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Inhibition of the contraction of the ductus arteriosus to oxygen by 1-aminobenzotriazole, a mechanism-based inactivator of cytochrome P450

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1 We have proposed that contractile tension of the ductus arteriosus is sustained by a cytochrome P450-linked mechanism acting as a limiting step in the synthesis of endothelin-1 (ET-1). In the present study, we have used the isolated ductus from near-term foetal lambs and guinea-pigs to investigate the effect on both muscle tone and ET-1 formation of 1-aminobenzotriazole (ABT), a suicide substrate for mono-oxygenase reactions.

2 ABT relaxed the lamb ductus at rest (2.5% O_2) and during the oxygen contraction (15 to 95% O_2). The effect was seen at 40 μM, and at 0.8 mM active tone was almost completely abolished. ABT (1 mM) also reversed the oxygen contraction in the guinea-pig ductus.

3 In the lamb ductus, the ABT response was not affected by removal of the endothelium or by treatment with 2.8 μM indomethacin (at 2.5% O_2) and the ensuing contraction.

4 At both low and high concentration, ABT relaxed marginally, or not at all, the potassium-contracted (55 mM) ductus from either species.

5 ET-1 release from either the intact or endothelium-denuded lamb ductus tended to decrease in the presence of ABT (1 mM), whilst during the same treatment cyclic GMP content of the tissue remained unchanged.

6 We conclude that ABT relaxation is due to suppression of a contractile mechanism and not to activation of prostaglandin- and NO-mediated relaxing mechanisms. This contractile mechanism has a cytochrome P450-based mono-oxygenase reaction as a key component.

Keywords: Foetus; ductus arteriosus closure; oxygen; cytochrome P450; endothelin-1; 1-aminobenzotriazole

Introduction

We proposed that constriction of the ductus arteriosus to oxygen and by extension normal closure of the vessel at birth, takes place through a multistep process, starting with the activation of a cytochrome P450 haemoprotein and terminating with the action of endothelin-1 (ET-1) on muscle cells (Coceani, 1994). The concept of a cytochrome P450 functioning as a signal transducer for oxygen in muscle cells of the ductus is based on several results, most of which have been obtained in the lamb. They are: the demonstration that CO potently relaxes the vessel, whether intact or trimmed to the medial layer (Coceani *et al.*, 1984; 1988); the attenuation of the CO response during exposure to light, peak photoreversal occurring characteristically at 450 nm (Coceani *et al.*, 1988); the capability of cytochrome P450 inhibitors of diverse structure to relax the ductus (Coceani *et al.*, 1984; 1988); and the immunochemical visualization on ductus muscle cells of a cytochrome P450 belonging to the CYP3A subfamily coupled with the demonstration that CYP3A inducers constrict the vessel *in utero* (Coceani *et al.*, 1994). Although these findings form collectively a strong case in support of our scheme, an issue still unsettled concerns the mechanism by which the ductal cytochrome is activated by oxygen with the attendant question of the nature of the signal being directed to the ET-1 synthetic system. Theoretically, the haemoprotein could play this transducing role in two ways, through a conformational change or by acting as a catalytic element in a mono-oxygenase reaction. The former possibility, however, is inconsistent with the finding that oxygen and CO have opposite effects on ductal tone when structural changes induced by the two ligands are expected to be similar, if not identical (Marks *et al.*, 1991). On the other hand, the attempt to demonstrate mono-oxygenase activity in the ductus by two conventional assays (Coceani *et al.*, 1994) or to implicate in the oxygen response natural vasoactive agents, such as the P450-derived products of arachidonic acid (Coceani *et al.*, 1988), has failed.

Availability of mechanism-based inhibitors of cytochrome P450, such as 1-aminobenzotriazole (ABT) (Halpert *et al.*, 1994), affords a new approach to this problem. Compared to other inhibitors, ABT has the advantage of becoming effective only after transformation to a suicide substrate by the target cytochrome (Ortiz de Montellano & Mathews, 1988). Thus, this agent does not act indiscriminately on all haemoproteins (Ortiz de Montellano & Mathews, 1981); yet, its inhibitor action is broad enough to encompass various isoforms of cytochrome P450 (Mathews *et al.*, 1985; Knickle & Bend, 1992; 1994).

The purpose of our investigation was to verify the existence of a mono-oxygenase reaction controlling the tone of the ductus arteriosus by testing ABT under basal conditions and during the contraction to oxygen. The action of the inhibitor was examined not only in the normal ductus, but also in the ductus pretreated with indomethacin or lacking the endothelium. The latter conditions were used to avoid a possible interference from relaxing agents, such as prostaglandin E_2 (PGE_2) and NO, which are known to be effective within the vessel (Coceani, 1994). Guanosine 3′:5′-cyclic monophosphate (cyclic GMP) was also measured in the tissue as an index for NO activity. In addition, we determined the effect of ABT on

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ET-1 release from the ductus with the intent of establishing a causal link between the synthesis of this peptide and a monooxygenease-type reaction. Experiments were carried out in both lamb and guinea-pig; however, the guinea-pig was used only to study ABT effects on muscle tension.

Methods

General procedure

Lamb Experiments were performed on near-term pregnant sheep (133–138 days gestation; term 145 days) of Targhee crossbreed. The procedures for anaesthesia, Caesarean delivery of the foetuses, and isolation of the ductus arteriosus have been published (Coceani *et al.*, 1986). The whole ductus or circular strips (two and, occasionally, three strips per ductus) were used depending on the protocol. In the former case, the vessel was split open and incubated in a shaker for measurement of ET-1 release (see below). Strips (9–14 mm long, 1.5–2 mm wide, 1–2 mm thick) were either mounted in individual organ baths (5 or 10 ml capacity) for recording mechanical tension or were incubated in a shaker for measuring cyclic GMP content (see below). The smaller bath enabled tissues to be freeze-clamped for the assay of cyclic GMP (see below). In certain experiments, the intimal surface was rubbed with filter paper (Whatman no. 41) to remove the endothelium, and the effectiveness of this procedure has been confirmed previously by morphological (Coceani & Kelsey, 1991) and pharmacological (Coceani *et al.*, 1994) means. Otherwise, care was taken not to damage the endothelial lining at any stage in the preparation.

Guinea-pig Pregnant guinea-pigs (Hartley crossbred) at term (>63 days) were anaesthetized with intraperitoneal urethane (2 g kg^{-1}) and their foetuses were delivered by Caesarean section. The weight of the foetuses (88–111 g) confirmed that animals were within a few days of delivery (see Draper, 1920). Surgical procedure and the procedure for isolation of a ring of the ductus arteriosus (2–2.5 mm in length) have been described previously (Bodach *et al.*, 1980). No attempt was made to remove the endothelium from any of the preparations. In all likelihood, however, insertion of the wires for mechanical recording through the lumen of the vessel (see below) caused variable damage to the intimal surface.

With both preparations, muscle tension was recorded isometrically with a force transducer (Grass FT-O3C) coupled to a Grass polygraph. Strips of the lamb ductus were mounted between a stationary glass rod and the transducer and they were then stretched by about 50% of the original length to obtain optimal tension output (Somlyo & Somlyo, 1964). This initial load was about 1.5 g (1 g weight = 9.8 mN). Rings of the guinea-pig ductus were suspended between two platinum hooks, one stable and the other connected to the transducer. The value for the applied tension (0.4–0.5 g) was derived from LaPlace's transformation of the transmural pressure *in vivo* (Ikeda *et al.*, 1973). Tissues were equilibrated in Krebs solution containing either no O_2(lamb) or 2.5% O_2 (lamb and guinea-pig), plus 5% CO_2 and balance N_2, to duplicate the condition *in utero*. In the course of the experiment, the oxygen content was increased to mimic the neonatal condition, as it is known that due to the thickness of the vessel, and the attendant steep gradient of the gas within the wall (Fay, 1973), effective concentrations for ductus contraction (15, 30 and 95% O_2) exceed the normal range (Fay, 1971; Coceani *et al.*, 1986). The partial pressure of O_2 (Po_2) of the medium was measured with an Instrumentation Laboratory gas analyzer (mod. 1301) and was 6–11, 13–23, 63–89, 185–187 and 531–596 mmHg when using, respectively, gas mixtures with 0, 2.5, 15, 30 and 95% O_2. The organ bath was supplied from several reservoirs and a system of three-way valves allowed a rapid change from one perfusion fluid to another. Perfusion rate was approximately 2 ml min^{-1}. The room was kept darkened throughout the experiment.

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. Potassium-Krebs solution (55 mM) was prepared by substituting NaCl with an equimolar amount of KCl. The pH of the solution was 7.4 after equilibration with gas mixtures containing 5% CO_2.

ABT was synthesized as the hydrochloride salt following the general procedure of Campbell & Rees (1969). Briefly, benzotriazole (11.9 g) and KOH (28 g) were dissolved in 100 ml of water, and heated to 60°C. Hydroxylamine-*O*-sulphonic acid (22.6 g; purity 97%) was then added to the solution over a 30-min period while keeping the temperature around 65°C. Once prepared, the reaction mixture was heated to 70°C for 1 h. Afterwards, the mixture was cooled and extracted twice with equal volumes of ethylacetate. Organic phases were combined, dried over magnesium sulphate, and evaporated to dryness. The resulting residue was purified by silica gel column chromatography using ethylacetate:hexane (2:1, v/v). Final purity was confirmed on this layer chromatography by developing silica gel G plates in the system ethylacetate-hexane (2:1, v/v; R_F for ABT, 0.34). The hydrochloride salt was prepared by bubbling HCl through a solution of ABT in dichloromethane. The inhibitor activity of ABT was assessed against human cytochrome P450-based reactions by standard procedures. For this purpose, vaccinia virus expression vectors were used to express CYP1A2, 2C8, 2C9 and 3A4 in Hep G2 cells (Gonzalez *et al.*, 1991). Lymphoblastoid cells containing CYP1A1 (Crespi, 1991) were obtained from Gentest Corp. (Woburn, MA, U.S.A.), and grown as described previously (Shou *et al.*, 1994). CHO cells expressing human aromatase (Zhou *et al.*, 1990) were a gift from Dr S. Chen (Beckman Research Institute, Duarte, CA, U.S.A.). Preparation of cell membrane fractions containing expressed cytochrome P450 enzymes have been described previously for the Hep G2 cell (Shou *et al.*, 1994), lymphoblastoid cell (Shou *et al.*, 1994), and CHO cell (Grogan *et al.*, 1993) systems. Likewise, general incubation and assay conditions have been published for the following reactions: CYP1A1- and CYP1A2-benzo[a]pyrene 7, 8-diol formation (Shou *et al.*, 1994); CYP2C8- and CYP2C9-tolbutamide hydroxylation (Rettie *et al.*, 1994); CYP3A4-testosterone 6β-hydroxylation (Buters *et al.*, 1994); and CYP19 (aromatase)-androstenedione aromatization (Grogan *et al.*, 1993). The enzyme source was preincubated over different time intervals (max, 30 min) with 1 mM NADPH, in the absence or presence of 50 μM ABT, prior to the addition of the substrate, and the incubation was then continued for 15 to 30 min depending on the reaction. Results of the assays are summarized in Figure 1. In the actual experiments, ABT was prepared as a stock solution in saline, and this solution was diluted with Krebs medium as required.

Indomethacin (Sigma) was dissolved in distilled ethanol (10 mg ml^{-1}) prior to preparation of the final solution in Krebs medium. Sodium nitroprusside (SNP) (Sigma) was dissolved directly in medium, and precautions were taken to protect this solution from light.

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

Measurement of ET-1 release

Specimens of the whole ductus were transferred into polypropylene tubes containing 0.5 ml of bovine serum albumin (BSA, Sigma)-supplemented (0.05%) Krebs medium (Coceani & Kelsey, 1991). Tubes were placed in a shaking water bath at 37°C and, to maximize the yield of ET-1, the medium was gassed with the 95% O_2 mixture and incubation was continued for 2 h (Coceani & Kelsey, 1991; Coceani *et al.*, 1992). ET-1 was assayed directly in the incubate by a radioimmunoassay against the human porcine-type (Coceani & Kelsey, 1991), and values of release refer to the wet weight of the tissue.

Measurement of cyclic GMP content

Cyclic GMP was measured with a radioimmunoassay kit (^{125}I-labelled ligand; DuPont) both in ductal strips that had been incubated in the shaker and in strips that had been set up in a special drop-away bath and freeze-clamped at an appropriate time during the recording. In the former case, the incubation lasted 1 h and, afterwards, tissues were also freeze-clamped. Brass plates precooled in liquid nitrogen were used for this rapid freezing. In both groups of experiments, Krebs medium was equilibrated with the 95% O_2 mixture. The frozen tissue was ground to a powder, dispersed in 6% trichloroacetic acid, and sonicated (Brenson model W-140, 120 at 20 KH_Z) at 4°C. The homogenate was left on ice for 1 h and then centrifuged (2500 g for 15 min). The resulting supernatant was extracted 5 times with 2 vol of water saturated diethyl ether and, after discarding the ether phase, the water phase was lyophilized. The dry residue was reconstituted in 0.2 ml of sodium acetate buffer (50 mM; pH 6.2) and aliquots of this solution were treated with a mixture of acetic anhydride and triethylamine (1:2 v/v) before the assay. Cyclic GMP standard was also acetylated for preparation of a reference curve. Extraction and radioimmunoassay procedures were validated by spiking with cyclic GMP (unlabelled or tritium-labelled) either the buffer or samples with a known nucleotide content. Recovery of added standard was quantitative, and consequently, assay values are given without correction. The limit of detection was 0.002 pmol/tube.

Analysis of data

Contractile tension, which varied depending on the preparation and the experimental condition (see Results), is given after correction for the applied tension. Values are expressed as the mean ± s.e.mean. Statistical comparison of two means was made with Student's *t* test for unpaired observations. Multiple comparisons were made with an analysis of variance (ANOVA), followed by Duncan's multiple range test and Bonferroni's protected *t* test, as appropriate, to determine differences within groups (control or treatment) and between groups (control *vs* treatment).

Results

Ductal strips from the lamb contracted to a variable degree during equilibration with either the O_2-free or the 2.5% O_2 mixture (Figure 2a, b). However, the tension output tended to be higher with the former mixture, hence confirming earlier results (Coceani *et al.*, 1986). A further contraction occurred when the oxygen tension of the medium was raised to levels reproducing the neonatal condition, and the magnitude of the response increased with the oxygen concentration over the range examined (Figure 2a, b). At its peak, this contraction equalled that resulting from treatment with excess potassium (Figure 2a, b). At any step in the response curve, contractions were sustained and persisted for as long as the oxygen concentration remained elevated (max. 70 min). No difference in contractile behaviour, whether related to the oxygen concentration or the presence of high potassium in the medium, was noted between intact and endothelium-denuded preparations (Figure 3). Indomethacin (2.8 μM) contracted gradually the ductus pre-equilibrated at 2.5% O_2 and, once the response was fully developed, tension output (4.7 ± 0.2 g, $n = 6$) did not differ appreciably from that of the untreated ductus exposed to 95% O_2.

The guinea-pig ductus, unlike the lamb ductus, did not develop any tension during equilibration in medium gassed with 2.5% O_2. However, the vessel contracted upon exposure to either higher oxygen concentrations or excess potassium (Figure 4a, b). Indomethacin was not tested, because the guinea-pig ductus is peculiarly unresponsive to non-steroidal anti-inflammatory drugs (Coceani *et al.*, 1978) and, hence, it seemingly lacks a prostaglandin-based relaxing mechanism.

Effect of ABT on contractile tension

ABT relaxed the lamb ductus over the entire range of oxygen concentrations, from 2.5% upwards (Figure 2a, c). This re-

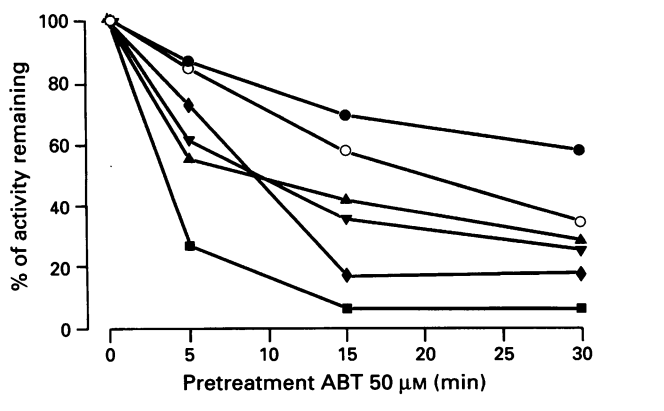

Figure 1 Time-dependent inhibition of expressed human cytochrome P450 activities by preincubation with 1-aminobenzotriazole (ABT, 50 μM). (●) CYP1A1- and (○) CYP1A2-benzo[a]pyrene 7,8-diol formation; (■) CYP2C8- and (◆) CYP2C9-tolbutamide hydroxylation; (▲) CYP3A4-testosterone 6β-hydroxylation; (▼) CYP19-androstenedione aromatization.

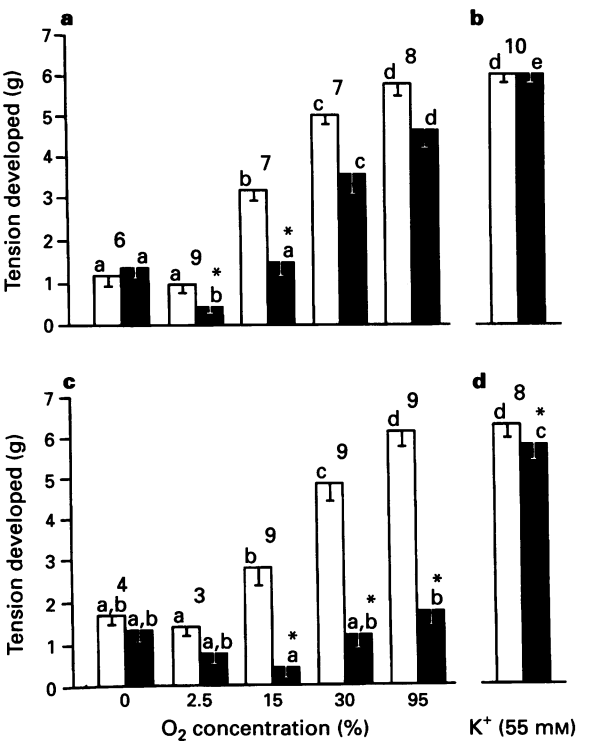

Figure 2 Effect of 1-aminobenzotriazole (ABT) on the contractile tension developed by the lamb ductus arteriosus at different O_2 concentrations (a, c) and in response to excess K^+ (b, d); open columns, control; solid columns, ABT 40 μM (a, b) or 0.8 mM (c, d). Responses to ABT refer to peak values. For each test condition, number of experiments (given above the columns) is the same in control and treatment groups. Note that potassium-Krebs was gassed with either 2.5% O_2 (b, $n = 9$; d, $n = 4$) or 95% O_2 (b, $n = 1$; d, $n = 4$); however, responses under the two conditions were not different and were pooled. In either the control or treatment sequence, different letters denote a significant difference. Significant differences between control and treatment groups are indicated with an asterisk.

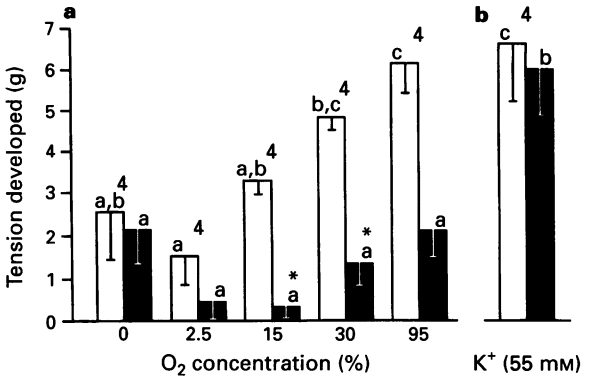

Figure 3 Effect of 1-aminobenzotriazole (ABT) on the contractile tension developed by the endothelium-denuded, lamb ductus arteriosus at different O_2 concentrations (a) and in response to excess K^+ (b); open columns, control; solid columns, ABT, 0.8 mM. Responses to ABT refer to peak values. For each test condition, number of experiments (given above each column) is the same in control and treatment groups. Note that potassium-Krebs was gassed with either 2.5% O_2 ($n=3$) or 95% O_2 ($n=1$); however, responses under the two conditions were not different and were pooled. In either the control or treatment sequence, different letters denote a significant difference. Significant differences between control and treatment groups are indicated with an asterisk.

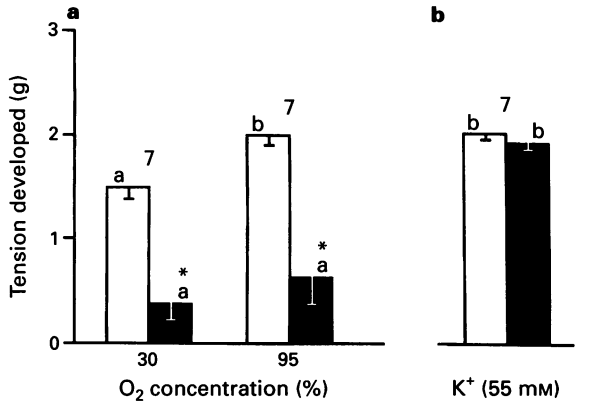

Figure 4 Effect of 1-aminobenzotriazole (ABT) on the contractile tension developed by the guinea-pig ductus arteriosus in response to O_2 (a) and excess K^+ (medium gassed with 2.5% O_2) (b); open columns, control; solid columns, ABT 1 mM. Responses to ABT refer to peak values. For each test condition, number of experiments (given above the columns) is the same in control and treatment groups. In either the control or treatment sequence, different letters denote a significant difference. Significant differences between control and treatment groups are indicated with an asterisk.

laxation was already evident at the 40-μM dose and, at the 0.8-mM dose, it was strong enough to cause nearly complete reversal of the contractile tension. Significantly, at either dose the compound had little or no effect on the ductus pre-equilibrated with the O_2-free gas mixture, despite the fact that the intrinsic tone was, if anything, greater than with the 2.5% O_2 mixture (Figure 2a, c). Whatever its magnitude, the ABT relaxation had an immediate onset and progressed rapidly to a peak (Figure 5a, b). Equally rapid was the reversal of the response after washing off the inhibitor from the bath, and the original tone was restored in full (Figure 5a, b). In some cases only, the relaxation persisted unabated throughout the treatment (22–46 min; up to 68 min in one case) (Figure 5b). More frequently, it subsided in part (Figure 5a), and this pattern was noted with either dose of ABT and regardless of the oxygen concentration of the medium. The endothelium-denuded duc-

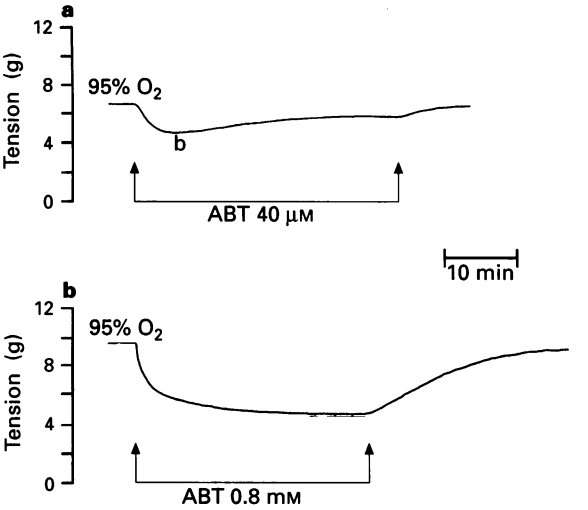

Figure 5 Typical response of the oxygen-contracted lamb ductus arteriosus to 1-aminobenzotriazole (ABT) 40 μM (a) and 0.8 mM (b). Values of tension above the zero baseline (1 g weight = 9.8 mN) include the load (1.5–1.6 g) applied at the start of the experiment.

tus behaved as the intact ductus in responding to 0.8 mM ABT (Figure 3). Likewise, the indomethacin-contracted, intact ductus at 2.5% O_2 was relaxed by 0.8 mM ABT (4.7±0.2 and 1.6±0.1 g tension before and after treatment, respectively; $n=6$) as markedly as the untreated ductus at 95% O_2 (see Figure 2a, c). In sharp contrast to the above findings, however, ABT relaxed marginally, or not at all, the potassium-contracted vessel (Figures 2 and 3).

In the guinea-pig, as in the lamb, ABT had a differential effect on the oxygen- versus the potassium-contracted ductus (Figure 4). As shown in a typical recording (Figure 6), ABT caused an immediate and marked relaxation if the tone of the vessel had been raised by oxygen, while, in contrast, the active tone resulting from treatment with excess potassium remained unchanged.

Effect of ABT on ET-1 release

Immunoreactive ET-1 (ir-ET-1) was consistently measurable in incubates of the lamb ductus and its concentration tended to be higher with intact than endothelium-denuded preparations (Table 1). Treatment with ABT resulted in lesser release of the peptide from both preparations, but changes failed to reach significance (Table 1).

Effect of ABT on cyclic GMP content

Cyclic GMP was measured in the lamb ductus at two points in time during the ABT treatment, as soon as the vessel had relaxed maximally in the organ bath (5–7 min) and after 1 h incubation in a tube with no tension applied. The former measurement allowed not only correlation of the cyclic GMP level with changes in contractile tension, but also observation of any transient rise in the nucleotide. This became important after finding that the ABT effect on ductal tone may subside partially with time (see above). In either set of experiments, however, ABT-treated tissues showed no difference in cyclic GMP content compared to tissues that had been handled in an identical manner without treatment (Table 2). In contrast, SNP promoted the accumulation of the nucleotide, thus confirming that the vessel had a viable guanylate cyclase (Table 2).

Discussion

The present investigation reaffirms the importance of a cytochrome P450-based mono-oxygenase reaction in the genera-

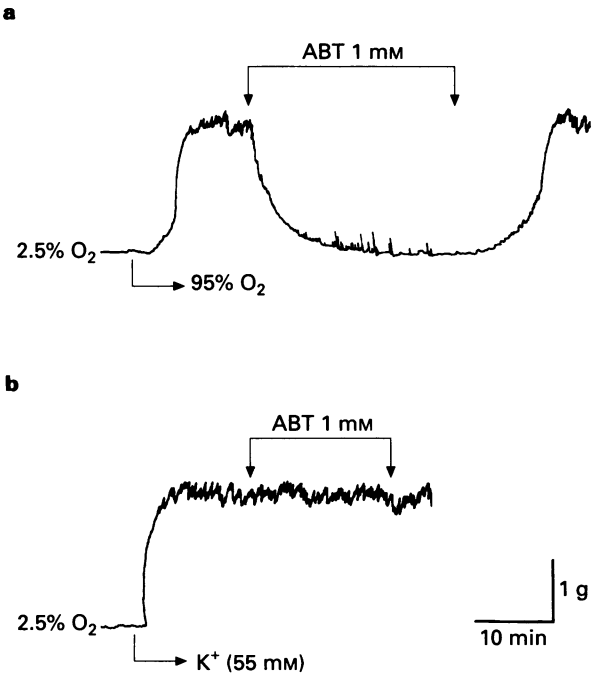

Figure 6 Comparison of the response of the oxygen- versus potassium-contracted guinea-pig ductus to 1-aminobenzotriazole (ABT). Calibration, 1 g weight = 9.8 mN.

Table 1 Release of immunoreactive endothelin-1 (ir-ET-1) from the lamb ductus arteriosus in the absence and presence of 1-aminobenzotriazole (ABT)

Ductus preparation	*ir-ET-1* (pg 100 mg^{-1} min^{-1}) *Control*	*ABT*
Intact	0.095 ± 0.02	0.068 ± 0.01
Endothelium-denuded	0.064 ± 0.007	0.049 ± 0.006

Albumin-supplemented (0.05%) Krebs medium was maintained at 37°C and gassed with 95% O_2:5% CO_2. Incubation continued for 2 h in the absence and presence of 1 mM ABT. Data are mean ± s.e.mean ($n = 5$ with the exception of the group, endothelium-denuded ductus/ABT, where $n = 3$).

Table 2 Effect of 1-aminobenzotriazole (ABT) and sodium nitroprusside (SNP) on cyclic GMP accumulation in the lamb ductus arteriosus

	Cyclic GMP (pmol g^{-1} tissue) *Control*	*ABT*	*SNP*
Condition a	11.3 ± 2.5	8.8 ± 1.6	–
Condition b	4 ± 0.8	5.1 ± 0.1	79.4 ± 2.6*

Ductal strips were freeze-clamped while recording mechanical tension in an organ bath (condition a) or after a 1-h incubation in a shaker (condition b). Measurements were taken before and during treatment with either 1 mM ABT or 10 μM SNP. Note that 'condition a' tissues were freeze-clamped at the peak of the ABT relaxation or after an equivalent time interval in the absence of the treatment (for details, see text). In either condition, Krebs medium was gassed with 95% O_2:5% CO_2. Data are mean ± s.e.mean ($n = 3$ for all groups except the SNP group where $n = 4$). *$P < 0.01$ vs control value.

tion of muscle tone in the ductus arteriosus and the contraction of the vessel to oxygen. In support of this concept are the following findings: (a) the demonstration that ABT potently relaxes the normal ductus, while leaving the potassium-contracted ductus virtually unaffected; in addition, the rapid time-course of responses and the range of effective doses accord with published data on the inhibitor (Mathews *et al.*, 1985); and (b) ABT relaxation is insignificant under extreme hypoxia when oxygen may become rate-limiting for the mono-oxygenase reaction and, by extension, may not be high enough to sustain the autocatalytic inactivation of the cytochrome P450 haemoprotein. Consistent with this concept is also the observation that the ABT effect occurs without apparently involving either PGE_2 or NO, from which one can infer that inhibition of a contractile process rather than activation of a relaxing process is the key event for the response. However, the attempt to link the ABT action with the reduced formation of ET-1, a presumptive effector of the oxygen contraction in the ductus (Coceani, 1994), was successful only in part, because the observed changes, though appropriate in their sign, did not reach significance. Based on this premise, the discussion will address two issues: the operation of the cytochrome P450 haemoprotein conditioning the contractile state of the ductus, and the implications of our findings for the mechanism of closure of the vessel at birth.

Although we have not been able to demonstrate mono-oxygenase activity in ductal tissue with certain assays (Coceani *et al.*, 1994), the existence of this activity is now documented, in a convincing albeit indirect manner, by the susceptibility of the vessel to the inhibitory action of ABT. The reason for the earlier negative result is not clear. The enzyme may be confined to a discrete site and may, therefore, have escaped detection. In fact, immunohistochemical examination has shown that only some muscle cells of the ductus exhibit in their plasma membrane a cytochrome P450 haemoprotein belonging to the CYP3A subfamily (Coceani *et al.*, 1994). Alternatively, considering the unique signalling function being assigned to this reaction, it may be that the enzyme utilizes a special substrate and may, consequently, not be amenable to identification by conventional assays. Important in this context is the fact that CYP3A haemoproteins are particularly versatile and may catalyze the metabolism of a wide range of substrates, both endogenous and exogenous (Wrighton & Stevens, 1992). These two possibilities are not mutually exclusive. Leaving aside this point, the finding that ABT is as effective on the guinea-pig ductus as it is on the lamb ductus, resolves an apparent inconsistency in our scheme. We have shown previously that the ductus in the guinea-pig is peculiarly unresponsive to CO at a concentration, relative to O_2(CO/O_2 ratio, 0.27), causing a marked relaxation of the lamb ductus (Coceani *et al.*, 1984). However, despite this differential sensitivity to CO, the two species share the capability to respond to a cytochrome P450 inhibitor (Coceani *et al.*, 1984). The lesser susceptibility of the guinea-pig ductus to CO, with the agent becoming effective only if its concentration greatly exceeds that of O_2(CO/O_2 ratio, 8.5) (Fay, 1971), was ascribed to low affinity of the target haemoprotein (Coceani *et al.*, 1984). The present result with ABT, coupled with the recent demonstration that well-characterized mono-oxygenase reactions are unevenly inhibited by CO (Leemann *et al.*, 1994), supports our original hypothesis on the exceptional behaviour of the guinea-pig ductus.

ET-1 release from the ductus was not inhibited by ABT, at least in an unequivocal manner, despite the many results implicating this peptide in the generation of contractile tone and the evidence linking causally its synthesis with a cytochrome P450-based mechanism (see Coceani, 1994). This unexpected result, however, may have a methodological cause. To collect enough ET-1 for the assay, tissues had to be incubated over a relatively long period of time (Coceani & Kelsey, 1991). During this time, the ABT effect may subside in part (see Results), so that the observed reduction in ET-1 could be an underestimate of the actual response. Furthermore, it has been recently reported that ET-1 undergoes a structural change, resulting in significant loss of both contractile activity and

immunoreactivity, when the oxygen tension of the medium is raised to levels similar to those used in our experiments (Yasuda *et al.*, 1994). This phenomenon has been linked to the formation of oxygen-derived free radicals (Yasuda *et al.*, 1994). If the same situation exists when testing ABT, inhibition of ET-1 formation could be offset, in part, by the greater stability of the released peptide in the medium. Mono-oxygenase reactions are, in fact, a major source of free radicals (Freeman & Crapo, 1982) and, by virtue of its action, ABT could also interfere with this particular process. This possibility warrants investigation.

A last point relates to the nature of the process sustaining the high tone of the ductus during hypoxia. The fact that ABT is marginally effective in this condition implies that the cytochrome P450 mechanism is not fully operational. Consistent with this idea is the observation that BQ123, an endothelin antagonist, mimics ABT in not relaxing the hypoxic ductus (Coceani *et al.*, 1992). Indeed, the actions of ABT and BQ123 coincide over the full range of oxygen concentrations and, likewise, the two agents share the lack of effect on the potassium-contracted vessel. While confirming the functional linkage between the cytochrome P450 and ET-1 systems, these findings suggest that another factor enters into play to maintain the high tone when oxygen levels are exceedingly low. Based on current knowledge of the mechanisms controlling ductal tone, with contractile and relaxing influences opposing each other (Smith & McGrath, 1991; Coceani, 1994), and the notion that the formation of relaxing agents (i.e. PGE_2 and NO) is also conditioned by oxygen (Needleman *et al.*, 1986; Kim *et al.*, 1993), it is conceivable that the contraction of the hypoxic ductus is determined, in part, by the withdrawal of a relaxing influence.

The realization that a cytochrome P450 haemoprotein plays a pivotal role in the development of contractile tone in the ductus arteriosus has important consequences, both conceptual and practical, for the process of closure of the vessel at birth. By its nature, this haemoprotein can be the target of inducers originating from inside or outside the body. Significant in this connection is the fact that dexamethasone and allied glucocorticoids, that is compounds sharing an inducing action on haemoproteins of the CYP3A subfamily, have been proven useful for the prevention of a persistent ductus in the premature (Clyman *et al.*, 1981a). Germane to this notion is the experimental finding that the same agents sensitize the ductus to the constrictor action of oxygen (Clyman *et al.*, 1981b, c). It is possible that, under certain conditions, this induction becomes strong enough to cause by itself constriction, and eventually closure, of the vessel. For example, it is well known that the ductus closes in cyanotic infants, although more gradually compared to healthy infants, despite the absence of the physiological trigger, namely, the postnatal rise in blood oxygen tension. A plausible explanation for this finding is that cortisol in the circulation, which is expected to be elevated in any state of stress in the infant as in the adult (Anand *et al.*, 1985; Hughes *et al.*, 1987), compensates for the lack of oxygen stimulation. In fact, it has been shown experimentally that dexamethasone may promote the contraction of the ductus *in utero* (Coceani *et al.*, 1994).

In conclusion, our study provides additional support to the concept that a cytochrome P450 haemoprotein, possibly belonging to the CYP3A subfamily, functions in the ductus arteriosus as a signal transducer for oxygen and a conditioning factor in the generation of contractile tone. This haemoprotein is viewed as a catalytic element in a, hitherto uncharacterized, mono-oxygenase reaction controlling the synthesis of endothelin, the ultimate effector for the contraction.

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References

ANAND, K.J.S., YACOUB, M.H., BROWN, M.J., SIPPELL, W.G. & AYNSLEY-GREEN, A. (1985). Endocrine and metabolic regulation in term neonates (TN) undergoing cardiac surgery (CS) with cardiopulmonary bypass (CPB). *Pediatr. Res.*, **19**, 628.

BODACH E., COCEANI, F., DUMBRILLE, A., OKPAKO, D.T. & OLLEY, P.M. (1980). The response of the isolated ductus arteriosus to transmural stimulation and drugs. *Br. J. Pharmacol.*, **71**, 419–427.

BUTERS, J.T.M., KORZEKWA, K.R., KUNZE, K.L., OMATA, Y., HARDWICK, J.P. & GONZALEZ, F.J. (1994). cDNA-directed expression of human cytochrome P450 CYP3A4 using baculovirus. *Drug Metab. Dispos.*, **22**, 688–692.

CAMPBELL, C.D. & REES, C.W. (1969). Reactive intermediates. Part 1. Synthesis and oxidation of 1- and 2-aminobenzotriazole. *J. Chem. Soc.*, **C**, 742–747.

CLYMAN, R.I., BALLARD, P.L., SNIDERMAN, S., BALLARD, R.A., ROTH, R., HEYMANN, M.A. & GRANBERG, J.P. (1981A). Prenatal administration of betamethasone for prevention of patent ductus arteriosus. *J. Pediatr.*, **98**, 123–126.

CLYMAN, R.I., MAURAY, F., ROMAN, C., HEYMANN, M.A., BALLARD, P.L., RUDOLPH, A.M. & PAYNE, B. (1981b). Effects of antenatal glucocorticoid administration on ductus arteriosus of preterm lambs. *Am. J. Physiol.*, **241**, H415–H420.

CLYMAN, R.I., MAURAY, F., ROMAN, C., RUDOLPH, A.M. & HEYMANN, M.A. (1981c). Glucocorticoids alter the sensitivity of the lamb ductus arteriosus to prostaglandin E_2. *J. Pediatr.*, **98**, 126–128.

COCEANI, F. (1994). Control of the ductus arteriosus - A new function for cytochrome P450, endothelin and nitric oxide. *Biochem. Pharmacol.*, **48**, 1315–1318.

COCEANI, F., BREEN, C.A., LEES, J.G., FALCK, J.R. & OLLEY, P.M. (1988). Further evidence implicating a cytochrome P-450-mediated reaction in the contractile tension of the lamb ductus arteriosus. *Circ. Res.*, **62**, 471–477.

COCEANI, F., HAMILTON, N.C., LABUC, J. & OLLEY, P.M. (1984). Cytochrome P450-linked monooxygenase: involvement in the lamb ductus arteriosus. *Am. J. Physiol.*, **246**, H640–H643.

COCEANI, F., HUHTANEN, D., HAMILTON, N.C., BISHAI, I. & OLLEY, P.M. (1986). Involvement of intramural prostaglandin E_2 in prenatal patency of the lamb ductus arteriosus. *Can. J. Physiol. Pharmacol.*, **64**, 737–744.

COCEANI, F. & KELSEY, L. (1991). Endothelin-1 release from lamb ductus arteriosus: relevance to postnatal closure of the vessel. *Can. J. Physiol. Pharmacol.*, **69**, 218–221.

COCEANI, F., KELSEY, L., ACKERLEY, C., RABINOVITCH, M. & GELBOIN, H. (1994). Cytochrome P-450 during ontogenic development: occurrence in the ductus arteriosus and other tissues. *Can. J. Physiol. Pharmacol.*, **72**, 217–226.

COCEANI, F., KELSEY, L. & SEIDLITZ, E. (1992). Evidence for an effector role of endothelin in closure of the ductus arteriosus at birth. *Can. J. Physiol. Pharmacol.*, **70**, 1061–1064.

COCEANI, F., OLLEY, P.M., BISHAI, I., BODACH, E. & WHITE, E.P. (1978). Significance of the prostaglandin system to the control of muscle tone of the ductus arteriosus. In *Prostaglandins and Perinatal Medicine, Advances in Prostaglandin and Thromboxane Research.* Vol IV. ed. Coceani, F. & Olley, P.M. pp. 325–333, New York: Raven Press.

CRESPI, C.L. (1991). Expression of cytochrome p450 cDNAs in human B lymphoblastoid cells: applications to toxicology and metabolite analysis. *Methods Enzymol.*, **206**, 123–129.

DRAPER, R.L. (1920). The prenatal growth of the guinea-pig. *Anat. Rec.*, **18**, 369–392.

FAY, F.S. (1971). Guinea-pig ductus arteriosus. I. Cellular and metabolic basis for oxygen sensitivity. *Am. J. Physiol.*, **221**, 470–479.

FAY, F.S. (1973). Biochemical basis for response of ductus arteriosus to oxygen. In *Fetal and Neonatal Physiology*. ed. Comline, K.S., Cross, K.W., Dawes, G.S. & Nathanielsz, P.W. pp. 136–140. Cambridge: Cambridge University Press.

FREEMAN, B.A. & CRAPO, J.D. (1982). Biology of disease. Free radicals and tissue injury. *Lab. Invest.*, **47,** 412–426.

GONZALEZ, F.J., AOYAMA, T. & GELBOIN, H.V. (1991). Expression of mammalian cytochrome p450 using vaccinia virus. *Methods Enzymol.*, **206,** 85–92.

GROGAN, J., SHOU, M., ZHOU, D., CHEN, S. & KORZEKWA, K.R. (1993). Use of aromatase (CYP19) metabolite ratios to characterize electron transfer from NADPH-cytochrome P450 reductase. *Biochemistry*, **32,** 12007–12012.

HALPERT, J.R., GUENGERICH, F.P., BEND, J.R. & CORREIA, M.A. (1994). Contemporary issues in toxicology. Selective inhibitors of cytochromes P450. *Toxicol. Appl. Pharmacol.*, **125,** 163–175.

HUGHES, D., MURPHY, J.F., DYES, J., ROBINSON, J.A., RIAD-FAHMY, D. & HUGHES, I.A. (1987). Blood spot corticoid concentrations in ill preterm infants. *Arch. Dis. Child.*, **62,** 1014–1018.

IKEDA, M., RUBINSTEIN, E.H. & SONNENSCHEIN, R.R. (1973). Interaction of three factors in the closure of the ductus arteriosus. *Proc. Soc. Exp. Biol. Med.*, **143,** 354–359.

KIM, N., VARDI, Y., PADMA-NATHAN, H., DALEY, J., GOLDSTEIN, I. & SAENZ DE TEJADA, I. (1993). Oxygen tension regulates the nitric oxide pathway. *J. Clin. Invest.*, **91,** 437–442.

KNICKLE, L.C. & BEND, J.R. (1992). Dose-dependent, mechanism-based inactivation of cytochrome P450 monooxygenases *in vivo* by 1-aminobenzotriazole in liver, lung, and kidney of untreated, phenobarbital-treated, and β naphthoflavone-treated guinea pigs. *Can. J. Physiol. Pharmacol.*, **70,** 1610–1617.

KNICKLE, L.C. & BEND, J.R. (1994). Bioactivation of arachidonic acid by the cytochrome P450 monooxygenases of guinea pig lung: the orthologue of cytochrome P4502B4 is solely responsible for formation of epoxyeicosatrienoic acids. *Mol. Pharmacol.*, **45,** 1273–1280.

LEEMANN, T., BONNABRY, P. & DAYER, P. (1994). Selective inhibition of major drug metabolizing cytochrome P450 isozymes in human liver microsomes by carbon monoxide. *Life Sci.*, **54,** 951–956.

MARKS, G.S., BRIEN, J.F., NAKATSU, K. & MCLAUGHLIN, B.E. (1991). Does carbon monoxide have a physiological function? *Trends Pharmacol. Sci.*, **12,** 185–188.

MATHEWS, J.A., DOSTAL, L.A. & BEND, J.R. (1985). Inactivation of rabbit pulmonary cytochrome P-450 in microsomes and isolated perfused lungs by the suicide substrate 1-aminobenzotriazole. *J. Pharmacol. Exp. Ther.*, **235,** 186–190.

NEEDLEMAN, P., TURK, J., JAKSCHIK, B.A., MORRISON, A.R. & LEFKOWITH, J.B. (1986). Arachidonic acid metabolism. *Annu. Rev. Biochem.*, **55,** 69–102.

ORTIZ DE MONTELLANO, P.R. & MATHEWS, J.M. (1981). Autocatalytic alkylation of the cytochrome P-450 prosthetic haem group by 1-aminobenzotriazole. *Biochem. J.*, **195,** 761–764.

RETTIE, A.E., WIENKERS, L.C., GONZALEZ, F.J., TRAGER, W.F. & KORZEKWA, K.R. (1994). Impaired (s)-warfarin metabolism catalyzed by the R144C allelic variant of CYP2C9. *Pharmacogenetics*, **4,** 39–42.

SHOU, M., KORZEKWA, K.R., CRESPI, C.L., GONZALEZ, F.J. & GELBOIN, H.V. (1994). Metabolism of benzo[a]pyrene by seven cDNA expressed human cytochromes p450. *Polycyclic Arom. Compounds*, **7,** 1–9.

SMITH, G.C.S. & MCGRATH, J.C. (1991). Prostaglandin E_2 and fetal oxygen tension synergistically inhibit response of isolated fetal rabbit ductus arteriosus to norepinephrine. *J. Cardiovasc. Pharmacol.*, **17,** 861–866.

SOMLYO, A.V. & SOMLYO, A.P. (1964). Vasomotor function of smooth muscle in the main pulmonary artery. *Am. J. Physiol.*, **206,** 1196–1200.

WRIGHTON, S.A. & STEVENS, J.C. (1992). The human hepatic cytochromes P450 involved in drug metabolism. *Crit. Rev. Toxicol.*, **22,** 1–21.

YASUDA, N., KASUYA, Y., YAMADA, G., HAMA, H., MASAKI, T. & GOTO, K. (1994). Loss of contractile activity of endothelin-1 induced by electrical field stimulation-generated free radicals. *Br. J. Pharmacol.*, **113,** 21–28.

ZHOU, D., POMPON, D. & CHEN, S. (1990). Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening. *Cancer Res.*, **50,** 6949–6954.

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