Hydrogen sulfide in the mouse ductus arteriosus: a naturally occurring relaxant with potential EDHF function

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1Institute of Life Sciences, Scuola Superiore Sant’Anna, Pisa, Italy; 2Institute of Clinical Physiology Consiglio Nazionale delle Ricerche, Pisa, Italy; and 3Laboratorio National Enterprise for nanoScience and nanoTechnology, Scuola Normale Superiore and Istituto Nanoscienze Consiglio Nazionale delle Ricerche, Pisa, Italy

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Hydrogen sulfide in the mouse ductus arteriosus: a naturally occurring relaxant with potential EDHF function. Am J Physiol Heart Circ Physiol 304: H927–H934, 2013. First published February 1, 2013; doi:10.1152/ajpheart.00718.2012.—We have previously reported that bradykinin relaxes the fetal ductus arteriosus via endothelium-derived hyperpolarizing factor (EDHF) when other naturally occurring relaxants (prostaglandin E2, nitric oxide, and carbon monoxide) are suppressed, but the identity of the agent could not be ascertained. Here, we have examined in the mouse whether hydrogen sulfide (H2S) is a relaxant of the ductus and, if so, whether it may also function as an EDHF. We found in the vessel transcripts for the H2S synthetic enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and the presence of these enzymes was confirmed by immunofluorescence microscopy. CSE and CBS were distributed across the vessel wall with the former prevailing in the intimal layer. Both enzymes occurred within the endoplasmic reticulum of endothelial and muscle cells, whereas only CSE was located also in the plasma membrane. The isolated ductus contracted to inhibitors of CSE (D,L-propargylglycine, PPG) and CBS (amino-oxyacetic acid), and PPG contraction was attenuated by removal of the endothelium. EDHF-mediated bradykinin relaxation was curtailed by both PPG and amino-oxyacetic acid, whereas the relaxation to sodium nitroprusside was not affected by either treatment. The H2S donor sodium hydrogen sulfide (NaHS) was also a potent, concentration-dependent relaxant. We conclude that the ductus is endowed with a H2S system exerting a tonic relaxation. In addition, H2S, possibly via an overriding CSE source, qualifies as an EDHF. These findings introduce a novel vasoregulatory mechanism into the ductus, with implications for antenatal patency of the vessel and its transitional adjustments at birth.

It is widely accepted that the ductus arteriosus is kept patent antenatally by a host of relaxing agents (22). Among them, prostaglandin (PG) E2 plays a prime role, and its action is likely supplemented by nitric oxide (NO) and carbon monoxide (CO). NO activity, in particular, changes through development in a reciprocal manner with PGE2 (18, 21, 26) and may also increase following any loss of PGE2 function (1, 23). Hydrogen sulfide (H2S), in contrast, has seemingly no role in the ductus, at least with the chicken embryo (28), despite substantial work in mammals documenting a vasoregulatory function for the compound in both adult (17, 29) and neonate (16).

In a previous investigation, we have found that the as-yet uncharacterized endothelium-derived hyperpolarizing factor (EDHF) is normally not detectable in the mouse ductus but manifests itself once constitutively active relaxants (i.e., PGE2, NO, and CO) are inhibited (2). As expected, this newly emerging EDHF is suppressed by excess potassium or the combined treatment with drugs interfering with small- and intermediate-conductance Ca2+-activated K+ channels, i.e., apamin plus 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (2). Based on these findings and data from other vascular districts, we then examined whether ductal EDHF may be identified with an epoxyeicosatrienoic acid or 12(S)-hydroxyeicosatetraenoic acid, but results were negative (4). Nevertheless, we speculated at the time on H2S playing such role, and this possibility has now become more plausible with the demonstration of EDHF-like properties in this agent (17, 19).

Based on this premise, our study had two distinct, yet complementary, objectives using the mouse fetal ductus as a model: 1) to assess whether H2S is a natural constituent of the vessel with a regulatory action on basal tone and 2) to determine whether the same agent also qualifies as the EDHF appearing upon suppression of the normally active relaxing mechanisms. Our approach is well suited for the latter task, because the ductal EDHF can be induced in a predictable, all-or-none fashion (2).

MATERIALS AND METHODS

Experiments were carried out in wild-type C57BL/6 mice (litter size, 3–10; Harlan, San Pietro al Natisone, Italy). Animals were housed in temperature- and humidity-controlled quarters with constant 12-h:12-h light-dark cycles and were given food and water ad libitum. Surgical procedures and experimental protocols have been approved by the Animal Care Committee of the Ministry of Health.

Solutions and drugs. The Krebs medium had the following composition: (in mM) 118 NaCl, 4.7 KCl, 1.0 KH2PO4, 0.9 MgSO4, 2.5 CaCl2, 11.1 dextrose, and 25 NaHCO3. Depending on the stage of the experiment, the solution was bubbled with gas mixtures containing no O2 or 2.5% O2 plus 5% CO2 and balance N2. The 2.5% concentration was chosen to mimic the fetal condition. PO2 of the medium was measured with a Chiron gas analyzer (model 248, Halstead, UK) and was 6.9 ± 0.1/0.89 ± 0.01 and 16.2 ± 0.13/2.1 ± 0.01 mmHg/kPa (pH 7.41 ± 0.002) when gas mixtures had 0 and 2.5% O2, respectively.

Polyclonal goat antibodies against the H2S-synthesizing enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), were obtained from Santa Cruz Biotechnology (Heidelberg, Germany).

The following compounds were used: bradykinin acetate (Sigma-Aldrich, St. Louis, MO); the H2S donor sodium hydrogen sulfide (NaHS, Sigma-Aldrich), as a suitable substitute for the free gas (33); the CSE inhibitor D,L-propargylglycine (PPG, Sigma-Aldrich); the CBS inhibitor amino-oxyacetic acid (AOAA, Sigma-Aldrich); the NO

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DUCTUS ARTERIOSUS, H₂S RELAXATION, AND EDHF FUNCTION

The heme oxygenase (i.e., CO synthesis) inhibitor zinc protoporphyrin (ZnP, Porphyrin Products, Carnforth, UK); the dual cyclooxygenase 1 and 2 inhibitor indomethacin (Sigma-Aldrich); the thromboxane A₂ analog 9,11-epithio-11,12-methano-thromboxane A₂ (ONO-11113, courtesy of ONO Pharmaceutical, Osaka, Japan); and sodium nitroprusside (SNP, Sigma-Aldrich). Concentrations of the inhibitors were derived from the literature with the aim of combining efficacy with selectivity.

ONO-11113 was dissolved in distilled ethanol (5 mg/ml), and aliquots (stored at −70°C) were diluted with Tris buffer (pH 7.4). Indomethacin was also dissolved in ethanol (10 mg/ml) before the final solution was prepared in Krebs medium. Likewise, ZnP was first prepared as a stock solution in 0.1 M NaOH (1 mM) on the day of the experiment. Ethanol in fluid bathing ductus preparations did not exceed 0.01% (indomethacin) or 0.001% (ONO-11113). Other substances dissolved in Krebs medium. However, in the case of NaHS a stock solution (1 mM) was prepared fresh before the test sequence, taking care to avoid any exposure to ambient oxygen. For this purpose, the medium was bubbled with the 5% CO₂-95% N₂ mixture before dissolving the compound, and the same gas mixture was flushed through the tube with the solution. This procedure also ensured a normal pH even when testing NaHS at its highest concentration (i.e., 1 mM). Solutions of ZnP and SNP were instead protected from light.

Concentrations of compounds are given in molar units and refer to their final value in Krebs medium.

In vitro recording. Term fetal mice of either sex (gestational age, 19 days; 1.1–1.4 g body wt) were delivered by Cesarean section under halothane anesthesia and were euthanized by cervical dislocation. The procedure for dissecting the ductus has been described in detail (6). In brief, the animal was secured in a chamber containing ice-cold Krebs solution, gassed with 5% CO₂ in N₂. Through a thoracotomy, the ductus was exposed, separated from the adjoining large blood vessels, and then suspended onto 25-μm tungsten wires (Cooner wire, Chatsworth, CA) inside an organ bath. In some experiments, the ductus was prepared without the endothelium. For this purpose, a cat whisker of suitable size, its surface coarse from polishing with fine-grain sandpaper (600 grit), was passed through the lumen before isolating the vessel. The procedure for preparing endothelium-denuded, small-caliber vessels has been described, and successful removal was confirmed by transmission electron microscopy and functional tests (2, 30). In all cases, the fluid was gassed with the 2.5% O₂ mixture, and the same mixture was flushed through a hood covering the bath. Preparations were then equilibrated (~60 min at 37°C) under minimum stretch (intact/endothelium-denuded, 0.04 ± 0.001/0.04 ± 0.002 mN/mm, n = 36 and 12, respectively) and the attendant internal circumference (C₀), with the related resting dimensions (in μm)

Table 1. Primer sequences for RT-PCR of H₂S-synthesizing enzymes

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product, bp</th>
</tr>
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<tbody>
<tr>
<td>CSE*</td>
<td>5’-CTGCCAGCATATTGACGTTACG-3’</td>
<td>5’-ATGACAGCCAGGAGCAGGAAAG-3’</td>
</tr>
<tr>
<td>CBS</td>
<td>5’-GAGTGTCAGGGGCGGATGCAA-3’</td>
<td>5’-CCCTGAGATGCGACACGATG-3’</td>
</tr>
<tr>
<td>MPST</td>
<td>5’-GATGACAGGAGGAGGAGTTGAG-3’</td>
<td>5’-GACGAGTGGACCTCTCTGCG-3’</td>
</tr>
</tbody>
</table>

Primer sequences were found in the literature (see asterisk, Ref. 13) or were designed by us. CSE, cystathionine-γ-lyase; CBS, cystathionine-β-synthase; MPST, mercaptopuruvate sulfurtransferase; bp, base pair.

Gene expression analysis. Total RNA was obtained [see Baragatti et al. (2)] from pooled preparations of the fetal ductus (∼30 preparations/group) using TriPure isolation reagent (Roche, Indianapolis, IN). Its yield was measured spectrophotometrically, and a quality control was performed by gel electrophoresis. Adult liver or kidney was taken as a positive control, depending on the gene. RNA was reverse transcribed with the QuantiTect Reverse Transcription kit (Qiagen, Milano, Italy) according to the manufacturer’s instructions. The cDNA product (0.1 or 0.2 μg/group) was used for amplification in semiquantitative PCR, and primer sequences for H₂S-synthesizing enzymes [CSE, CBS, and mercaptopuruvate sulfurtransferase (MPST)] were retrieved from the literature or were designed on purpose (Table 1). Electrophoresis of the PCR product was performed on agarose gel (1.5% wt/vol) stained with ethidium bromide, and bands from the ductus and reference tissue were visualized in parallel. Ductus cDNA was collected from the designated zone of the gel, purified with QiAQuick Gel Extraction kit (Qiagen), and sequenced (BRM Genomics, Padova, Italy) to confirm gene identity.

Immunohistochemistry. Transversal sections (5 μm thick) of frozen ductus were processed, as previously reported (3), to assess the subcellular localization of H₂S-synthesizing enzymes appearing most...
prominent from the gene analysis (i.e., CSE and CBS; see RESULTS). Briefly, CSE and CBS were exposed with polyclonal goat antibodies (dilution, 1:50 for both), and chicken anti-goat IgG (dilution, 1:150 and 1:100 for CSE and CBS, respectively), conjugated to FITC, served as secondary antibody (Santa Cruz). Specificity of binding was verified by omitting the primary antibody. Colocalization of immunoreactive sites with the plasma membrane and endoplasmic reticulum was examined using, respectively, the markers wheat germ agglutinin conjugated with marine blue and BODIPY 558/568 Brefeldin A (Molecular Probes, Eugene, OR). Images were acquired on a confocal microscope (TCS-SP2 on module DM IRE 2, Leica), and colocalization of signals was confirmed on three-dimensional (3-D) reconstructions obtained from the deconvolution of a z-stack of xy-images (i.e., series of images along the z-axis) (3). Further elaboration with the Huygens Professional 3.2.0p7 software (Scientific Volume Imaging, Hilversum, The Netherlands) yielded the Pearson coefficient proving presence (values, 0 to 1) or absence (values, 0 to –1) of colocalization. The same software provided a colocalization map rendered with iso-colocalization surfaces. Quantification of individual CSE and CBS immunostaining was also performed with Imaris 7.3.0 software (Bitplane, Zürich, Switzerland) on selected deconvoluted 3-D stacks with the aim of determining the relative intensity of signals in the intimal versus medial layer.

Statistical analysis. Data are presented as means ± SE. Comparisons were made with Student’s t-test or ANOVA, as required. Differences are considered significant for P < 0.05.

RESULTS

Gene expression analysis. We detected transcripts for both CSE and CBS in the ductus (Fig. 1, A and B), and their identity was confirmed by comparing the nucleotide sequence of the purified material with an authentic reference. Conversely, the signal for MPST was exceedingly weak (Fig. 1 C), and, consequently, we decided not to consider this enzyme any further.

Localization of CSE and CBS. Using fluorescence microscopy, we found CSE and CBS immunostaining in the ductus. Its distribution encompassed both intimal and medial layers in either case (Fig. 2, A and B), but CSE staining was more intense in the intima (Fig. 2 A). In particular, we recorded a CSE signal about 45% higher in the intima, possibly the endothelium, than in the muscle. Conversely, the CBS signal was about 15% higher in muscle. Furthermore, while CSE was more diffuse, CBS appeared to be confined mostly within distinct zones identifiable with the cell cytoplasm (Figs. 3 A and 4 A). Indeed, with appropriate markers, we found a colocalization of both CSE and CBS with the endoplasmic reticulum, whereas only CSE was also located within the plasma membrane (Figs. 3 and 4). The latter findings were quantified by calculating the Pearson coefficient on 3-D images obtained after the deconvolution procedure (Fig. 5, A–D). Accordingly, we recorded values for CSE (endoplasmic reticu-
lum, 0.46 ± 0.07; and plasma membrane, 0.2 ± 0.04; n = 3) and CBS (endoplasmic reticulum, 0.22 ± 0.03; and plasma membrane, 0.007 ± 0.015; n = 4) with the expected differential magnitude in connection to the two cell components. In a few instances, the Pearson coefficient could be measured separately in muscle and endothelial cells, and the resulting values presented the same profile (data not shown). With either enzyme, we did not detect any immunoreactivity in the absence of the primary antibody.

**In vitro study.** The ductus, whether intact or endothelium-denuded, developed a variable degree of tension during equilibration (with/without endothelium, 0.12 ± 0.01/0.11 ± 0.04 mN/mm) with transient contractions of uneven amplitude (0.1–0.9 mN/mm) and/or low-amplitude fast discharges often superimposed. In agreement with previous reports (2, 3), the vessel contracted promptly to the reference spasmogen ONO-11113 (at 0.1 M), the actual values being slightly lower in the absence of the endothelium (intact/endothelium-denuded, 1.08 ± 0.1/0.66 ± 0.12 mN/mm, n = 10 and 6). The smaller size of preparations in the latter group (see MATERIALS AND METHODS) may account for this difference. Equally consistent was the contraction to the inhibitor cocktail (intact ductus; see MATERIALS AND METHODS), and a stable tone was attained with all preparations (0.82 ± 0.11 mN/mm, n = 15).

When tested on the intact ductus at rest, the CSE inhibitor PPG (10 μM) elicited a contraction in four of five experiments (Fig. 6A). In the remaining experiment, there was no obvious change in baseline throughout treatment. When present, the response started after some delay (4–18 min; and mean, 10) and then progressed to a maximum (peak in 4–23 min; and mean, 14) to subside partially thereafter. Phasic activity was often superimposed on the tonic contraction, becoming either faster or appearing ex novo in response to the inhibitor. Similarly developed was the peak contraction of the resting ductus to the CBS inhibitor AOAA (1 mM) (Fig. 6A), and the time course of the response (latency 6–18 min; mean, 12; and peak in 22–79 min; mean, 35) did not depart greatly from that seen with PPG. Both compounds, on the other hand, did not increase any further the ductus contraction to the inhibitor cocktail, nor did they change its pattern in any apparent way (data not shown). Removal of the endothelium resulted in a small, although not significant, reduction of the peak response to PPG, whereas the response to AOAA remained substantially unchanged (Fig. 6B). Furthermore, the PPG contraction required a longer time for full development (peak in 9–87 min; mean, 48).

Bradykinin relaxed dose-dependently the intact ductus precontracted with the inhibitor cocktail. As shown in Fig. 7, threshold was at 10–100 pM and a maximum around 100 μM. Pretreatment with either PPG (Fig. 7A) or AOAA (Fig. 7B) curtailed the relaxation and, in the higher dose range of bradykinin, both inhibitors often converted the original relaxation into a modest contraction. SNP (at 100 μM), on the other hand, was always a relaxant and reversed completely, or nearly completely, the contractile tone of the ductus both in the absence (tone reversal, 0.92 ± 0.1 mN/mm; 89 ± 9%; n = 11) and presence of PPG (tone reversal, 0.72 ± 0.03 mN/mm; 75 ± 5%; n = 8) or AOAA (tone reversal, 0.54 ± 0.04 mN/mm; 100 ± 16%; n = 4).

**Fig. 3.** Immunofluorescence micrographs of the mouse ductus arteriosus. Confocal images stained, in the order, with CSE antibody (green channel; A), endoplasmic reticulum marker (red channel; B), plasma membrane marker (blue channel; C), and CSE antibody plus superimposed markers for the endoplasmic reticulum (D) and plasma membrane (E). et, Endothelium; smc, smooth muscle. Arrowheads show examples of spots with high colocalization. Scale bar = 10 μm.
NaHS also potently relaxed the intact ductus and its effect progressively increased over the range 1 nM–1 mM (Fig. 8). Relaxation was immediate in onset and, at the highest concentration, was often preceded by a small and transient contraction (maximum, 0.1 mN/mm).

DISCUSSION

Contrary to findings in the chicken ductus (28), we demonstrate that H2S is naturally present in the mouse ductus and exerts a tonic relaxant action. As expected from previous studies (16, 17, 29), CSE is confirmed as a prime enzyme for its synthesis. Our data, however, depart from current knowledge (16, 17, 29) inasmuch as they show, on one hand, the occurrence in the vessel of CBS, being normally viewed as an extravascular enzyme and, on the other, the insignificant expression of MPST. At the same time, they give evidence of an endothelial source for H2S, hence validating a concept that has experienced some uncertainty through the years (17, 32, 34). In fact, we found some loss of the contractile response to the CSE inhibitor in the endothelium-denuded ductus. Moreover, immunofluorescence microscopy showed a preferential expression of CSE in the intimal layer of the ductus and, conceivably, the endothelium therein, as one would expect from any EDHF candidate. Based on this premise, our discussion will address two main issues: the possible role of H2S as a novel regulator of ductal tone and the feasibility of the same agent serving as EDHF under certain conditions.

H2S emerges as the latest addition to a host of compounds relaxing the ductus and, collectively, contributing to its patency through fetal life. Consistent with this concept are several aspects of our study: 1) the notable potency of H2S as relaxant that sets the ductus apart from most blood vessels, requiring instead high, conceivably nonphysiological, concentrations of the compound for a response (25, 33); 2) the notion that the reported potency of the compound is, if anything, an underestimate since under our experimental conditions, only a fraction of the parent NaHS is bound to generate H2S (8); 3) the lack of a contractile component in the H2S response, which again distinguishes the ductus from several other vessels (7, 9, 17, 20) and, at the same time, proves a coincidence of effects between the applied compound and the putative endogenous agent; 4) the occurrence of the H2S-forming CSE also in the plasma membrane of cells, hence at an optimal location for dynamic responses to any challenge; and, most importantly, 5) the contractile action of CSE and CBS inhibitors on the ductus at rest, hence implying the normal operation of a H2S-based relaxing mechanism within the vessel wall. An unspecific action of the inhibitors, due to a concomitant rise of homocysteine (29), may be ruled out, because this compound is a relaxant rather than a contractile agent on blood vessels and is only effective at exceedingly high concentrations (27). On the other hand, the alternative possibility of homocysteine interfering with the dilator function of the endothelium is equally unfausible since, when compared with the finding with
H₂S inhibitors, such response takes a longer time to unfold (5, 24). Significant in this context is also the prominent lipophilic character of H₂S, ensuring a facile diffusion of the compound within the cellular milieu despite its intrinsic reactivity with diverse substrates including oxygen (10, 15). Summing up, H₂S qualifies as a novel ductal regulator with distinctive physicochemical properties. Its activity may be favored antenatally by the relatively low-oxygen environment and, as a corollary, may abate postnatally with the physiological rise in blood oxygen tension. Any such transitional change, if confirmed, would assign to the H₂S mechanism an additional, facilitatory role in closure of the ductus at birth. [Note: H₂S may also react with oxygen and generate a, hitherto unidentified, vasoconstrictor product (15). Hence, ductus closure could be facilitated by either or both H₂S-linked processes: withdrawal of a tonic relaxation and enhancement of the drive for contraction.]

While contributing to tone regulation thanks to its diffuse formation across the vessel wall, H₂S may also qualify as an EDHF-like agent. A key premise to this concept is found in our

![Diagram](image_url)
previous work showing that bradykinin relaxation meets the criteria for an EDHF-mediated event inasmuch as it unfolds upon removal of both NO and CO mechanisms and is also liable to inhibition by excess potassium or treatment with compounds interfering with appropriate Ca\(^{2+}\)-activated K\(^+\) channels (2). Hence, the observation that CSE and CBS inhibitors interfere with a bradykinin relaxation having such prerequisites establishes a causal link between H\(_2\)S and EDHF. Added support, however, comes from the occurrence of the H\(_2\)S-synthetic enzymes in the intimal layer—indeed, the prevailing expression of CSE at that site—with some loss in the response to PPG after removal of the endothelium. Not minor in this context is also the demonstration of a plasmalemmal location for CSE, ensuring the direct access of H\(_2\)S to the intercellular milieu as one would expect from any candidate for the quickly acting EDHF (11, 12). In brief, H\(_2\)S qualifies as an intercellular milieu as one would expect from any candidate for offered at this time for this apparent inconsistency, although curtailing the bradykinin relaxation. No explanation can be that the CBS inhibitor is equally, if not more, effective in outwardly incongruent with the latter conclusion is the finding appear more important as a source of the agent. However, EDHF being formed in response to bradykinin. CSE would\(\)uisites establishes a causal link between H\(_2\)S and EDHF. Added support, however, comes from the occurrence of the compounds interfering with appropriate Ca\(^{2+}\) channels (2). Hence, the observation that CSE and CBS inhibitors interfere with a bradykinin relaxation having such prerequisites establishes a causal link between H\(_2\)S and EDHF. 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Concentration-response curve to NaHS in preparations precontracted with the combination indomethacin (2.8 \(\mu\)M)/\(\text{N}^\text{\textasterisk}4\)-nitro-L-arginine methyl ester (100 \(\mu\)M)/zinc protoporphyrin (10 \(\mu\)M) before (\(n = 4\)). Wall tension before NaHS was 0.62 ± 0.08 mN/mm. Note that tone reversal for sodium nitroprusside (100 \(\mu\)M) was 0.68 ± 0.05 mN/mm (115 ± 16% reversal from the precontraction value).
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