

Sodium nitroprusside potentiates H₂S-induced contractions in body wall muscle from a marine worm.

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RESEARCH NOTE

Hydrogen sulfide (H_2S) at concentrations of ca. 0.05 to 1 $mmol\ l^{-1}$ appears to function as a gasotransmitter in vertebrates, analogous to nitric oxide (NO) and carbon monoxide [1-5], but the actions of H_2S in invertebrate tissue have not been well studied. In this study, we investigated the role of H_2S in modulating body wall muscle tone in the marine echiuran worm Urechis caupo (Echiuridae). We first determined that U. caupo body wall homogenates produce H_2S upon addition of L-cysteine and pyridoxal-5'-phosphate (PLP), and that the rate is increased by addition of 2-mercaptoethanol, suggesting the presence of an activated L-serine sulphydrase pathway. We then measured the contractile response of U. caupo body wall circular muscle strips to NaHS (which produces H_2S in solution) and the NO donor sodium nitroprusside (SNP), both with and without subsequent application of acetylcholine (ACh). We found that NaHS alone stimulated contraction in muscle strips equivalent to about one-third the force of ACh alone, whereas SNP alone had no effect on muscle tone. However, simultaneous addition of NaHS with SNP elicited a much stronger contraction, reaching more than twice that of ACh alone, which could be increased further by subsequent application of ACh.

Free H_2S is present in tissues of both vertebrates [1, 6, 7] and invertebrates [8, 9] at concentrations as high as 0.02 to 0.07 $mmol\ l^{-1}$. This finding was initially surprising, given the well-known toxicity of H_2S , which includes inhibition of mitochondrial cytochrome c oxidase at low micromolar levels [10]. H_2S can be produced from L-cysteine by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine beta-synthase (CBS) and cystathionine gamma-lyase (CSE). Endogenous enzymatic production has been shown in tissues from mammals [1, 11-13], the clam Tapes philippinarum and the annelid Arenicola marina [14], and the clam Mercenaria mercenaria [15].

The identification of both H_2S and enzymatic pathways for H_2S production in tissues has stimulated an ongoing search for potential actions of H_2S as an endogenous signaling molecule. In mammalian smooth muscle, 0.025 to 0.1 $mmol\ l^{-1}$ H_2S elicits concentration-dependent relaxation in aorta,

ileum, portal vein and vas deferens [11, 16, 17] and decreases spontaneous contractility in uterus [18]. In mammalian cardiac muscle, H₂S decreases contractility both in vivo and in vitro [12]. The net cardiovascular effect of H₂S in mammals is decreased blood pressure, similar to the action of NO. At least some of these actions of H₂S are likely mediated through direct activation of ATP-sensitive K⁺ (K_{ATP}) channels [13]. Two studies have addressed the action of H₂S on nonmammalian vertebrates [5, 7], with the most recent of these being a careful, phylogenetic survey of responses among the vertebrates [5], and have shown that many smooth muscles have complex, multiphasic responses to H₂S (e.g., relaxation-constriction-relaxation).

In contrast to recent work on mammalian smooth and cardiac muscle, there has been little information about potential signaling actions of H₂S in invertebrate muscle. At concentrations above ca. 5 mmol l⁻¹, H₂S inhibits body wall muscle contraction in U. caupo, but this is more likely a toxic effect [19]. H₂S at 0.05 to 0.1 mmol l⁻¹ also impairs or prevents associative learning and long-term memory in the freshwater snail Lymnaea [20], but whether this affected muscle tone was not tested. However, Gainey and Greenberg [15] reported that, in branchial muscles of the clam M. mercenaria, NaHS potentiates the contractile action of 5-hydroxytryptamine (5HT), although it does not directly affect muscle tone. This effect on 5HT-induced contractions is similar to the action of NO on 5HT-induced contractions in the same muscle. The effect is seen at NaHS concentrations as low as 10 nmol l⁻¹, with a maximal effect at 1 μmol l⁻¹, and appears to act through a soluble guanylate cyclase (sGC) cascade. In mammals, by comparison, 0.3 to 1 mmol l⁻¹ H₂S can almost completely reverse the response to exogenous ACh and norepinephrine in guinea pig ileum and portal vein but not thoracic aorta, and H₂S greatly enhances the smooth muscle relaxant effect of SNP on thoracic aorta [11]. This latter effect was also noted over a decade earlier in guinea pig ileum by Kruszyna et al. [21], but this was prior to the recognition that H₂S and NO have signaling functions.

In this study, we investigated H₂S production and the effects of NaHS on muscle contraction in vitro in U. caupo, which is a large (60 g or more) worm that inhabits U-shaped burrows in mudflats along

the California coast [22]. We collected U. caupo by hand at low tides during winter at Pillar Point Harbor in San Mateo County, California. The worms were maintained unfed in filtered, aerated, recirculating seawater for no more than 3 weeks before they were used for experiments. To confirm that U. caupo tissues have the capacity to produce H₂S, body wall homogenates were prepared and assayed for H₂S production in the presence and absence of 10 mmol l⁻¹ L-cysteine with 2 mmol l⁻¹ PLP, as described previously for T. philippinarum [14]. U. caupo body wall tissue alone produced no detectable H₂S, but this increased to 0.2 nmol H₂S g⁻¹ min⁻¹ (n=2) when L-cysteine and PLP were added. These results are comparable to previously reported production rates for adductor muscle, siphon and gill tissue from T. philippinarum and body wall from A. marina [14]. Addition of 2.5 mmol l⁻¹ 2-mercaptoethanol increased H₂S production to 0.5 nmol g⁻¹ min⁻¹, suggesting the presence of an “activated L-serine sulfhydryase pathway”, similar to tissues of T. philippinarum, but in contrast to A. marina, which does not show evidence of this pathway in body wall [14].

To investigate the effect of NaHS and SNP on muscle contraction, strips of body wall circular muscle were mounted to a force transducer and muscle tension was recorded during bath exposure to 0.1 mmol l⁻¹ ACh to elicit contraction [23], 1 mmol l⁻¹ NaHS and 1 mmol l⁻¹ SNP, as well as simultaneous application of NaHS and SNP (1 mmol l⁻¹ each) followed by ACh. In 19 of 22 strips, ACh alone elicited a slow contraction with a mean force of 3.3 g ± 0.56 g (Fig. 1A). The remaining 3 strips did not respond to ACh. NaHS alone elicited a contraction in 5 of 8 strips, although the response was smaller than with ACh (Fig. 1B). Therefore, unlike mammalian smooth muscle, in which H₂S causes relaxation, H₂S (as NaHS) causes contraction of U. caupo muscle. The only previous demonstration of H₂S-induced muscle contraction is the triphasic response of trout branchial artery [7]. In contrast to NaHS, SNP had no effect on muscle tone (Fig. 1C). However, simultaneous application of NaHS and SNP elicited a much larger contraction than from NaHS alone, and this was further increased by subsequent application of ACh (Fig. 1D).

The possibility of an interaction was investigated further by testing muscle strips at 16 combinations of NaHS×SNP, each at four levels (0, 0.01, 0.1 and 1 mmol l⁻¹). For each muscle strip, 0.1 mmol l⁻¹ ACh was also added after the contraction from NaHS×SNP reached a plateau (or after it was clear there would be no contraction). We found that the lowest concentration of NaHS (0.01 mmol l⁻¹) had no effect on muscle tension, whereas 0.1 and 1 mmol l⁻¹ NaHS produced a contraction equivalent in force to approximately 0.3 times that of ACh alone (Fig. 2A, note the “0 mM SNP” bars at each NaHS concentration), with a range of 0.03 to 0.9. In contrast, SNP alone had no effect at any concentration (Fig. 2A, i.e., cluster of bars “0 mM NaHS”). However, simultaneous application of NaHS with SNP elicited a strong contraction. At 1 mmol l⁻¹ NaHS, the average force (relative to 0.1 mmol l⁻¹ ACh) was 2.4 ± 0.7 and 2.7 ± 0.19 for 0.1 and 1 mmol l⁻¹ SNP, respectively (Fig. 2A, note the shaded bars in cluster at “1.0 mM NaHS”). To test for interactions, the data were analyzed by two-factor within-subjects ANOVA, followed by the Tukey HSD multiple comparisons test (Statistica v5.5). The effect of NaHS on force was significant (P = 0.0063, F₃ = 8.1), whereas the effect of SNP alone was not (P = 0.15, F₃ = 2.3), and there was a significant interaction between NaHS and SNP (P = 0.032, F₃ = 2.5). When ACh was added to the strips, the resulting contraction had a tendency to increase with increasing SNP but not with increasing NaHS (Fig. 2B), but statistical analyses (performed as above) did not detect any significant differences among the treatments (probably due at least in part to large variation in the response to ACh and comparatively small sample size). By comparison, in rat aortic helical strips precontracted with norepinephrine, the vasorelaxant effect of SNP is enhanced with NaHS [11], whereas in rings of the same tissue precontracted with phenylephrine, NaHS inhibits the effect of SNP [16], with the reason for the discrepancy in these two studies being unclear [16]. It should also be noted that although the NaHS concentrations necessary to produce an effect in U. caupo body wall were similar to [11] or only slightly higher than [16] that required to contract rat aorta and smooth muscle of other vertebrate vessels [5], the SNP concentrations necessary for interaction with NaHS in U. caupo muscle were at least an order of magnitude higher than that required in rat aorta.

The data in this study indicate that U. caupo body wall tissue homogenate produces H₂S upon addition of cysteine, and that NaHS elicits a contraction in body wall muscle that is potentiated by SNP. The nature of the interaction between SNP and NaHS in this study and in three studies of mammalian smooth muscle [11, 16, 21] is not known. However, since SNP alone had no effect on U. caupo body wall muscle tone, the interaction between NaHS and SNP is not simply additive, as may occur in rat aorta [11]. SNP donates NO in solution, raising the possibility that H₂S and NO act on separate signaling receptor sites [e.g., 4, 13, 24]. On the other hand, SNP reacts directly with thiols in vitro to form nitrosothiol complexes and free radicals via nucleophilic attack on the NO ligand, which is accompanied by a color change to purple-red [25]. Presumably, this is also the basis of NO release in vivo, for which potential thiols include cysteine, homocysteine and glutathione [25, 26], but could also include H₂S. Therefore, NaHS may enhance the release of NO from SNP. Furthermore, reaction of SNP with thiols may also produce S-nitrosothiols [26], which can act as storage and transport forms of NO [27] and may also directly modulate vessel tone in mammals [28]. This suggests that reaction of NaHS with SNP may not only increase the release of NO, but may additionally produce S-nitrosothiols that then release NO over time and may also act directly. In support of this, an aerated solution of NaHS and SNP (1 mmol l⁻¹ each) retains its capacity to modulate trout vascular smooth muscle tone for 30 min or more, even after the purple-red color has disappeared (R.A. Dombkowski and K.R. Olson, personal communication).

Interestingly, H₂S signaling in M. mercenaria appears to have a seasonal component [15]; the effect of NaHS on 5HT action was present in February and March but absent in August, whereas H₂S production was highest in summer months. We only tested the effect of NaHS on U. caupo collected in winter, so it is unknown whether the response would have differed in other seasons. It is clear, however, that the apparent potentiation of NaHS by SNP in U. caupo differs in at least two ways from the effect of NaHS on M. mercenaria [15]: First, no direct effect of NaHS on muscle tone was observed in M. mercenaria, whereas NaHS alone induced contraction in U. caupo. Second, NaHS and NO can each potentiate 5HT alone in M. mercenaria, whereas in U. caupo there was no significant action of NaHS or

SNP, either together or alone, on the action of ACh. Nonetheless, taken together our data provide strong evidence that H₂S acts as an endogenous signaling transmitter in invertebrates, and that H₂S, whether alone or in combination with ACh or 5HT, has the capacity to stimulate contraction, which is opposite to the relaxation elicited by H₂S in mammalian smooth muscle. It is worth noting, however, that both U. caupo and M. mercenaria are found in habitats with environmental H₂S, and indeed multiple “sulfide-detoxification mechanisms” have been described in U. caupo [29, 30] and other so-called “sulfide-adapted” animals [31]. Whether other invertebrates respond to endogenous H₂S in the same way as sulfide-adapted invertebrates remains to be determined.

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Figure Legends

Figure 1. Contraction force in strips of U. caupo body wall circular muscle in vitro in response to bath application of acetylcholine (ACh), NaHS and sodium nitroprusside (SNP). The scale bar indicates tension (vertical axis) and time (horizontal axis). **A:** Reference contraction in response to 0.1 mmol l^{-1} ACh (at arrow). **B:** Small, extended contraction in response to 1 mmol l^{-1} NaHS (at arrow). **C:** Application of 1 mmol l^{-1} SNP had no effect on muscle tension. **D:** Simultaneous application of 1 mmol l^{-1} SNP and 1 mmol l^{-1} NaHS (at first arrow) caused a contraction almost equivalent to that of ACh alone, while subsequent application of 0.1 mmol l^{-1} ACh (at second arrow) elicited an even larger contraction.

Methods: Circular muscle strips of uniform length, thickness and width were cut from the body wall of U. caupo using a tissue chopper consisting of 20 razor blades bolted together with 5mm distance between each blade. The strips were immediately placed in aerated buffer (37 g l^{-1} Instant Ocean, 50 mmol l^{-1} Na-Hepes and 1.0 g l^{-1} dextrose, pH 7.4) at 9°C for no more than 4 h before being used. For each trial, a single strip was mounted between stainless steel hooks and connected to an isometric force transducer (S10, World Precision Instruments) on a micromanipulator. The strip was then lowered into a chamber containing 6 ml aerated buffer at room temperature ($20\text{-}22^\circ\text{C}$) and slowly stretched to a preload tension of 2.75 g. Digital recording was then initiated at 5 Hz, and ACh, NaHS and SNP were added alone or in combination, as indicated in the text. Throughout each trial, the chamber solution was kept well stirred with a magnetic stirrer. Stock solutions of 10 mmol l^{-1} NaHS and 10 mmol l^{-1} SNP were prepared fresh in buffer, stored on ice (in darkness for SNP) and used within 2 hours.

Figure 2. Contraction force in strips of U. caupo body wall circular muscle in vitro in response to simultaneous bath application of SNP (0.01 , 0.1 and 1 mmol l^{-1}) and NaHS (0.01 , 0.1 and 1 mmol l^{-1}), followed by 0.1 mmol l^{-1} acetylcholine (ACh). Data are presented as mean \pm SEM, with 4 independent replicates for each of the 16 NaHS \times SNP combinations. **A: Change in muscle tension immediately following simultaneous addition of NaHS and SNP.** Contraction force is relative to the force elicited by 0.01 mmol l^{-1} ACh alone in reference strips. **B: Change in muscle tension following addition of ACh to strips pre-contracted by NaHS and SNP.** Force is presented as the tension generated *in addition* to that elicited by NaHS and SNP, and is relative to the force elicited by 0.01 mmol l^{-1} ACh alone in reference strips. **Methods:** Strips from 4 animals were prepared as described in Figure 1, with each strip being used for only one treatment. The order of treatments was randomized for each animal, and every fifth strip was

exposed to ACh alone as a reference (≥ 4 reference strips per animal). For analysis, the contraction force of each NaHS \times SNP strip was normalized to the average force of the reference ACh strips for that animal.

Figure 1

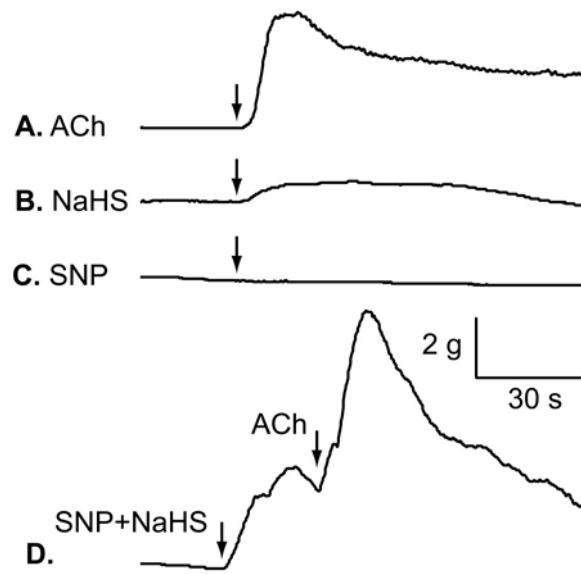


Figure 2

