The Journal of Experimental Biology 214, 4030-4040
© 2011. Published by The Company of Biologists Ltd
doi:10.1242/jeb.061473

RESEARCH ARTICLE

Hydrogen sulfide (H_2S) and hypoxia inhibit salmonid gastrointestinal motility: evidence for H_2S as an oxygen sensor

Ryan A. Dombkowski[1], Marie G. Naylor[1], Emma Shoemaker[1], Michelle Smith[1], Eric R. DeLeon[2,4], Gilbrian F. Stoy[3,4], Yan Gao[4] and Kenneth R. Olson[4,*]

[1]Department of Biology, Saint Mary's College, Notre Dame, IN 46556, USA, [2]Department of Chemistry, University of Notre Dame, Notre Dame, IN 46556, USA, [3]Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA and [4]Indiana University School of Medicine – South Bend, South Bend, IN 46617, USA

*Author for correspondence (kolson@nd.edu)

Accepted 30 August 2011

SUMMARY

Hydrogen sulfide (H_2S) has been shown to affect gastrointestinal (GI) motility and signaling in mammals and O_2-dependent H_2S metabolism has been proposed to serve as an O_2 'sensor' that couples hypoxic stimuli to effector responses in a variety of other O_2-sensing tissues. The low P_{O_2} values and high H_2S concentrations routinely encountered in the GI tract suggest that H_2S might also be involved in hypoxic responses in these tissues. In the present study we examined the effect of H_2S on stomach, esophagus, gallbladder and intestinal motility in the rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) and we evaluated the potential for H_2S in oxygen sensing by examining GI responses to hypoxia in the presence of known inhibitors of H_2S biosynthesis and by adding the sulfide donor cysteine (Cys). We also measured H_2S production by intestinal tissue in real time and in the presence and absence of oxygen. In tissues exhibiting spontaneous contractions, H_2S inhibited contraction magnitude (area under the curve and amplitude) and frequency, and in all tissues it reduced baseline tension in a concentration-dependent relationship. Longitudinal intestinal smooth muscle was significantly more sensitive to H_2S than other tissues, exhibiting significant inhibitory responses at 1–10 μmol l^{-1} H_2S. The effects of hypoxia were essentially identical to those of H_2S in longitudinal and circular intestinal smooth muscle; of special note was a unique transient stimulatory effect upon application of both hypoxia and H_2S. Inhibitors of enzymes implicated in H_2S biosynthesis (cystathionine β-synthase and cystathionine γ-lyase) partially inhibited the effects of hypoxia whereas the hypoxic effects were augmented by the sulfide donor Cys. Furthermore, tissue production of H_2S was inversely related to O_2; addition of Cys to intestinal tissue homogenate stimulated H_2S production when the tissue was gassed with 100% nitrogen (~0% O_2), whereas addition of oxygen (~10% O_2) reversed this to net H_2S consumption. This study shows that the inhibitory effects of H_2S on the GI tract of a non-mammalian vertebrate are identical to those reported in mammals and they provide further evidence that H_2S is a key mediator of the hypoxic response in a variety of O_2-sensitive tissues.

Key words: H_2S, hypoxia, visceral smooth muscle, gastrointestinal system, fish.

INTRODUCTION

Recent studies have shown that exogenous hydrogen sulfide (H_2S) initiates pharmacophysiological responses in most, if not all, organ systems (Li et al., 2011; Olson, 2011). These studies have been instrumental in the hypothesis that hydrogen sulfide is a biologically relevant signaling molecule thereby joining nitric oxide (NO) and carbon monoxide (CO) as 'gasotransmitters' (Wang, 2002).

H_2S is of particular interest in the gastrointestinal (GI) tract as it is both produced by GI tissues and generated in large quantities by bacterial flora in the lumen of the gut (Blachier et al., 2010; Hosoki et al., 1997; Linden et al., 2008; Martin et al., 2010; Teague et al., 2002; Wallace, 2010). Both pathological and physiological attributes have been ascribed to H_2S in the GI tract (Wallace, 2010). H_2S has been suggested to contribute to a variety of intestinal disorders including ulcerative colitis, inflammatory bowel disease and colorectal cancer (Attene-Ramos et al., 2010; Medani et al., 2010; Rowan et al., 2009). Conversely, H_2S has been shown to have anti-inflammatory and antinociceptive actions in the GI tract and H_2S 'releasing' compounds have been added to non-steroidal anti-inflammatory drugs (NSAIDs) to prevent gastric irritation and promote healing of ulcers (Wallace, 2010; Wallace et al., 2007). H_2S has even been shown to serve as an energy source for ATP production by mucosal epithelial cells (Blachier et al., 2010; Bouillaud and Blachier, 2011; Goubern et al., 2007; Lagoutte et al., 2010).

Exogenous H_2S has been observed to affect both GI smooth muscle and the enteric nervous system. H_2S directly relaxes GI smooth muscle in the mouse stomach and colon (Dhaese and Lefebvre, 2009; Dhaese et al., 2010) and rat ileum (Nagao et al., 2010). In the guinea-pig stomach, 'low doses' of H_2S (0.1–0.3 mmol l^{-1}) enhance resting tension and slightly reduce contractile amplitude, whereas at higher concentrations H_2S suppresses the amplitude of spontaneous contractions (Zhao et al., 2009). The mechanism of H_2S-mediated relaxation is poorly understood and most common mediators of muscle relaxation have in fact been ruled out. In the mouse, H_2S-induced relaxations are independent of potassium (K^+) channels, NO, cAMP and cGMP pathways, and nerve inhibition, and do not involve membrane

ATPase, calcium (Ca^{2+}) channels, internal calcium stores or Rho-kinase; H_2S may, or may not, act *via* activation of myosin light chain phosphatase (Dhaese and Lefebvre, 2009; Dhaese et al., 2010). In the rat ileum, H_2S-mediated relaxations are independent of intrinsic, enteric and visceral afferent nerves, NO and both ATP- (K_{ATP}) and calcium-sensitive (K_{Ca}) potassium channels (Nagao et al., 2010). Low-dose activation of guinea-pig gastric smooth muscle may be mediated *via* inhibition of voltage-gated K^+ channels and high-dose inhibition may be mediated *via* K_{ATP} channel activation (Zhao et al., 2009). In the mouse intestine, H_2S inhibits pacemaker amplitude and frequency of interstitial cells of Cajal at high concentrations (0.5–1.0 $mmol\,l^{-1}$), but may slightly stimulate at low concentrations (50–100 $\mu mol\,l^{-1}$); these effects appear to be *via* modulation of intracellular calcium (Parajuli et al., 2010). H_2S also stimulates proliferation of interstitial cells of Cajal (Huang et al., 2010).

Linden and colleagues critically evaluated the criteria for H_2S as a biologically relevant gasotransmitter in the GI tract (Linden et al., 2010). They concluded that, while there is considerable evidence that H_2S is generated by GI tissues and that H_2S can affect a variety of GI functions, H_2S cannot yet be considered an endogenous signaling molecule because, among other things, there is no evidence to date to show that the production of H_2S by GI tissue is regulated.

An alternative to regulating H_2S production to control H_2S signaling would be to actively regulate H_2S metabolism in the face of constitutive H_2S production. We have previously shown that bovine lung tissue, cultured pulmonary arterial smooth muscle cells and isolated mitochondria inactivate H_2S by a P_{O_2}-dependent process at physiologically relevant P_{O_2} values (Olson et al., 2010). This balance between constitutive H_2S production and O_2-dependent inactivation in cells forms the basis for the hypothesis that H_2S metabolism is the crucial step coupling hypoxia to tissue response, i.e. the 'O_2 sensor' (Olson and Whitfield, 2010). Evidence for this mechanism has been found in systemic and respiratory vascular smooth muscle (Olson et al., 2006; Olson et al., 2008a; Olson et al., 2010), urinary bladder smooth muscle (Dombkowski et al., 2006), chromaffin cells (Perry et al., 2009) and O_2-sensing chemoreceptor cells (Li et al., 2010; Olson et al., 2008b; Peng et al., 2010; Telezhkin et al., 2009; Telezhkin et al., 2010).

The GI tract is an intriguing candidate for O_2 regulation of H_2S signaling. Not only is H_2S produced by GI tissue and luminal bacteria but also both tissue and bacterial metabolism can affect P_{O_2}. In the mouse, for example, tissue extraction and bacterial O_2 consumption lower luminal P_{O_2} from nearly 60 mmHg in the stomach to 11 mmHg in the small intestine and ultimately down to 3 mmHg at the sigmoid–rectal junction (He et al., 1999). A decrease in the tissue perfusion/metabolism ratio, the hallmark of ischemic bowel disease, can halve these values within 5 min (He et al., 1999). These P_{O_2} values are sufficiently low to inhibit H_2S oxidation and tip the scale in the intracellular environment from net H_2S consumption to H_2S production (Olson et al., 2010).

Although the effects of H_2S on the mammalian GI tract have been examined in some detail, information on H_2S effects in the GI tract of non-mammalian vertebrates is lacking. The present study was designed to examine the effects of this signaling molecule on the GI tract of fish as they are perhaps more intensively studied than any other non-mammalian vertebrate and they provide a broad phylogenetic and evolutionary perspective of vertebrate development. These studies also allowed us to examine the hypothesis that H_2S is involved in O_2 sensing in the GI tract. We first examined the effects of H_2S and hypoxia on the intrinsic rhythmicity and contractile activity of esophagus, stomach, gall bladder, and circular and longitudinal GI smooth muscle of the rainbow trout (*Oncorhynchus mykiss*). Next, we compared the intestinal responses from rainbow trout with those of a much smaller and technically more manageable salmonid, the juvenile coho salmon (*Oncorhynchus kisutsch*). We then used the coho intestine to examine the hypothesis that H_2S mediates the hypoxic responses by directly comparing the effects of these two stimuli on mechanical and/or enteric communication in the intestine and by examining the effect of inhibitors of H_2S biosynthesis and a sulfide donor as a precursor of H_2S synthesis on the hypoxic responses. Confirmation of the inverse relationship between H_2S and O_2 was obtained by direct measurement, in real time, of intestinal H_2S production and its O_2-dependent consumption using polarographic H_2S and O_2 electrodes.

MATERIALS AND METHODS

Animals

Rainbow trout [*O. mykiss*, Kamloops strain (Jordan 1892), 400–800 g] of either sex were purchased from local hatcheries (Harrietta Hills Trout Farm, Harrietta, MI, USA, and Sweetwater Springs Fish Farm, Peru, IN, USA) and kept in circulating 2000 l tanks containing well-water at 12–15°C, aerated with filtered room air, and exposed to 12 h:12 h light:dark cycles. The fish were fed a maintenance diet of commercial trout pellets (Purina, St Louis, MO, USA). The trout were stunned by a blow to the head and segments of the esophagus, stomach, gall bladder, anterior intestine and posterior intestine were dissected out and placed in cold (~4°C) Cortland buffered saline (for composition, see below). Anterior intestinal segments were taken from a 2 cm portion of intestine starting ~1 cm distal to the pyloric cecae. Posterior intestinal segments, 2 cm long, were taken 1 cm from the vent. The dissected tissues were cleaned and placed in fresh Cortland buffered saline at 4°C until use. All segments were used for experimentation within 24 h of removal.

Coho salmon [*O. kisutch* (Walbaum 1792), 10–20 cm, ~10–30 g] juveniles were obtained from the Bodine State Fish Hatchery, Mishawaka, IN, USA, and kept in a 500 l rectangular tank containing continuously flowing well-water at 12–15°C for several weeks prior to experimentation. Coho were stunned by a blow to the head and the intestines were removed and prepared for myography as described above.

All procedures followed NIH guidelines and were approved by the local Institutional Animal Care and Use Committee (IACUC).

Myography

Circular smooth muscle rings (~5 mm long) from the trout esophagus, stomach, gall bladder, anterior intestine and posterior intestine, and from the coho anterior intestine and posterior intestine were mounted on 280 μm diameter stainless steel wire hooks through the tissue lumen and suspended in 5 ml water-jacketed smooth muscle baths filled with 14°C Cortland buffered saline and aerated with room air. Segments from both the trout and coho anterior and posterior intestine were also mounted in a longitudinal direction by puncturing the intestinal walls with the same hooks. The cylindrical structure of the intestinal segments was not compromised in either setup. The bottom hooks were stationary; the upper hooks were connected to Grass model FT03C force-displacement transducers (Grass Instruments, West Warwick, RI, USA). Tension was measured on a Grass Model 7E or 7F polygraph (Grass Instruments). Polygraph sensitivity was calibrated prior to the beginning of each experiment and was able to detect changes as small as 5 μN. Data were archived on a PC computer at 1 Hz

using Softwire software (Measurement Computing, Middleboro, MA, USA). The chart recorders and software were calibrated for zero tension and 2 mN loads prior to each experiment.

Baseline (resting) tension of ~5–7 mN for trout gall bladders and ~8–1.2 mN for the remaining tissues was applied and continuously adjusted for at least 1 h prior to experimentation as the tissues exhibited substantial stress relaxation. Gall bladders and intestinal segments from both fish established baseline resting loads ranging from 2 to 6 mN, and trout stomach and esophagus maintained baseline tension from ~4 to 8 mN. All tissues were allowed to establish baseline activity in an undisturbed state for a minimum of 1 h prior to the beginning of experimentation. In the absence of external stimuli, trout gall bladder rings and circular and longitudinal intestinal smooth muscle from both fish usually (>90%) exhibited large spontaneous contractions that were approximately 2–5 times resting load. Spontaneous contractions were less frequently observed (<30%) in circular smooth muscle from trout stomach and esophagus. Tissues that did not exhibit spontaneous activity or stretch-induced relaxation were not examined.

H_2S responses

Cumulative H_2S concentration–response profiles were obtained for all tissues in the absence of other stimulation. NaHS was used in initial experiments whereas Na_2S was used in later studies as it was more readily available and has fewer sulfur impurities (Doeller et al., 2005). Both salts form HS^- and H_2S when dissolved and we have not noticed any obvious differences in the effects produced by these salts (Dombkowski et al., 2006). In these studies 'H_2S' refers to total free sulfide, i.e. dissolved H_2S plus HS^- based on the molarities of the dissolved salts.

H_2S concentration–response profiles were obtained from circular smooth muscle of trout esophagus, stomach and gall bladder, as well as from circular and longitudinal smooth muscle of both trout and coho intestine. Concentration–response profiles were also obtained from trout esophagus, stomach, gall bladder and circular smooth muscle and coho intestinal longitudinal smooth muscle pre-contracted with $10\,\mu mol\,l^{-1}$ carbamylcholine chloride (carbachol).

Hypoxic responses

The effects of hypoxia, produced by gassing with 100% N_2, were examined in otherwise unstimulated and carbachol ($10\,\mu mol\,l^{-1}$) pre-contracted anterior and posterior circular and longitudinal intestinal smooth muscle from both trout and coho. Unstimulated or carbachol pre-contracted intestinal segments were exposed to either two or three 20 min bouts of hypoxia, separated by 20 min of aeration with room air.

Relationship between H_2S and hypoxia

Two protocols were employed to examine the role of H_2S in hypoxic responses. In the first, the effects of hypoxia were examined in coho longitudinal intestinal smooth muscle in the presence of inhibitors of H_2S biosynthesis. The cystathionine γ-lyase (CSE) inhibitor propargylglycine (PPG, $10\,mmol\,l^{-1}$) or the cystathionine β-synthase (CBS) inhibitor amino-oxyacetic acid (AOA, $1\,mmol\,l^{-1}$) was added to the tissues 30 min prior to hypoxia. In another group of experiments, the tissues were exposed to the inhibitor for 30 min then pre-contracted with $10\,\mu mol\,l^{-1}$ carbachol and hypoxia was administered when the carbachol response plateaued. In a third group of experiments the sulfide donor cysteine (0.1, 1 or $10\,mmol\,l^{-1}$) was added 30 min prior to the $10\,\mu mol\,l^{-1}$ carbachol pre-contraction and subsequent hypoxia.

Effects of O_2 on H_2S production

H_2S production by homogenized trout intestine was measured in real time using an amperometric (polarographic) H_2S sensor constructed in the laboratory (Whitfield et al., 2008). Approximately 1 g of anterior intestine was homogenized, on ice, in 9 ml of Cortland buffered saline and gassed on a rotary tonometer with humidified 100% nitrogen to remove the oxygen. A 1.5 ml sample of homogenate was placed in a closed chamber with ports for simultaneous measurement of oxygen with a standard Clark-type electrode and H_2S. H_2S production was measured in the absence and in the presence of 1 and $10\,mmol\,l^{-1}$ cysteine. Room air was administered through an injection port and the relationship between P_{O_2} and P_{H_2S} recorded. The relationship between P_{H_2S} and H_2S concentration was derived from a standard curve using Na_2S; the rate of H_2S production was obtained from the slope of the H_2S concentration as a function of time.

Data analysis

Text files were converted to .acq files for analysis with Biopac Lab Pro (AcqKnowledge, Biopac Systems Inc., Goleta, CA, USA). Four different parameters were captured for each tissue segment using this software: (1) baseline tension, defined by the average tension in between spontaneous contractions; (2) spontaneous contraction amplitude, defined by the difference between the highest and lowest tension measured during each cycle; (3) spontaneous contraction frequency, if present; and (4) total contractile activity, determined as the area under the contractile curve (AUC) from zero tension, which essentially captures baseline tension and spontaneous activity combined. For comparison, all data were averaged over a 600 s (10 min) time frame immediately before treatment and during the final 600 s (10 min) of each respective H_2S concentration or hypoxic exposure. Data presented are all from the initial exposure to hypoxia. For all H_2S concentration–response experiments on carbachol pre-contracted segments, only the total contractile activity was examined for comparison with otherwise unstimulated tissues. All four parameters were analyzed in the coho intestinal $1\,mmol\,l^{-1}$ Na_2S bolus experiments and hypoxic experiments with PPG, AOA and cysteine. Esophageal and gastric rings infrequently displayed spontaneous contractions; therefore, only baseline tension and total contractile activity were examined for these two tissues. Because H_2S and hypoxia reduced all parameters, the H_2S and hypoxic responses are presented as a percentage of pretreatment values. The H_2S concentration–response curves were truncated at $1\,mmol\,l^{-1}$ NaHS or Na_2S as higher concentrations are quite likely irrelevant physiologically. The effective concentration for half-maximal response (EC_{50}) was calculated using TableCurve® (Jandel Corp., Chicago, IL, USA).

In several instances the relative responses in different intestinal segments to H_2S or hypoxia were not significantly different and these data were combined. In coho, only two significant differences were found between anterior and posterior intestinal segments. The amplitude of spontaneous contractions in both anterior circular and anterior longitudinal smooth muscle was nearly twice the amplitude observed in posterior tissues (Fig. 1). This was likely due to the degree of muscularity, which was considerably greater in the anterior segments. Because the relative (as a percentage of control) responses of these segments to either H_2S or hypoxia were not significantly different, the percentage change in anterior and posterior intestinal data were combined for EC_{50} calculations and for comparisons between circular and longitudinal smooth muscle preparations. In trout, H_2S and hypoxia produced a significantly greater relaxation of baseline tension in posterior longitudinal

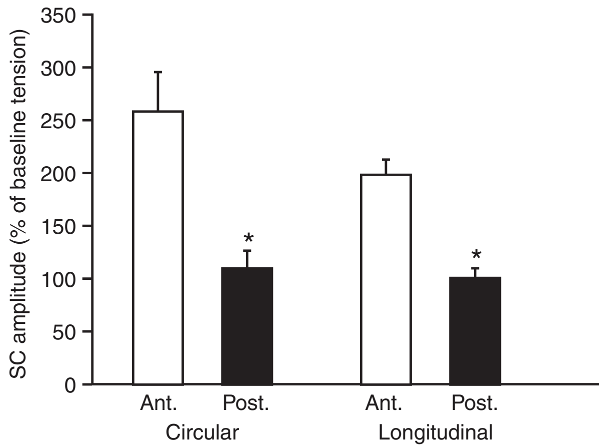

Fig. 1. Comparison of the amplitude of spontaneous contractions (SC) in circular and longitudinal smooth muscle from the anterior (Ant.) and posterior (Post.) intestine of coho. Spontaneous contractions are significantly lower (*) in both posterior segments. Means + s.e.m. (N=4 fish).

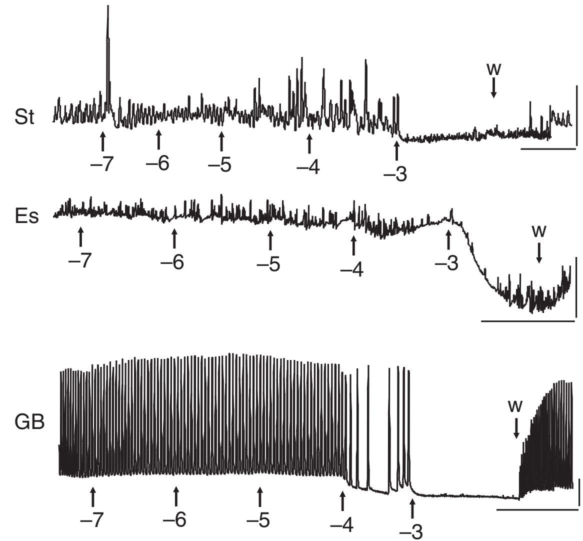

Fig. 3. Traces showing the effect of H_2S (log molar concentration) on trout stomach (St), esophagus (Es) and gallbladder (GB). Spontaneous contractions were infrequently observed in ES and ST (shown here). When present, they were inhibited by 1 mmol l^{-1} H_2S (–3). H_2S inhibited baseline tension in all tissues. Note in the gallbladder that 100 µmol l^{-1} (–4) greatly decreased the frequency of spontaneous contractions and lowered baseline tension but did not affect the amplitude of spontaneous contractions. All parameters began to return to normal after the H_2S was washed out (W). Tension and time scale, 10 mN and 15 min, respectively.

intestinal smooth muscle than in anterior longitudinal intestinal smooth muscle (Fig. 2). However, all of the other responses to H_2S and hypoxia expressed as a percentage of pretreatment values (total contractile activity, spontaneous contraction amplitude and frequency) were not significantly different and these data were pooled for calculation of EC_{50} values and for comparisons between circular and longitudinal intestinal smooth muscle. Student's *t*-tests and one-way ANOVA were used for comparisons between groups with SigmaStat® (Jandel Corp.). Results are presented as means ± s.e.m. Significance was assumed at $P \leq 0.05$.

Chemicals

Unless otherwise stated all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). Na_2S and NaHS were purchased from Fisher Scientific (Pittsburgh, PA, USA). Cortland buffered saline (pH 7.8) was: 124 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 1.1 mmol l^{-1} $MgSO_4 \cdot 7H_2O$, 2 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 5.55 mmol l^{-1} glucose, 12 mmol l^{-1} $NaHCO_3$, 0.09 mmol l^{-1} NaH_2PO_4 and 1.8 mmol l^{-1} Na_2HPO_4. NaHS and Na_2S stock solutions were made fresh daily under nitrogen and used within 8 h of preparation. By convention, H_2S is used hereafter to indicate either Na_2S or NaHS.

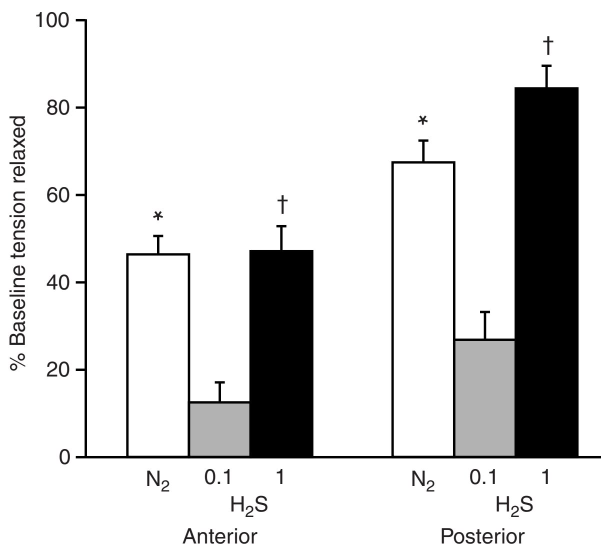

Fig. 2. Effects of hypoxia (N_2) and hydrogen sulfide (H_2S, 0.1 or 1 mmol l^{-1}) on baseline (resting) tension in anterior and posterior longitudinal intestinal smooth muscle from trout. Posterior segments were relaxed significantly more than anterior segments by both hypoxia (*) and 1 mmol l^{-1} H_2S (†). Means + s.e.m. (N=4 fish).

RESULTS

There was no significant deterioration in any of the measured parameters in any of the control tissues for up to 4 h (not shown). All experimental protocols were completed in 4 h or less.

Effects of H_2S on trout GI tract

The effects of H_2S on total contractile activity (AUC), baseline tone, spontaneous contraction amplitude and frequency of spontaneous contractions for trout GI tissues are shown in Table 1 and in Figs 3–5. Longitudinal intestinal smooth muscle was the most sensitive to H_2S, with significant decreases in all but spontaneous contraction amplitude occurring at 1 µmol l^{-1}. In other tissues half of the measured parameters were significantly reduced at 10 µmol l^{-1} H_2S and all parameters were significantly reduced at 100 µmol l^{-1} H_2S. The effects of high concentrations of H_2S (100 µmol l^{-1} to 1 mmol l^{-1}) were often significantly greater in longitudinal smooth muscle than they were in circular smooth muscle. In a number of tissues, but especially noticeable in longitudinal smooth muscle of trout intestine (Fig. 4), application of 1 mmol l^{-1} H_2S produced an initial increase in baseline tension and amplitude of spontaneous contractions which was then followed by a decrease in baseline tension and spontaneous contractions. Application of 1 mmol l^{-1} H_2S produced one or several spontaneous contractions in anterior intestinal circular smooth muscle but did not increase baseline tension. H_2S also produced a

Table 1. Effects of H_2S and hypoxia (N_2) on contractile activity in otherwise unstimulated tissue from *Oncorhynchus mykiss*

	H_2S					
	100 nmol l^{-1}	1 μmol l^{-1}	10 μmol l^{-1}	100 μmol l^{-1}	1 mmol l^{-1}	N_2
Esophagus (*N*=8)						
Total contractile activity (AUC)	111±12	97±14	91±7	72±10*	17±50*	–
Baseline tone	112±10	101±5	94±2*	77±5*	41±20*	–
Stomach (*N*=8)						
Total contractile activity (AUC)	101±12	99±4	96±5	91±4*	51±14*	–
Baseline tone	99±5	99±7	97±2	94±1*	49±11*	–
Gallbladder (*N*=8)						
Total contractile activity (AUC)	104±11	96±4	89±1*	71±4*	25±11*	–
SC amplitude	100±6	99±7	97±3	85±10*	7±9*	–
Baseline tone	97±15	101±8	96±2	87±5*	52±4*	–
Frequency	101±4	94±6	93±2*	41±4*	10±8*	–
Circular (*N*=15)						
Total contractile activity (AUC)	102±4	99±7	92±7	78±7*	44±7*	56±6*
SC amplitude	98±4	101±5	95±6	90±3*	15±12*	51±14*
Baseline tone	99±3	99±5	91±4*	86±5*	67±9*	69±12*
Frequency	102±7	95±16	96±6	91±4*	22±20*	48±22*
Longitudinal (*N*=8)						
Total contractile activity (AUC)	97±5	92±2*	86±4*	59±5*,†	16±9*,†	31±10*,†
SC amplitude	101±3	89±7	76±9*	59±16*,†	21±8*	23±11*
Baseline tone	100±7	91±4*	82±6*	78±9*	35±7*	41±9*
Frequency	99±7	90±3*	79±4*	49±7*,†	26±13*	29±11*

Values expressed as a percentage of pretreatment values; means ± s.e.m.; *N* is the number of animals.
AUC, area under the curve; SC, spontaneous contraction.
*Significantly reduced from pretreatment (100%) values; †significantly different from circular smooth muscle at similar treatment.

concentration-dependent relaxation of 10 μmol l^{-1} carbachol pre-contracted esophagus and anterior intestinal circular smooth muscle (Fig. 5), as well as stomach and gall bladder (not shown). Spontaneous activity and baseline tension were restored when the tissues were rinsed with H_2S-free buffer.

The EC_{50} values for the H_2S effect on contractile activity (AUC) for both trout and coho tissues are shown in Table 2. Longitudinal intestinal smooth muscle preparations from both fish were significantly more sensitive to H_2S than the respective circular smooth muscle preparations; there were no other significant differences between or within species, tissues or unstimulated *vs* pre-contracted preparations.

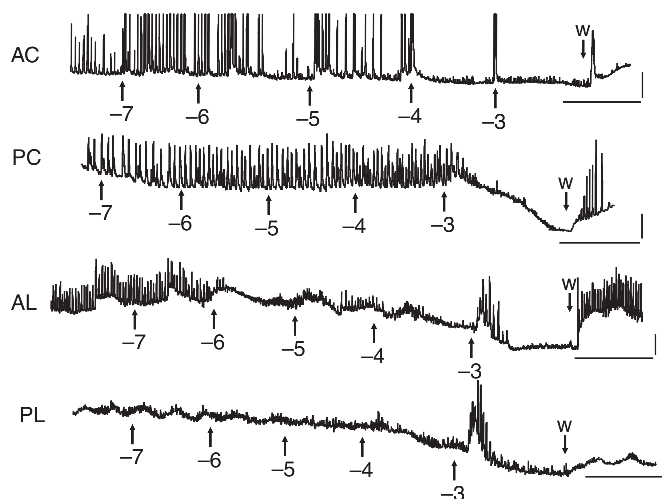

Fig. 4. Traces showing the effect of H_2S (log molar concentration) on trout intestine. AC and PC, anterior and posterior circular smooth muscle, respectively; AL and PL, anterior and posterior longitudinal smooth muscle, respectively. Note the transient increase then decrease in spontaneous contractions and baseline tension following 1 mmol l^{-1} H_2S (–3). Tension and time scale, 10 mN and 15 min, respectively. W, H_2S wash out.

Effects of hypoxia on trout intestine

The effects of hypoxia (gassing with 100% N_2) on AUC, spontaneous contraction amplitude, baseline tone and frequency in trout circular and longitudinal intestinal smooth muscle are shown in Table 1 and Fig. 6. The effects of hypoxia were similar, if not identical, to those produced by H_2S. Hypoxia significantly reduced all parameters in circular and longitudinal intestinal smooth muscle. Hypoxia was significantly more efficacious in decreasing AUC in longitudinal smooth muscle than it was in circular smooth muscle and, although not significant, there was a tendency for a greater effect on spontaneous contraction amplitude, baseline tone and frequency as well. In longitudinal smooth muscle of trout intestine,

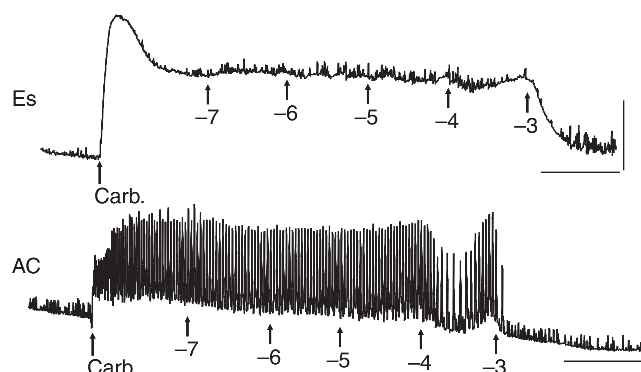

Fig. 5. Traces showing the effect of H_2S (log molar concentration) on carbachol (Carb., 10 μmol l^{-1}) pre-contracted trout esophagus (Es) and anterior circular intestine (AC). Tension and time scale, 10 mN and 15 min, respectively.

Table 2. Effective H_2S concentration eliciting half-maximal (EC_{50}) reduction of total contractile activity (AUC) of unstimulated or 10 μmol l^{-1} carbachol pre-stimulated tissue

Species	Tissue	EC_{50}	*N*	H_2S salt
O. mykiss (pre-stimulation)	Stomach	171±60	3	NaHS
	Espohagus	120±21	4	NaHS
	Gall bladder	101±20	4	NaHS
	Intestine (circular)	125±19	8	NaHS
O. mykiss	Stomach	155±61	8	NaHS
	Espohagus	111±21	8	NaHS
	Gall bladder	93±41	8	NaHS
	Intestine (circular)	146±21	15	Na_2S
	Intestine (longitudinal)	78±10*	8	Na_2S
O. kisutch (pre-stimulation)	Intestine (longitudinal)	99±21	8	Na_2S
O. kisutch	Intestine (circular)	156±35	8	Na_2S
	Intestine (longitudinal)	88±12*	8	Na_2S

Values are expressed as μmol l^{-1} H_2S; means ± s.e.m.; *N* is the number of animals.
AUC, area under the curve.
*Significantly different from circular smooth muscle.

the onset of hypoxia produced an initial increase in baseline tension and spontaneous contraction amplitude followed by a subsequent decrease in these parameters (Fig. 6). These responses were strikingly similar to those produced by 1 mmol l^{-1} H_2S (Fig. 4). Spontaneous activity returned and baseline tension partially recovered when the tissues were aerated with room air.

Effects of H_2S on coho intestine

As in trout, H_2S caused a concentration-dependent reduction in AUC, spontaneous contraction amplitude, baseline tone and frequency of spontaneous contractions in coho circular and longitudinal intestinal smooth muscle (Table 3, Fig. 7). Longitudinal intestinal smooth muscle was also more sensitive to H_2S than circular smooth muscle (Table 2). Application of high concentrations (100 μmol l^{-1} to 1 mmol l^{-1}) of H_2S often resulted in a transient burst of contractile activity followed by a stable relaxation and essentially complete loss of spontaneous activity (Fig. 7). Spontaneous activity and baseline tension were restored when the tissues were rinsed with H_2S-free buffer.

Effects of carbachol and inhibitors of H_2S biosynthesis on coho intestine

Carbachol (10 μmol l^{-1}) increased AUC, spontaneous contraction amplitude and frequency of spontaneous contractions in longitudinal smooth muscle of the coho intestine (Table 4). Pretreatment with the CSE inhibitor PPG had no effect on any of the variables, whereas pretreatment with the CBS inhibitor AOA significantly reduced AUC. Addition of H_2S (1 mmol l^{-1}) significantly decreased the carbachol-induced effect on AUC, spontaneous contraction amplitude and frequency of spontaneous contractions (Table 4).

Effects of hypoxia on coho intestine

Hypoxia (N_2) significantly reduced AUC, spontaneous contraction amplitude, baseline tone and frequency of spontaneous contractions in otherwise unstimulated coho anterior and posterior, circular and longitudinal smooth muscle (Table 5, Fig. 8). As shown in Fig. 8, the onset of hypoxia often resulted in a transient burst of contractile activity, followed by a prolonged relaxation that persisted until normal aeration was restored. This response was similar to the effects

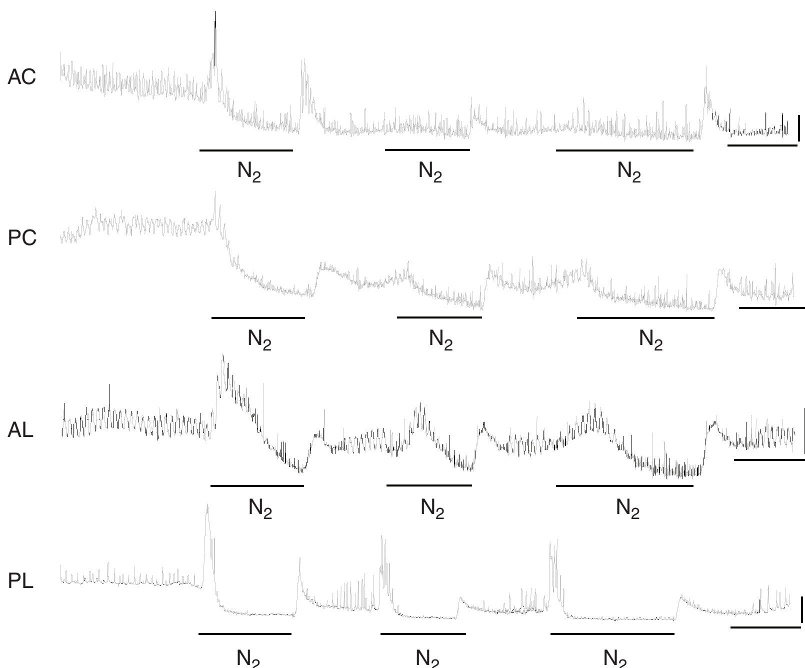

Fig. 6. Traces showing the effects of three consecutive exposures to hypoxia (N_2) on trout intestinal anterior and posterior circular smooth muscle (AC and PC, respectively) and anterior and posterior longitudinal smooth muscle (AL and PL, respectively). Note the transient increase then decrease in spontaneous contractions and baseline tension immediately following onset of hypoxia. These responses are similar to those produced by H_2S (Fig. 4). Tension and time scale, 10 mN and 15 min, respectively.

Table 3. Effect of H_2S on contractile activity in combined anterior and posterior intestinal segments from *O. kisutch*

	100 nmol l^{-1}	1 μmol l^{-1}	10 μmol l^{-1}	100 μmol l^{-1}	1 mmol l^{-1}
Circular (*N*=8)					
AUC	102±3	97±1	91±1*	73±4*	40±4*
SC amplitude	100±6	99±7	95±3	75±4*	17±2*
Baseline tone	101±2	100±5	97±2	92±1*	75±4*
Frequency	99±2	98±4	93±2*	77±4*	47±4*
Longitudinal (*N*=8)					
AUC	99±2	95±1*	84±2*	55±2*,†	15±4*,†
SC amplitude	102±4	90±9	83±5*	36±5*,†	11±7*
Baseline tone	100±3	97±3	92±2*	85±2*	40±6*,†
Frequency	101±9	98±7	92±3*	86±5*	31±9*

Values are expressed as a percentage of pretreatment values; means ± s.e.m.; *N* is the number of animals.
AUC, area under the curve; SC, spontaneous contraction.
*Significantly reduced from pretreatment (100%) values; †significantly different from circular for similar treatment.

of elevated H_2S (Fig. 7). The effects of hypoxia on AUC and baseline tone were significantly more pronounced in longitudinal than in circular intestinal smooth muscle. There were no significant differences between hypoxia and 1 mmol l^{-1} H_2S for any measured parameter in either circular or longitudinal smooth muscle.

Relationship between H_2S and hypoxia in coho longitudinal intestinal smooth muscle

Incubation with the CSE inhibitor PPG had no effect on the hypoxic relaxation or inhibition of spontaneous contractions in unstimulated coho longitudinal intestinal smooth muscle, whereas incubation with the CBS inhibitor AOA significantly inhibited the hypoxic effect on AUC, spontaneous contraction amplitude and frequency (Table 5). In carbachol-precontracted longitudinal smooth muscle, PPG significantly inhibited the hypoxic effect on AUC, spontaneous contraction amplitude and baseline tone while AOA inhibited spontaneous contraction amplitude and frequency (Fig. 9).

Prior addition of 0.1 mmol l^{-1} cysteine did not affect the hypoxic response of carbachol pre-contracted intestinal strips whereas 1 and 10 mmol l^{-1} cysteine augmented the hypoxic effect on AUC, baseline tone and frequency of spontaneous contractions (Fig. 9, Table 5). The effects of 1 and 10 mmol l^{-1} of cysteine on the hypoxic response were not significantly different.

O_2 dependency of H_2S production in the trout intestine

Addition of 1 mmol l^{-1} cysteine to deoxygenated, homogenized trout intestine slightly stimulated H_2S production (not shown). Subsequent addition of 10 mmol l^{-1} cysteine greatly increased the rate of H_2S formation (10.5±6.3 μmol min^{-1} g^{-1} wet mass, *N*=5 fish). H_2S production quickly reverted to net H_2S consumption upon addition of room air to the reaction chamber. After the oxygen was removed, presumably by tissue consumption, H_2S production returned (Fig. 10).

DISCUSSION

Our findings show that exogenous H_2S relaxes intestinal smooth muscle and inhibits spontaneous contractions in multiple areas of the fish GI tract, indicative of effects on either contractile activity of GI smooth muscle or enteric signaling, or both. Longitudinal smooth muscle of intestine from both trout and coho was consistently more sensitive to H_2S than circular smooth muscle, which may indicate differential regulation of function. Further evidence that H_2S is the oxygen-sensitive couple linking tissue hypoxia to physiological responses was provided by observations that: (1) hypoxic responses of the GI tract exactly mimicked those produced by H_2S, (2) hypoxic responses were inhibited by inhibitors of H_2S biosynthesis and were augmented by the sulfide donor cysteine, and

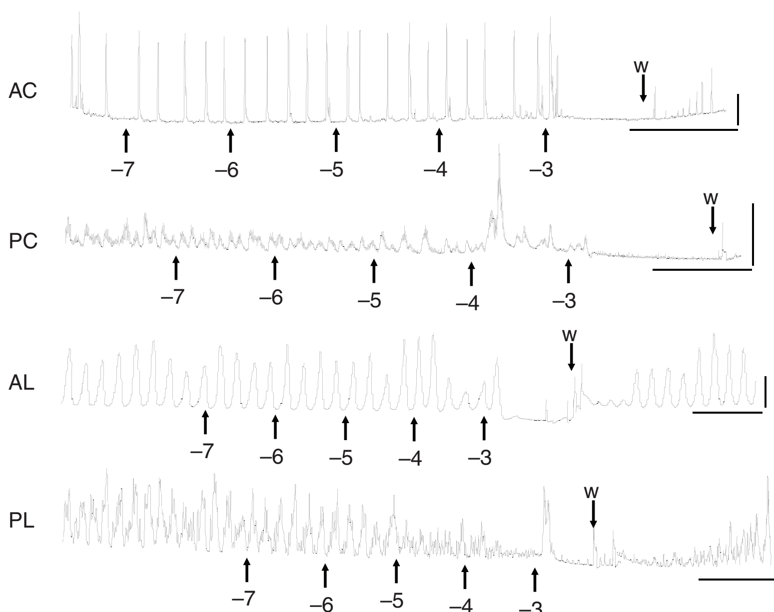

Fig. 7. Traces showing the effect of H_2S (log molar concentration) on coho intestine. AC and PC, anterior and posterior circular smooth muscle, respectively; AL and PL, anterior and posterior longitudinal smooth muscle, respectively. Tension and time scale, 10 mN and 15 min, respectively. W, H_2S wash out.

Table 4. Effects of 10 μmol l^{-1} carbachol alone, carbachol after treatment with inhibitors of H_2S biosynthesis and application of H_2S (1 mmol l^{-1}) during carbachol stimulation of longitudinal intestinal smooth muscle from *O. kisutch*

	Carb.	Carb. + PPG	Carb. + AOA	Carb. + H_2S
AUC	217±31†	235±34	117±12*	79±21*
SC amplitude	111±3†	109±3	131±12	11±7*
Frequency	97±6†	92±5	101±11	25±3*

Values are expressed as a percentage of pretreatment values (100%); means ± s.e.m.; *N* is the number of animals.

Carbachol, carb.; PPG, propargylglycine (10 mmol l^{-1}; inhibitor of cystathionine γ-lyase); AOA, amino-oxyacetic acid (1 mmol l^{-1}; inhibitor of cystathionine β-synthase); AUC, area under the curve; SC, spontaneous contraction.

As an example, carbachol increased AUC 117% (217–100%) whereas with Carb + AOA, AUC only increased by 17% (117–100%); thus, nearly all of the carbachol effect was inhibited by AOA.

†All carbachol responses are significantly different from control (unstimulated); *significantly different from carbachol alone.

(3) H_2S production by the GI tract is stimulated by cysteine and inversely related to oxygen availability.

Comparison of H_2S effects in mammals and trout

Our study shows that the effects of H_2S on AUC, spontaneous contractions, baseline tone and rhythmicity in the GI tract of fish are qualitatively, if not quantitatively, identical to those observed in a number of mammalian preparations. Salmonid tissues, however, appear to be more sensitive to H_2S.

Although few studies have provided detailed concentration–response characterization of the effects of H_2S in the mammalian GI tract, most studies suggest that the threshold for a response is 10 μmol l^{-1} or above and EC_{50} values (either provided by the authors or estimated from their data) are usually well above 300 μmol l^{-1}. In the mouse gastric fundus pre-contracted with prostaglandin F2α ($PGF_{2\alpha}$), relaxation was first observed at 10 μmol l^{-1} and EC_{50} values ranged from 350 to 429 μmol l^{-1} (Dhaese and Lefebvre, 2009). In the guinea-pig stomach, 10 μmol l^{-1} H_2S significantly enhanced resting tension and slightly reduced the contractile amplitude, whereas 300 μmol l^{-1} H_2S suppressed spontaneous contractions (Zhao et al., 2009). EC_{50} values estimated from the inhibitory effect of H_2S appeared to be ~400 μmol l^{-1}. Relaxation of the mouse distal colon was observed at 100 μmol l^{-1} H_2S, although this was the lowest concentration tested, and the EC_{50} was 444 μmol l^{-1} (Dhaese et al., 2010). In longitudinal muscle from rat ileum, 10 μmol l^{-1} H_2S reduced AUC, average amplitude of spontaneous activity and baseline tone by 7%, 3% and 6%, respectively (Nagao et al., 2010). These parameters were reduced 7%, 3% and 5% by 100 μmol l^{-1} H_2S (Nagao et al., 2010). As the responses to 10 and 100 μmol l^{-1} H_2S were not different, this does not appear to be a concentration-dependent response (each tissue served as its own control; time-matched controls were not employed, making it impossible to determine whether these effects were due to slight deterioration or stabilization of the preparation). However, 500 μmol l^{-1} H_2S reduced these parameters by 60%, 20% and 32%, suggesting that the threshold may be above 100 μmol l^{-1}. Based on the responses at 100, 500 and 1000 μmol l^{-1} (Nagao et al., 2010), the EC_{50} appears to be ~500 μmol l^{-1}. Similarly high concentrations (500 μmol l^{-1} H_2S) were necessary to affect pacemaker currents in interstitial cells of Cajal from mouse small intestine (Parajuli et al., 2010).

Significant changes in either motility or frequency in the trout and coho GI tract were observed between 1 and 10 μmol l^{-1} H_2S and the EC_{50} values for tissue response was between 78 and 171 μmol l^{-1} (overall mean was 120 μmol l^{-1}). These concentrations are around 5-fold (or more) lower than those commonly necessary to achieve physiological responses in mammalian preparations. This suggests that the GI tract of the fish is exquisitely sensitive to this

Table 5. Effects of hypoxia (N_2), hypoxia in the presence of inhibitors of H_2S biosynthesis and hypoxia in the presence of the sulfide donor cysteine on contractile activity of longitudinal intestinal smooth muscle from *O. kisutch*

	Longitudinal			Circular	N_2 + cysteine		
	N_2	N_2 + PPG	N_2 + AOA	N_2	0.1 mmol l^{-1}	1 mmol l^{-1}	10 mmol l^{-1}
Unstimulated (*N*=8)							
AUC	17±3‡,§	25±4	42±9*	47±5			
SC amplitude	15±5‡	16±6	35±5*	19±5			
Baseline tone	41±6‡,§	47±7	62±9	80±6			
Frequency	37±9‡	41±12	67±7*	49±7			
Pre-contracted (*N*=8)							
AUC	84±17†	167±21*	101±11		91±13	47±16*	61±22
SC amplitude	21±7†	37±3*	78±14*		23±7	19±9	19±4
Baseline tone	73±4†	141±26*	111±17		81±11	36±4*	56±11
Frequency	36±2†	56±13	89±16*		29±19	19±4*	22±11
% Pre-stimulus relaxed	97±12†	56±14*	67±11*		101±31	165±21*	147±17*

Longitudinal smooth muscle strips were examined in otherwise unstimulated conditions and in tissues pre-contracted with 10 μmol l^{-1} carbachol. The effect of hypoxia on unstimulated circular intestinal smooth muscle is shown for comparison.

Values for unstimulated and pre-contracted area under the curve (AUC), spontaneous contraction (SC), baseline tone and frequency are expressed as a percentage of pretreatment (control, 100%); % pre-stimulus is expressed as the percentage of 10 μmol l^{-1} carbachol-induced tension that was relaxed by hypoxia; means ± s.e.m.; *N* is the number of animals.

PPG, propargylglycine (inhibitor of cystathionine γ-lyase); AOA, amino-oxyacetic acid (inhibitor of cystathionine β-synthase).

‡Significantly reduced from control; †significantly reduced from carbachol effect (Table 4); §significantly different from circular for similar treatment; *significantly different from hypoxia alone.

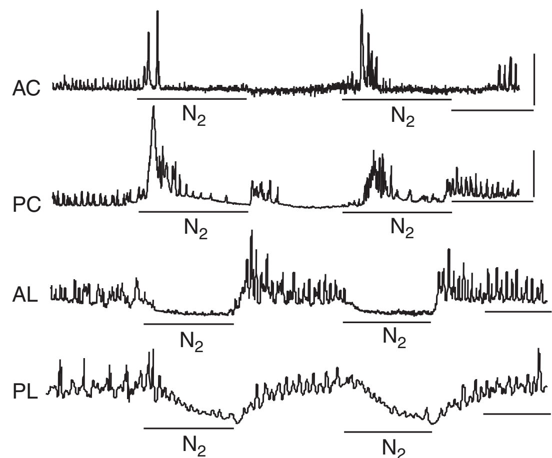

Fig. 8. Traces showing the effects of two consecutive exposures to hypoxia (N_2) on coho intestinal anterior and posterior circular smooth muscle (AC and PC, respectively) and anterior and posterior longitudinal smooth muscle (AL and PL, respectively). The effects of hypoxia on spontaneous contractions and baseline tension are similar to those produced by H_2S (Fig. 7). Tension and time scale, 10 mN and 15 min, respectively.

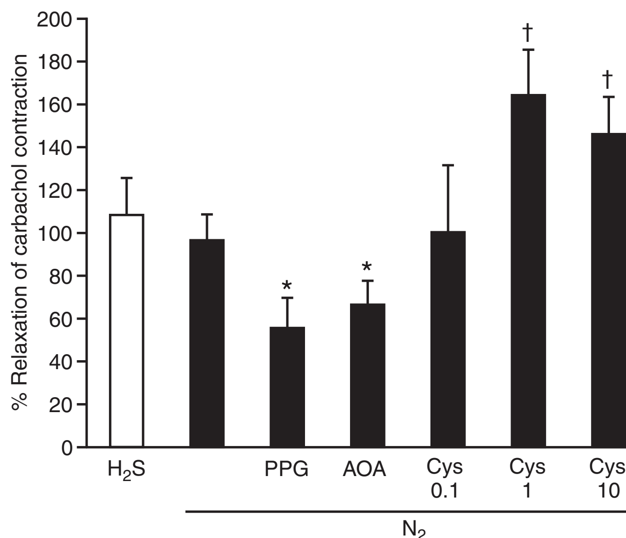

Fig. 9. Effects of H_2S (1 mmol l^{-1}), hypoxia alone (N_2) or hypoxia in the presence of inhibitors of H_2S synthesis or a sulfide donor on carbachol (10 μmol l^{-1}) pre-contracted longitudinal smooth muscle of coho intestine. The carbachol contraction was equally and completely relaxed by H_2S and hypoxia. Inhibition of cystathionine γ-lyase with D,L-propargylglycine (PPG; 20 mmol l^{-1}) or inhibition of cystathionine β-synthase with amino-oxyacetic acid (AOA; 1 mmol l^{-1}) significantly (*) inhibited the effect of hypoxia, whereas addition of the sulfide donor, cysteine (1 and 10 mmol l^{-1}) significantly (†) augmented the hypoxic response and the tissues relaxed below the initial resting tension. Means + s.e.m. (*N*=8 fish).

signaling molecule. However, the reason for this difference is not clear. Tissue (body) temperature could influence H_2S metabolism or kinetics of downstream mediators, although one would expect that the intrinsically lower metabolic rate of exothermic fish would decrease responsivness, not increase it. It is also possible that there is not as much background H_2S from bacterial production of H_2S in the lumen of the fish GI tract as there is in mammals. To our knowledge bacterial H_2S production has not been measured in the fish GI tract.

Are the effects of exogenous H_2S 'physiological'?

It is not clear whether the effects of H_2S produced in this study (or others) are 'physiological' responses. This is due in large part to uncertainties surrounding: (1) the concentration of endogenous H_2S in and around cells, (2) the actual concentration of exogenous H_2S at the effector site, and (3) whether tissue H_2S concentration can be regulated by physiologically relevant stimuli.

Although there are many reports of plasma and blood H_2S concentration between 30 and 300 μmol l^{-1}, these may be experimental artifacts (reviewed in Olson, 2009). In fact, H_2S may not even exist in the circulation (Whitfield et al., 2008). Recent measurements have also shown that the concentration of H_2S in a variety of tissues appears to be well below 1 μmol l^{-1} (Furne et al., 2008). Furthermore, as H_2S is rapidly oxidized at H_2S concentrations below 20 μmol l^{-1}, yet higher concentrations of H_2S inhibit mitochondrial cytochrome oxidase, and hence oxidative phosphorylation (Lagoutte et al., 2010), one could argue that H_2S most likely does not exist in appreciable amounts in the GI tract and that it would be physiologically disadvantageous to allow H_2S concentrations to even approach 20 μmol l^{-1}. Passive loss of H_2S from experimental apparatus due to volatility could be another confounding factor in the discrepancy between endogenous and physiologically effective exogenous H_2S concentrations. We (DeLeon et al., 2011) recently observed that the half-time for H_2S in a smooth muscle myograph of the type used in the present study was under 5 min, and this was due to volatilization not oxidation of H_2S. More accurate assessment of EC_{50} values awaits continuous monitoring of H_2S concentrations throughout an experiment.

The distal GI tract, however, may be exposed to high H_2S concentrations as a result of bacterial H_2S generation in the lumen. But here it is difficult to envisage how lumenal H_2S production could have a signaling function. It is more likely that bacterially generated H_2S is a potentially toxic metabolite to be disposed of or, perhaps, used as an energy source (Blachier et al., 2010). Collectively, this suggests that if H_2S is a physiologically relevant signal, then it acts in a paracrine or more probably autocrine manner, and it is most likely of tissue, not lumenal, origin. Exact details of H_2S signaling activities await detailed measurement of intracellular H_2S concentrations.

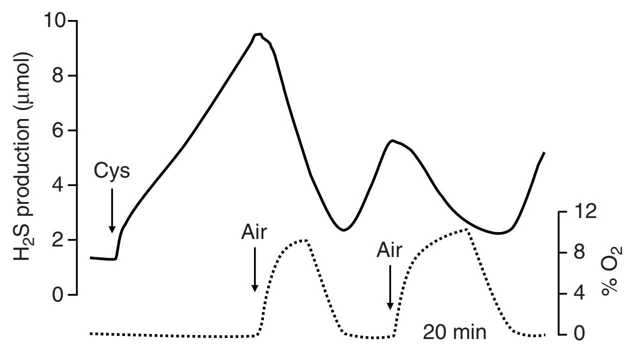

Fig. 10. Trace showing the relationship between H_2S production and O_2 in real time in homogenized trout intestine. Addition of 10 mmol l^{-1} cysteine (Cys) greatly augments H_2S production (solid line) in the absence of O_2, whereas injection of air (arrow) increases P_{O_2} (dotted line) and the tissue is converted from net H_2S production to net H_2S consumption. Net H_2S production resumes when the O_2 is depleted.

Comparison of effects of H_2S and hypoxia in the GI tract: the role of H_2S in oxygen sensing

Rapid oxidation of H_2S by the colon has been well documented (see Furne et al., 2001; Lagoutte et al., 2010; Levitt et al., 1999). This supports the argument that not only are tissue H_2S concentrations minimal but also removal of H_2S requires oxygen. The corollary to this is that an increase in H_2S is predicated upon the reciprocal decrease in O_2. Thus, the inverse relationship between H_2S and O_2 may be the physiological couple involved in H_2S signaling.

As described above, we have proposed that O_2-dependent metabolism of H_2S serves as an O_2 'sensor' in a variety of mammalian and non-mammalian tissues (Dombkowski et al., 2006; Olson et al., 2006; Olson et al., 2008a; Olson et al., 2008b; Olson et al., 2010; Whitfield et al., 2008) (reviewed in Olson and Whitfield, 2010). Our hypothesis is based on the following observations: (1) H_2S and hypoxia produce essentially identical responses in a variety of tissues, (2) tissues enzymatically generate H_2S, (3) tissue production of H_2S, or the concentration of exogenous H_2S, is inversely related to oxygen availability, i.e. P_{O_2}, (4) inhibitors of H_2S synthesis inhibit hypoxic responses, and (5) potential sulfide donors augment the hypoxic responses. Recent studies on the mammalian carotid body (Li et al., 2010; Peng et al., 2010; Telezhkin et al., 2009; Telezhkin et al., 2010) and kidney (Beltowski, 2010) have supported this hypothesis and our assertion that this is a phylogenetically ancient and ubiquitous O_2-sensing mechanism. The present study extends these observations to the fish GI tract and supports the hypothesis that O_2-dependent H_2S metabolism is a fundamental oxygen-sensing mechanism.

In the fish GI tract essentially all of the effects of hypoxia are similar, if not identical, to those produced by H_2S. Both stimuli relax visceral smooth muscle, inhibit spontaneous contractions and decrease their frequency. Furthermore, the magnitude of the effects of these two stimuli is also similar and they vary from tissue to tissue. Both hypoxia and H_2S are more efficacious in longitudinal than in circular intestinal smooth muscle and, within a preparation (longitudinal or circular smooth muscle), the magnitude of inhibition produced by these two stimuli is virtually identical. Even more striking is the transient stimulatory effect observed in both trout and coho intestine at the onset of hypoxia or H_2S application. This is reminiscent of the signature biphasic hypoxic constriction of rat pulmonary arteries that is uniquely mimicked by exogenous H_2S (Olson et al., 2006).

Fig. 10 clearly shows that the trout intestine generates H_2S in the presence of cysteine. This is consistent with the demonstrated production of H_2S by CBS and CSE in the mouse colon (Linden et al., 2008). By simultaneously measuring P_{O_2}, we were also able to determine that H_2S was only generated in the absence of O_2 or, at the very least, at very low P_{O_2} values. Furthermore, the reversal from net H_2S production by intestinal tissue to net H_2S consumption when oxygen was injected into the intestinal homogenate supports our previous observations in other tissues that H_2S production and/or tissue concentrations are inversely related to oxygen availability.

Given that the effects of hypoxia and H_2S on the trout and coho GI tract were virtually identical, and that the GI tissue produces H_2S when hypoxic, one would expect that inhibition of H_2S biosynthesis would inhibit the hypoxic response and that an increase in H_2S biosynthesis by addition of the sulfide donor cysteine would enhance the hypoxic response. This is precisely what we observed (Fig. 9). Interestingly, however, the hypoxic response was equally inhibited by inhibition of CSE with PPG and of CBS with AOA. The effects of PPG on the hypoxic response appeared to be direct, whereas AOA may have an indirect effect on the hypoxic response as it also inhibited the carbachol contraction independent of hypoxia. It remains to be determined whether H_2S generated by CBS has other effects on the intestine that are independent of the hypoxic response.

What is the physiological function of H_2S in the GI tract?

The present results show that H_2S inhibits GI activity and provide considerable evidence that this may be the mechanism through which the hypoxic response is expressed. This raises the question, what is the physiological significance of either H_2S or hypoxia in GI function? As both of these stimuli would presumably decrease the GI motility and transit time through the gut, they may serve to also decrease oxygen demand by GI tissues in the face of an oxygen deficit or perhaps permit more thorough processing of lumenal contents. Alternatively, these responses may be an evolutionary relic. As H_2S and hypoxia have similar effects on vascular smooth muscle in a broad range of vessels, and on smooth muscle in the urinary bladder, and as shown here in a variety of GI tissues, it may be that this response was at one time employed in a different signaling capacity during the early evolution of eukaryotes. However, as tissues became more specialized it was no longer linked to a specific physiological function. Clearly, this remains to be investigated.

ACKNOWLEDGEMENTS

The authors wish to thank D. Meunick, B. Bell and the staff at the Richard Clay Bodine State Fish Hatchery, Indiana Department of Natural Resources, for their help in obtaining coho tissues.

FUNDING

This work was supported in part by the National Science Foundation [grant nos IOS 0641436 and IOS 1051627].

REFERENCES

Attene-Ramos, M. S., Nava, G. M., Muellner, M. G., Wagner, E. D., Plewa, M. J. and Gaskins, H. R. (2010). DNA damage and toxicogenomic analyses of hydrogen sulfide in human intestinal epithelial FHs 74 Int cells. *Environ. Mol. Mutagen.* **51**, 304-314.

Beltowski, J. (2010). Hypoxia in the renal medulla: implications for hydrogen sulfide signaling. *J. Pharmacol. Exp. Ther.* **334**, 358-363.

Blachier, F., Davila, A. M., Mimoun, S., Benetti, P. H., Atanasiu, C., Andriamihaja, M., Benamouzig, R., Bouillaud, F. and Tome, D. (2010). Luminal sulfide and large intestine mucosa: friend or foe? *Amino Acids* **39**, 335-347.

Bouillaud, F. and Blachier, F. (2011). Mitochondria and sulfide: a very old story of poisoning, feeding and signaling? *Antioxid. Redox. Signal.* **15**, 379-391.

DeLeon, E. R., Stoy, G. F. and Olson, K. R. (2011). Passive loss of hydrogen sulfide in biological experiments. *Anal. Biochem.* (in press).

Dhaese, I. and Lefebvre, R. A. (2009). Myosin light chain phosphatase activation is involved in the hydrogen sulfide-induced relaxation in mouse gastric fundus. *Eur. J. Pharmacol.* **606**, 180-186.

Dhaese, I., van Colen, I. and Lefebvre, R. A. (2010). Mechanisms of action of hydrogen sulfide in relaxation of mouse distal colonic smooth muscle. *Eur. J. Pharmacol.* **628**, 179-186.

Doeller, J. E., Isbell, T. S., Benavides, G., Koenitzer, J., Patel, H., Patel, R. P., Lancaster, J. R., Darley-Usmar, V. M. and Kraus, D. W. (2005). Polarographic measurement of hydrogen sulfide production and consumption by mammalian tissues. *Anal. Biochem.* **341**, 40-51.

Dombkowski, R. A., Doellman, M. M., Head, S. K. and Olson, K. R. (2006). Hydrogen sulfide mediates hypoxia-induced relaxation of trout urinary bladder smooth muscle. *J. Exp. Biol.* **209**, 3234-3240.

Furne, J., Springfield, J., Koenig, T., DeMaster, E. and Levitt, M. D. (2001). Oxidation of hydrogen sulfide and methanethiol to thiosulfate by rat tissues: a specialized function of the colonic mucosa. *Biochem. Pharmacol.* **62**, 255-259.

Furne, J., Saeed, A. and Levitt, M. D. (2008). Whole tissue hydrogen sulfide concentrations are orders of magnitude lower than presently accepted values. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R1479-R1485.

Goubern, M., Andriamihaja, M., Nubel, T., Blachier, F. and Bouillaud, F. (2007). Sulfide, the first inorganic substrate for human cells. *FASEB J.* **21**, 1699-1706.

He, G., Shankar, R. A., Chzhan, M., Samouilov, A., Kuppusamy, P. and Zweier, J. L. (1999). Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral EPR imaging. *Proc. Natl. Acad. Sci. USA* **96**, 4586-4591.

Hosoki, R., Matsuki, N. and Kimura, H. (1997). The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem. Biophys. Res. Commun.* **237**, 527-531.

Huang, Y., Li, F., Tong, W., Zhang, A., He, Y., Fu, T. and Liu, B. (2010). Hydrogen sulfide, a gaseous transmitter, stimulates proliferation of interstitial cells of Cajal via phosphorylation of AKT protein kinase. *Tohoku J. Exp. Med.* **221**, 125-132.
Lagoutte, E., Mimoun, S., Andriamihaja, M., Chaumontet, C., Blachier, F. and Bouillaud, F. (2010). Oxidation of hydrogen sulfide remains a priority in mammalian cells and causes reverse electron transfer in colonocytes. *Biochim. Biophys. Acta* **1797**, 1500-1511.
Levitt, M. D., Furne, J., Springfield, J., Suarez, F. and DeMaster, E. (1999). Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. *J. Clin. Invest.* **104**, 1107-1114.
Li, L., Rose, P. and Moore, P. K. (2011). Hydrogen sulfide and cell signaling. *Annu. Rev. Pharmacol. Toxicol.* **51**, 169-187.
Li, Q., Sun, B., Wang, X., Jin, Z., Zhou, Y., Dong, L., Jiang, L. H. and Rong, W. (2010). A crucial role for hydrogen sulfide in oxygen sensing via modulating large conductance calcium-activated potassium channels. *Antioxid. Redox Signal.* **12**, 1179-1189.
Linden, D. R., Sha, L., Mazzone, A., Stoltz, G. J., Bernard, C. E., Furne, J. K., Levitt, M. D., Farrugia, G. and Szurszewski, J. H. (2008). Production of the gaseous signal molecule hydrogen sulfide in mouse tissues. *J. Neurochem.* **106**, 1577-1585.
Linden, D. R., Levitt, M. D., Farrugia, G. and Szurszewski, J. H. (2010). Endogenous production of H_2S in the gastrointestinal tract: still in search of a physiologic function. *Antioxid. Redox Signal.* **12**, 1135-1146.
Martin, G. R., McKnight, G. W., Dicay, M. S., Coffin, C. S., Ferraz, J. G. and Wallace, J. L. (2010). Hydrogen sulphide synthesis in the rat and mouse gastrointestinal tract. *Dig. Liver Dis.* **42**, 103-109.
Medani, M., Collins, D., Docherty, N. G., Baird, A. W., O'Connell, P. R. and Winter, D. C. (2010). Emerging role of hydrogen sulfide in colonic physiology and pathophysiology. *Inflamm. Bowel Dis.* **17**, 1620-1625
Nagao, M., Linden, D. R., Duenes, J. A. and Sarr, M. G. (2010). Mechanisms of action of the gasotransmitter hydrogen sulfide in modulating contractile activity of longitudinal muscle of rat ileum. *J. Gastrointest. Surg.* **15**, 12-22.
Olson, K. R. (2009). Is hydrogen sulfide a circulating gasotransmitter in vertebrate blood? *Biochim. Biophys. Acta* **1787**, 856-863.
Olson, K. R. (2011). The therapeutic potential of hydrogen sulfide: separating hype from hope. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R297-R312.
Olson, K. R. and Whitfield, N. L. (2010). Hydrogen sulfide and oxygen sensing in the cardiovascular system. *Antioxid. Redox Signal.* **12**, 1219-1234.
Olson, K. R., Dombkowski, R. A., Russell, M. J., Doellman, M. M., Head, S. K., Whitfield, N. L. and Madden, J. A. (2006). Hydrogen sulfide as an oxygen sensor/transducer in vertebrate hypoxic vasoconstriction and hypoxic vasodilation. *J. Exp. Biol.* **209**, 4011-4023.
Olson, K. R., Forgan, L. G., Dombkowski, R. A. and Forster, M. E. (2008a). Oxygen dependency of hydrogen sulfide-mediated vasoconstriction in cyclostome aortas. *J. Exp. Biol*, **211**, 2205-2213.
Olson, K. R., Healy, M., Qin, J., Skovgaard, N., Vulesevic, B., Duff, D. W., Whitfield, N. L., Yang, G., Wang, R. and Perry, S. F. (2008b). Hydrogen sulfide as an oxygen sensor in trout gill chemoreceptors. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R669-R680.
Olson, K. R., Whitfield, N. L., Bearden, S. E., St Leger, J., Nilson, E., Gao, Y. and Madden, J. A. (2010). Hypoxic pulmonary vasodilation: a paradigm shift with a hydrogen sulfide mechanism. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R51-R60.
Parajuli, S. P., Choi, S., Lee, J., Kim, Y. D., Park, C. G., Kim, M. Y., Kim, H. I., Yeum, C. H. and Jun, J. Y. (2010). The inhibitory effects of hydrogen sulfide on pacemaker activity of interstitial cells of Cajal from mouse small intestine. *Korean J. Physiol. Pharmacol.* **14**, 83-89.
Peng, Y. J., Nanduri, J., Raghuraman, G., Souvannakitti, D., Gadalla, M. M., Kumar, G. K., Snyder, S. H. and Prabhakar, N. R. (2010). H_2S mediates O_2 sensing in the carotid body. *Proc. Natl. Acad. Sci. USA* **107**, 10719-10724.
Perry, S. F., McNeill, B., Elia, E., Nagpal, A. and Vulesevic, B. (2009). Hydrogen sulfide stimulates catecholamine secretion in rainbow trout (*Oncorhynchus mykiss*). *Am. J. Physiol. Regul. Integr. Comp. Physiol*, **296**, R133-R140.
Rowan, F. E., Docherty, N. G., Coffey, J. C. and O'Connell, P. R. (2009). Sulphate-reducing bacteria and hydrogen sulphide in the aetiology of ulcerative colitis. *Br. J. Surg.* **96**, 151-158.
Teague, B., Asiedu, S. and Moore, P. K. (2002). The smooth muscle relaxant effect of hydrogen sulphide *in vitro*: evidence for a physiological role to control intestinal contractility. *British J. Pharmacol.* **137**, 139-145.
Telezhkin, V., Brazier, S. P., Cayzac, S., Muller, C. T., Riccardi, D. and Kemp, P. J. (2009). Hydrogen sulfide inhibits human BK(Ca) channels. *Adv. Exp. Med. Biol.* **648**, 65-72.
Telezhkin, V., Brazier, S. P., Cayzac, S. H., Wilkinson, W. J., Riccardi, D. and Kemp, P. J. (2010). Mechanism of inhibition by hydrogen sulfide of native and recombinant BKCa channels. *Respir. Physiol. Neurobiol.* **172**, 169-178.
Wallace, J. L. (2010). Physiological and pathophysiological roles of hydrogen sulfide in the gastrointestinal tract. *Antioxid. Redox Signal.* **12**, 1125-1133.
Wallace, J. L., Dicay, M., McKnight, W. and Martin, G. R. (2007). Hydrogen sulfide enhances ulcer healing in rats. *FASEB J.* **21**, 4070-4076.
Wang, R. (2002). Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? *FASEB J.* **16**, 1792-1798.
Whitfield, N. L., Kreimier, E. L., Verdial, F. C., Skovgaard, N. and Olson, K. R. (2008). A reappraisal of H_2S/sulfide concentration in vertebrate blood and its potential significance in ischemic preconditioning and vascular signaling. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1930-R1937.
Zhao, P., Huang, X., Wang, Z. Y., Qiu, Z. X., Han, Y. F., Lu, H. L., Kim, Y. C. and Xu, W. X. (2009). Dual effect of exogenous hydrogen sulfide on the spontaneous contraction of gastric smooth muscle in guinea-pig. *Eur. J. Pharmacol.* **616**, 223-228.