel wet wt; n = 6, respectively) in otherwise untreated vessels.

**NaHS dose response in precontracted stEBA.** As with unstimulated vessels, the NaHS response in stEBA precontracted with 10^{-5} M carbachol was usually triphasic with an inconsistent phase 1 relaxation, a predominant phase 3 relaxation at lower NaHS concentrations (Fig. 2C), and a predominant phase 2 contraction at higher concentrations of NaHS. Both phase 2 and phase 3 were dose dependent (Fig. 3). Phase 3 relaxations appeared \sim 3.7 \times 10^{-6} M NaHS, with an EC_{50} of 6.7 \pm 0.9 \times 10^{-5} M. The threshold for phase 2 was also quite low, 1.1 \times 10^{-5} M, but the contractions remained small until NaHS was increased to 6.7 \times 10^{-4} M. The EC_{50} for phase 2 was 1.1 \pm 1.2 \times 10^{-3} M (n = 7). The highest NaHS concentration examined on carbachol-stimulated stEBA was 6.7 \times 10^{-2} M. This did not appear to have any toxic effect on the vessels.

**Effects of NaHS on stEBA precontracted with different agonists.** The ability of NaHS to relax stEBA (phase 3) precontracted with different agonists is shown in Fig. 7. NaHS was significantly less effective in relaxing 30 mM KCl-induced contractions (29 \pm 5% relaxation, n = 7) than contractions produced by either 3 \times 10^{-6} M NE (75 \pm 18% relaxation, n = 6) or 10^{-5} M Carb (105 \pm 12% relaxation, n = 28). Vessels precontracted with 15 mM K_2SO_4 were relaxed significantly less (41 \pm 9% relaxation, n = 8) than Carb-contracted vessels. There were no differences between the two potassium-contracted groups or between the NE- and Carb-contracted groups.

**Treatment effects on Carb-precontracted stEBA.** Phase 3 in Carb-precontracted vessels was not affected by Indo (77 \pm 15% relaxation, n = 7; not shown) or Gly (69 \pm 33% relaxation, n = 7; not shown). Phase 1 and phase 2 in Carb-precontracted vessels were not significantly affected by ODQ, whereas phase 3 was significantly reduced (n = 8; Fig. 5).

**NaHS effects on other steelhead trout vessels.** NaHS (3.7 \times 10^{-4} M) produced a triphasic response in Carb (10^{-5} M)-precontracted CMA and ACV that was similar to that observed in EBA (not shown). NaHS only produced vasodilation in Carb-precontracted BA (not shown).

**cGMP production in rtEBA.** Figure 8 shows the effect of NaHS on resting tension and cGMP production by rtEBA. Although vessels were slightly relaxed by low (3.7 \times 10^{-5} M) and contracted by high (3.7 \times 10^{-3} M) NaHS concentrations, tissue cGMP production was significantly decreased by both treatments. Sodium nitroprusside, by comparison, was even more efficacious in lowering resting tension and produced a ninefold increase in cGMP (Fig. 8).

**Plasma [H_2S].** The total sulfide concentration (equivalent to NaHS concentration used in vitro) in rainbow trout plasma was 4.0 \pm 0.3 \times 10^{-3} M, n = 4 (colorimetric method) and 3.8 \pm 0.4 \times 10^{-5} M, n = 9 (ion selective electrode method).

**DISCUSSION**

Our study shows that the H_2S donor, NaHS, is a triphasic, dilator-constrictor-dilator of efferent branchial arteries in both stEBA and rtEBA. All phases in stEBA are variously affected by inhibitors of putative endothelium-derived relaxing substances and guanylyl cyclase, suggesting multiple and complex
activating processes. The first phase is the least consistent of the three and may be mediated to a large extent by signals from the endothelium. The second (contractile) phase in stEBA is unaffected by the L-type calcium channel inhibitor, whereas it is reduced by both nifedipine and removal of extracellular calcium in rtEBA. Phase 2 in stEBA depends in part on activation of light-chain kinase. Phase 2 is dose dependent and it is the predominant phase at supraphysiological concentrations. The third phase predominates at lower, physiological NaHS concentrations. It is dose dependent, decreases resting tone in unstimulated vessels, and it relaxes ligand- and, to a lesser extent, depolarization-contracted vessels. Potassium channels, possibly K\(^{\text{ATP}}\), may contribute to this phase and in rtEBA it is independent of cGMP. NaHS has triphasic, or predominantly dilatory, effects on other conductance vessels as well, indicative of its generalized vasoactivity. Plasma titers of H\(_2\)S (equivalent to NaHS) obtained from unanesthetized rainbow trout are sufficiently high to produce the observed in vitro effects and we propose that H\(_2\)S is a tonically active endogenous vasoregulatory molecule in these fish. Our findings suggest that not only does H\(_2\)S have a long phylogenetic lineage, but that industrial or agricultural release of H\(_2\)S may have debilitating effects on cardiovascular homeostasis of aquatic vertebrates, especially considering the potential ease with which this gas could cross the respiratory membranes.

The precedent for H\(_2\)S as an endogenous signaling molecule in the cardiovascular system has been established in mammals. H\(_2\)S is produced locally in blood vessels (6, 24), it relaxes both arteries and veins (8, 23, 24), and endogenous levels appear to be physiologically active (22, 24). Two of these three components have now been identified in trout, and the general aspects of H\(_2\)S regulation may be quite similar between the two vertebrate groups. However, the effects of H\(_2\)S on trout vessels appears more complex, and this may be related to a greater dependency of fish on H\(_2\)S for cardiovascular regulation.

Both H\(_2\)S gas and NaHS produce an equivalent monophasic relaxation of mammalian vascular smooth muscle (23), whereas NaHS has a triphasic effect in trout (Fig. 2). Each of the three phases appears distinct, and they may be initiated by, or controlled through, different mechanisms.

Phase 1 relaxation. Phase 1 is the most variable of the three phases. When present, it begins within minutes of NaHS application, and the relaxation is small, only ~5% of resting tension (500 mg). The inhibitory effect of Indo-Encu-Clot (Fig. 4) suggests that phase 1 may be due to an H\(_2\)S-mediated release of an intrinsic inhibitory factor in the vessel. Most likely this an arachidonic acid metabolite, probably a prostanoid, released from the endothelium; this mechanism has been demonstrated in trout (13). Furthermore, endothelium-derived NO is not present in trout conductance vessels (7, 8, 10, 13). Phase 1 is also inhibited by the K\(^{\text{ATP}}\) channel antagonist Gly (Fig. 4). Because Gly also inhibits phase 3, whereas indomethacin only inhibits phase 1, it is probable that Gly is acting directly on the vascular smooth muscle, not the endothelium. The endothelium also contributes to (augments) H\(_2\)S relaxation of rat aortas, although this response is monophasic (23).

Potentiation of stEBA phase 1 relaxation by the soluble guanylate cyclase (sGC) inhibitor ODQ (Fig. 5) is counterintuitive because activation of this enzyme is generally associated with relaxation. However, it is possible that the ODQ effect is nonspecific. NS-2028 did not affect phase 1 in uncontracted stEBA, nor did ODQ significantly affect phase 1 in precontracted vessels (Fig. 5). Furthermore, NaHS did not increase cGMP production by rtEBA (see below). Zhao and Wang (23) found that ODQ and another sGC inhibitor, NS-2028, potentiated the H\(_2\)S relaxation of rat aortas. They (23) attributed the augmented ODQ effect to generation of dilatory free radicals, but did not explore the NS-2028 response. Although the phase 1 response requires further examination, it does not appear to directly involve cGMP production.

Phase 1 in uncontracted stEBA was not affected by either the L-type calcium channel blocker, D-600, or the myosin light chain kinase inhibitor, ML-9. This is as expected because both agents should inhibit contraction and not affect relaxation. However, H\(_2\)S relaxation of rat aortas was enhanced by removal of extracellular calcium or application of the L-type calcium channel blocker nifedipine, and this was not affected by inhibition of K\(_{\text{Ca}}\) channels (23). Thus phase 1 in the stEBA appears different from H\(_2\)S dilation of the rat aorta.

Phase 2 contraction. H\(_2\)S-mediated contraction (Fig. 2) has not been described in mammalian (8, 23, 24) vessels and may be unique to fish or other nonmammalian vertebrates. This contraction is independent of preexisting tone, it is dose dependent, and it becomes the predominant response at high H\(_2\)S levels. It also involves classical calcium activation of myosin light-chain kinase because it is reduced in the presence of ML-9. Calcium for this contraction may come in part from intracellular stores (12) or it may enter via voltage-dependent or voltage-independent calcium channels. The inability of D-600 to affect phase 2 in stEBA suggests that voltage-dependent, L-type channels are not involved; however, similar inhibition of contraction by nifedipine and zero extracellular calcium in rtEBA argues against this (see below).

Phase 2 contractions are dose dependent, and in both otherwise unstimulated and precontracted stEBA it appears that there are two components to the response: a high-sensitivity, low-ef‌cacy component and one that is ~100-fold less sensitive, but four to five times stronger (Fig. 3). It should be noted, however, that the NaHS concentration midway between these two components is also the concentration range at which phase 3 becomes most prevalent, and it is possible that phase 3 relaxation may have artificially created the two components. This needs further examination.

It is not clear if phase 2 contraction is physiologically relevant in vivo because the concentration of NaHS at which phase 2 becomes predominant (~10\(^{-3}\)) is well above plasma levels (4 \(\times\) 10\(^{-3}\)). H\(_2\)S is synthesized in the blood vessel wall, but not endothelium, of rat vessels (24). If H\(_2\)S synthesis also occurs in fish vessels, the H\(_2\)S concentration in the milieu around the vascular smooth muscle could be significantly higher than it is in the plasma and it may be possible to attain vasconstrictory levels. This would be especially relevant if H\(_2\)S synthesis in trout vessels is regulated, which has been shown to be the case in the rat aorta (24).

Phase 3 relaxation. Phase 3 relaxation of unstimulated or precontracted stEBA in vitro predominates in both magnitude and duration at H\(_2\)S concentrations similar to those found in plasma of unanesthetized fish. This suggests that H\(_2\)S is a tonically active vasodilator in trout and perhaps all fish. Phase 3 is less efficacious in relaxing vessels precontracted with either KCl or K\(_2\)SO\(_4\) than it is with ligand-contracted vessels,

\(\text{AJP-Regul Integr Comp Physiol} \cdot \text{VOL. 286} \cdot \text{APRIL 2004} \cdot \text{www.ajpregu.org}\)
(Fig. 7). Although the contribution of chloride channels cannot be ruled out, it appears that elevated potassium is responsible for the reduced NaHS efficacy, suggesting that potassium channels are an integral component of phase 3. The inhibitory effect of the K<sub>ATP</sub> channel inhibitor Gly on both phase 1 and phase 3 implicates K<sub>ATP</sub> channels in the H<sub>2</sub>S effect. This is somewhat surprising to us because we have performed extensive studies on both stEBA and rtEBA at rest and under a variety of stimuli, using both Gly and the K<sub>ATP</sub> channel opener pinacidil, and up until now both drugs have been ineffective (M. P. Smith and K. R. Olson, unpublished).

Inhibition of phase 3 by ODQ and NS-2028 implicates sGC in H<sub>2</sub>S-mediated vasodilation of phase 3 in stEBA. However, we did not find any evidence for cGMP production in rtEBA exposed to either low (3.7 × 10<sup>-4</sup> M) or high (3.7 × 10<sup>-3</sup> M) NaHS, although the lower concentration appeared to produce a slight relaxation and clearly the vessels were capable of generating cGMP when stimulated with SNP (Fig. 8). It remains to be determined if the effects of ODQ are nonspecific in stEBA or if there are other conditions that might have affected cGMP production in rtEBA, such as NaHS concentration, time dependence of NaHS exposure, or the necessity for prestimulation. Although it seems less likely, there may also be differences between stEBA and rtEBA.

The steepness and relative proximity of the concentration-dependent phase 2 contraction and phase 3 relaxation (Fig. 3) and their apposition to plasma H<sub>2</sub>S titers raise an interesting question: can fish actively increase or decrease vessel tone with a single signaling molecule? If so, this would be quite novel because we are unaware of any paracrine factor synthesized by mammalian vascular smooth muscle with this dual capability. Alternatively, is the H<sub>2</sub>S-mediated constriction a defense mechanism to prevent circulatory collapse in the event that environmental H<sub>2</sub>S increases? Our results indicate that, in vitro, vascular smooth muscle is not adversely affected by elevated H<sub>2</sub>S and that isolated vessels remain functional at NaHS levels as high as 0.1 M, although these levels in the environment are extremely toxic to fish (16). It remains to be determined what impact elevated H<sub>2</sub>S has on the intact vascular system.

Environmental implications. Natural production of H<sub>2</sub>S can become toxic to fish, especially near the soil-water interface in water with high sulfur content, low O<sub>2</sub> saturation, and high concentrations of H<sub>2</sub>S producing bacteria (16). H<sub>2</sub>S can also be derived from artificial fiber synthesis, food production, paper and pulp manufacturing, rooting material production, sewage treatment, and livestock, the latter especially evident as runoff from manure pits (3, 16). Trout are one of the most sensitive aquatic vertebrates to H<sub>2</sub>S (10× more than goldfish, <i>Crassius auratus</i>), and the toxicity-concentration relationship is quite steep; the 48 h lethal threshold concentration for juvenile rainbow trout is 0.0087 mg/l H<sub>2</sub>S and the concentration that is lethal to one-half the trout at 48 h (LC<sub>50</sub>) is 0.015 mg/l H<sub>2</sub>S (16). Assuming that 15.5% of NaHS in solution exists as H<sub>2</sub>S (see below), the 48 h lethal threshold of NaHS is 1.0 × 10<sup>-6</sup> M and the LC<sub>50</sub> is 1.7 × 10<sup>-6</sup> M. The EC<sub>50</sub> for phase 3 is only 6.7 × 10<sup>-5</sup> M, and plasma levels are 4 × 10<sup>-5</sup> M, roughly 50 times critical levels in the water. We are not aware of the mechanism(s) of H<sub>2</sub>S (or sulfide) excretion in trout; however, it appears that at least a 100-fold gradient from blood to water is necessary for survival. It remains to be determined if the cardiovascular system is the target of H<sub>2</sub>S toxicity; the proximity of plasma levels and vascular sensitivity to lethal ambient concentrations suggest that this is possible.

NaHS solution chemistry. A few comments on the chemistry of H<sub>2</sub>S are in order. The present study, like those on the rat (23), uses the hydrosulfide ion (HS<sup>-</sup>), provided by NaHS in solution, to produce H<sub>2</sub>S. NaHS has been proven to be quantitatively as efficacious as a solution prepared by bubbling H<sub>2</sub>S gas (23). Assuming that molecular H<sub>2</sub>S is the vasoactive species, bath conditions (pH, temperature, etc.) will affect the H<sub>2</sub>S concentration. Earlier studies (6, 20, 21, 23) made the assumption that H<sub>2</sub>S in solution at the mammalian pH of 7.4, exists as ~30–33% H<sub>2</sub>S and 67–70% HS<sup>-</sup>. Although it is reasonable to neglect the third species, S<sup>2-</sup>, due to the high pK<sub>2</sub> (11.96), and where this 30–33% assumption does hold true for a standard Henderson-Hasselbach calculation at 20°C, it ignores the effect of temperature on pK<sub>A</sub>. Mammalian vessels are routinely examined at their physiological temperature, 37°C. Using the equation established experimentally (3), the pK<sub>A</sub> at 37°C is 6.755, not 7.04 for standard conditions (20°C). This reduces the estimated %H<sub>2</sub>S from 30–33% to 18.5%. In our experiments at 14°C the pK<sub>A</sub> is ~7.06. With the bath (or trout plasma) pH of 7.8, the estimated %H<sub>2</sub>S is 15.5%. Thus the effects of temperature on pK and the physiological pH of these two vertebrates offset and there is only a small difference between our estimated [H<sub>2</sub>S] and those used in mammalian studies. Furthermore, adding NaSH to the bath has a negligible effect on bath pH. Assuming that the %H<sub>2</sub>S in trout and mammalian studies are essentially similar, then it appears that trout arteries (phase 3, Fig. 3) are twice as sensitive as rat aortas (24) to H<sub>2</sub>S (EC<sub>50</sub> of 6.7 × 10<sup>-3</sup> vs. 12.5 × 10<sup>-3</sup> M, respectively). In addition, H<sub>2</sub>S is more efficacious in trout; although it typically relaxes precontracted rat aortas by 50–60% (24), it produces ~100% relaxation of Carb-contracted efferent branchial arteries (Fig. 2C).

Perspectives

Hydrogen sulfide is the third gasotransmitter with vasoactive activity described in mammals and it is now the first such molecule to be identified in fish. Few, if any, vasodilator molecules, other than H<sub>2</sub>S, have been found in fish plasma at physiologically relevant concentrations and this is perhaps indicative of a premiere role for H<sub>2</sub>S in piscine cardiovascular homeostasis. In addition to its cardiovascular aspects, this study opens a number of other interesting investigative venues. H<sub>2</sub>S has a variety of physiological functions in the mammalian central nervous system and its effects on organ systems in fish need to be examined. Synthesis, metabolism, and excretion of H<sub>2</sub>S, and their regulation in fish need to be addressed and the conundrum of how plasma H<sub>2</sub>S levels can exceed toxic environment concentrations must be resolved. Finally, the problem of integrating this endogenous regulatory system in the face of potentially disrupting H<sub>2</sub>S from the environment may provide insight into both H<sub>2</sub>S homeostasis and toxicity.

ACKNOWLEDGMENTS

The authors thank Dr. R. Wang for helpful suggestions, Dr. A. Schulman and M. Doellman for technical assistance, and D. Munnick and the staff at the Richard Clay Bodine State Fish Hatchery, Indiana Department of Natural Resources, for help in obtaining steelhead tissues.
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GRANTS

This work was supported in part by National Science Foundation Grant No. IBN-0235223.