Vertebrate phylogeny of hydrogen sulfide vasoactivity

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HYDROGEN SULFIDE (H2S) is found in both aquatic and terrestrial environments where it can be produced by both natural and industrial processes (15). Long known for its toxic properties (16, 38), reproductive (34, 38), pulmonary (33, 44), and heart tissues, including brain (1), vascular (16, 45), gastrointestinal and have physiological actions on, a variety of mammalian classes to determine whether H2S is universally vasoactive and to

Dombkowski, Ryan A., Michael J. Russell, Alexis A. Schulman, Meredith M. Doellman, and Kenneth R. Olson. Vertebrate phylogeny of hydrogen sulfide vasoactivity. Am J Physiol Regul Integr Comp Physiol 288: R243–R252, 2005. First published September 2, 2004; doi:10.1152/ajpregu.00324.2004.—Hydrogen sulfide (H2S) is a recently identified endogenous vasodilator in mammals. In steelhead/rainbow trout (Oncorhynchus mykiss, Osteichthyes), H2S produces both dose-dependent dilation and a unique dose-dependent constriction. In this study, we examined H2S vasoactivity in all vertebrate classes to determine whether H2S is universally vasoactive and to identify phylogenetic and/or environmental trends. H2S was generated from NaHS and examined in unstimulated and precontracted systemic and, when applicable, pulmonary arteries (PA) from Pacific hagfish (Aptostichus stoumi, Agnatha), lamprey (Petromyzon marinus, Agnatha), sandbar shark (Carcharhinus milberti, Chondrichthyes), marine toad (Bufo marinus, Amphibia), American alligator (Alligator mississippiensis, Reptilia), Pekin duck (Anas platyrhynchos domesticus, Aves), and white rat (Rattus rattus, Mammalia). In otherwise unstimulated vessels, NaHS produced 1) a dose-dependent relaxation in Pacific hagfish dorsal aorta; 2) a dose-dependent constriction in sea lamprey dorsal aorta, marine toad aorta, alligator aorta and PA, duck aorta, and rat thoracic aorta; 3) a threshold relaxation in shark ventral aorta, dorsal aorta, and afferent branchial artery; and 4) a multiphasic contraction-relaxation-contraction in the marine toad PA, duck PA, and rat PA. Precontraction of these vessels with another agonist did not affect the general pattern of NaHS vasoactivity with the exception of the rat aorta, where relaxation was now dominant. These results show that H2S is a phylogenetically ancient and versatile vasoregulatory molecule that appears to have been opportunistically engaged to suit both organ-specific and species-specific homeostatic requirements.

cardiovascular; fish; amphibian; reptile; bird; mammal; evolution levels are sufficiently high to produce vasoactive effects (44, 47). The pathway(s) through which H2S exerts its dilatory activity has not been completely established, although it is evident that potassium channels, especially those sensitive to ATP (KATP), are involved, and, when opened by H2S, the resulting hyperpolarization initiates relaxation (45, 47).

In some instances, H2S appears to interact with NO, thereby augmenting relaxation in rat thoracic aorta and portal veins in vitro (16). Blockade of NO synthase via nitro-l-arginine methyl ester (l-NAME) also reduces the relaxation efficacy of NaHS (45). Similar synergy has been demonstrated in vivo. l-NAME-induced-hypertensive rats display decreased mRNA transcription of cystathionine γ-lyase and lower plasma H2S levels, and, although exogenous H2S is able to counteract the hypertensive effects of l-NAME treatment, it does not alter NO production (48). Pretreatment of rat aorta with low concentrations of sodium nitroprusside (SNP) does not significantly affect NaHS-induced relaxation; however, low-dose NaHS significantly reduces SNP-induced vasorelaxation, suggesting that NO likely stimulates the H2S pathway, whereas the opposite does not appear to be the case. It is evident that the relationship between these two gases affords an additional level of cardiovascular integration.

If H2S is a physiologically relevant vasoregulatory molecule, it is likely to display a long phylogenetic history. Recent work in our laboratory has indicated that H2S may also be an important vasoregulatory signal in trout (7). However, unlike that observed in mammals, H2S produces a triphasic relaxation-contraction-relaxation in isolated conductance arteries (efferent branchial artery and celiacomesenteric artery) and veins (anterior cardinal vein) from steelhead trout (Oncorhynchus mykiss, Skamania strain). Although phase 1 relaxation is relatively minor, phase 2 contraction and phase 3 relaxation are quite pronounced. Both phase 2 and phase 3 are dose-dependent, with EC50 values of 1.1 × 10−3 M and 6.7 × 10−5 M, respectively; the apposition of the phase 3 EC50 with plasma titers of H2S (4.0 × 10−5 M) in trout indicates that this gas may be tonically vasoactive (7).

A H2S-mediated contraction, such as observed in trout, is unprecedented in mammalian smooth muscle. This suggests that H2S may have functions in nonmammalian vertebrates that are different from or in addition to its vasodilatory action in mammals. However, the physiological effects of H2S have not been examined in nonmammalian vertebrates, other than trout (7), and it has not been possible to determine whether the differences between trout and mammals

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reflect evolutionary trends, environmental constraints, or other factors.

The purpose of the present study was to examine the ubiquity of \( \text{H}_2\text{S} \) as a vasoactive “gasotransmitter” in vertebrates and to provide initial insight into the factors that may have affected its physiological expression. To this end, we examined the response of systemic and, when possible, pulmonary arteries (PA) from each vertebrate class to \( \text{H}_2\text{S} \) using in vitro myography. We found \( \text{H}_2\text{S} \)-mediated vasoactivity in all vertebrate vessels; in many species, we found these responses to be vessel specific and often multiphasic.

**MATERIALS AND METHODS**

**Animals**

All animal procedures received IACUC approval.

Pacific hagfish (**Eptatretus stouti**, 30–60 g) were captured off the coast of California and airlifted overnight to the University of Notre Dame (UND) in cold (5–8°C) seawater. The animals were housed at UND in 15-gallon saltwater aquaria at ~5°C and exposed to a 12:12-h light-dark photoperiod. They were not fed. Hagfish were anesthetized in benzocaine (1:5,000, wt/vol), and the dorsal aortas (DA) were dissected out and placed in hagfish buffered saline at 4°C until use.

Sea lampreys (**Petromyzon marinus**, 130–450 g) were trapped in streams feeding into the Great Lakes during the spring-summer spawning season and airlifted to UND where they were maintained in 500-liter rectangular tanks with aerated, flowing well water (15°C) and exposed to a 12:12-h light-dark photoperiod. They were not fed. Lampreys were anesthetized in benzocaine (1:5,000, wt/vol), and the DA were dissected out and placed in lamprey buffered saline at 4°C until use.

Sandbar sharks (**Carcharhinus milberti**) were captured off the Virginia coast by hook and line and held in captivity at the Virginia Institute of Marine Sciences for ~8 wk. They were then anesthetized with pentobarbital sodium (1–2 ml of a 390 mg/ml solution), and the DA, ventral aorta (VA), and afferent branchial arteries (ABA) were dissected out and placed in cold (4°C) elasmobranch buffered saline.

The vessels were then anesthetized overnight to UND and used within 24 h of arrival.

Marine toads (**Bufo marinus**) were purchased from a commercial supplier and maintained at Portland State University before shipment to UND. At UND, they were maintained under 12:12-h light-dark cycles with ready access to water and were fed mealworms twice per week. The animals were anesthetized with benzocaine, and the aorta and PA were dissected out and placed in 4°C amphibian saline until use.

Female American alligators (**Alligator mississippiensis**, Reptilia) were maintained at the University of California-Irvine. They were anesthetized with 5% isofluorane and injected with 100 United States Pharmacopoeia units heparin/ml blood. Visceral organs were removed intact and shipped overnight in cold (4°C) saline to UND. On arrival, the aorta and PA were immediately removed and placed in cold reptilian saline until use.

Pekin ducks (**Anas platyrhynchos domesticus**, Aves) were obtained from a local abattoir. The birds were killed by cervical dislocation, and the visceral organs were immediately placed in cold (4°C) avian saline. At UND, the aorta and PA were removed and stored in cold avian saline until use.

White rats (**Rattus rattus**, Mammalia) were anesthetized with 50 mg/animal pentobarbital sodium, and the visceral organs were removed and placed in cold (4°C) Krebs-Henseleit mammalian saline. The aorta and PA were then dissected out and stored at 4°C until use. In a few experiments, rats were euthanized with CO2, and the vessels were removed as described above.

**Smooth Muscle Isolation**

The vessels were cut into 0.5- to 1.0-cm-long segments and mounted on 280-μm-diameter stainless steel wire hooks and suspended in 5-ml water-jacketed smooth muscle baths filled with the appropriate buffer at the animal’s physiological temperature (hagfish: 14°C, lamprey: 14°C, shark: 23°C, toad: 25°C, alligator: 25°C, duck: 40°C, rat: 37°C). They were aerated with room air (HEPES buffers) or 95% air-5% CO2 (bicarbonate buffers). 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 Grass Instruments model 7E or 7F polygraph. Polygraph sensitivity was set to detect changes as small as 5 mg. We archived data on a personal computer at 1 Hz using Labtech notebook software (Laboratory Technologies, Andover, MA) or SoftWire (Measurement Computing, Middleboro, MA). The chart recorders and software were calibrated before each experiment.

Length-tension relationships have been derived from KCl-contracted vessels from most species in other studies (unpublished observations) in this laboratory. These data, plus general vessel size and physical characteristics, were taken into account to apply a reasonable baseline (resting) tension (~500–1,500 mg) for 0.5–1 h before experimentation. The vessels were then contracted with 80 mM KCl and washed twice, and resting tension was reestablished. The vessels were contracted a second time with 80 mM KCl and washed twice, and resting tension was reestablished for a minimum of 30 min before further experimentation. This procedure has been used to achieve optimum in vitro vessel activity (Olson, unpublished observations).

The cumulative dose-response characteristics of NaHS, which forms \( \text{H}_2\text{S} \) in solution (45), were examined in otherwise unstimulated vessels and in vessels precontracted with another ligand. The ligands were selected for optimal contractile properties, i.e., for hagfish, 3 × 10−4 M carbachol; for lamprey, shark, and alligator, 10−6 M norepinephrine (NE); and for duck, 10−7 M arginine vasotocin (AVT). Vessels precontracted with NE were treated with 10−6 M propranolol 10 min before NE addition. Because of limited tissue availability, NaHS dose-response relationships were not obtained from precontracted marine toad aorta and PA and rat PA.

**Data Analyses**

We fit dose-response curves for each vessel using Table Curve (Jandel, Chicago, IL). In a few instances, the vessels were still responding at the highest NaHS concentrations (>10−2 M), and the EC50 was estimated. Student’s t-tests were used for comparisons between groups of vessels (SigmaStat, Jandel). Results are expressed as means ± SD. Significance was assumed at \( P < 0.05 \).

**Chemicals**

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Physiological Salines**

**Hagfish HEPES-buffered saline.** This saline solution (pH 7.8) contained (in mM) 474 NaCl, 8.05 KCl, 5.1 CaCl2, 2H2O, 9 MgCl2 + 6H2O, 5.55 glucose, 3 HEPES acid, and 7 HEPES Na+ salt.

**Lamprey and trout HEPES-buffered saline.** This saline solution (pH 7.8) contained (in mM) 145 NaCl, 3 KCl, 0.57 MgSO4 + 7H2O, 2 CaCl2 + 2H2O, 5 glucose, 3 HEPES acid, and 7 HEPES Na+ salt.

**Elasmobranch bicarbonate/phosphate-buffered saline.** This saline solution (pH 7.6) contained (in mM) 280 NaCl, 6 KCl, 3 MgSO4 + 7H2O, 5 CaCl2 + 2H2O, 5 glucose, 0.5 Na2SO4, 1 NaH2PO4 + 2H2O, 8 NaHCO3, 350 urea, 72 trimethylamine-n-oxide, and 5 glucose.

**Amphibian bicarbonate-buffered saline.** This saline solution (pH 7.4) contained (in mM) 112 NaCl, 2 KCl, 1.8 CaCl2 + 2H2O, and 2.4 NaHCO3.
Reptilian bicarbonate-buffered saline. This saline solution (pH 7.4) contained (in mM) 80 NaCl, 2 CaCl₂, 2.5 KCl, 0.5 MgCl₂, 2.5 CaCl₂ × 2H₂O, 8.3 glucose, 0.8 NaH₂PO₄ × 2H₂O, 10 NaHC₂O₃, 0.2 NaHPO₄, 25 NaHCO₃, 5 alanine, and 0.11 ascorbic acid.

Avian bicarbonate/phosphate-buffered saline. This saline solution (pH 7.4) contained (in mM) 115 NaCl, 5 KCl, 0.5 MgCl₂, 2.5 CaCl₂ × 2H₂O, 8.3 glucose, 0.8 NaH₂PO₄ × 2H₂O, 10 NaHC₂O₃, 0.2 NaHPO₄, 25 NaHCO₃, 5 alanine, and 0.11 ascorbic acid.

Mammalian Krebs-Henseleit bicarbonate-buffered saline. This saline solution (pH 7.4) contained (in mM) 115 NaCl, 2.51 KCl, 2.46 MgSO₄, 1.91 CaCl₂ × 2H₂O, 5.56 glucose, 1.38 NaH₂PO₄, 25 NaHCO₃, and 25 NaHCO₃.

All buffers were stored in the refrigerator and used within 72 h of preparation. The effects of NaHS on pH of the buffered salines were measured with a standard pH electrode at 22°C during step-wise addition of NaHS.

RESULTS

The effects of NaHS on unstimulated and precontracted vessels and their EC₅₀ values are summarized in Tables 1 and 2, respectively. Figures 1–13 illustrate typical responses, and Fig. 14 is a cladogram of NaHS vasoactivity.

Pacific Hagfish DA

A few unstimulated hagfish DA displayed a small contraction at NaHS concentrations of <3 × 10⁻⁵ M; however, concentrations greater than this produced a predominant dose-dependent relaxation (Fig. 1). Vessels were still relaxing at concentrations greater than this produced a predominant dose-addition of NaHS.

Reptilian bicarbonate-buffered saline. This saline solution (pH 7.4) contained (in mM) 115 NaCl, 5 KCl, 0.5 MgCl₂, 2.5 CaCl₂ × 2H₂O, 8.3 glucose, 0.8 NaH₂PO₄ × 2H₂O, 10 NaHC₂O₃, 0.2 NaHPO₄, 25 NaHCO₃, 5 alanine, and 0.11 ascorbic acid.

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Pacific Hagfish DA

A few unstimulated hagfish DA displayed a small contraction at NaHS concentrations of <3 × 10⁻⁵ M; however, concentrations greater than this produced a predominant dose-dependent relaxation (Fig. 1). Vessels were still relaxing at 10⁻¹ M NaHS, at which point the experiment was terminated. Carbachol-precontracted DA also contracted slightly at low (<3 × 10⁻⁵ M) NaHS concentrations and relaxed at higher concentrations. At ~10⁻³ M NaHS, nearly 100% of the carbachol-induced contraction was abolished. The vessels did not return to baseline tension after the NaHS was removed, and they were largely unresponsive to further carbachol or NaHS exposure.

Rat AO (CO₂) C 6.0 ± 0.2 × 10⁻³ (7)

Table 1. Responses of otherwise unstimulated vessels to NaHS

<table>
<thead>
<tr>
<th>Animal</th>
<th>Artery</th>
<th>Response</th>
<th>EC₅₀ (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hagfish</td>
<td>DA</td>
<td>R</td>
<td>3.0 ± 0.3 × 10⁻³ (3/8)*</td>
</tr>
<tr>
<td>Lamprey</td>
<td>DA</td>
<td>C</td>
<td>1.7 ± 10⁻⁴ ± 0.7 × 10⁻⁴ (7)</td>
</tr>
<tr>
<td>Shark</td>
<td>DA</td>
<td>R</td>
<td>3 × 10⁻⁴ (5)†</td>
</tr>
<tr>
<td>VA</td>
<td>R</td>
<td>3 × 10⁻⁴ (5)†</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>R</td>
<td>3 × 10⁻⁴ (5)†</td>
<td></td>
</tr>
<tr>
<td>Trout‡</td>
<td>EBA</td>
<td>R₁-C₂-R₃</td>
<td>C₂: 1.8 ± 0.07 × 10⁻³ (7)</td>
</tr>
<tr>
<td>Toad</td>
<td>AO</td>
<td>C</td>
<td>7.0 ± 2.1 × 10⁻⁴ (2/4)†</td>
</tr>
<tr>
<td>PA</td>
<td>C₁-R₂-C₃</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Alligator</td>
<td>AO</td>
<td>C</td>
<td>4.0 ± 3.9 × 10⁻⁴ (2/7)*</td>
</tr>
<tr>
<td>PA</td>
<td>C</td>
<td>1.0 ± 0.8 × 10⁻³ (2/7)*</td>
<td></td>
</tr>
<tr>
<td>Duck</td>
<td>AO</td>
<td>C</td>
<td>3.0 ± 2.0 × 10⁻³ (7)*</td>
</tr>
<tr>
<td>PA</td>
<td>C₁-R₂-C₃</td>
<td>R₂: 8.9 ± 3.2 × 10⁻⁴ (8)</td>
<td></td>
</tr>
<tr>
<td>C₃: 2.3 ± 1.2 × 10⁻³ (8)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>AO (CO₂)</td>
<td>C</td>
<td>6.0 ± 0.2 × 10⁻³ (7)</td>
</tr>
<tr>
<td>AO (PB)</td>
<td>C</td>
<td>1.3 ± 0.3 × 10⁻³ (8)</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values for EC₅₀ are mean NaHS (in mol/l) ± SD; n, no. of animals; n₁/n₂, no. of animals/no. of vessels when n₁ < n₂. *Estimated EC₅₀; † single threshold dose; ‡from Ref. 3; ND, not determined.

Table 2. Responses of pre-contracted vessels to NaHS

<table>
<thead>
<tr>
<th>Animal</th>
<th>Artery</th>
<th>Response</th>
<th>EC₅₀ (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hagfish</td>
<td>DA</td>
<td>R</td>
<td>3.0 ± 0.3 × 10⁻³ (3/8)*</td>
</tr>
<tr>
<td>Lamprey</td>
<td>DA</td>
<td>C</td>
<td>1.8 ± 0.4 × 10⁻⁴ (7)</td>
</tr>
<tr>
<td>Shark</td>
<td>DA</td>
<td>R</td>
<td>3 × 10⁻⁴ (5)†</td>
</tr>
<tr>
<td>VA</td>
<td>R</td>
<td>3 × 10⁻⁴ (5)†</td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>R</td>
<td>3 × 10⁻⁴ (5)†</td>
<td></td>
</tr>
<tr>
<td>Trout‡</td>
<td>EBA</td>
<td>R₁-C₂-R₃</td>
<td>C₂: 1.1 ± 0.4 × 10⁻³ (7)</td>
</tr>
<tr>
<td>Toad</td>
<td>AO</td>
<td>C</td>
<td>7.0 ± 2.1 × 10⁻⁴ (4)*</td>
</tr>
<tr>
<td>PA</td>
<td>C₁-R₂-C₃</td>
<td>R₂: 6.5 ± 4.9 × 10⁻³ (2/4)*</td>
<td></td>
</tr>
<tr>
<td>Alligator</td>
<td>AO</td>
<td>C</td>
<td>4.0 ± 3.9 × 10⁻³ (2/7)*</td>
</tr>
<tr>
<td>PA</td>
<td>C</td>
<td>1.0 ± 0.8 × 10⁻³ (2/7)*</td>
<td></td>
</tr>
<tr>
<td>Duck</td>
<td>AO</td>
<td>C</td>
<td>3.0 ± 2.0 × 10⁻³ (7)</td>
</tr>
<tr>
<td>PA</td>
<td>C₁-R₂-C₃</td>
<td>R₂: 4.7 ± 3.1 × 10⁻³ (8)</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>AO (CO₂)</td>
<td>R</td>
<td>1.3 ± 0.4 × 10⁻³ (8)*</td>
</tr>
<tr>
<td>PA (PB)</td>
<td>C₁-R₂-C₃</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Values for EC₅₀ are mean NaHS (in mol/l) ± SD; n, no. of animals; n₁/n₂, no. of animals/no. of vessels when n₁ < n₂. *Estimated EC₅₀; † single threshold dose, ‡ from Ref. 3; § Zhao et al. (47); ND, not determined.

Sea Lamprey DA

NaHS contracted otherwise unstimulated (Fig. 2) and NE-precontracted sea lamprey (not shown) DA with a threshold slightly above 10⁻⁵ M. In other experiments, a single application of 10⁻³ M NaHS to NE-contracted DA produced an initial steep contraction, followed by a delay (or slight relaxation), and then an even greater contraction (Fig. 3A). The vessels returned to baseline tension after removal of NaHS. When these vessels were contracted a second time with NE and exposed to 10⁻³ M NaHS, they developed a similar amount of total tension, but the initial contraction and relaxation were essentially lost (Fig. 3B).

Sandbar Shark DA, VA, and ABA

All unstimulated shark vessels, including spontaneously contracting DA, displayed a threshold relaxation at 3 × 10⁻⁴

Fig. 1. Typical records of the cumulative NaHS dose-response characteristics in both unstimulated (A) and 10⁻⁴ M carbachol (CARB)-precontracted (B) Pacific hagfish dorsal aorta (DA). Values indicate log M NaHS concentration ([NaHS]); unlabeled arrows represent 3× the previous [NaHS]. W, wash of agonists.
M NaHS (Fig. 4). This did not appear to be dose dependent because further increases in NaHS concentration did not affect vessel tension.

As has been shown in other elasmobranchs (27), precontraction often increases the baseline tension as well as frequency and amplitude of spontaneous contractions. These parameters in spontaneously active DA and ABA were inhibited with NaHS at the same 3 × 10⁻⁴ M threshold (Fig. 5). Conversely, most VA displayed little response to NE stimulation or NaHS, although removal of NE and NaHS produced several large transient contractions (Fig. 5). A few additional differences between DA and ABA were noted. The spontaneous contractions of NE-precontracted ABA had greater amplitude and lower frequency than those observed in DA (Fig. 5). In ABA, 3 × 10⁻⁴ M NaHS nearly eliminated the spontaneous contrac-

![Fig. 2. NaHS dose-response curve for unstimulated sea lamprey DA (n = 7). Inset: typical record. Values indicate log M [NaHS]; unlabeled arrows represent 3 × the previous [NaHS].](image)

![Fig. 3. Typical record of a 10⁻⁶ M norepinephrine (NE)-precontracted sea lamprey DA after an initial exposure to 10⁻³ M NaHS in which an initial transient contraction and possible relaxation are observed (A); this was followed by a wash and then a second NaHS exposure (B), which eliminated all but the last phase. Prop, propranolol.](image)

![Fig. 4. Typical records of the cumulative NaHS dose-response characteristics of an unstimulated afferent branchial artery (ABA), ventral aorta (VA), and DA from the sandbar shark. Values indicate log [NaHS]; unlabeled arrows represent 3 × the previous [NaHS]. Large arrows at the end of the records (W) represent 2 consecutive washes. Entire response occurs at essentially 3 × 10⁻⁴ M NaHS.](image)

![Fig. 5. Typical tracings of the cumulative NaHS dose-response characteristics of a 10⁻⁶ M NE-precontracted ABA, VA, and DA from the sandbar shark. Vessels were pretreated with 10⁻⁵ M Prop before NE. Values indicate log [NaHS]; unlabeled arrows represent 3 × the previous [NaHS]. Large arrows at the end of the records (W) represent 2 consecutive washes. The NE precontraction produced spontaneous contractions in ABA and DA, which were inhibited by a threshold dose of 3 × 10⁻⁴ M NaHS. The frequency of spontaneous contractions in ABA also appeared to be increased by 3 × 10⁻⁵ M NaHS.](image)
**Marine Toad Aorta and PA**

NaHS produced essentially identical dose-dependent monophasic contractions of both unstimulated (Fig. 6, *top*) and NE-precontracted marine toad aorta with a threshold concentration of $3 \times 10^{-4}$ M. The strongest contraction was produced at the highest dose used ($10^{-2}$ M NaHS), and it was difficult to establish an accurate EC$_{50}$. At this dose ($10^{-2}$ M), considerable tension was produced in otherwise unstimulated vessels, >1 g of tension/10 mg vessel wet wt. The vessels did not recover after NaHS washout, and their response to KCl was reduced by over 90%.

The responses of both unstimulated (Fig. 6, *bottom*) and NE-precontracted PA appeared multiphasic. At low doses ($10^{-4}$ M NaHS), there was a monophasic contraction that changed to a transient contraction superseded by relaxation at $10^{-3}$ M. At $3 \times 10^{-3}$ M, only a relaxation was observed, whereas at $10^{-2}$ M NaHS, there was a transient contraction, relaxation, and then a sustained contraction. The vessels did not recover after NaHS removal. We could only obtain a total of four PA from two animals, and, because these vessels were small (~3 mg), as was the magnitude of the response, the dose-response characteristics of the individual phases in precontracted vessels could not be determined.

**American Alligator Aorta and PA**

Unstimulated American alligator AP and PA displayed dose-dependent contraction to NaHS (Fig. 7). The vessels were still contracting significantly at the highest dose examined ($10^{-1}$ M), and EC$_{50}$ values were estimated. Aortas and PA precontracted with $10^{-6}$ M NE also displayed a similar NaHS dose-dependent contraction (data not shown). In other experiments on NE-precontracted vessels, a single treatment with $10^{-3}$ M NaHS produced a contraction in aortas that was of similar magnitude to the NE contraction, whereas the PA response to NaHS was fourfold greater than the NE contraction (Fig. 8).

**Pekin Duck Aorta and PA**

NaHS produced a dose-dependent contraction in unstimulated duck thoracic aorta with a threshold around $10^{-4}$ M (Fig. 9). The EC$_{50}$ values were estimated because aortas were still responding at the highest dose ($10^{-2}$ M); however, the vessels recovered when NaHS was removed. Aortas precontracted with $10^{-6}$ M AVT displayed a brief and small relaxation to $3 \times 10^{-5}$ M NaHS, but subsequent doses resulted in contraction on top of the AVT plateau (Fig. 10). To examine whether this relaxation was a single event or dependent on the NaHS concentration, another group of aortas were precontracted with $10^{-6}$ M AVT or 80 mM KCl, and doses exceeding $3 \times 10^{-5}$ M NaHS were applied. Invariably, if NaHS concentration was $3 \times 10^{-5}$ M, it produced a contraction (not shown).

NaHS produced multiphasic responses in unstimulated PA (Fig. 9). At low ($\sim 10^{-5}$ M) concentrations, NaHS was mainly contractile. At $10^{-4}$ M, a brief contraction was followed by relaxation and a small contraction, and, at $3 \times 10^{-4}$ M, the initial brief contraction was followed by a sustained relaxation.
NaHS concentrations of $10^{-3}$ and $3 \times 10^{-3}$ M produced monophasic contractions, and the triphasic response reappeared at $10^{-2}$ M NaHS. Spontaneous contractions appeared in AVT-precontracted PA, and, although they were inhibited by NaHS, it was not possible to examine NaHS effects at low ($<10^{-4}$ M) concentrations. Higher concentrations of NaHS produced a triphasic contraction-relaxation-contraction (from $10^{-4}$ to $3 \times 10^{-4}$ M) and a monophasic contraction at NaHS in excess of $10^{-3}$ M NaHS. Both unstimulated and AVT-precontracted vessels completely recovered when the compounds were washed out.

The dose-response curves for phase 2 relaxation and phase 3 contraction of unstimulated and precontracted PA are shown in Fig. 11. The predominant dose-dependent relaxation at lower NaHS concentrations may in fact be masking a portion of the dose-dependent constriction, or there may be two distinct contractile responses.

**Rat Thoracic Aorta**

Unstimulated rat thoracic aortas displayed little activity to NaHS until doses were elevated to $10^{-3}$ M, and monophasic contractions were observed thereafter (Fig. 12). The method of euthanasia appeared to affect the vessel’s response, as the EC$_{50}$ for aorta from rats euthanized with CO$_2$ was significantly ($P < 0.05$) lower than for rats euthanized with pentobarbital (Fig. 13). NaHS produced a dose-dependent relaxation of aorta precontracted with $10^{-7}$ NE (not shown).

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**Fig. 9.** NaHS dose-dependent responses of unstimulated thoracic aorta (top) and PA (bottom) from the Pekin duck. Values indicate log [NaHS]; unlabeled arrows represent 3× the previous [NaHS]. Aortic responses are monophasic; PA show a transient contraction and then relaxation at lower [NaHS] that gives way to a contraction and then triphasic contraction-relaxation-contraction at high ($10^{-3}$ M) NaHS.

**Fig. 10.** Traces of the cumulative NaHS dose-response characteristics of $10^{-6}$ M arginine vasotocin (AVT)-precontracted thoracic aorta (top) and PA (bottom) from the Pekin duck. Values indicate log [NaHS]; unlabeled arrows represent 3× the previous [NaHS]. NaHS produced a monophasic contraction of aortas, except for a slight relaxation at the lowest dose. Low doses of NaHS produced a dose-dependent relaxation of PA, which was replaced by contraction at higher doses.

**Fig. 11.** Cumulative dose-response curves for the contraction (squares) and relaxation (circles) of unstimulated (open symbols) and AVT-precontracted (solid symbols) duck PA. EC$_{50}$ values for unstimulated and AVT-precontracted relaxation were $8.86 \pm 3.19 \times 10^{-3}$ M ($n = 8$) and $4.73 \pm 3.10 \times 10^{-3}$ M NaHS ($n = 8$), respectively. EC$_{50}$ values for unstimulated and AVT-precontracted contraction were $2.34 \pm 1.19 \times 10^{-3}$ M ($n = 8$) and $1.78 \pm 0.99 \times 10^{-3}$ M NaHS ($n = 8$), respectively. SD omitted for clarity.

**Fig. 12.** Typical record of the monophasic, dose-dependent contractile response of unstimulated rat aorta to NaHS and the triphasic response (in parentheses) of $10^{-6}$ M NE-precontracted rat PA to $10^{-3}$ M NaHS. Values for aorta indicate log [NaHS]; unlabeled arrows represent 3× the previous [NaHS].
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Fig. 13. NaHS dose-response curve for unstimulated rat thoracic aortas from animals euthanized with CO2 (○) or pentobarbital (PB; □). EC50 values for CO2 and PB (5.96 ± 2.03 × 10^-3 M, n = 7, and 1.34 ± 0.25 × 10^-2 M, n = 8, respectively) are significantly (P < 0.05) different.

Rat PA precontracted with 10^-7 NE displayed a triphasic contraction-relaxation-contraction when exposed to 10^-3 M NaHS (n = 7, Fig. 12). No other studies have been performed on rat pulmonary vessels.

DISCUSSION

The results from the present study show that H2S has vasoactive properties in at least one species from each class of vertebrates; results also support the hypothesis that this gasotransmitter is a phylogenetically ancient vasoregulatory molecule. However, unlike the monophasic vasorelaxation that has been reported previously in the rat (45, 47) our studies show that H2S has both vasodilatory and vasoconstrictory properties. Furthermore, these disparate responses may occur in different vascular beds within a single species or even in the same vessel at different levels of H2S exposure. This level of complexity and versatility suggests that H2S may be unparalleled as a vasoregulatory molecule and that it may have been opportunistically adapted to suit diverse homeostatic functions.

It is difficult to unequivocally establish evolutionary patterns or environmental factors that may have been involved in the determination of H2S vasoactivity because of the variety of H2S-mediated responses and the fact that we only examined a single species from each class of vertebrates. However, there are some interesting points that deserve discussion and certainly further investigation.

Antiquity of the H2S Response

H2S joins NO as the second putative gasotransmitter with demonstrated vasoactivity in all nonmammalian vertebrates examined to date, but this is not proof that they are endogenous vasoregulators. There is still considerable controversy regarding whether NO is produced by fish vascular endothelium and whether it acts as a local messenger. NO, or NO donors such as SNP, are vasoconstrictors in Agnatha and Chondrichthyes (12, 17, 23, 32, 37), although studies in vivo or in perfused organ preparations have supported NO as an endothelium-derived dilator in Osteichthyes (12, 17, 23, 32, 37), studies on isolated blood vessels have failed to support an endothelial source of NO from any fish (9, 11, 19, 28), and the possibility remains that NO signaling from the vascular endothelium originated in Amphibia (22).

Information regarding vascular H2S production is limited, although there is support for H2S as a vascular signaling molecule in Osteichthyes. In preliminary studies, our laboratory found that rainbow trout vessels produce H2S (26) and that H2S circulates in the plasma at physiologically relevant levels (7). Although additional studies on Agnatha and Chondrichthyes are needed, it is possible that H2S predates NO as an endogenous vasoregulatory gasotransmitter.

The “Primordial” Response

With the exception of Chondrichthyes, we found that H2S has some contractile activity in all vertebrates (Fig. 14), which suggests that vasoconstriction may be the most phylogenetically ancient and prevalent response. Earlier reports of the effects of H2S on precontracted rat aortas indicated a monophasic, dose-dependent relaxation, without any contraction (16, 45, 47). However, as shown in Fig. 12, H2S produces essentially a monophasic contraction in unstimulated rat aortas, and the threshold for this contraction, 3 × 10^-3 M (Fig. 12), is 15-fold greater than the threshold for relaxation of precontracted aortas (45, 47). A substantial contraction is also evident in NE-precontracted rat PA exposed to 10^-3 M H2S (Fig. 12).

The weakest contractile response of nonmammalian vertebrates was observed in hagfish aortas (Fig. 1), and no contraction was observed in shark aortas. Unlike the rat aorta, H2S-mediated relaxations in the hagfish and shark were independent of prestimulation. The latter two were also the only two marine vertebrates examined and the only two with plasma osmolarity above 350 mosM. It remains to be determined whether there is an environmental correlate with H2S vasoreactivity. A similar case for an environmental component could be made for the aortas of terrestrial vertebrates, all of which contract when exposed to H2S, in the absence of a precontraction, and all but the rat contract in the presence of precontractile stimulation. The shark response was also unique in that it occurred at a single threshold dose. It is possible that a dose-dependent

![Cladogram illustrating the responses of systemic (symbols above thick line) and either pre-gill or pulmonary (symbols below line) vessels to NaHS. +, Contraction; -, relaxation. Groups of symbols represent multiphasic responses. Responses in parentheses indicate those observed only in precontracted vessels.](http://ajpregu.physiology.org/)
response falls between the doses used (10^{-4} and 3 \times 10^{-4} M H_2S), although this seems unlikely. One could also argue that relaxation and constriction may have appeared nearly simultaneously during vertebrate evolution because the responses of the two Agnathans used in this study were essentially the opposite. In addition, multiphasic responses were observed in all but two of the vertebrates (Fig. 14).

**Tissue Specificity and Multiphasic Responses**

A striking finding of this study is the dissimilarity between the H_2S responses of systemic and respiratory vessels in trout, toads, birds, and mammals. In all but the trout, the aortic responses were monophasic and pulmonary responses were triphasic and consistently a contraction-relaxation-contraction. Even in the alligator, where H_2S contracted both systemic and pulmonary vessels, H_2S was considerably more potent in the latter (Fig. 8) and the threshold was 10-fold lower. The general differences between H_2S responses in systemic and respiratory vessels strongly suggest that H_2S performs tissue-specific functions and that it is more versatile in the systemic circulation of trout and the pulmonary circulation of most terrestrial vertebrates.

The multiphasic response itself is both unusual and versatile. It is technically difficult to separate all three phases and to identify the dose dependency of each. However, we (7) have previously characterized the second (constriction) and third (dilation) phases in trout efferent branchial arteries and found that there is only a 16-fold difference in the EC_{50} between the two phases. Although we can only estimate the EC_{50} values for the third (constriction) phase of the toad and duck, the difference in otherwise unstimulated toad PA is only 3-fold and in unstimulated and precontracted duck PA the difference is 26- and 38-fold, respectively. These estimates can be refined and extended to other vertebrates once specific inhibitors of the different phases are identified; nevertheless, they strongly suggest that the outcome of H_2S vasoactivity can be readily reversed with modest adjustments in either H_2S production or inactivation.

**Physiological Relevance of H_2S Doses**

Plasma H_2S concentrations in trout and rats are between 4 \times 10^{-5} and 3 \times 10^{-4} M (7, 43, 44, 47), and because H_2S is synthesized by vascular smooth muscle it is likely that tissue levels exceed those in the plasma. To our knowledge, plasma H_2S concentrations have not been reported for any other vertebrate, but they are probably similar to the trout and rat. Nearly all of the vessels that we examined responded to these physiologically relevant H_2S levels, and we assume that this is indicative of a tonic vasoregulatory activity of H_2S. We also observed substantial vasoactivity at higher H_2S concentrations, but the physiological relevance of this will not be known until intravascular and intracellular H_2S concentrations have been determined.

**Potential Sources of Variability/Error**

Although we feel that the responses to NaHS observed in the present study are typical for an individual species, there are a number of factors that could contribute to some variability in the pattern of response or the sensitivity of the vessel. Some of these factors are methodological, such as the variety of euthanasia methods and incubation media used and/or delays incurred during shipping and storage. Other variability could be attributable to indirect effects that H_2S might have on extracellular pH (pH_{e}) and intracellular pH (pH_{i}), Po_2, and H_2S toxicity. These points are briefly considered in the following paragraphs.

**Method of euthanasia.** The variety of anesthetics used during euthanasia could contribute to variability in the H_2S response, although we believe that the differences, if present, may be more in terms of H_2S sensitivity rather than substantive changes in the overall response pattern. In previous studies (7, and Dombkowski, Russell, and Olson, unpublished observations), we observed a similar triphasic H_2S response in efferent branchial arteries from steelhead trout anesthetized with ethyl-m-aminoenzoate (MS-222) and from rainbow trout (both *O. mykiss*) killed by a blow to the head. However, there were slight differences between these two strains in the dependence of phase 2 contraction on extracellular calcium, and it is not known whether this is characteristic of the different strains of trout or the effect of MS-222. Anesthetic effect is apparent in the H_2S-mediated contraction of unstimulated aortas from carbon dioxide- vs. pentobarbital sodium-euthanized rats (Table 1), where the former appears twice as sensitive to H_2S. Nevertheless, H_2S produced a monophasic contraction in both groups, whereas H_2S consistently relaxed precontracted aortas (47) from rats also killed after pentobarbital sodium anesthesia (Wang, personal communication). Duck vessels were not exposed to any anesthetic, yet they exhibited vessel-specific and, in the case of pulmonary vessels, multiphasic responses to NaHS. Thus it seems unlikely that anesthetics affect the general NaHS response.

**Vessel storage.** It has been our experience that vessels stored in cold physiological buffers remain viable and fully responsive to a variety of agonists for several days and vessels from ectothermic vertebrates even longer. Because vessels from both endothermic vertebrates were used within 24 h, little, if any, degradation in H_2S response is expected.

**Buffer characteristics.** A variety of salines and buffers was used in the present studies for several reasons: they were used in the laboratory that supplied the tissue, they were commonly cited in the literature, and, in the case of the HEPES buffers, they were specifically developed in this laboratory for use with isolated fish vessels. However, these salines could affect the vessels’ response, either because the chemical composition was not ideal or because the buffering capacity was insufficient at elevated NaHS concentrations. It is beyond the scope of this study to attempt to optimize the salines, the effects of NaHS and H_2S on pH_e and pH_i and the potential for pH-mediated effects on vascular tone need consideration.

NaHS could potentially affect both pH_e and pH_i. In the medium, formation of HS^- from the dissociation of NaHS to Na^+ + HS^- and subsequent formation of H_2S would be expected to raise pH_e whereas the gaseous H_2S, after rapidly diffusing into the cell and dissociating into H^+ and HS^-, would paradoxically lower pH_i. The degree of change in either pH_e or pH_i depends of course on the buffering capacity of the respective environments.

The effects of a wide range of NaHS concentrations on buffer pH is shown in Fig. 15. The pH of all media was relatively stable until NaHS concentrations exceed 1 mM. At 1 mM, pH of most salines increased by 0.1 pH unit or less; even
at 10 mM NaHS, most salines maintained pH within ~0.5 of the initial value. The amphibian and elasmobranch salines were the least effective, and 10 mM NaHS increased pH by nearly two units in the former and almost one unit in the latter. The effects of NaHS on pH are beyond the scope of this study; however, as described below, they may not mirror changes in pHo.

In mammalian vessels, alkalization of either extracellular or intracellular milieu generally produces contraction and acidification produces relaxation (2). The variety of NaHS responses observed in this study suggests that either 1) different vessels react differently to a common change in pH, 2) NaHS has variable effects on pHo and pHi in different vessels and/or media, or 3) pH is not a major mediator of the NaHS response. Sorting out these possibilities will be difficult because even the response of nonmammalian vessels to pH remains unclear. However, several points favor the third option. First, pHo remains relatively constant until NaHS exceeds 1 mM; therefore, contractions at lower NaHS concentrations are unlikely to be pH mediated. Second, NaHS-mediated relaxations are not due to a change in pH because NaHS only alkalizes the medium. Third, only a fraction (~25%; Ref. 7) of the dissolved NaHS exists as H2S at physiological pH; therefore, the effects of NaHS on intracellular H2S and pHi would be expected to be less than the effects on pHo. Furthermore, as pH increases, the H2S fraction decreases. For example, at 20°C, the pK\text{a} is nearly 7 (5, 26), and, if pH is 7.5, then a solution of NaHS is nearly 25% H2S. If pH increases to 8.5, then only 3% of the dissolved NaHS is H2S; this falls to 1% at pH 9. Experimentally, Jacques (18) has shown that H2S entry into cells of the marine alga, Valonia macrophysa, decreases by 80% as ambient alkalinity increases from a pH of 7.0 to 8.2. This creates an interesting dichotomy; buffers such as HEPES are better at preventing an increase in pH, but are predicted to favor a decrease in pH. Thus, on the basis of pH changes alone, vessels in well-buffered media would be predicted to relax at high NaHS concentrations due to the fall in pH, whereas, in poorly buffered media, the rise in pHo at elevated NaHS concentrations should favor contraction. There was no consistent trend to support this hypothesis in the present experiments, suggesting that, although some of the NaHS vasoactivity may be due to pH effects, a change in pH per se is not a major mechanism of NaHS action in vertebrate vessels. This also suggests that the lower EC\text{50} for contractions observed in aortas from rats euthanized with CO\textsubscript{2} was not a residual effect of hypercapnic acidosis, as this would be predicted to decrease a NaHS contraction. A residual effect of CO\textsubscript{2} on other cellular processes appears more likely.

**H2S oxidation-O2 reduction.** Oxidation of H2S by ambient or intracellular oxygen can potentially lower H2S levels and bias H2S dose-response estimates. Determination of intracellular H2S concentration is further complicated by the pH-dependent equilibrium of H2S across cell membranes (described above) and by tissue production of H2S (14, 16, 43, 46, 47). Thus the actual sensitivity of tissues to H2S may be either lower or greater than published values.

Conversely, reduction of tissue oxygen by H2S could produce hypoxia, which in itself could be vasoactive. Although spontaneous oxidation of H2S in the presence of O2 is rather slow (the half-time is in the order of 0.4 to 65 h in air-saturated seawater), catalyzed oxidation in cells is undoubtedly faster (3). With the oxygen solubility coefficient ranging between 1.25 and 2 \(\mu\text{mol}\cdot\text{l}^{-1}\cdot\text{Tor}^{-1}\) at 37 and 10°C, respectively (4), oxygen and H2S would be present in approximately equimolar amounts at a tissue Po\textsubscript{2} of 20 Torr. Even if Po\textsubscript{2} was greater, addition of exogenous H2S could produce hypoxic conditions. In mammals, hypoxia generally relaxes systemic vessels and contracts pulmonary vessels (42). Hypoxia also contracts DA of both hagfish and lamprey (29). Although in some tissues the vasoactive effects of H2S and hypoxia were similar (for example, the precontracted rat aorta and lamprey DA), in many other vessels they were the opposite (hagfish, toad, alligator, duck, and unstimulated rat aortas). A H2S-induced hypoxia cannot be ruled out in vessels that displayed multiphasic responses; however, like pH, the hypoxic component of H2S vasoactivity appears to be secondary in many vessels.

**H2S toxicity.** Toxic effects of H2S have been attributed to the ability of H2S to inhibit cytochrome-c oxidase at low micromolar concentrations and to open the mitochondrial permeability transition pore at half-millimolar H2S concentrations (20, 24, 39, 40). The H2S concentration that inhibits cytochrome-c oxidase is well below the doses we used, but they are also lower than plasma levels in both trout and rats. Mitochondrial oxidation of H2S in intact tissues raises the toxic threshold two to three orders of magnitude above that of tissue homogenates or purified enzyme; in fact, mitochondria of some fish can use up to 20 \(\mu\text{M}\) sulfide for ATP production (3). Our experiments indicate that some vascular smooth muscle is also quite resistant to elevated H2S, as many vessels appeared to completely recover from exposure to millimolar levels of H2S. How this is accomplished is not known, but it does not appear to be due to adaptation of cytochrome-c oxidase because the enzyme is equally H2S sensitive in both H2S-sensitive and H2S-tolerant organisms (3). Furthermore, the lack of correlation between H2S-mediated and hypoxia-mediated vascular responses (see above) suggests that cytochrome-c oxidase is not inhibited in those blood vessels tolerant to high H2S concentrations. It remains to be determined how H2S protection is conveyed.

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