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Vasoactivity of the gasotransmitters hydrogen sulfide and carbon monoxide in the chicken ductus arteriosus

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van der Sterren S, Kleikers P, Zimmermann LJI, Villamor E. Vasoactivity of the gasotransmitters hydrogen sulfide and carbon monoxide in the chicken ductus arteriosus. *Am J Physiol Regul Integr Comp Physiol* 301: R1186–R1198, 2011. First published August 3, 2011; doi:10.1152/ajpregu.00729.2010.—Besides nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) is a third gaseous messenger that may play a role in controlling vascular tone and has been proposed to serve as an O_2 sensor. However, whether H_2S is vasoactive in the ductus arteriosus (DA) has not yet been studied. We investigated, using wire myography, the mechanical responses induced by Na_2S (1 μM–1 mM), which forms H_2S and HS^- in solution, and by authentic CO (0.1 μM-0.1 mM) in DA rings from 19-day chicken embryos. Na_2S elicited a 100% relaxation (pD_2 4.02) of 21% O_2-contracted and a 50.3% relaxation of 62.5 mM KCl-contracted DA rings. Na_2S-induced relaxation was not affected by presence of the NO synthase inhibitor L-NAME, the soluble guanylate cyclase (sGC) inhibitor ODQ, or the K^+ channel inhibitors tetraethylammonium (TEA; nonselective), 4-aminopyridine (4-AP, K_V), glibenclamide (K_{ATP}), iberiotoxin (BK_{Ca}), TRAM-34 (IK_{Ca}), and apamin (SK_{Ca}). CO also relaxed O_2-contracted (60.8% relaxation) and KCl-contracted (18.6% relaxation) DA rings. CO-induced relaxation was impaired by ODQ, TEA, and 4-AP (but not by L-NAME, glibenclamide, iberiotoxin, TRAM-34 or apamin), suggesting the involvement of sGC and K_V channel stimulation. The presence of inhibitors of H_2S or CO synthesis as well as the H_2S precursor L-cysteine or the CO precursor hemin did not significantly affect the response of the DA to changes in O_2 tension. Endothelium-dependent and -independent relaxations were also unaffected. In conclusion, our results indicate that the gasotransmitters H_2S and CO are vasoactive in the chicken DA but they do not suggest an important role for endogenous H_2S or CO in the control of chicken ductal reactivity.

ductus arteriosus; oxygen sensing

NITRIC OXIDE SYNTHESIZED from L-arginine by nitric oxide synthase (NOS) and carbon monoxide (CO) synthesized from heme by heme oxygenase (HO) are well-known gaseous messengers that, among several other functions, play a pivotal role in the regulation of vascular tone (39). Recent studies indicate that another gas, hydrogen sulfide (H_2S), is also produced in substantial amounts in a variety of cells and exerts many physiological effects, suggesting its potential role as a regulatory mediator (39, 45, 51). Two key enzymes in the transsulfuration pathway, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), have been consistently shown to produce H_2S, using L-cysteine as substrate (39, 45, 51, 57). The expression of CBS is more abundant in liver and neuronal tissues, while CSE is the dominant H_2S-generating enzyme in the cardiovascular system. A growing amount of research supports that by modulating vascular tone, promoting apoptosis of vascular smooth muscle cells, and inhibiting proliferation-associated vascular remodeling, H_2S participates in the regulation of both function and structure of the circulatory system (39, 41, 45, 49, 51, 57, 69, 70).

The ductus arteriosus (DA) is a large fetal shunt connecting the pulmonary artery to the aorta, allowing most of the right ventricular output to bypass the unexpanded lungs (10, 56). Although the cyclooxygenase pathway, with PGE_2 as its major effector, is assigned a prime role in the active maintenance of fetal DA patency, the gasotransmitters NO and CO are viewed as additional effectors acquiring prominence under certain conditions (7). However, whether the third gaseous mediator, i.e., H_2S, is also a modulator of DA tone has not been investigated so far.

The failure of the DA to close after birth is a neonatal complication, often associated with premature birth, which can negatively impact the outcome of preterm infants (56). Therefore, advancing our knowledge on the mechanisms that regulate vascular tone in the DA and its developmental biology may have direct clinical significance. Recently, the chicken embryo has emerged as a suitable model for the study of DA developmental biology (56). Our group and another laboratory have characterized the responsiveness of the chicken DA to several mediators that participate in the control of ductal tone in mammals, including O_2, NO, CO_2, PGs, sex hormones, and catecholamines (2–4, 9, 20, 33, 34, 46, 61). Since the gasotransmitters H_2S and CO are important endogenous signaling molecules in numerous vascular beds, we hypothesized that they would be vasoactive also in the chicken DA. Moreover, since H_2S has been proposed as vascular O_2 sensor, we hypothesized its role in the process of O_2 sensing/signaling in the DA. To test these hypotheses, we analyzed, using wire myography, the mechanical responses induced by Na_2S, which forms H_2S and HS^- in solution (26), and by authentic CO (62) in chicken DA rings. We also evaluated the effects of the precursors and inhibitors of the endogenous synthesis of either H_2S or CO on ductal reactivity and responsiveness to O_2. The DA was compared with femoral and pulmonary arteries, which respond in disparate ways to changes in O_2 tension (2, 20, 53, 72).

MATERIALS AND METHODS

Embryo incubation and vessel isolation. All experimental procedures were carried out according to the regulations of the Dutch Law on Animal Experimentation and the European Directive for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (86/609/EU) and were approved by the Committee on Animal Experimentation of the University of Maastricht. Fertilized

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eggs of White Leghorn chickens (Het Anker, Ochten, The Netherlands) were incubated at a temperature of 37.8°C/45% air humidity and automatically rotated once every hour over an angle of 90 degrees (incubator model 25HS; Masalles Comercial, Spain). Embryos incubated for 15 and 19 days of the 21-day incubation period were studied. The majority of the experiments were performed in 19-day (noninternally pipped) embryos, while for the study of developmental changes, the 15-day and the 19-day vessels were compared. The experiments involving pulmonary arteries were performed in externally pipped 21-day embryos, because pulmonary arteries from 19-day embryos show, in general, weak vasomotor responses (63). On the experimental day, the embryos were taken out, immediately killed by decapitation, and a midline laparotomy and sternotomy were performed. With the aid of a dissecting microscope, the right and the left DA were carefully dissected free from surrounding tissue, severed distal to the origin in the right or left pulmonary artery and proximal to the insertion into the aorta, and divided in two segments referred to as pulmonary side and aortic side (PulmDA and AoDA, respectively). The boundary between pulmonary and aortic side was determined based on the marked differences in diameter observed along the chicken DA (2, 4). In some embryos, rings of the femoral and the caudomedial intrapulmonary arteries were also obtained, as previously described (63, 72).

Recording of arterial reactivity. Two stainless steel wires (diameter 40 μm) were inserted into the lumen of the vessels, which were mounted as ring segments (mean length, 1.72 mm, SD 0.31) between an isometric force transducer and a displacement device in a myograph (model 610M; Danish Myo Technology, Aarhus, Denmark). The myograph organ bath (5 ml volume) was filled with Krebs-Ringer bicarbonate (KRB) buffer maintained at 39°C. After an equilibration period of 30 min, the vessels were distended to a resting tension corresponding to a transmural pressure of 10 mmHg (15-day embryos) or 20 mmHg (19-day and 21-day embryos). These pressures correspond to the mean arterial blood pressure reported in chicken embryos at the corresponding age (6) and elicit the highest contractile response to KCl, as determined in previous experiments (33, 46). After 30 min of incubation at basal tone, a control contraction was elicited by raising the K^+ concentration of the buffer to 62.5 mM (in exchange for Na^+). During the first phase of mounting and stabilization, DA rings were maintained in KRB buffer aerated with 95% N_2/5% CO_2 (P_{O_2}, 2.48 kPa, SD 0.34, $n = 12$, measured with an ABL 510 blood gas analyzer; Radiometer Copenhagen, Denmark). Afterward, the gas mixture was switched to 21% O_2/74% N_2/5% CO_2 (P_{O_2}, 19.16 kPa, SD 1.15, $n = 12$) to induce a normoxic contraction of the PulmDA (2, 3). The relaxations evoked by H_2S and CO were mainly studied during this normoxic contraction, but some experiments (see RESULTS) were performed in vascular rings (DA, femoral, and pulmonary arteries) precontracted with KCl (62.5 mM) or phenylephrine (10 μM) and aerated with 5% O_2/90% N_2/5% CO_2 (P_{O_2}, 6.96 kPa, SD 0.52, $n = 12$) or with 95% N_2/5% CO_2. The latter gas mixture was also used to induce a direct hypoxic contraction in AoDA and pulmonary artery rings.

Response of chicken DA to exogenous H_2S and CO. Na_2S (1 μM–1 mM), which forms H_2S and HS^- in solution, was used to analyze the response to H_2S because of its availability with a reduced amount of elemental sulfur impurities (26, 27). Na_2S was dissolved in deoxygenated HEPES buffer under 100% N_2 and titration to pH 7.4 with HCl.

Concentration-response curves to CO (0.1 μM-0.1 mM) were conducted by addition of increasing volumes of a HEPES buffer solution (pH 7.4) saturated with CO, as previously described (62). The concentration of CO in the saturated solution was estimated from the solubility of CO in water at 25°C and 1 atm of pressure (62). We assumed that the loss of added CO from the HEPES solution at the time of measuring relaxation was negligible. Because this assumption was not strictly correct, actual concentrations of CO in the organ chamber might be somewhat lower than estimated (62).

To assess the mechanisms involved in the response of the chicken DA to the different vasoactive mediators, some experiments were performed in the presence of the following pharmacological tools: the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM), the soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), the nonselective K^+ channel inhibitor tetraethylammonium (TEA, 5 mM), the voltage-gated K^+ channel (K_V) inhibitor 4-aminopyridine (4-AP; 10 mM), the ATP-sensitive K^+ channel (K_{ATP}) inhibitor glibenclamide (10 μM), the large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) inhibitor iberiotoxin (100 nM), the intermediate-conductance K_{Ca} (IK_{Ca}) channel inhibitor TRAM-34 (1 μM), the small-conductance K_{Ca} (SK_{Ca}) channel inhibitor apamin (50 nM), the TP receptor antagonist SQ29548 (10 μM), the dual endothelin (ET) receptor antagonist bosentan (10 μM), the L-type Ca^{2+} channel blocker nifedipine (10 μM), and the sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (2 μM). Parallel control experiments were always carried out to correct for the possible effects of the different vehicles used to dissolve the drugs. In another group of experiments, the endothelium was removed by gentle rubbing of the vessel lumen with a horse tail, as previously described (4). The absence of a functional endothelium was verified by the failure of acetylcholine (1 μM) to induce relaxation of the vascular tissues precontracted with phenylephrine (10 μM) (4).

Effects of endogenous H_2S and CO on DA reactivity. In this group of experiments, the effects of inhibiting or stimulating H_2S and CO synthesis on the response of the PulmDA to normoxia and hypoxia were examined. O_2-induced contraction in the chicken DA is easily reversible when returning to hypoxia and highly reproducible in two consecutive challenges (20). Thus, each vessel was exposed twice to a cycle of hypoxia-normoxia-hypoxia and the second exposure was elicited in the presence of vehicle (control), the CSE-inhibitor D,L-propargylglycine (PPG; 1 mM), the CBS-inhibitor amino-oxyacetate (AOA; 1 mM), the substrate for endogenous H_2S production (L-cysteine; 1 mM), the HO-inhibitor zinc protoporphyrin IX (ZnPP IX; 10 μM), or the substrate for endogenous CO production hemin (10 μM). Experiments involving ZnPP or hemin were carried out in the dark (19). Due to the scarce information on the effects of the above mentioned compounds in chicken tissues, doses were selected based on their effects in mammalian tissues (7, 19, 52, 65).

In previous studies, we demonstrated that acetylcholine (ACh) evoked an endothelium-dependent (NO-mediated) relaxation, whereas the NO donor sodium nitroprusside (SNP) evoked an endothelium-independent relaxation in the chicken DA (4). To analyze the possible role of H_2S and CO in these relaxations, concentration response curves to ACh (10 nM-10 μM) and SNP (10 nM–0.1 mM) were constructed in O_2-contracted DA rings in the absence or presence of PPG (1 mM), AOA (1 mM), L-cysteine (1 mM), or ZnPP IX (10 μM).

Drugs and solutions. KRB buffer contained (in mmol/l): 118.5 NaCl, 4.75 KCl, 1.2 $MgSO_4 \cdot 7\ H_2O$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 5.5 glucose. HEPES buffer contained (in mmol/l): 142.9 NaCl, 4.75 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 5.5 glucose, 15.0 HEPES. Solutions containing different concentrations of K^+ were prepared by replacing NaCl by an equimolar amount of KCl. CO was obtained from Lindegas Benelux. Tetraethylammonium chloride, glibenclamide, and L-cysteine were obtained from Alexis Biochemicals (Lausen, Switzerland). SQ29548 was obtained from Cayman Chemical (Ann Arbor, MI). All other drugs were obtained from Sigma (St. Louis, MO). All drugs were initially dissolved in distilled deionized water, except ODQ, TRAM-34, ZnPP IX, hemin, and SQ29548, which were dissolved in DMSO, nifedipine, which was dissolved in ethanol and 4-AP, which was directly dissolved in KRB buffer.

Data analysis. Results are presented as the mean (SD) of measurements in the number of (n) embryos. For clarity of figures, results are shown as means ± SE. Contractions are expressed in terms of active wall tension, calculated as the force divided by twice the length of the arterial segment (mN/mm). Relaxations are expressed as the percentage of reduction of the contraction induced by 21% O_2, KCl or

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phenylephrine. Sensitivity/potency (expressed as $pD_2 = -\log EC_{50}$) and efficacy (expressed as E_{max}) were calculated by nonlinear regression analysis of the concentration-response curves. Differences between mean values were assessed by unpaired *t*-tests or one-way ANOVA, followed by post hoc Bonferroni *t*-test. Differences were considered statistically significant at $P < 0.05$. All analyses were performed using a commercially available statistics package (GraphPad Prism version 5, GraphPad InStat version 3.00; GraphPad Software, San Diego, CA).

RESULTS

Response of chicken embryo vessels to contractile stimuli. In endothelium-intact PulmDA rings from 19-day chicken embryos, exposure to 21% O_2 evoked a mean contraction of 0.342 mN/mm (SD 0.07, $n = 48$). KCl (62.5 mM, in the presence of 5% O_2) evoked a mean contraction of 0.121 mN/mm (SD 0.04, $n = 14$) in PulmDA rings from 15-day embryos, 0.462 mN/mm (SD 0.04, $n = 18$) in PulmDA rings from 19-day embryos, 0.512 mN/mm (SD 0.16, $n = 12$) in AoDA rings from 19-day embryos, 1.423 mN/mm (SD 0.22, $n = 16$) in femoral artery rings from 19-day embryos, and 0.543 mN/mm (SD 0.12, $n = 9$) in pulmonary artery rings from 21-day embryos. KCl (62.5 mM, in the presence of 0% O_2) evoked a mean contraction of 0.503 mN/mm (SD 0.12, $n = 6$) in PulmDA rings from 19-day embryos. Finally, phenylephrine (10 μM) evoked, in PulmDA rings from 19-day embryos, mean contractions of 0.587 mN/mm (SD 0.18, $n = 6$) and 0.596 mN/mm (SD 0.19, $n = 6$) under 0% and 5% O_2, respectively.

Response to Na_2S. Na_2S relaxed O_2-contracted PulmDA rings (19-day) in a dose-dependent manner (Figures 1*A* and 2*A*; pD_2 4.02, SD 0.12, $n = 13$). When the PulmDA rings (19-day) were precontracted with KCl (62.5 mM in the presence of 0% or 5% O_2), Na_2S also evoked a concentration-dependent relaxation (Figs. 1*C* and 2*A*) with similar potency (0% O_2: pD_2 3.97, SD 0.38, $n = 6$; 5% O_2: pD_2 4.16, SD 0.42, $n = 9$) but lower efficacy than the observed in O_2-contracted rings (E_{max} KCl-contracted 0% O_2: 40.96%, SD 27.2; E_{max} KCl-contracted 5% O_2: 50.34%, SD 27.2; E_{max} O_2-contracted: 100.71%, SD 18.4; $P < 0.01$ vs. KCl-contracted in the presence of 0% or 5% O_2). When PulmDA rings (19-day) were precontracted with phenylephrine (10 μM, in the presence of 0% or 5% O_2), Na_2S also evoked a concentration-dependent relaxation (Fig. 2*A*) with similar potency (0% O_2: pD_2 4.48, SD 0.44, $n = 6$; 5% O_2: pD_2 4.51, SD 0.41, $n = 6$) and efficacy as the ones observed in O_2-contracted rings.

As can be observed in Fig. 1, *A* and *C*, the relaxations evoked by the lowest effective concentrations (0.03–0.1 mM) of Na_2S were transient and followed by a progressive return of the tone toward the precontraction level. To assess whether this phenomenon was due to a biphasic effect (i.e., relaxation followed by contraction), we analyzed the time pattern of a more prolonged (>30 min) exposure to Na_2S (0.1 mM, Figs. 1, *B* and *D*). The level of tension reached after > 30 min of exposure to Na_2S was 0.373 mN/mm (SD 0.14, $n = 6$) in the O_2-contracted PulmDA rings and 0.493 mN/mm (SD 0.16, $n = 6$) in the KCl-contracted vessels. This level of tension was not significantly different from the one observed in time-control experiments (O_2-contracted: 0.358 mN/mm, SD 0.18, $n = 4$; KCl-contracted: 0.511, SD 0.21, $n = 4$). This suggests that Na_2S did not evoke an actual contraction and that the concentration of H_2S might have been reduced due to constant bubbling of O_2, CO_2, and N_2 in the organ bath, inducing the progressive return of active wall tension towards the precontraction line.

KCl-contracted PulmDA rings from 15-day embryos were relaxed by Na_2S (Fig. 2*B*) with similar potency (pD_2 4.24, SD 0.14, $n = 6$) and efficacy (E_{max} 34.25%, SD 13.6) as observed for 19-day PulmDA rings. Na_2S also relaxed KCl-contracted 19-day AoDA rings (pD_2 3.98, SD 0.18, $n = 9$), 19-day femoral artery rings (pD_2 4.23, SD 0.34, $n = 8$), and 21-day pulmonary artery rings (pD_2 4.29, SD 0.28, $n = 9$) with similar potency and efficacy as observed for 19-day PulmDA rings (Fig. 2*B*).

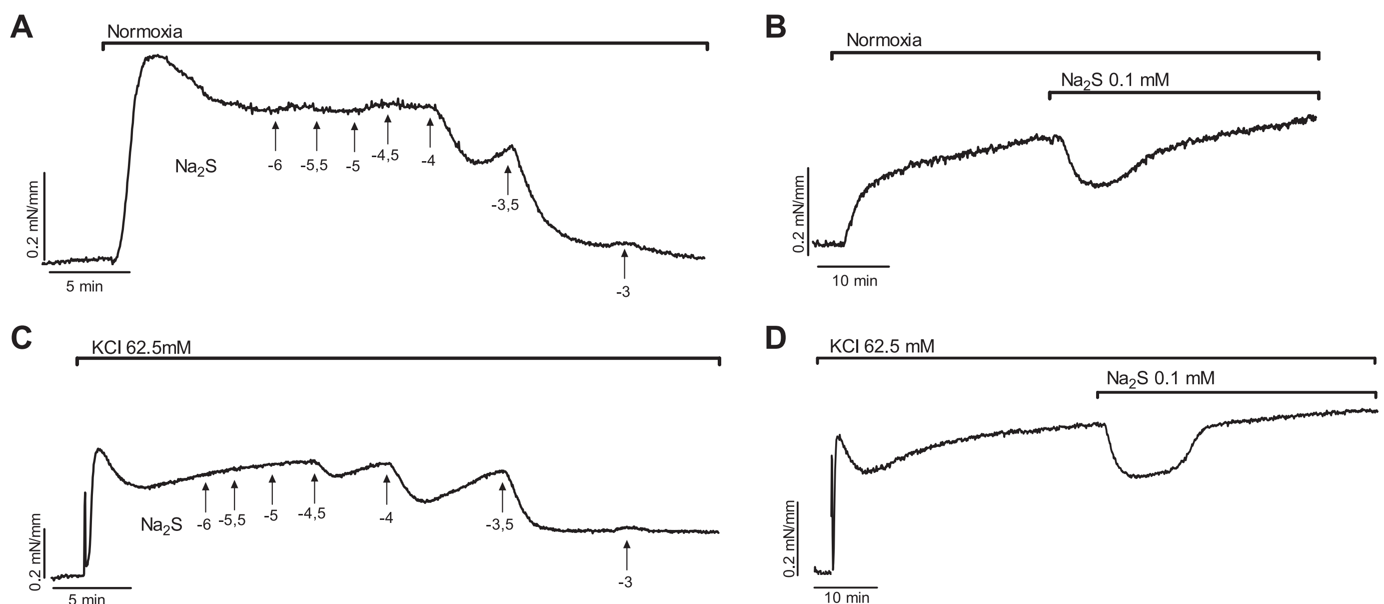

Fig. 1. Relaxant effects of Na_2S in chicken embryo ductus arteriosus (DA). Representative tracing of active wall tension vs. time showing the response of 2 O_2-contracted DA rings (19-day, pulmonary side; *A* and *B*) and two 62.5 mM KCl-contracted DA rings (19-day, pulmonary side; *C* and *D*) to cumulative (*A* and *C*) or single (*B* and *D*) concentrations of Na_2S. Values indicate log M [Na_2S]. KCl-induced contractions were performed under 5% O_2. The deflections observed in the traces *C* and *D* (at the beginning of KCl-induced contractions) correspond to the replacement of the buffer.

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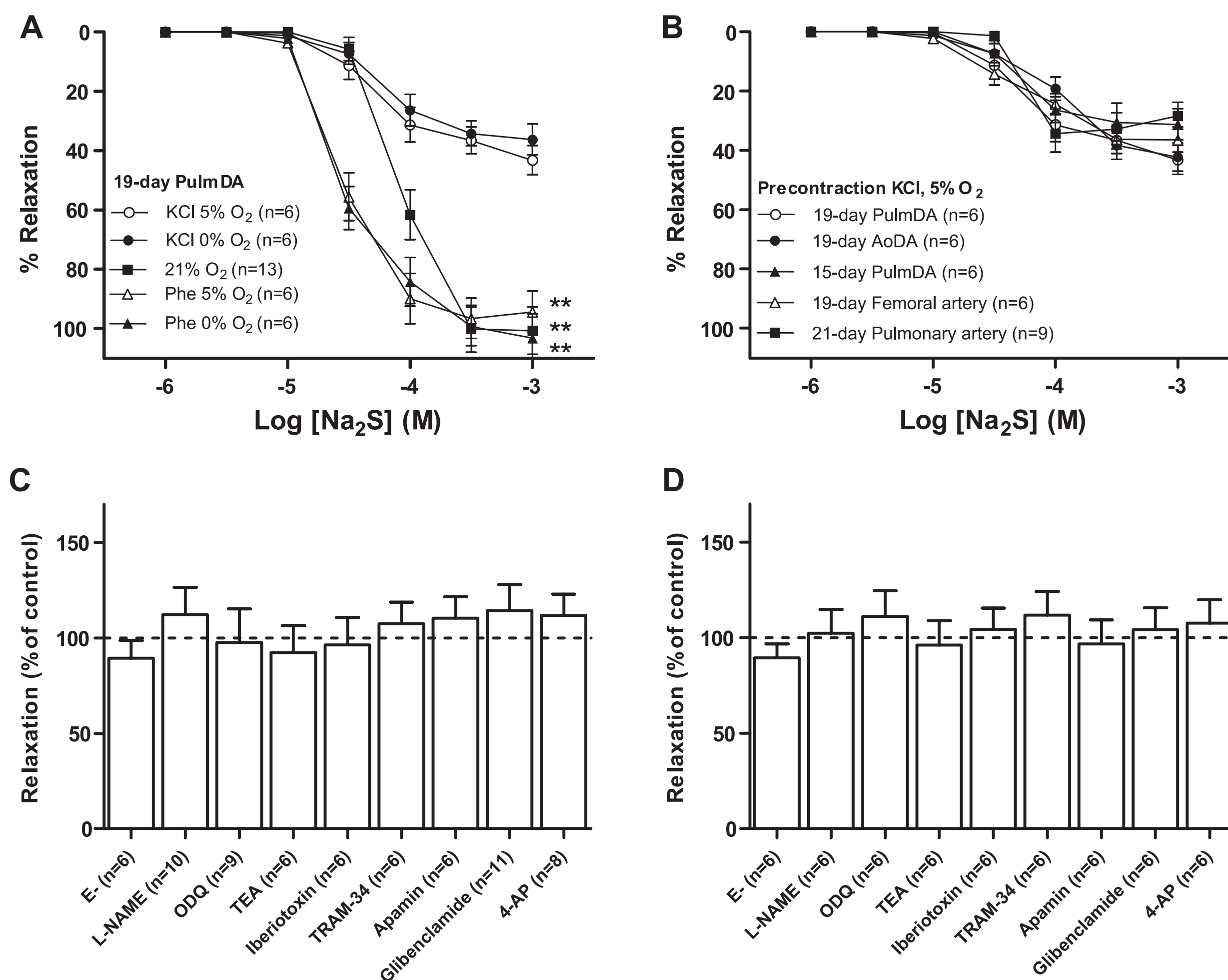

Fig. 2. *A*: mean ± SE cumulative dose-response curves for Na_2S in 21% O_2-, 62.5 mM KCl-, or 1 μM phenylephrine (Phe)-contracted 19-day DA (pulmonary side) rings. KCl and Phe contractions were performed under 0% and 5% O_2. $^{**}P < 0.01$ for difference in E_{max} compared with KCl-contracted. *B*: mean ± SE cumulative dose-response curves for Na_2S in KCl-contracted DA (PulmDA, pulmonary side; AoDA, aortic side), femoral and pulmonary artery rings from 15-day, 19-day, and 21-day chicken embryos. Contractions were performed under 5% O_2. *C* and *D*: maximal relaxation (mean ± SE) evoked by Na_2S (1 μM–1 mM) in O_2-contracted (*C*) and 10 μM Phe-contracted (*D*) PulmDA rings (19-day) after endothelium removal (E-) or in the presence of the nitric oxide (NO) synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 0.1 mM), the soluble guanylate cyclase inhibitor ODQ (10 μM), the nonselective K^+ channel inhibitor tetraethylammonium (TEA; 5 mM), the voltage-gated K^+ channel (K_V) inhibitor 4-aminopyridine (4-AP; 10 mM), the ATP-sensitive K^+ channel (K_{ATP}) inhibitor glibenclamide (10 μM), the large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) inhibitor iberiotoxin (100 nM), the intermediate-conductance K_{Ca} (IK_{Ca}) channel inhibitor TRAM-34 (1 μM), or the small-conductance K_{Ca} (SK_{Ca}) channel inhibitor apamin (50 nM). The experiments involving Phe (*D*) were performed under 0% O_2 (P_{O_2}, ~2.5 kPa). The data are expressed as % response observed in the control (endothelium intact, vehicle-treated) group.

The efficacy (Fig. 2*C*) and the potency of Na_2S to relax O_2-contracted PulmDA rings (19-day) were not affected by endothelium removal (pD_2 3.89, SD 0.22, $n = 6$), or by the presence of the NOS inhibitor L-NAME (pD_2 4.11, SD 0.13, $n = 10$), the sGC inhibitor ODQ (pD_2 3.92, SD 0.17, $n = 8$), the nonselective K^+ channel inhibitor TEA (pD_2 4.07, SD 0.12, $n = 6$), the K_V channel inhibitor 4-AP (pD_2 4.01, SD 0.14, $n = 8$), the K_{ATP} channel inhibitor glibenclamide (pD_2 4.08, SD 0.23, $n = 11$), the BK_{Ca} channel inhibitor iberiotoxin (pD_2 4.14, SD 0.16, $n = 6$), the IK_{Ca} channel inhibitor TRAM-34 (pD_2 4.02, SD 0.18, $n = 6$), or the SK_{Ca} channel inhibitor apamin (pD_2 3.96, SD 0.18, $n = 6$). A significant contraction ($P < 0.05$ vs. the respective vehicle) was observed following the 20- to 30-min incubation with L-NAME (0.101 mN/mm, SD 0.08), ODQ (0.132 mN/mm, SD 0.09), and 4-AP (0.124 mN/mm, SD 0.05), but not following incubation with TEA, iberiotoxin, TRAM-34, or apamin. The subsequent contraction induced by O_2 was significantly reduced (0.246 mN/mm, SD 0.09, $n = 8$, $P < 0.05$ vs. control) when the PulmDA rings were incubated with 4-AP. The other experimental conditions did not affect O_2-induced contraction in PulmDA rings. Finally, the efficacy (Fig. 2*D*) and the potency (data not shown) of Na_2S to relax 19-day PulmDA rings precontracted with phenylephrine (10 μM, in the presence of 0% O_2) were not significantly affected by any of the above-described experimental conditions.

Role of H_2S in oxygen sensing in chicken embryo vessels. To investigate whether endogenous H_2S affected the response to O_2 of chicken DA, PulmDA rings (19-day) were exposed twice to a cycle of hypoxia-normoxia-hypoxia, and the second exposure was elicited in the presence of vehicle or the substrate for endogenous H_2S production L-cysteine, the CSE inhibitor PPG, or the CBS inhibitor AOA. As shown in Fig. 3, *A–D*, L-cysteine, PPG, and AOA did not affect the subsequent

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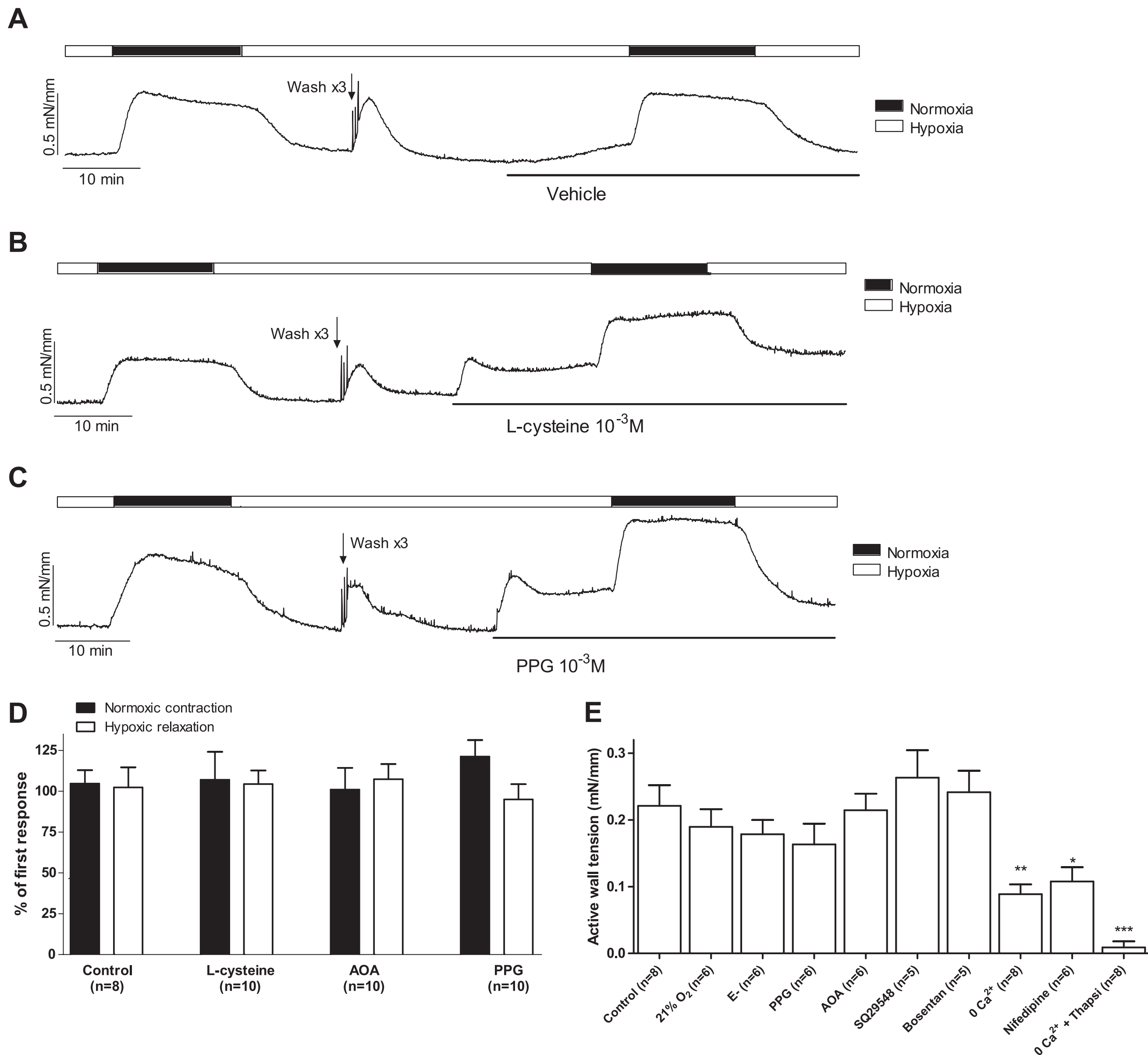

Fig. 3. Lack of role for endogenous H_2S in the response to normoxia/hypoxia of chicken DA. *A–D*: DA rings (19-day, pulmonary side) were exposed twice to a cycle of hypoxia-normoxia-hypoxia and the second exposure was elicited in the presence of vehicle, the substrate for endogenous H_2S production L-cysteine (1 mM), the cystathionine γ-lyase inhibitor inhibitor D,L-propargylglycine (PPG; 1 mM), or the cystathionine β-synthase inhibitor amino-oxyacetate (AOA; 1 mM). Normoxic contraction and hypoxic relaxation in *D* are expressed as % first maximal response in the same vessel. *E*: mean ± SE active wall tension developed by DA rings (19-day, pulmonary side) in response to L-cysteine (1 mM). Except when otherwise stated, all the experiments were performed under 0% O_2. L-Cysteine-induced contraction was not significantly affected by O_2 tension, endothelium removal (E-), or by the presence of PPG, AOA, the TP receptor antagonist SQ29548 (10 μM), or the dual endothelin receptor antagonist bosentan (10 μM). In contrast, when vessels were incubated in a Ca^{2+}-free medium (containing 1 mM EGTA, 0 Ca^{2+}) or with the L-type Ca^{2+} channel blocker nifedipine (10 μM), the contraction evoked by L-cysteine was significantly impaired. The remaining component of the L-cysteine-induced tonic contraction in the absence of Ca^{2+} was almost completely inhibited by pretreatment with the sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (2 μM). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control.

contraction evoked by normoxia or the relaxation evoked by hypoxia. A significant contraction ($P < 0.05$ vs. the respective vehicle) was observed following the incubation with PPG (0.176 mN/mm, SD 0.12) and L-cysteine (0.210 mN/mm, SD 0.07) (see Fig. 4, *A–C*), but not following incubation with AOA.

In a separate group of experiments, we examined the mechanisms involved in L-cysteine-induced contraction of 19-day PulmDA rings. As shown in Fig. 3*E*, L-cysteine-induced contraction was not significantly affected by O_2 tension, endothelium removal, or by the presence of PPG, AOA, the TP receptor antagonist SQ29548, or the dual ET receptor antagonist bosentan. In contrast, when 19-day PulmDA rings were incubated in a Ca^{2+}-free medium (containing 1 mM EGTA) or with the L-type Ca^{2+} channel blocker nifedipine, the contraction evoked by L-cysteine was significantly impaired. The remaining component of the L-cysteine-induced tonic contraction in the absence of Ca^{2+} (0 mM Ca^{2+} + 1 mM EGTA) was almost completely inhibited by pretreatment with thapsigargin, which depletes internal Ca^{2+} stores (44).

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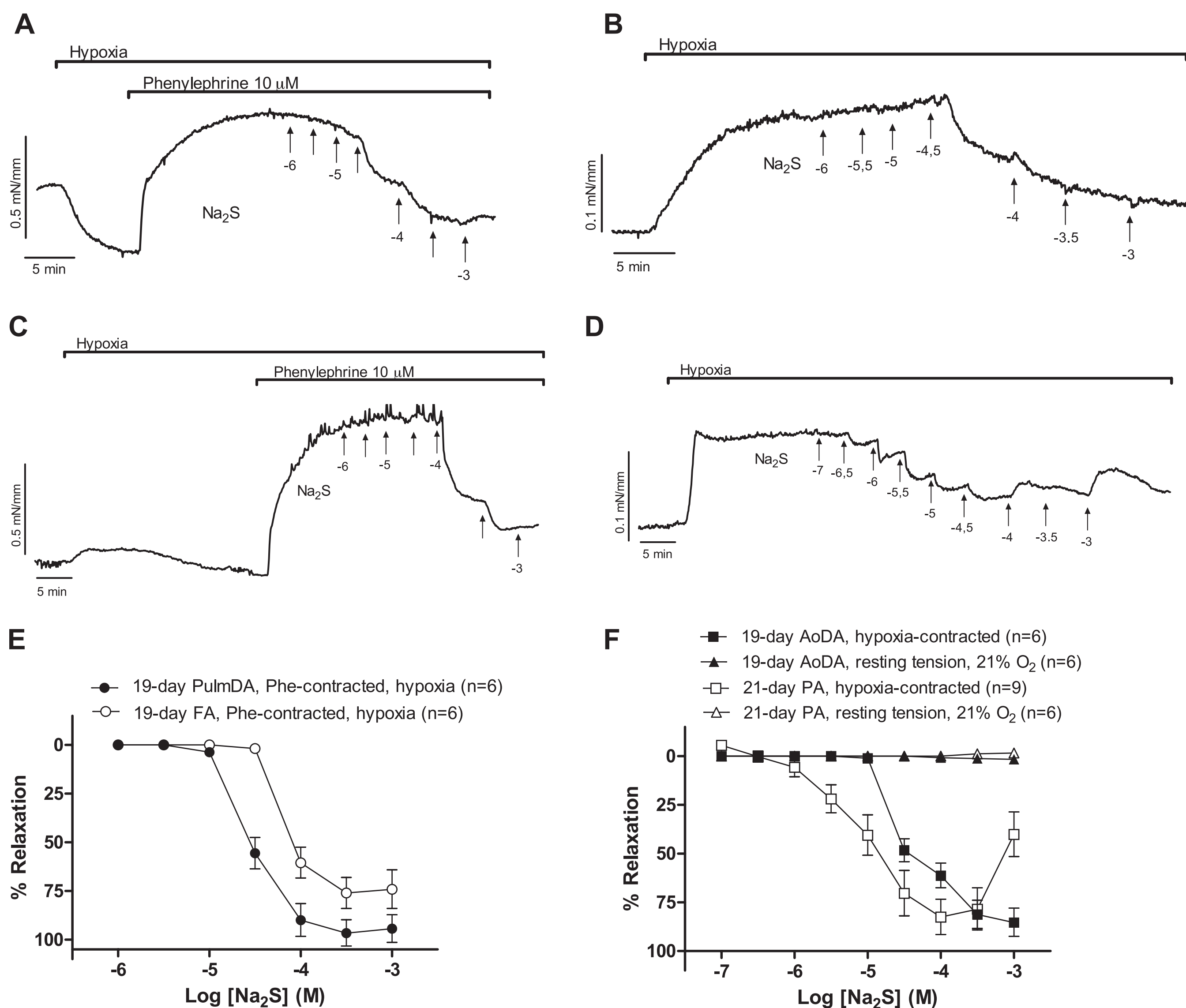

Fig. 4. Relaxant effects of Na_2S in chicken embryo vessels under hypoxic conditions (P_{O_2}, ~2.5 kPa). Hypoxia evoked a relaxation in the pulmonary side (PulmDA; *A*) and a tonic contraction in the aortic side (AoDA; *B*) of the DA and in the pulmonary artery (PA; *D*). In the femoral artery (FA; *C*), hypoxia induced a transient contraction. The PulmDA and the femoral artery rings were subsequently precontracted with phenylephrine (10 μM) to evaluate the response to Na_2S. In the experiments involving AoDA and PA rings, the precontraction was the one evoked by hypoxia itself. *E–F*: mean ± SE cumulative dose-response curves for Na_2S in chicken embryo vessels exposed to the experimental conditions described above. The responses to Na_2S of nonprecontracted (resting tension) AoDA and PA rings (bubbled with 21% O_2) are also depicted (*F*).

Olson et al. (52) reported that H_2S and hypoxia produced the same mechanical response in vessels from at least one species in every vertebrate class and that the effects of H_2S and hypoxia were competitive. As described above, hypoxia and H_2S evoked relaxation in the chicken PulmDA rings. However, this effect did not appear to be competitive since Na_2S relaxed KCl- and phenylephrine-contracted PulmDA rings under hypoxic conditions (Figs. 2*A* and 4, *A* and *E*). To assess whether hypoxia and H_2S evoked similar responses in other chicken embryo vessels, we studied AoDA, femoral, and pulmonary artery rings. Hypoxia evoked a tonic contraction in the AoDA (0.162 mN/mm, SD 0.11, $n = 6$) and the pulmonary artery rings (0.109 mN/mm, SD 0.10, $n = 9$) (Fig. 4), as previously described (2, 20, 73). This hypoxic contraction was relaxed by Na_2S in a concentration-dependent manner (pD_2 AoDA: 4.41, SD 0.32, $n = 8$; pD_2 pulmonary artery: 5.1, SD 0.41, $n = 6$). Interestingly, high concentrations (≥ 0.1 mM) of Na_2S evoked a transient contraction in the hypoxia-contracted pulmonary artery rings (Figs. 4, *D* and *F*). In contrast, in the quiescent (nonprecontracted) pulmonary artery and AoDA rings (exposed to 21% O_2), Na_2S did not elicit significant mechanical effects (Fig. 4*F*). In the quiescent femoral arteries, hypoxia induced a transient contraction (Fig. 4*C*), as previously described (53). The tone of the vessels was subsequently increased with phenylephrine (10 μM) to evaluate the response to Na_2S under hypoxic conditions. Na_2S relaxed the hypoxic, phenylephrine-contracted femoral artery rings in a concentration-dependent manner (pD_2: 4.11, SD 0.36, $n = 6$, Fig. 4*E*).

Response to CO. As shown in Fig. 5, *A* and *D*, CO relaxed O_2-contracted PulmDA rings (19-day) in a dose-dependent

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manner. However, a maximum relaxation was not achieved with the highest concentration of CO tested (0.1 mM), and a pD_2 value could therefore not be calculated. When the PulmDA rings (19-day) were contracted with KCl (62.5 mM in the presence of 5% O_2), CO also evoked a concentration-dependent relaxation (Figs. 5, *B* and *D*), which was significantly lower than the relaxation observed when the vessels were precontracted with 21% O_2. For example, the relaxation evoked by 0.1 mM CO was 61.86% (SD 30.57, $n = 11$) in O_2-contracted PulmDA rings and 18.55% (SD 14.74, $n = 21$) in KCl-contracted vessels ($P < 0.001$ vs. O_2-contracted). CO also relaxed KCl-contracted AoDA (Figs. 5, *C* and *D*) and femoral artery rings from 19-day embryos and PulmDA rings from 15-day embryos with similar efficacy as the one observed in PulmDA rings from 19-day embryos (Fig. 5*D*).

As shown in Fig. 6*A*, the relaxation induced by CO (0.1 mM) was markedly impaired in the presence of the sGC inhibitor ODQ, the nonselective K^+ channel inhibitor TEA or the K_V channel inhibitor 4-AP. Endothelium removal or the presence of the K_{ATP} channel inhibitor glibenclamide, the BK_{Ca} channel inhibitor iberiotoxin, the IK_{Ca} channel inhibitor TRAM-34, or the SK_{Ca} channel inhibitor apamin did not significantly affect CO-induced relaxation (Fig. 6*A*). To investigate whether the inhibition of K_V channels also affected other relaxations mediated through the sGC/cGMP pathway, we analyzed the effects of 4-AP on the relaxation induced by the NO donor SNP in O_2-contracted PulmDA rings. As shown in Fig. 6*B*, the presence of 4-AP significantly impaired the potency (pD_2 5.16, SD 0.21, $n = 7$ vs. 5.58, SD 0.30, $n = 6$; $P < 0.05$) and the efficacy (E_{max} 29.35%, SD 13.78 vs. 67.13%, SD 21.86, $P < 0.01$) of SNP.

In another group of experiments, we analyzed the effects of the HO-inhibitor zinc protoporphyrin (ZnPP IX; 10 μM), and the substrate for endogenous CO production hemin (10 μM) on the response of the PulmDA to normoxia. Incubation for 20–30 min of PulmDA rings (19-day) with ZnPP IX did not affect basal tone or O_2-induced contraction (data not shown). A more prolonged incubation (4 h) was performed with hemin (10 μM). During this prolonged incubation a transient increase in the tone of the PulmDA rings was observed, but this transient increase in wall tension was also observed in the vessels incubated for 4 h in the presence of vehicle (DMSO). In the hemin group the second response to normoxia was 30.61% (SD 37.6, $n = 5$) of the first contraction. However, a similar percentage of the first contraction was observed in the control group (25.22%, SD 22.97, $n = 4$).

Role of endogenous H_2S and CO in the relaxations evoked by ACh and SNP. In this group of experiments, we analyzed the effects of L-cysteine, PPG, AOA, and ZnPP IX on the relaxations evoked by ACh and SNP in O_2-contracted PulmDA rings (19-day). As shown in Fig. 7, no significant effects were observed. In contrast, the NOS inhibitor L-NAME (0.1 mM) impaired ACh-induced relaxation (pD_2 control: 6.95, SD 0.27, $n = 9$; pD_2 L-NAME: 6.47, SD 0.28, $n = 8$; $P < 0.05$, Fig. 7*C*). The presence of ZnPP IX did not produce a further effect on the inhibition elicited by L-NAME (pD_2 ZnPP IX + L-NAME: 6.39, SD 0.27, $n = 8$).

DISCUSSION

Besides NO and CO, H_2S is a third gaseous autocrine/paracrine messenger, which has recently been shown to play

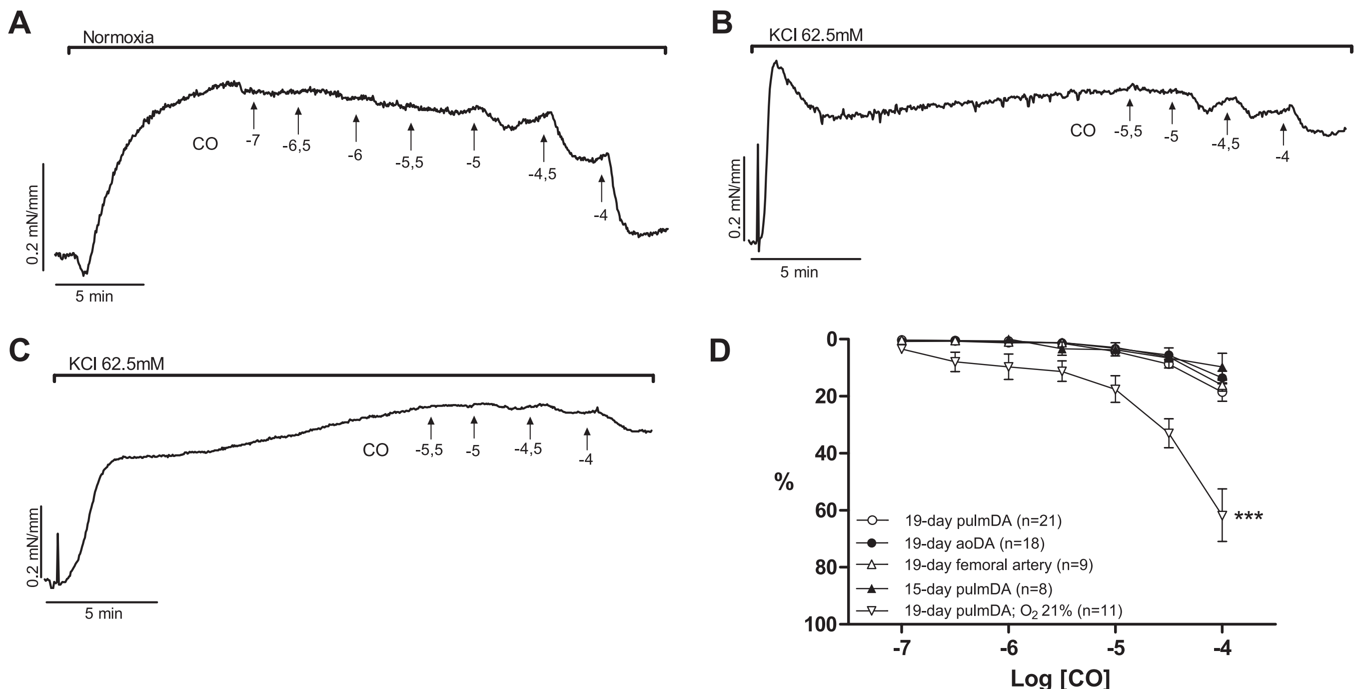

Fig. 5. Relaxant effects of authentic CO in chicken embryo vessels. *A*–*C*: representative tracing of active wall tension vs. time showing the response of an O_2-contracted DA ring (19-day, pulmonary side) (*A*), a 62.5 mM KCl-contracted DA ring (19-day, pulmonary side) (*B*), and a 62.5 mM KCl-contracted DA ring (19-day, aortic side) (*C*) to increasing concentrations of CO. KCl contractions were performed under 5% O_2. Values indicate log M [CO]. *D*: mean ± SE cumulative dose-response curves for CO in O_2- or KCl-contracted DA (PulmDA, pulmonary side; AoDA, aortic side) and femoral artery rings from 15- and 19-day chicken embryos. ***$P < 0.001$ for difference in maximal relaxation when compared with KCl-contracted/19-day/PulmDA.

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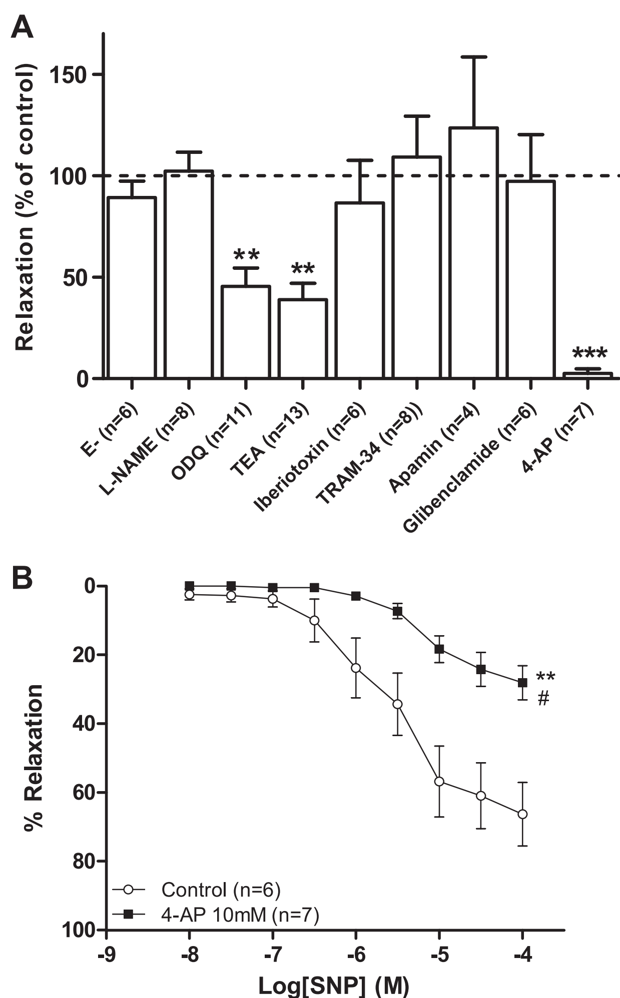

Fig. 6. *A*: maximal relaxation (mean ± SE) evoked by authentic CO (0.1 μM–0.1 mM) in O_2-contracted DA rings (pulmonary side, 19-day) after endothelium removal (E-) or in the presence of the NO synthase inhibitor L-NAME (0.1 mM), the soluble guanylate cyclase inhibitor ODQ (10 μM), the nonselective K^+ channel inhibitor tetraethylammonium (TEA; 5 mM), the voltage-gated K^+ channel (K_V) inhibitor 4-aminopyridine (4-AP; 10 mM), the ATP-sensitive K^+ channel (K_{ATP}) inhibitor glibenclamide (10 μM), the large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) inhibitor iberiotoxin (100 nM), the intermediate-conductance K_{Ca} (IK_{Ca}) channel inhibitor TRAM-34 (1 μM), or the small-conductance K_{Ca} (SK_{Ca}) channel inhibitor apamin (50 nM). The data are expressed as % response observed in the control (endothelium intact, vehicle-treated) group. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control. *B*: mean ± SE cumulative dose-response curves for the NO donor sodium nitroprusside (SNP) in O_2-contracted DA rings (19-day, pulmonary side) in the absence (control) or the presence of 4-AP (10 mM). $^{**}P < 0.01$, for difference (vs. control) in E_{max}; #$P < 0.05$ for difference (vs. control) in pD_2.

important physiological roles (39, 45, 50, 51, 57). The effects of H_2S have been investigated in numerous mammalian and nonmammalian blood vessels but not in the DA. In the present work, using isolated DA rings from chicken embryos, we show that Na_2S, which forms H_2S and HS^- in solution, induces a concentration-dependent relaxation of this vessel. Although we could not elucidate the mechanisms underlying this relaxation, the pathway mainly involved in mammalian vessels (i.e., K_{ATP} channel activation) does not appear to play a role in H_2S-induced relaxation of the chicken DA. On the other hand, exogenous CO relaxed the chicken DA by a pathway that involves sGC and K_V channel activation. The presence of precursors/inhibitors of H_2S and CO synthesis did not significantly affect the response of the chicken DA to normoxia/hypoxia and did not affect endothelium-dependent or -independent relaxation. Therefore, our results indicate that the gasotransmitters H_2S and CO are vasoactive in the chicken DA but they do not suggest an important role for endogenous H_2S or CO in the control of chicken ductal reactivity.

H_2S-induced relaxation of chicken DA. H_2S relaxed chicken DA rings with similar potency (pD_2 ~4) as the one reported, for example, in rat aorta (pD_2 3.86) (69), or rat mesenteric artery (pD_2 3.98) (15). These concentrations of H_2S have been considered as physiologically relevant by numerous investigators but it should be noted that recent studies employing different analytical techniques have not verified micromolar concentrations of H_2S in blood or tissues (see Ref. 50 for review). The exhaustive study of Dombkowski et al. (29) demonstrated that H_2S (in the high-micromolar range) has vasoactive properties in at least one species from each class of vertebrates. Interestingly, they show that H_2S induces either vascular relaxation or contraction and that these disparate responses may occur in different vascular beds within a single species or even in the same vessel at different levels of H_2S exposure (29). When they analyzed the response to H_2S in avian blood vessels (Pekin duck aorta and pulmonary arteries), they observed that H_2S was mainly contractile in either unstimulated or precontracted aorta, whereas in the pulmonary artery, H_2S produced multiphasic responses (29). In contrast, we observed that in chicken embryo vessels H_2S is mainly a relaxing agent. The information about the effects of H_2S in human vascular smooth muscle is limited, but H_2S-induced relaxation has been demonstrated in the internal mammary artery (65) and the corpus cavernosum (22). However, low concentrations of H_2S (50 μM) evoked a contraction in the human mammary artery that appeared to be related to the inactivation of NO (65). As discussed below, our experimental data do not support the presence of an interaction between NO and H_2S in chicken embryo DA.

Na_2S was less efficacious in relaxing chicken DA rings contracted with 62.5 mM K^+ than it was in vessels contracted with O_2 or phenylnephrine, suggesting the involvement of K^+ channels in the relaxation. The currently available data indicate that H_2S relaxes mammalian blood vessels mostly by opening K_{ATP} channels in smooth muscle cells (45). The K_{ATP} channel antagonist glibenclamide attenuated H_2S-induced relaxation in several mammalian and nonmammalian vessels, such as rat thoracic aorta (41, 59, 70), rat mesenteric arteries (15), human internal mammary artery (65), and trout efferent branchial artery (28). In addition, patch-clamp studies have demonstrated that H_2S increases K_{ATP}-dependent current and induces hyperpolarization in mammalian vascular smooth muscle cells (59, 70). However, glibenclamide failed to affect the relaxing effect of H_2S in mouse aorta (37) and rat coronary artery (14). In the latter vessel, H_2S-induced relaxation was impaired by the presence of the K_V channel blocker 4-AP (14). In the chicken DA, neither glibenclamide, nor 4-AP or inhibitors of other major known K^+ channels altered H_2S-induced relaxation. This result is in correspondence with the lack of effect of K^+ channel blockers on H_2S-induced relaxation in mammalian bronchial (36) and gastrointestinal smooth muscles (24, 60) and suggests that the mechanisms underlying H_2S-mediated relaxation are strongly tissue- and species-dependent.

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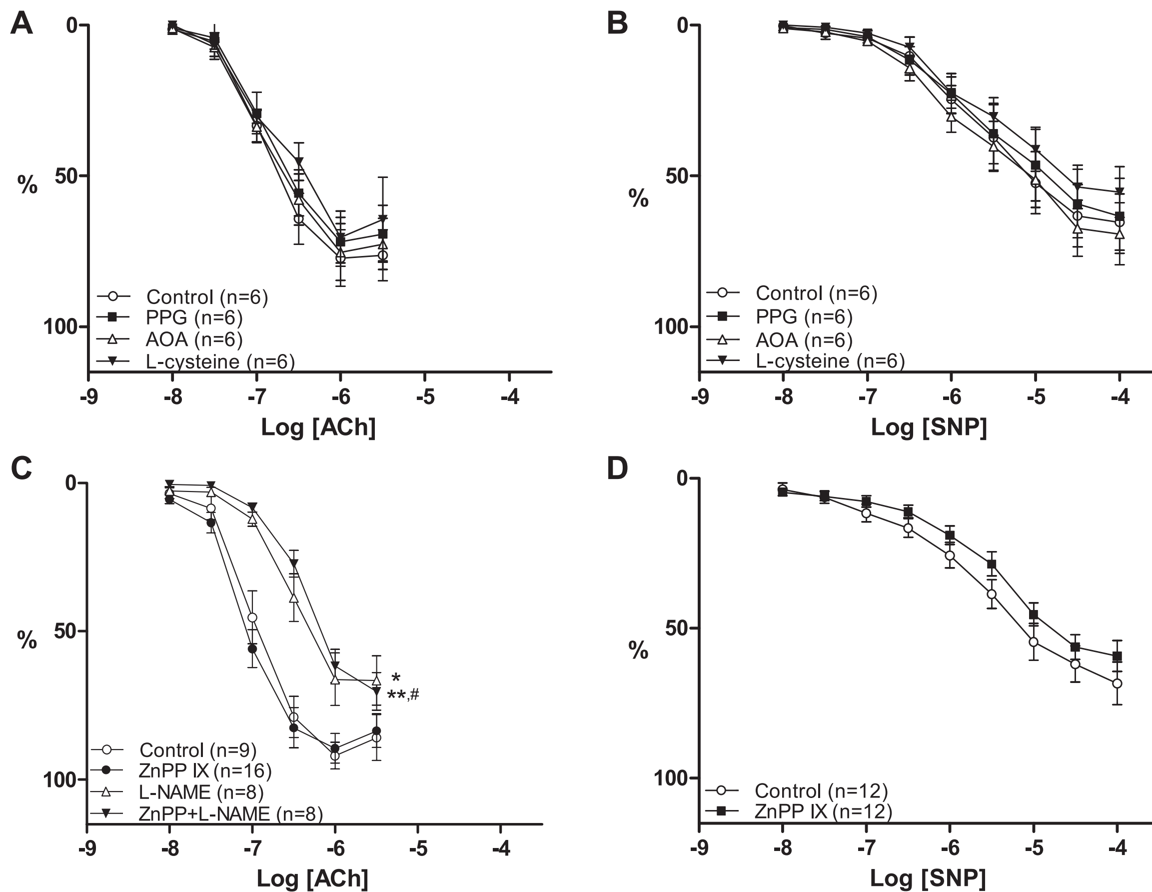

Fig. 7. Mean ± SE cumulative dose-response curves for acetylcholine (ACh; *A* and *C*) and sodium nitroprusside (SNP; *B* and *D*) in O_2-contracted DA rings (19-day, pulmonary side, endothelium intact) in the absence (control) or presence of L-cysteine (1 mM, H_2S substrate) D,L-propargylglycine (PPG; 1 mM, cystathionine γ-lyase inhibitor), amino-oxyacetate (AOA; 1 mM, cystathionine β-synthase inhibitor), zinc protoporphyrin IX (ZnPP IX; 10 μM, heme oxygenase inhibitor), and L-NAME (0.1 mM, NO synthase inhibitor). **P* < 0.05, ***P* 0.01 for difference (vs. control) in pD_2. #*P* < 0.05 for difference (vs. ZnPP IX) in pD_2.

It has been suggested that the reduced efficacy of H_2S to relax smooth muscles precontracted with KCl could be the result of the interference between Cl^- and HS^- (27). HS^-, which at physiological pH accounts for ~80% of the total H_2S + HS^-, may interfere with a Cl^- channel or transporter and this interference would be diminished when transmembrane Cl^- gradients are changed by using high concentrations of KCl (27). However, this could not explain the difference in efficacy we found since, in our experiments, solutions containing different concentrations of K^+ were prepared by replacing NaCl by an equimolar amount of KCl. Therefore, the transmembrane Cl^- gradient was not changed and could not play a role in H_2S induced relaxation.

A crosstalk between H_2S and NO has been suggested in some vascular tissues, but the mechanisms are still not clear (64). H_2S has been shown to either enhance (69) or attenuate (35) the relaxant effect of NO in the rat aorta. It has been reported that H_2S may impair NO-induced relaxation through the inhibition of endothelial NOS (eNOS) (37), the scavenging of NO with the formation of a nitrosothiol compound (66), the decrease in the sensitivity of the cGMP pathway to NO (64), or the modification of K_{Ca} channels to decrease their sensitivity to NO (64). On the other hand, NO may increase the expression of CSE or the cellular uptake of cysteine (64). Our present results in the chicken embryo DA do not suggest an interaction between H_2S and NO in this vessel, since inhibition of NOS and sGC did not affect H_2S-induced relaxation. Furthermore, the presence of inhibitors of H_2S synthesis or the H_2S precursor L-cysteine did not affect the relaxation evoked by ACh or SNP.

Unlike NO or CO, specific receptors for H_2S have not been identified, and putative molecular targets appear to be both tissue and species dependent (42). Therefore, other possible mechanisms involved in the smooth muscle effects of H_2S, such as changes in intracellular pH (38), production of reactive oxygen species (48), downregulation of cAMP (41), activation of adenylate cyclase (48), inhibition of phosphodiesterase activity (12), activation of phospholipase A_2 (25), activation of myosin light chain phosphatase (23), or sulfhydration of actin (47) remain to be explored in chicken embryo vessels.

H_2S as O_2 sensor in the chicken DA. Olson et al. (49) recently proposed that H_2S is an O_2 sensor in vascular smooth muscle. This hypothesis is based on their observations that *1*) H_2S and hypoxia produce the same mechanical response in vessels from at least one species in every vertebrate class; *2*) the effects of H_2S and hypoxia are competitive; and *3*) blood vessels enzymatically generate H_2S and inhibitors of H_2S synthesis inhibit hypoxic responses, whereas the H_2S precursor

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L-cysteine augments it. The DA is exquisitely sensitive to O_2 (56), but the role of H_2S in its O_2 responsiveness had not yet been investigated. The present study was performed in the chicken DA, a vessel that is the result of the fusion of two vessels with different embryological origin, morphology, and functionality. The PulmDA consists almost exclusively of neural crest-derived cells, shows the structure of a muscular artery, and responds to O_2 with contraction (and to hypoxia with relaxation), whereas the AoDA is of mesodermal origin, shows the morphology of an elastic artery, relaxes in response to normoxia, and contracts in response to hypoxia (2, 9, 10, 20, 34, 61). Previous studies have shown the involvement of the mitochondrial electron transport chain as sensor, H_2O_2 as mediator, and K_V channels and Rho kinase as effectors of O_2-induced contraction in the chicken PulmDA (20, 34). This indicates the presence of a common mechanism for O_2 sensing/signaling in mammalian and nonmammalian DA (56). The O_2-induced relaxation of the AoDA, on the contrary, appears to be dependent on the NO/cGMP pathway mediated by endothelial cells (34).

In agreement with the results of Olson et al. (49, 52), we observed that H_2S and hypoxia produce the same mechanical response (i.e., relaxation) in the PulmDA. However, the response to H_2S and hypoxia was not competitive because PulmDA rings, when precontracted with KCl or phenylephrine under hypoxic conditions, still show Na_2S-evoked relaxation. Moreover, Na_2S was also a relaxing agent in the immature 15-day PulmDA, which is not sensitive to the effects of normoxia/hypoxia (2, 20, 33). In addition, neither hypoxic relaxation nor normoxic contraction of the 19-day PulmDA was affected by the presence of precursors/inhibitors of H_2S synthesis. It should be noted that the enzyme inhibitors used (AOA and PPG) lack specificity and are often poorly absorbed by intact tissues (57). What is more, to the best of our knowledge, there are no data available on the inhibitory effects of AOA and PPG in chicken tissues, and the doses that we used were based on studies in mammalian tissues. Despite these limitations, the present study suggests that H_2S is not involved in O_2 sensing/signal transduction in the PulmDA.

In an effort to clarify the possible role of H_2S in O_2 sensing in chicken embryo vasculature, we decided to further investigate the effects of Na_2S in two vessels, the AoDA and the pulmonary artery, which respond to hypoxia with contraction, and one vessel, the femoral artery, which (when precontracted) responds to hypoxia with relaxation (2, 20, 53, 72). We observed that in these vessels, under hypoxic conditions, Na_2S evoked a concentration-dependent relaxation (Fig. 4). Moreover, while hypoxia induced a tonic contraction in quiescent (nonprecontracted) AoDA and pulmonary artery rings, Na_2S did not elicit significant mechanical effects. Therefore, the ability of exogenous H_2S to mimic hypoxic responses was not found in the chicken embryo vessels that respond to hypoxia with contraction. The only partial exception to this pattern was the hypoxia-contracted pulmonary artery, which, although relaxed in response to low concentrations of Na_2S, was contracted by higher (>0.1 mM) concentrations. This observation is in agreement with that by Olson et al. (52) who demonstrated that H_2S evoked two dose-dependent effects in bovine pulmonary arteries. In these vessels, H_2S produced a dose-dependent relaxation between 10 nM and 10 μM, whereas concentrations > 10 μM produced a dose-dependent contraction. They hypothesize that much of the H_2S produced by the pulmonary vascular smooth muscle cells during normoxia will be oxidized and the resultant low H_2S concentration dilates the vessels and reduces vascular resistance. In contrast, during hypoxia, the concentration of H_2S will increase and result in vasoconstriction. However, this leads back to the question of whether the pulmonary vascular smooth muscle cells are able to produce the large amount of H_2S that may be required to induce vasoconstriction. Nevertheless, as Olson (51) has recently stressed, to date no study has identified any stimulus for H_2S production in cells in real time and under physiological conditions, other than showing an inverse relationship between H_2S production and P_{O_2}. The demonstration of this relationship in chicken embryo vessels warrants further investigation.

L-Cysteine, the precursor of H_2S, evoked a tonic contraction in the 19-day PulmDA rings. This contraction was endothelium-independent and was significantly inhibited either by omission of extracellular Ca^{2+} or by inclusion of the L-type Ca^{2+} channel blocker nifedipine. The remaining component of the L-cysteine-induced tonic contraction in the absence of Ca^{2+} was almost completely inhibited by pretreatment with thapsigargin, which depletes internal Ca^{2+} stores (44). Taken together, these findings suggest that L-cysteine elicits contraction of PulmDA rings by promoting Ca^{2+} influx via nifedipine-sensitive Ca^{2+} channels and intracellular Ca^{2+} release from the sarcoendoplasmic reticulum. Our results are consistent with the data obtained in both smooth and striated muscle, which indicate that L-cysteine is an important modulator of Ca^{2+} homeostasis (13, 21, 54). We also observed that neither the CSE inhibitor PPG nor the CBS inhibitor AOA significantly affected L-cysteine-induced contraction of PulmDA rings. This, together with the opposite effects of H_2S (relaxation) and L-cysteine (contraction) in this preparation, suggests that the contractile effect of L-cysteine was not related to H_2S production. In contrast to our findings, several studies in mammalian vessels demonstrated H_2S-mediated relaxation to be elicited by L-cysteine (5, 15).

CO-induced relaxation of chicken DA. The endogenous production of CO occurs through the activity of HOs, enzymes that catalyze the degradation of heme to CO, iron, and biliverdin. Three isoforms of HO have been identified: the highly inducible HO-1 and the constitutively present HO-2 and HO-3 (11). HO-3 is not enzymatically active in heme degradation, although it may function as a heme-sensing or -binding protein (11). CO participates in the regulation of vascular tone, smooth muscle proliferation and platelet aggregation. These effects of CO are mediated via multiple pathways, including activation of sGC, activation of K^+ channels, and interference with the cytochrome P450 (CYP450)-based mono-oxygenase reaction, limiting the synthesis of ET-1 (11, 18, 67). CO-induced relaxation has been described in pulmonary and systemic vessels as well as in the DA from several mammalian species (1, 7, 16–19, 32, 62). Dombkowski et al. (30) demonstrated that the CO-releasing molecule CORM-3 relaxed sea lamprey and rainbow trout systemic arteries, suggesting that CO vasoactivity has been well conserved throughout vertebrate evolution. To our knowledge, the effects of exogenous CO in avian vessels had not been analyzed before. In the present work we show that the chicken embryo DA and femoral arteries are relaxed by exogenous CO. The relaxant potency and efficacy

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of CO in these chicken vessels were comparable to the ones reported in mammalian vessels (1, 62).

The response of the mammalian DA to exogenous CO has been studied in the guinea pig and in the lamb (16–19, 32). In 1971, Fay (32) reported CO-induced relaxation of O_2-contracted guinea pig DA rings. This finding was not confirmed by Coceani et al. (17) but they demonstrated CO-induced relaxation as well as HO-1 and HO-2 expression in endothelial and smooth muscle cells of the lamb DA (17, 18). Interestingly, they showed that CO-induced relaxation in the lamb DA is not mediated through a significant stimulation of sGC but through inhibition of the functional complex CYP450/ET-1 (18). In contrast, we observed that in the chicken DA CO-induced relaxation involves sGC and K_V channel stimulation.

The sGC/cGMP system is commonly regarded as main messenger for CO in smooth muscle cells (16). Accordingly, we observed that CO-induced relaxation in the chicken DA was markedly impaired in the presence of the sGC inhibitor ODQ. However, as mentioned above, CO can induce relaxation by acting on different targets including K^+ channels (8, 68). The dependence of CO-induced relaxation of chicken DA on transmembrane K^+ gradient suggests that K^+ efflux through K^+ channels is involved in the response. Furthermore, 4-AP, which blocks K_V channels, significantly inhibited CO-induced relaxation. Electrophysiological and pharmacomechanical studies in a variety of mammalian vascular tissues have pointed to a pivotal role of K_V channels in sGC/cGMP-mediated relaxation (58, 71). To analyze whether in the chicken DA the involvement of K_V channels was a particularity of CO-induced relaxation, we tested the effects of the NO donor SNP in the presence of 4-AP. Previously, we demonstrated that SNP induced a sGC-dependent relaxation of chicken DA (4). Herein, we observed that 4-AP inhibited the relaxation evoked by SNP. This suggests that activation of K_v channels occurs downstream from sGC activation. Therefore, a plausible mechanism for CO-induced relaxation in the chicken DA would be sGC/cGMP-mediated activation of K_v channels rather than direct CO-mediated channel regulation.

In the mouse DA, inhibition of HO with ZnPP induces a contraction that is curtailed in eNOS-deleted fetuses, indicating a crucial role for NO in CO-mediated ductal tone (7). Moreover, the endothelium-dependent relaxation evoked by bradykinin in the mouse DA was suppressed by either L-NAME or ZnPP. This has also been found in the lamb DA (19) and suggests that, in these vessels, NO and CO act sequentially in mediating bradykinin-induced relaxation. In the chicken DA, bradykinin is not vasoactive (4) but ACh evokes an endothelium-dependent relaxation that is partially impaired by L-NAME (4). We observed that incubation with ZnPP did not induce a significant contraction of the chicken DA and did not affect ACh-induced relaxation either in the absence or presence of L-NAME. Accordingly, we previously demonstrated the lack of effect of ZnPP in ACh-induced relaxation of chicken embryo pulmonary arteries, suggesting an absent role for endogenous production of CO in chicken embryo vessels (63). However, HO is expressed in the chicken (31, 55) and Leo et al. (40) demonstrated a partial role for endogenous CO in the endothelium-derived relaxation evoked by ACh in carotid arteries from 6- to 8-wk-old chicken. This indicates the possibility of developmental and/or tissue-specific differences in the role of CO in the chicken vasculature.

Perspectives and Significance

Of the many gases with biological roles, O_2 is the most relevant with regard to the physiological regulation of the DA, but others, such as reactive O_2 species, CO_2, NO, and CO are also signaling molecules in this vessel. The present study shows, for the first time, the vasoactive properties of H_2S in the chicken DA. There are still numerous questions remaining before H_2S can be accepted as a biologically relevant signaling molecule (42, 51) and, therefore, our findings warrant further confirmation in the DA from other species. Blood vessels belong to a specialized homeostatic O_2-sensing system, which responds rapidly to moderate changes in O_2 tension (49). This has been extensively studied in mammals where hypoxia contracts pulmonary vessels, relaxes systemic vessels, and relaxes the DA, whereas normoxia evokes the opposite effect. The search for an evolutionary-conserved, ubiquitous O_2-sensing mechanism represents an important and exciting area of research but the identity of the O_2 sensor(s) is still elusive. Hypoxic constriction of pulmonary (73) and chorioallantoic (43) arteries and the aortic side of the DA (2, 9, 20, 34, 61), as well as hypoxic relaxation of femoral arteries (53, 72) and the pulmonary side of the DA (2, 9, 20, 34, 61) are present in the chicken embryo vasculature that represents an interesting model to challenge the putative ubiquity of such vascular O_2 sensing/signaling mediators like H_2S. Finally, as the response to H_2S is strongly tissue- and species-dependent (29); future studies, comparing vessels from embryonic/fetal and adult animals, will elucidate whether the response to the gasotransmitter is also age-dependent.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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