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Carbon monoxide formation in the ductus arteriosus in the lamb: implications for the regulation of muscle tone

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1 We have previously shown that carbon monoxide (CO) potently relaxes the lamb ductus arteriosus and have ascribed this response to inhibition of a cytochrome P450-based mono-oxygenase reaction controlling the formation of endothelin-1 (ET-1). In the present study, we have examined whether CO is formed naturally in the vessel.

2 The CO-forming enzyme, haem oxygenase (HO), was identified in ductal tissue in its constitutive (HO-2) and inducible (HO-1) isoforms by Western immunoblotting and immunological staining procedures (both light and electron microscopy). HO-1 was localized to endothelial and muscle cells, while HO-2 was found only in muscle cells. Inside the muscle cells, HO-1 and HO-2 immunoreactivity was limited to the perinuclear region, and the Golgi apparatus in particular. However, upon exposure to endotoxin, HO-1 became more abundant, and both HO isoforms migrated towards the outer region of the cytoplasm close to the sarcolemma.

3 CO was formed enzymatically from added substrate (hemin, 50 μM) in the 10,000 *g* supernatant of the ductus and its formation was inhibited by zinc protoporphyrin IX (ZnPP, 200 μM).

4 ZnPP (10 μM) had no effect on the tone of the ductus under normal conditions (2.5 to 95% O_2), but it contracted the endotoxin-treated ductus (at 2.5% O_2). At the same concentration, ZnPP also tended to contract the hypoxic vessel (zero O_2).

5 ZnPP (10 μM) curtailed the relaxant response of the oxygen (30%)/indomethacin (2.8 μM)-contracted ductus to bradykinin (35 nM), while it left the sodium nitroprusside (35 nM) relaxation unchanged.

6 We conclude that CO is formed in the ductus and may exert a relaxing influence when its synthesis is upregulated by an appropriate stimulus.

Keywords: Ductus arteriosus; heme oxygenase; carbon monoxide; nitric oxide; cytochrome P450; endothelin-1; guanylyl cyclase/cyclic GMP; endotoxin; bradykinin

Introduction

Although it has been known for some time that carbon monoxide (CO) is formed in the body under normal conditions, only recently has its potential as signalling agent been recognized (Marks *et al*., 1991). Two facts may be regarded as critical for the development of this concept: the identification of endothelium-derived relaxing factor (EDRF) as nitric oxide (NO) with the attendant realization that gaseous agents constitute a new class of biological messengers (Moncada *et al*., 1991) and a better knowledge of the functional organization of the CO-forming enzyme, haem oxygenase (HO) (Maines, 1992). HO exists in two forms, constitutive (HO-2) and inducible (HO-1), which are encoded by distinct genes and are amenable to differential induction (Maines, 1992). While HO-2 may be activated by natural compounds, such as acetylcholine, which are also effective on NO synthase (Zakhary *et al*., 1996), HO-1 is upregulated by noxious stimuli including hyperthermia (Ewing *et al*., 1994), hypoxia (Morita & Kourembanas, 1995), and pyrogens (Maines, 1992). The two isozymes are widely distributed (Maines, 1992) and, consistent with a role of CO in vasoregulation, they are also present in vascular tissue (Ewing *et al*., 1994; Zakhary *et al*., 1996). Additional support for the latter mechanism comes from the demonstration that vascular HO is catalytically active (Cook *et al*., 1995; Grundemar *et al*., 1995); that blood vessels are dilated, albeit unevenly (Utz & Ullrich, 1991; Brian *et al*., 1994), by CO (Coburn *et al*., 1979; Coceani *et al*., 1984; 1988; McGrath & Smith, 1984; Vedernikov *et al*., 1989; Adeagbo *et al*., 1990; Gräser *et al*., 1990; Furchgott & Jothianandan, 1991) and that HO inhibitors exert a pressor effect *in vivo* (Johnson *et al*., 1995).

In previous investigations, we have shown that CO potently relaxes the ductus arteriosus and have attributed this response to inhibition of a cytochrome P450-based mono-oxygenase reaction conditioning the formation of the constrictor endothelin-1 (ET-1) (Coceani *et al*., 1988; 1996a). In view of the new role being assigned to CO, our present aim was to ascertain if this agent qualifies as a regulator of muscle tone in the ductus. For this purpose, we examined whether HO isozymes are present in the tissue and, if so, whether they are catalytically active and able to generate CO in an effective concentration. Western immunoblotting and immunocytochemistry were used to detect the enzyme proteins, while CO was measured by a gas chromatographic method (Vreman & Stevenson, 1988). Concomitantly, we studied the effect on ductal tone of substances which either promote or inhibit the HO reaction. Selected experiments were also performed in other blood vessels, both in the foetus and the adult, to verify any specificity of findings in the ductus arteriosus.

Methods

General procedure

Experiments were performed on near-term pregnant sheep (133–139 days gestation; term, 145 days) of Southdown or Dorset crossbreed. The procedures for anaesthesia, Caesarean delivery of the foetuses, and isolation of the ductus arteriosus

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with the adjacent large vessels have been described previously (Coceani *et al*., 1986). The ductus arteriosus, the main pulmonary artery with part of its branches, and a segment from the descending aorta (thoracic tract) of the foetus plus a portion of the thoracic aorta from the mother were collected in ice-cold Krebs solution gassed with 5% CO_2 in N_2 and were freed of loose connective tissue. Vessels were always used fresh, the only exception being few specimens for the HO assay and Western blot analysis (see below) which were used after storage at $-70°C$.

Morphological analysis

For immunofluorescence microscopy, freshly dissected blood vessels were processed immediately or following incubation (2 h; 37°C) in Krebs medium containing endotoxin (100 ng ml^{-1}). The medium was gassed with 2.5% O_2 : 5% CO_2 in N_2, and incubation without endotoxin served as a reference. Vessels were flash frozen in OCT (Miles Diagnostics) by use of liquid nitrogen and were then cut in a cryostat. In all cases, the plane of cutting was perpendicular to the main axis of the vessel. Cryostat sections were fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer (PBS, pH 7.2). Before the labelling, the fixative was removed by thorough rinsing with PBS (four times, 5 min each), and any residual aldehyde was neutralized by treatment with 0.15% glycine-0.5% bovine serum albumin (BSA) in PBS. After an additional rinsing in PBS, tissues were incubated (1 h; room temperature) with antiserum against HO-1 (dilution, 1 : 200) or HO-2 (dilution, 1 : 100) with BSA-supplemented PBS as a vehicle. The antiserum was subsequently washed off with PBS, and another incubation was carried out in the dark (1 h; room temperature) with a secondary antibody (goat antirabbit IgG) conjugated to FITC (dilution, 1 : 50). Controls included the use of an irrelevant antiserum (goat antimouse IgG), preabsorption of the primary antiserum with enzyme protein, or omission of the primary antiserum. In addition, as a positive control for the visualization of muscle, certain specimens were treated with an antiserum against α-actin (dilution, 1 : 600). Sections were then examined with a Reichert Jung Polyvar epifluorescence microscope or a Leica TCDS confocal microscope, and representative images were saved.

For transmission immunoelectron microscopy, blood vessels, whether untreated or endotoxin-treated, were fixed in 2% paraformaldehyde – 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). The fixed specimens were then cut in 1-mm^3 blocks and kept in fixative for 2 h. Afterwards, they were stored in PBS containing sodium azide (20 mM) and, before being cut, they were infused with 2.3 M sucrose overnight. Tissue blocks were mounted on aluminium pins and plunge frozen in liquid nitrogen. Cryosections ($-100°C$) of approximately 100-nm thickness were prepared with glass knives in a Reichert Jung Ultracut E microtome equipped with a FC4D cryochamber. These ultrathin sections were transferred to glow-discharged carbon-Formvar nickel grids, by use of a loop of molten sucrose. After any residual aldehyde had been neutralized by treatment with 0.15% glycine – 0.5% BSA in PBS, sections were washed repeatedly with BSA-supplemented PBS (0.5%) and were then incubated with antiserum against either HO-1 (dilution 1 : 200) or HO-2 (dilution, 1 : 100). The antiserum was subsequently removed by repeated rinsing with BSA-supplemented PBS, and specimens were treated for 60 min with gold-labelled goat antirabbit immunoglobulin (dilution, 1 : 20). After another thorough wash with PBS and distilled water in succession, the sections were embedded in a thin film of methylcellulose containing 0.2% uranyl acetate and then were viewed and photographed in a JEOL model 1200 Ex II transmission electron microscope. Specificity of binding was confirmed in the same way as in the immunofluorescence study (see above). After an initial screening, a fixed number of cells (between 25 and 29) were chosen in every specimen for particle quantification. Furthermore, in each of these cells, ten fields of equal magnification (25,000 ×) were selected at random within immunoreactive areas (i.e. perinuclear and peripheral cytoplasm; see Results) and photographed. Negative images were digitized, and the number of particles in the whole cell as well as in discrete regions of each cell was assessed by use of a morphometric programme (NIH Image 1.53).

Pharmacological analysis

Only the ductus arteriosus was used in these experiments. As previously shown (Coceani *et al*., 1986), the vessel was opened and then cut perpendicularly to the main axis to yield two, or even three, strips, depending on its length. Ductal strips were prepared intact or were trimmed down to the medial layer, taking advantage of natural cleavage planes to remove both intima and adventitia (Coceani *et al*., 1986). The resulting preparation, whole or trimmed, was mounted in a 10 ml bath between a stationary glass rod and an isometric tension transducer (Grass FT-03C) coupled to a Grass polygraph. The initial load was applied in a single step (intact ductus, 1.5–1.8 g; 1 g weight = 9.8 mN) or a series of steps (ductal muscle alone, 0.7–0.9 g), and preparations were stretched by about 50% of the original length to obtain optimal tension output (Somlyo & Somlyo, 1964). Throughout this procedure care was taken not to damage the endothelium in preparations of the whole ductus.

Tissues were equilibrated in Krebs solution gassed with either 5% CO_2 in N_2 or 2.5% O_2 : 5% CO_2 in N_2, and the same gas mixture or mixtures containing a higher oxygen content (15, 30, and 95%) were employed in the actual experiment. The partial pressure of O_2 (PO_2) was measured with an Instrumentation Laboratory gas analyser (mod. 1301) and was 7–13, 14–23, 77–84, 188–211, and 635–661 mmHg (pH 7.4 in all cases) when gas mixtures with 0, 2.5, 15, 30 and 95% O_2, respectively, were used. Both fluid reservoir and organ bath were continuously bubbled with the required gas mixture. The perfusion rate was approximately 2 ml min^{-1} and temperature was set at 37°C. The room was kept darkened throughout the whole study.

During experiments, the Krebs solution was equilibrated with the 2.5% O_2 mixture to mimic the condition *in utero*, while higher oxygen concentrations (15, 30, and 95% O_2) duplicated the neonatal condition. The latter concentrations, exceeding the normal range for the neonate, were selected as the ductus is thick and, consequently, there is a steep oxygen gradient across its wall (Fay, 1973). Several procedures were used to enhance HO activity in the vessel. Acetylcholine, which has been recently described as an activator of vascular HO-2 (Zakhary *et al*., 1996), does not dilate the ductus (Bodach *et al*., 1980) and, hence, is not suitable. In its place we tested bradykinin, even though its dilator action has been shown to be in the ductus curtailed by N^G-nitro-L-arginine (Coceani *et al*., 1994) which would argue against a significant role of HO-2. To prove that the vessel is responsive to bradykinin without inducing tachyphylaxis, the compound was tested only once at an appropriate concentration (i.e. 35 nM, see Coceani *et al*., 1994) under control conditions before proceeding to a full dose-range (i.e. 10 pM to 1 μM) in the presence of ZnPP (10 μM). Hypoxia (i.e. exposure to the zero-oxygen mixture) and treatment with endotoxin (100 ng ml^{-1}) were chosen instead as a means to upregulate HO-1. The zero-oxygen mixture was used based on the assumption that oxygen in the bathing fluid (see above), though low enough to induce HO-1, does not become rate-limiting for the HO reaction. Endotoxin was added to the perfusion fluid in two periods, separated by a 10-min interval during which normal Krebs was circulated through the bath. The duration of each treatment was the same (1 h) in one group of experiments (ZnPP 10 μM tested after the second endotoxin application), but was different (1 and 1–4 h, respectively) in the other (ZnPP 10 μM tested during the second endotoxin application). Only once was endotoxin used a single time or was used more than twice (i.e. four successive applications), and in both cases ZnPP was tested after the

treatment. Control tests in which endotoxin was measured in the bathing fluid by the Limulus assay confirmed the absence of any loss through incubation (unpublished data). ZnPP (10 μM) was tested three successive times during hypoxia (60- to 106-min applications at a 60- to 70-min interval). In certain experiments, the Krebs solution was supplemented with the HO substrate, haemin (10 μg ml^{-1}), with the idea of promoting CO formation regardless of the isozyme involved.

Analytical methods

Measurement of vascular HO activity Enzyme activity was determined by measuring the CO generated in the course of the NADPH-dependent degradation of heme (Cook *et al*., 1995). For this purpose, fresh vessels were minced with a razor blade, while frozen vessels were ground to a powder. In either case, tissues were homogenized (Polytron; 3×30 s) in 10 (ductus arteriosus) or 5–10 (other blood vessels) vol of 0.1 M potassium phosphate buffer (pH 7.4). The resulting homogenate was centrifuged for 10 min at 600 *g* to collect the lipids into a floating cap which was discarded, and then was centrifuged again for 15 min at 10,000 *g*. The whole procedure was carried out at 4°C, and the supernatant was separated and stored at −70°C until the assay (normally <2 weeks and exceptionally for a longer time; max. 6 weeks). Aliquots of the supernatant, differing in volume depending on whether they originated from an intact vessel (0.35 ml, 2 mg protein ml^{-1}) or the medial layer of the ductus (0.4–0.5 ml, 0.6–1.2 mg protein ml^{-1}), were transferred to amber vials (Chromatographic Specialties). They were then mixed with 0.1 ml of a haemin-albumin (methaemalbumin) complex (0.25 : 0.025 mM; for preparation, see Cook *et al*., 1995) plus 0.1 ml of 5 mM NADPH, and the final volume was adjusted to 1 ml with phosphate buffer. The whole preparation was carried out on ice, and vials were then sealed with screw caps fitted with Teflon/silicon septa. The samples were preincubated in a shaking water bath set at 37°C for 5 min; the headspace was purged with CO-free air; and the incubation was continued for another 15 min. The reaction was stopped by placing the tubes on solid carbon dioxide, where they remained for 15–60 min until the headspace gas was analysed. Incubations were carried out in duplicate and were run in parallel with incubations in the presence of ZnPP (0.2 mM) (duplicate), in the absence of NADPH (single), and in the absence of NADPH but with ZnPP added (single). Only exceptionally was the tissue not incubated with ZnPP. CO in the headspace was quantitated by gas chromatography, with a mixture of CO in air (Scott Specialty Gases) as a reference (Cook *et al*., 1995). HO activity in the sample was calculated by subtracting CO formation in the incubate without NADPH (blank) from total CO formation and was expressed as a pmol CO formed mg^{-1} protein min^{-1}. Specificity of the reaction was verified by measuring the amount of CO being generated in the presence of ZnPP. Preliminary trials proved that the reaction rate is linear over the interval used and, moreover, they excluded any loss of enzyme activity in blood vessels stored at −70°C. Likewise, the activity remained unchanged in the frozen tissue supernatant.

Western immunoblot analysis of HO The analysis was carried out in the microsomal fraction from foetal (ductus arteriosus, pulmonary artery, aorta) and adult (aorta) blood vessels by the procedure of Trakshel *et al*. (1986). Briefly, specimens, whether freshly dissected or frozen (see above), were homogenized (Polytron; 4×20 s) in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 135 mM KCl. The homogenate was centrifuged at 10,000 *g* (20 min) and the resulting supernatant was centrifuged at 150,000 *g* (60 min). The microsomal pellet was resuspended in 20 mM potassium phosphate buffer with 1 M KCl and 10 mM EDTA added (final volume, 1 ml) and was centrifuged again at 150,000 *g* (60 min). The resulting pellet was resuspended in 20 mM Tris-HCl buffer containing 1 mM EDTA and 20% (v/v) glycerol to a final protein concentration between 2 and 10 mg ml^{-1}. For Western blot analysis, protein samples (200 μg) were fractionated by sodium dodecyl sulphate : polyacrylamide gel electrophoresis under denaturing conditions and then were transferred to a Millipore Immobilon PVDF membrane with a Biorad transfer apparatus. Dyed molecular weight markers (Helix Technologies, Novex) were run along the appropriate HO isozyme standard in each gel. Membranes were incubated with HO antibodies diluted (HO-1, 1 : 300; HO-2, 1 : 150) in TPBS (phosphate-buffered saline: 0.05% (v/v) Tween 20, pH 7.4) containing 5% nonfat dry milk. Horseradish peroxidase conjugated goat antirabbit antibody was used to visualize immunopositive proteins. Membranes were washed with TPBS, treated for enhanced chemiluminescence (Amersham), and exposed to film at room temperature (Kodak X-OMA-TAR).

Measurement of endothelin-1 release Specimens of the whole ductus were transferred into polypropylene tubes containing 0.5 ml of BSA-supplemented (0.05%) Krebs medium (Coceani & Kelsey, 1991) gassed with a 95% O_2 : 5% CO_2 mixture and with ZnPP added (10 μM). A high oxygen content was selected to ensure that the synthetic system was fully operational (see Coceani *et al*., 1992), and incubation lasted 2 h. ET-1 was measured in the incubate after extraction on SepPak C18 cartridges (Waters), by use of a modification (see Löffler *et al*., 1992) of a previously published radioimmunossay procedure (Coceani & Kelsey, 1991). Extraction was necessary to avoid interference from ZnPP. Briefly, samples were spiked with [^{125}I]-ET-1 (about 500 d.p.m., 0.5–0.7 pg; 1 d.p.m. = 60 Bq) and eluted from the cartridge with 2.5 ml of methanol-water (9 : 1, by vol) (recovery of radioactivity 83 ± 2%, $n = 5$). The eluate was then dried and the residue was dissolved in buffer (100 μl) for the assay. Values of ET-1 release apply to the wet weight of the tissue, and reference values for the untreated tissue are taken from a previous study (Coceani *et al*., 1996b).

Measurement of cyclic GMP content Cyclic GMP was measured with a radioimmunoassay kit (^{125}I-labelled ligand; DuPont) in ductal strips that had been incubated in a shaker and then frozen. Incubation lasted 1 h (without endotoxin) or 2 h (with endotoxin) and was carried out in Krebs medium gassed with 2.5% O_2 : 5% CO_2 in N_2. Treatments included ZnPP (10 μM), endotoxin (100 ng ml^{-1}), or ZnPP and endotoxin combined. Work-up of specimens and assay procedure with its validation have been described elsewhere (Coceani *et al*., 1996b). Assay values are given without correction, because recovery of nucleotide was quantitative (Coceani *et al*., 1996b) and there was also no interference from added ZnPP.

Protein measurement Protein content of samples was measured according to Lowry *et al*. (1951).

Solutions and drugs

The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1, $MgSO_4$ 0.9, dextrose 11.1 and $NaHCO_3$ 25. The pH of the solution was 7.4 after equilibration with gas mixtures containing 5% CO_2.

Recombinant rat HO-1 and HO-2 proteins and their relative antisera were obtained from StressGen. Antisera were polyclonal and had been generated in the rabbit. Colloidal gold-labelled (particle diameter, 10 nm) goat antirabbit immunoglobulin and an antibody against α-actin were purchased from Amersham, while FITC-conjugated goat anitrabbit immunoglobulin and goat antimouse immunoglobulin were supplied by, respectively, Molecular Probes and Miles Pharmaceuticals.

The following compounds were used: bradykinin acetate (Sigma); sodium nitroprusside (SNP; Sigma); endotoxin (lipopolysaccharide W, *E. coli*, serotype O55:B5; Difco); hemin chloride (Sigma); indomethacin (Sigma) and zinc protoporphyrin IX (ZnPP; Porphyrin Products Inc). Indomethacin and ZnPP inhibit, respectively, cyclo-oxygenase and HO, and

their concentration was selected to combine efficacy with selectivity (Coceani & Olley, 1982; Zakhary *et al*., 1996). ZnPP may also inhibit guanylyl cyclase (Ignarro *et al*., 1984) and, to rule out this possibility, SNP action and cyclic GMP content were examined in some of the treated tissues (see above). All other chemicals and solvents were of analytical grade purity.

Indomethacin was dissolved in distilled ethanol (10 mg ml^{-1}) before the preparation of the final solution in Krebs medium. Likewise, hemin and ZnPP were first prepared as a stock solution in 0.1 M NaOH (1 mM for both) on the day of the experiment. Other substances dissolved readily in saline or Krebs medium. Solutions of certain compounds (SNP, hemin, ZnPP) were protected from light.

Doses of all compounds are given in molar concentrations and refer to their final concentration in the bath.

Analysis of data

Contractile tension of ductal strips *in vitro* varied with the preparation and the experimental condition (see Results), and is given after the applied tension had been subtracted.

Data are expressed as the mean $\pm$ s.e.mean. When HO activity was being measured, incubations were carried out in duplicate and the results were averaged before statistical analysis. Statistical comparison of two means was done by Student's *t* test for paired or unpaired observations. Multiple comparisons were made with an analysis of variance (ANOVA). Differences are considered significant for $P<0.05$.

Results

Western immunoblot analysis

The microsomal fraction from all blood vessels gave a clear double band coinciding with the molecular mass of HO-2 (Figure 1a). Conversely, the 30-kDa signal corresponding to HO-1 was faint (foetal vessels; Figure 1b) or not detectable (adult aorta; data not shown). No positive reaction for either HO isoform was noted when antisera preabsorbed with the appropriate antigens were used.

Localization of haem oxygenase

In control experiments, it was found that a 2-h incubation period in Krebs medium does not cause by itself any significant structural change in the vessels. Specifically, vascular muscle cells, though often contracted and with clumps of filaments, had the plasma membrane intact. The only apparent anomaly in these cells was the widening of the mitochondrial cristae and a dilated sarcoplasmic reticulum.

Epifluorescence microscopy showed that HO isoforms are similarly distributed in different blood vessels of the foetus (ductus arteriosus, aorta) and that the staining pattern does not change with age (foetus vs. adult aorta). In addition, no difference was seen between vessels processed immediately after dissection and vessels that had been incubated in Krebs medium. In all cases, HO-1 was localized to both endothelial and smooth muscle cells, while HO-2 was found only in muscle cells (Figure 2a and b). Treatment with endotoxin did not alter this pattern. By contrast, immunoreactivity to either enzyme was absent when an irrelevant antibody (goat antimouse IgG) or an antibody which had been preincubated with the appropriate antigen (HO-1 or HO-2 protein) was used. A difference between control and endotoxin-treated tissues was found instead when immunoreactive proteins were viewed inside the muscle cells by confocal microscopy. While the untreated tissue showed a relatively uniform distribution for both HO-1 and HO-2 (Figure 2c and d), the treated tissue had the enzymes clustered at discrete sites in the form of bright punctuations (Figure 2e and f).

When viewed by transmission imunoelectron microscopy, ductus muscle cells exhibited greater immunogold reactivity

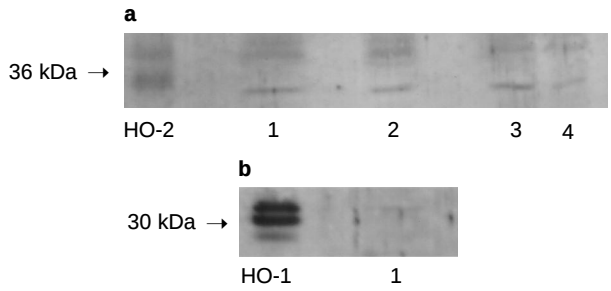

Figure 1 Western immunoblot of the microsomal fraction from foetal and adult blood vessels. Each lane contained 200 μg of microsomal protein (for details, see Methods section). (a) HO-2; lane 1, ductus arteriosus; lane 2, foetal aorta; lane 3, foetal pulmonary artery; lane 4, adult aorta. (b) HO-1; lane 1, ductus arteriosus. A similarly faint signal was obtained with other foetal blood vessels. Note that, as indicated by the supplier, authentic HO-1 and HO-2 do not give a single immunoreactive band.

for HO-2 than HO-1 under control conditions, regardless of whether the tissue had been processed fresh (198 ± 42 and 148 ± 30 gold particles/cell for HO-2 and HO-1, respectively) or after incubation in Krebs medium (194 ± 63 and 121 ± 30 gold particles/cell for HO-2 and HO-1, respectively). This unequal expression of the two isozymes disappeared after treatment with endotoxin (100 ng ml^{-1}), and the total number of HO-1-linked gold particles rose to that of HO-2 (189 ± 58 and 192 ± 22 gold particles/cell for HO-2 and HO-1, respectively). Nevertheless, differences remained in the distribution of both isozymes between the untreated tisuses, whether or not incubated in Krebs medium, and the tissues treated with endotoxin. As shown in Figure 3 (a and b) and Figure 4 (a and c), in the untreated tissue antigenicity was predominant in the perinuclear region and was distinctly evident in the Golgi apparatus. No such staining pattern was noted in the endotoxin-treated tissue. In that case, gold particles were located in the peripheral rather than the perinuclear cytoplasm and, in certain sections, also appeared to be clustered along profiles of the sarcolemma (Figure 3c and d, Figure 4b and d). Table 1 provides the actual number of particles in different regions of the cell under the two conditions.

CO formation

Foetal vascular tissue, whether from the ductus arteriosus or the main pulmonary artery and the aorta, generated CO and, as expected from an HO-catalyzed reaction, this process was inhibited by ZnPP (Figure 5). As shown in Figure 5, the actual yield of CO did not differ among vessels. Likewise, preparations of the ductus consisting of muscle only were as active as preparations of the whole vessel (Figure 5). Conversely, the aorta from the adult tended to have a less efficient CO-generating system and, moreover, its inhibition by ZnPP did not reach significance (Figure 5).

Effect of haemin and ZnPP on vascular tone

Ductal strips developed active tension during equilibration in medium gassed with 2.5% O_2. A further contraction occurred upon exposure to higher oxygen concentrations and a maximum was attained with 95% O_2. Strips of the whole ductus and strips of muscle alone behaved identically, though in the latter case the tension output was smaller and also more variable (see Coceani *et al*., 1986). Both preparations contracted to indomethacin (2.8 μM) and, as shown previously (Coceani *et al*., 1986), this response was observed at all oxygen concentrations.

Haemin (10 μM) lowered the tone of the intact ductus at 2.5% but not 30% O_2 (Table 2). However, this effect was not seen after treating the tissue with indomethacin (tension output

was 4.9 ± 0.5 and 4.9 ± 0.4 g, respectively, in the absence and presence of haemin; $n = 4$) and, hence, it was ascribed to activation of cyclo-oxygenase (see Lands, 1979) rather than haem oxygenase.

ZnPP (10 μM) was ineffective under normal oxygen concentrations (2.5 to 95%), regardless of whether the preparation was intact or had been trimmed down to the muscle (Table 2). On the contrary, the inhibitor tended to be a constrictor, or was clearly a constrictor, when tested, respectively, on the hypoxic ductus (Table 3) or the normally oxygenated ductus after priming with endotoxin (Table 4). In the latter case, the response was seen during the treatment, when the tissue was fully relaxed, and could not be elicited after endotoxin had been washed out from the bath and the original tone had been restored (Table 4). When present, the ZnPP-induced contraction developed gradually (peak in 2–4 h), was sustained after reaching the peak, and subsided upon washing. This response is unlikely to be nonspecific, because guanylyl cyclase, a possible alternative target for ZnPP (Ignarro *et al*., 1984), showed no sign of inhibition whether under control conditions or after exposure to endotoxin. Guanosine 3′ : 5′-cyclic monophosphate (cyclic GMP) content was 5.96 ± 0.87 ($n = 6$) and 7.96 ± 2.78 ($n = 5$) pmol g^{-1} tissue, respectively, in the absence and presence of endotoxin (100 ng ml^{-1}), and neither value was modified by ZnPP treatment (6.85 ± 0.35 and 7.41 ± 1.43 pmol g^{-1} tissue with the naive and endotoxin-primed ductus, respectively; $n = 4$ and $n = 5$ in the order). Furthermore, basal release of ET-1, a putative constrictor agent in the ductus (Coceani, 1994), was not changed significantly by ZnPP and, if anything, was lower rather than higher (0.095 ± 0.02 and 0.052 ± 0.01 pg 100 mg^{-1} min^{-1} for control and treatment, respectively; $n = 5$ for both groups).

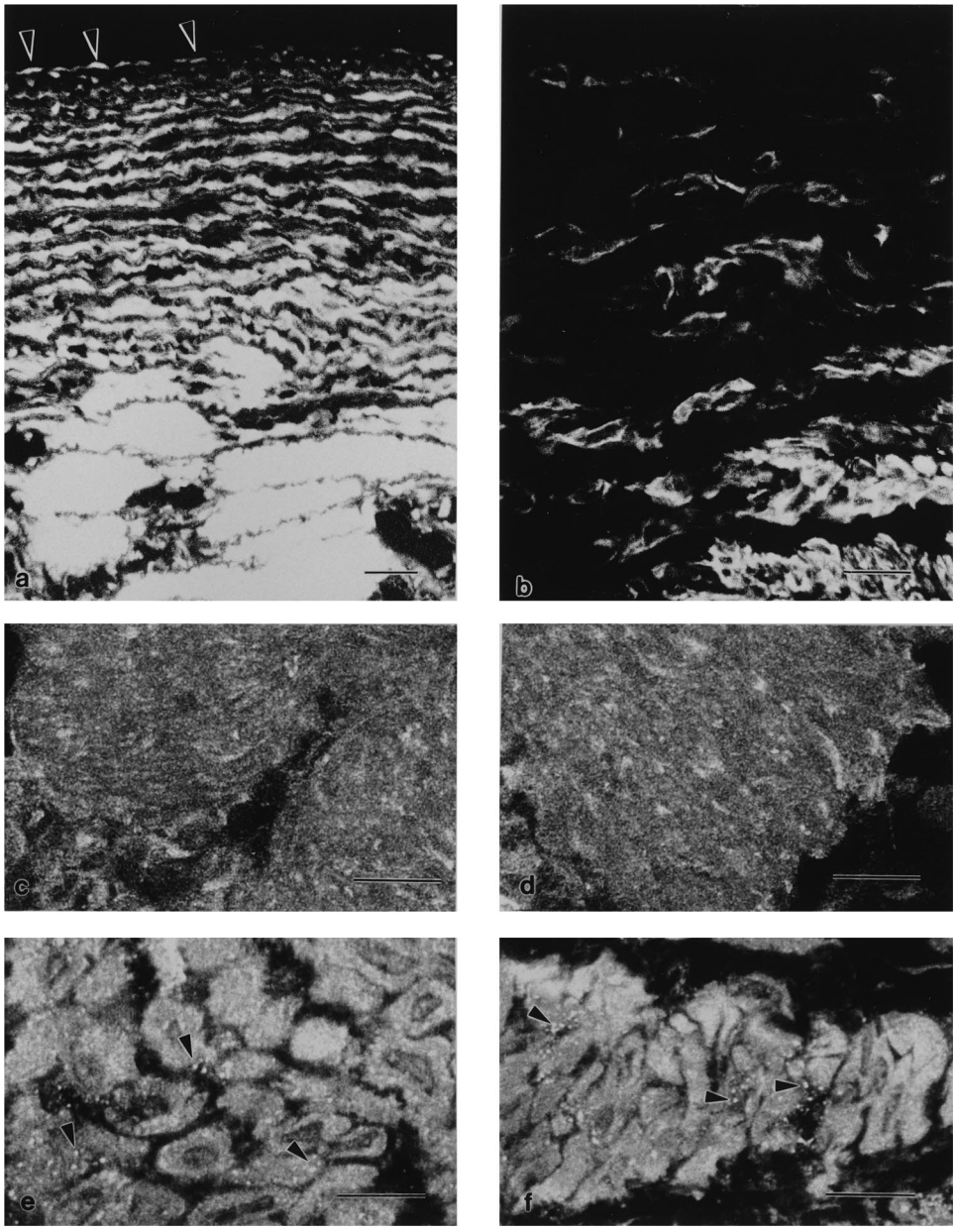

Figure 2 Immunofluorescence micrographs of the lamb ductus arteriosus. (a) section incubated with HO-1 antiserum; note the pronounced labelling of endothelial (arrowheads) and smooth muscle cells. (b) Same section as (a) incubated with HO-2 antiserum; labelling is confined to the muscle cells. (c) Confocal micrograph showing HO-1 immunoreactivity; note homogeneous distribution of label throughout the sarcoplasm. (d) Confocal micrograph showing HO-2 immunoreactivity; label is uniformly distributed in the sarcoplasm. (e) Confocal micrograph of endotoxin-treated tissue showing HO-1 immunoreactivity; note the punctiform distribution (arrowheads) of the reaction product in this contracted cell. (f) Confocal micrograph of endotoxin-treated tissue showing HO-2 immunoreactivity; label has a spot-like distribution. Bar represents 150 μm (panels a and b) or 1.75 μm (panels c to f).

Bradykinin is known to relax dose-dependently the oxygen-contracted, indomethacin-treated ductus (Coceani *et al*., 1994) and its effectiveness was confirmed here by showing a relaxation of appropriate magnitude to the 35 nM dose (i.e. 1.3 ± 0.2 g, $n = 8$; see Coceani *et al*., 1994). However, the compound lost most of its activity after ZnPP (10 μM) was added to the bath, and only at the highest concentrations was it able to produce a small relaxation (0.15 ± 0.08 and 0.31 ± 0.1 g, respectively, at 0.1 and 1 μM; $n = 8$). Furthermore, contrary to controls the relaxation was often preceded by a transient contraction. To rule out a nonspecific effect of ZnPP, particularly an interference with guanylyl cyclase (see Ignarro *et al*., 1984); in some experiments SNP (35 nM) was tested before and during treatment with the inhibitor. However, in no instance, was the SNP relaxation reduced by ZnPP 10 μM (control, 3.7 ± 0.1 g; treatment, 3.9 ± 0.2 g; $n = 4$).

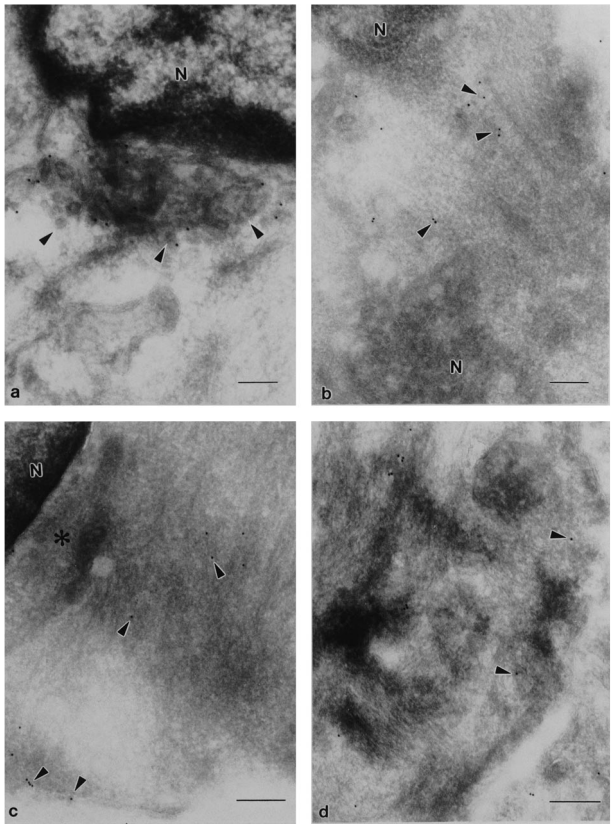

Figure 3 Transmission electron micrograph of ultrathin cryosections from the muscle layer of the foetal ductus arteriosus. In all panels, sections have been reacted with antiserum against HO-1. (a) Immunogold labelling of the perinuclear region and the Golgi apparatus in the untreated tissue; note that label is confined to the Golgi apparatus and Golgi-derived vesicles (arrowheads). (b) Immunogold labelling of the perinuclear region (N, nucleus) in the untreated tissue; gold particles are seen in the cytoplasm (arrowheads). (c) Immunogold labelling in the endotoxin-treated tissue; note that the cytoplasm adjacent to the nucleus (N) is almost void of any label (asterisk) and that the label is evident instead in the peripheral cytoplasm and along the plasma membrane (arrowheads). (d) Immunogold labelling in the endotoxin-treated tissue; note label in the peripheral cytoplasm (arrowheads). Bar represents 0.25 μm.

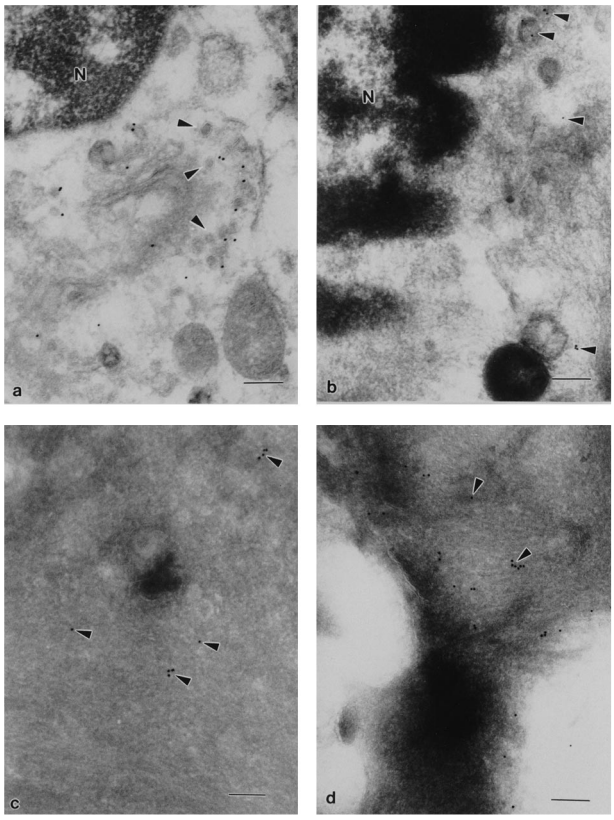

Figure 4 Transmission electron micrograph of ultrathin cryosections from the muscle layer of the foetal ductus arteriosus. In all panels, sections have been reacted with antiserum against HO-2. (a) Immunogold labelling of the perinuclear region and the Golgi apparatus in the untreated tissue; note label on Golgi-derived vesicles (arrowheads). (b) Immunogold labelling of the perinuclear region in the endotoxin-treated tissue; note the sparse distribution of label. (c) Immunogold labelling of the peripheral cytoplasm in the untreated tissue; note the scarcity of reactive sites. (d) Immunogold labelling of the peripheral cytoplasm in the endotoxin-treated tissue; note the greater density of label (arrowheads) compared to (c). Bar represents 0.25 μm (a and b) or 0.4 μm (c and d).

Table 1 Immunogold labelling of muscle cells of the lamb ductus arteriosus with antibody against heme oxygenase 1 (HO-1) or haem oxygenase 2 (HO-2): intracellular distribution of gold particles

Region of the cell	*Condition of tissue*	*No. of gold particles*	
		HO-1	*HO-2*
Cytoplasm, perinuclear	No incubation in Krebs	18 ± 4.9	21 ± 4.1
	Incubation in Krebs alone	15 ± 3.3	14 ± 1.8
	Incubation in Krebs plus endotoxin	7.8 ± 0.9*	8.4 ± 1.7*
Cytoplasm, peripheral	No incubation in Krebs	4 ± 0.9	3 ± 1.4
	Incubation in Krebs alone	2.3 ± 0.8	2 ± 0.08
	Incubation in Krebs plus endotoxin	8 ± 0.6*	7 ± 3.2*

Gold particles were counted in transmission electron micrographs of ultrathin cryosections from the muscle layer of the foetal ductus arteriosus. Tissues were used immediately after dissection or were incubated at 37°C in Krebs medium gassed with 2.5% O_2: 5% CO_2 in N_2. Incubation continued for 2 h in the absence and presence of endotoxin (100 ng ml^{-1}). A constant number of fields ($n = 10$) of equal magnification were examined in each region of the cell (for details, see Methods). *$P < 0.05$ vs. untreated tissue.

Discussion

This study shows that CO qualifies as a natural regulator of muscle tone in the ductus arteriosus and, by extension, proves that any contribution of this dilator agent to general haemodynamics is already manifest *in utero*. Indeed, judging from the greater activity of the HO reaction in foetal blood vessels compared to the adult aorta, it would appear that this regulatory mechanism is potentially more effective during the perinatal period. A role for CO in the ductus is supported by several facts: (a) the identification of HO-1 and HO-2 in the tissue together with the observation that the enzymes are expressed in relevant cells, such as the endothelium and smooth muscle; (b) the demonstration that HO proteins are catalytically active and are amenable to upregulation by appropriate stimuli; and (c) the finding that an inhibitor of HO-1/HO-2 reactions contracts the vessel under conditions, such as the treatment with endotoxin, which are known to promote the activity of these enzymes. Based on this premise, the discussion will address two issues: the arrangement of the CO synthetic system in the wall of the ductus, and the importance of the CO mechanism for regulation of ductal tone vis-a-vis other active agents and NO in particular.

Our investigations confirms, and extends, previous findings (Ewing *et al*., 1994; Zakhary *et al*., 1996) insofar as it shows that HO-1 and HO-2 are present in the sheep vasculature from an early age. However, the actual distribution of the two enzymes across the vessel wall has special features. HO-2 could not be detected in the endothelial layer, which is at variance with data from both rat (Ewing *et al*., 1994) and pig (Zakhary *et al*., 1996). HO-1, on the other hand, was found in both endothelial and muscle cells, while in a previous study in the pig (Zakhary *et al*., 1996) the enzyme appeared to be confined to the muscle layer. The

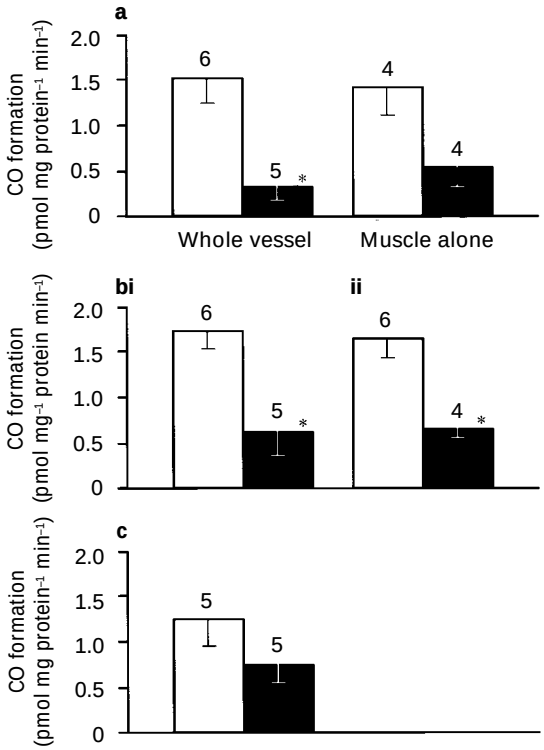

Figure 5 CO formation in the 10,000 *g* supernatant fraction of foetal and adult blood vessels from sheep, (a) ductus arteriosus, (b) foetal (i) aorta and (ii) pulmonary artery, (c) adult aorta; open columns, control; solid columns, ZnPP 0.2 mM (for details, see Methods). For each group, number of experiments are given above the columns, and a significant difference ($P<0.01$) between control and treatment groups is indicated with an asterisk. Note that ZnPP inhibition was more variable in preparations of the ductus limited to the muscle than in those comprising the whole vessel.

Table 3 Effect of ZnPP on the contractile tension of the hypoxic ductus arteriosus in the lamb

No. of ZnPP test	*Steady-state tension (g)* *Before ZnPP*	*During ZnPP*
First	0.33±0.11 (6)	0.56±0.08 (6)
Second	0.08±0.08 (6)	0.81±0.34 (6)
Third	0.41±0.27 (6)	0.65±0.29 (6)

Tissues were made hypoxic by gassing the Krebs medium with the 0% O_2 mixture, and ZnPP (10 μM) was tested for 60–106 min at 60– to 70–min intervals. Data are mean±s.e.mean for the number of experiments given in parentheses. Difference between control and treatment groups does not reach significance.

Table 4 Effect of ZnPP on the contractile tension of the endotoxin-treated ductus arteriosus in the lamb

Time of ZnPP test	*Steady-state tension (g)* *Without ZnPP*	*With ZnPP*
During endotoxin treatment	0 (9)	1.9±0.4 (9)*
After endotoxin treatment	1.7±0.6 (7)	2.0±0.6 (7)

Endotoxin (100 ng ml^{-1}) was added to the Krebs medium (gassed with 2.5% O_2) in two successive periods, and ZnPP (10 μM) was tested during or after the second treatment (for details, see Methods). The 'after endotoxin' group includes two experiments in which the endotoxin treatment was given, respectively, once and four successive times, and results did not differ from those of the remaining experiments. Data are mean±s.e.mean for the number of experiments given in parentheses. Note that in the presence of endotoxin the ductus lost completely its tone and that, after washing out the pyrogen, 1 to 4 h were required for the original tension to be restored. *$P<0.01$ vs. control (note that ZnPP had no effect in 1 of the 9 experiments).

Table 2 Effect of haemin and ZnPP on the contractile tension (g) of the ductus arteriosus in the lamb

Compound	*Ductus preparation*	*2.5% O_2* *C*	*T*	*15% O_2* *C*	*T*	*30% O_2* *C*	*T*	*95% O_2* *C*	*T*
Hemin	intact strip	0.65±0.25 (4)	0.2±0.11 (4)*	–	–	4.6±0.5 (4)	4±0.6 (4)	–	–
ZnPP	intact strip	0.35±0.13 (6)	0.2±0.11 (6)	–	–	3.6±0.3 (7)	3.6±0.3 (7)	–	–
	muscle bundle	0.25±0.1 (3)	0.13±0.13 (3)	0.6 (2)	0.4 (2)	0.7 (2)	0.87 (2)	0.75±0.07 (3)	1.01±0.39 (3)

Haemin and ZnPP (both at 10 μM) were added to the perfusion fluid and kept in contact with the tissue for 0.5–1.5 h and 0.5–2.5 h, respectively. In each experiment, contractile tension was measured before (C) and during (T) treatment. Data are mean (if only two experiments) or mean±s.e.mean for the number of experiments given in parentheses. Here and in the following two tables, values of tension refer to the entire tension developed by the preparation (i.e. total tension minus applied tension, see Methods). *$P<0.05$ vs. control (note that the haemin effect was not seen after treating tissues with 2.8 μM indomethacin, see text).

reason for these differences is not clear. Species may vary in the expression pattern of these enzymes and, indeed, we have confirmed that the adult pig aorta, unlike the adult sheep aorta, shows HO-2 immunoreactivity in the endothelium (unpublished data), Furthermore, the way tissue specimens are collected may be important. No endothelial staining for HO-2 could be found even in the pig if animals were killed by electrical stunning, as is the case in the abattoir, rather than a barbiturate overdose (unpublished data). Leaving aside these differences and the question of the relative importance of intrinsic vs methodological factors in their occurrence, the HO complex seems quite dynamic in reacting to challenges, as exemplified by the changes taking place in muscle cells exposed to endotoxin. Not only is HO-1 upregulated as one would expect with any inducible enzyme, but both the inducible and constitutive HO isoforms are also translocated towards the outer regions of the cell wherefrom their product, CO, can more easily diffuse to extracellular targets. A comparable set of events has been described several years ago in liver cells following treatment with cobalt, though at the time HO was considered a single enzyme and its apparent redistribution was ascribed to differential induction in subcellular organelles (Hino *et al*., 1979). No information is available on the reversibility of the endotoxin-induced changes, nor is it known whether the same changes occur upon exposure to agents acting only on HO-2. However, with regard to the first question, the observation that the endotoxin-treated ductus loses its capacity to contract to ZnPP upon returning to a normal medium implies that HO isozymes can regain fairly rapidly their native arrangement.

Despite its potential for activation, the HO system in the ductus becomes functional only under certain conditions, at least *in vitro*. Specifically, as judged from the susceptibility to ZnPP, CO formation does not reach the threshold for a vasodilator effect at either foetal or neonatal P_{O_2}, unless the vessel is exposed to substances promoting both HO isoforms (endotoxin) or HO-2 alone (conceivably bradykinin). Besides keeping its tone unaltered, the ZnPP-treated ductus shows no change in ET-1 release, and the coexistence of these two negative findings accords with the proposed mechanism of action of CO (see below). However, findings *in vitro* do not necessarily apply to the condition *in vivo* where agents activating HO-2 may be released spontaneously from adventitial nerves or HO-2 itself may be present in the nerves and be activated by incoming impulses (Zakhary *et al*., 1996). In fact, it has been shown that an *in vivo* preparation, unlike the *in vitro* preparations used by us and others (Johnson *et al*., 1995; Ny *et al*., 1995; Zakhary *et al*., 1996), exhibits a vasoconstrictor response to HO inhibitors in the absence of any conditioning treatment (Johnson *et al*., 1995). The situation is expectedly more complex with the hypoxic ductus, because, on the one hand, the HO (specifically HO-1) protein undergoes upregulation (Morita & Kourembanas, 1995) and, on the other hand, oxygen may become rate-limiting for catalytic activity. The overlap between these opposing processes may explain the failure of the ZnPP contraction to reach significance (see Table 3).

Regardless of whether CO activity is continuous or intermittent, the mechanism by which this agent dilates the ductus arteriosus needs to be defined. In theory, CO could exert its effect in two ways, by activating guanylyl cyclase or by interfering with the cytochrome P450-based mono-oxygenase reaction limiting the synthesis of ET-1 (Coceani *et al*., 1996a,b). However, in practice, the former mechanism is unlikely to play a major role since the relaxation of the ductus to CO develops virtually unabated in the presence of guanylyl cyclase inhibitors and the magnitude of this relaxation is not correlated with levels of cyclic GMP in the tissue (Coceani *et al*., 1996a). In this context, it must be noted that binding of CO to guanylyl cyclase is weak and with an unusually high dissociation rate (Stone & Marletta, 1994; Kharitonov *et al*., 1995), which argues against the importance of this process for signal transduction. Significantly, NO, which is also formed in the ductus (Coceani *et al*., 1994), differs from CO in clearly causing relaxation via the guanylyl cyclase/cyclic GMP system (Coceani *et al*., 1994; 1996a).

From the foregoing, we conclude that CO dilates the ductus arteriosus by reducing the formation of the constrictor ET-1. In addition, we assume that CO is formed in the vessel and contributes, together with NO (Coceani *et al*., 1994) and particularly prostaglandin E_2 (PGE_2) (Coceani, 1994), to the relaxation of the muscle. The two gas-based relaxing mechanisms appear to be complementary in several ways: the chemical stability of the active agent (CO, relatively stable; NO, labile) and, by extension, the ability of the agent to diffuse within the tissue (see Steinhorn *et al*., 1994), the nature of the main target in the tissue (CO, cytochrome P450/ET-1 system; NO, guanylyl cyclase/cyclic GMP system) and the way in which relaxation is elicited (CO, inhibition of a contractile process; NO, stimulation of a relaxant process). Moreover, these mechanisms are amenable to activation by the same agent (e.g. endotoxin, see Maines, 1992; Moncada *et al*., 1991) and, through their combined action, may ensure an optimal response in the target. In this connection, our observation that ZnPP curtails the relaxation of the ductus to bradykinin as effectively as a NO synthase inhibitor (see Coceani *et al*., 1994) is unexpected and warrants a comment. An unspecific effect of ZnPP on NO synthase and guanylyl cyclase, though feasible (Ignarro *et al*., 1984; Meffert *et al*., 1994; Ny *et al*., 1995), appears unlikely, because treated tissues showed no reduction in the cyclic GMP content, whether under basal conditions or during exposure to endotoxin, nor did they exhibit a smaller relaxation to SNP. Furthermore, based on previous findings with true inhibitors (Coceani *et al*., 1994), interference with either enzyme should have led to a rise in the tone of the ductus without the need of any priming by endotoxin. However, no such response to ZnPP was noted even when a fully relaxed vessel (i.e. at 2.5% O_2) was used. Any explanation for this seemingly paradoxical finding is necessarily speculative. One possibility is that NO promotes the formation of CO by stimulating the synthetic enzyme system (Motterlini *et al*., 1996). Alternatively, NO could facilitate the binding of CO to the target haem (Maines, 1993). Either scheme, implying a functional cooperation between these two agents, would require experimental verification.

In conclusion, the present study demonstrates that CO is formed in the ductus arteriosus where it exerts a relaxing influence on the muscle. CO may therefore contribute to patency of the vessel *in vivo* in combination with PGE_2 and NO. The actual importance of this mechanism is not known, nor is it known whether our data are applicable to the clinical situation. However, if it is assumed that the same arrangement occurs in man as in sheep, it is conceivable that CO, in concert with NO, provides to the unborn child protection against any drug interfering with PGE_2 function (e.g. non-steroidal anti-inflammatory drugs) taken by the mother. At the same time, its presence may complicate the course of a persistent ductus in the premature, particularly in the case of intervening infections (see Gonzalez *et al*., 1996), and may ultimately condition the efficacy of non-steroidal antiinflammatory drugs as therapeutic agents.

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