Endothelin A receptor is necessary for O₂ constriction but not closure of ductus arteriosus

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Coceani, F., Y.-A. Liu, E. Seidlitz, L. Kelsey, T. Kuwaki, C. Ackerley, and M. Yanagisawa. Endothelin A receptor is necessary for O₂ constriction but not closure of ductus arteriosus. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H1521-H1531, 1999.—In vitro and in vivo techniques were developed with genetically modified mice to determine whether endothelin-1 (ET-1) functions as an O₂ mediator in closure of the ductus arteriosus (DA) at birth. Wild-type CD-1 and 129/SvEv mice with ETA receptor −/−, ++/−, and ++/++ genotypes were used. Isolated DA from term ET-A ++/++ fetuses contracted to O₂ (5–95%) and a thromboxane A₂ analog (ONO-11113, 0.1 µM). Instead, ET-1 elicited a dual response with weak relaxation (0.1 nM) preceding contraction (1–100 nM). Indomethacin (2.8 µM) was also a constrictor. ETA −/− DA, unlike ETA ++/− DA, contracted marginally to O₂ and ET-1 but responded to ONO-11113. O₂ contraction was also reduced in ETA ++/− DA. In vivo, DA constricted equally in tracheotomized ETA −/− and ETA ++/− newborns. Conversely, no DA constriction was seen in hyperoxic ETA −/− fetuses in utero, although it occurred in ETA ++/− and ++/++ littermates. We conclude that ET-1 mediates the DA constrictor response to O₂. Without ET-1, however, the vessel still closes postnatally, conceivably caused by the withdrawal of relaxing influence(s).

The ductus arteriosus is a large muscular shunt in the fetus, connecting the pulmonary artery with the aorta and allowing blood to bypass the unexpanded lungs. At birth, as the lungs acquire their ventilatory function and blood PO₂ rises to extrauterine values, the ductus constricts and within hours undergoes functional closure. Both prenatal patency and postnatal closure of the ductus are known to be actively induced, but questions remain about the identity of the effector agents (3, 10, 29). Although there is good evidence implicating PGE2 (3, 10, 29), either alone or in combination with nitric oxide (NO) (1, 7) in ductus patency, the mechanism of ductus closure is being debated (29, 33).

We have proposed that ductus closure takes place through a multistep process in which O₂ is the trigger, a cytochrome P-450-based monoxygenase reaction the signal transducer, and endothelin-1 (ET-1) the effector (4, 8, 9), acting via the ETA receptor subtype. Coincidently, we have assumed that subsidence of the relaxing influence of PGE2 at birth, secondary to the fall in blood PGs and the reduced sensitivity of ductal muscle to PGE2 (3, 10, 29), is an accessory event. However, recent work in mice lacking the EP4 receptor subtype, which is the putative target for PGE2 in the ductus (22, 30), points to a major role of this PG mechanism in ductus closure (22). Furthermore, a group (15) has questioned our scheme involving ET-1 as O₂ messenger in the ductus, whereas other investigators (21, 32) have linked the O₂ constriction to inhibition of the K⁺ channels.

The development during the past few years of mice strains with the targeted deletion of genes encoding distinct components of the ET-1 system (2, 19, 35) provides the means of directly assessing the role of the peptide in ductus closure. Among the available mutations, we opted to use animals lacking the ETA receptor (2) rather than ET-1 itself (19, 35) to avoid any confounding action of the maternally derived peptide on the null mutant fetus (18). However, before addressing this question, certain methodological issues had to be solved. The mouse fetal ductus had never been studied in vitro, and experiments were required to verify the viability of the preparation. To assess the behavior of the ductus in vivo, specifically its closure, steps had to be taken to overcome the occurrence in ETA null mutants of craniofacial anomalies precluding respiratory function and, hence, survival after birth (2). Once these issues had been settled, our specific aim was to ascertain whether deletion of the ETA receptor, with the attendant loss of ET-1 contractile action, interferes with O₂ constriction of the fetal ductus in vitro and the postnatal closure of the vessel in vivo.

METHODS

Experiments were carried out in inbred 129/SvEv mice with ET-A −/−, ++/−, and ++/++ genotypes (litter size 3–12, mean 9). Wild-type CD-1 mice (litter size 7–18, mean 13) formed a separate control. Fetuses were used near term, and their gestational age was 18 days in all but a few early experiments in which age was 19 days. However, results were identical at the two ages and were pooled. ETA −/− mice were identified by their characteristic craniofacial anomaly (2), whereas analysis of tail genomic DNA was necessary with the heterozygous and wild-type littermates (2). About 25% of the ET-A −/− fetuses could not be used because of gross cardiovascular malformations (2). Surgical procedures and experimental protocols were approved by the Animal Care Committee of our institutions.
Solutions and Drugs

Krebs medium had the following composition (in mM): 118 NaCl, 4.7 KCl, 1 KH2PO4, 0.9 MgSO4, 2.5 CaCl2, 11.1 glucose, and 25 NaHCO3. Potassium-Krebs solution (55 mM) was prepared by substituting NaCl with an equimolar amount of KCl. Solutions were bubbled with gas mixtures containing either no O2 or O2 in various concentrations (2.5, 5, 12.5, 30, and 95%) plus 5% CO2 and, when required, balance N2. In the actual experiment, a single mixture (2.5% O2) was chosen to mimic the fetal condition, whereas several mixtures (5, 12.5, 30, and 95%) were used to ascertain the susceptibility of the vessel to O2 with the aim of duplicating the neonatal condition (optimally at 12.5% O2). O2 was measured with an Instrumentation Laboratory gas analyzer (model 1610, Lexington, MA) and was 1.17 ± 0.05, 2.35 ± 0.04, 3.1 ± 0.07, 7.51 ± 0.06, 26.6 ± 0.2, and 92.7 ± 0.5 kPa (pH 7.4 ± 0.003) when gas mixtures were used with 0, 2.5, 12.5, 30, and 95% O2, respectively.

ET-1 (human and porcine type; Peninsula, Belmont, CA) was dissolved under aseptic conditions in sterile water containing 0.05% human serum albumin. Aliquots of this stock solution (0.25 mg/ml) were stored at −20°C and, on the day of the experiment, were diluted with bovine serum albumin-supplemented saline (0.05%). The thromboxane A2 (TxA2) analog 9,11-epithio-11,12-methano-TxA2 (ONO-11113, courtesy of ONO Pharmaceutical, Osaka, J apan) was dissolved in distilled ethanol (5 mg/ml), and aliquots (stored at −70°C) were diluted with Tris buffer (pH 7.4). The cyclooxygenase inhibitor indomethacin (Sigma, St. Louis, MO) was also dissolved in ethanol (10 mg/ml) before preparation of the final solution in Krebs medium. Ethanol in the fluid bathing vessels, and its length was measured in situ. Any animal with a ductus < 300 µm in length could not be used. Afterward, the pulmonary artery trunk was split partially open at the junction with the ductus arteriosus from CD-1 and 129/SvEv strain can be bred more easily. Subsequent experiments in preparation. This was done in the CD-1 mouse since this strain can be bred more easily. Subsequent experiments in CD-1 fetuses (0.9–1.35 g body wt, mean wt 1.1). Table 1. Resting dimensions of isolated ductus arteriosus from CD-1 and 129/SvEv (ETA-deficient) full-term fetal mice

<table>
<thead>
<tr>
<th></th>
<th>CD-1 +/-</th>
<th>--/--</th>
<th>+/-</th>
<th>+/+</th>
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<tbody>
<tr>
<td>n</td>
<td>33</td>
<td>6</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Internal diameter, µm</td>
<td>139 ± 1</td>
<td>142 ± 3</td>
<td>138 ± 1</td>
<td>143 ± 2</td>
</tr>
<tr>
<td>Vessel length, mm</td>
<td>506 ± 16</td>
<td>562 ± 35</td>
<td>578 ± 23</td>
<td>593 ± 36</td>
</tr>
<tr>
<td>Short side</td>
<td>525 ± 15</td>
<td>575 ± 30</td>
<td>600 ± 20</td>
<td>599 ± 36</td>
</tr>
<tr>
<td>Wall thickness, µm</td>
<td>19 ± 0.5</td>
<td>22 ± 3</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is no. of vessels with each vessel obtained from a different animal. *Vessel length of ductus arteriosus is uneven because of its insertion into aorta at an angle. Short side was taken for normalization of internal circumference and calculation of tension output (see In Vitro Studies).
In Vivo Studies

Experiments were carried out in fetuses or newborns depending on the protocol. In the former case, animals were delivered by caesarean section under halothane anesthesia unless otherwise specified, whereas in the latter case they were delivered (under halothane or after cervical dislocation, see EXPERIMENTS IN CD-1 MICE, below) at different intervals after a vaginal delivery. Any animal surviving after birth was placed on a warm metal plate (−3°C) and was also heated with a lamp.

Experimental design. Changes in ductus caliber occurring in vivo during the transition from the pre- to postnatal condition were assessed by fixing the vessel in situ with the whole body freezing technique (16). Because the ETA null mutant does not survive postnatally because of mechanical obstruction in the upper airway (2), separate experiments were performed in CD-1 mice to verify whether the neonatal condition could be reproduced in utero by making the dam hyperoxic and, whether closure of the ductus progresses normally in fetuses tracheotomized immediately after caesarean delivery. The latter procedure has been used to maintain living mice lacking the ET-1 gene (20).

EXPERIMENTS IN CD-1 MICE. Ductus caliber was measured in fetuses, whether delivered from a normoxic or hyperoxic dam, in newborns at different ages (1- to 12-h old), and in fetuses that had been subjected to tracheotomy and survived for 3 h.

Pregnant mice were made hyperoxic by breathing 100% O₂ inside a box for 3 h. Throughout this procedure, animals were anesthetized with chloral hydrate (35–70 mg/100 g ip, supplemented as required) and kept warm with water-filled bags at 39°C. Some fetuses were frozen for morphological analysis, whereas others were used for measurement of blood PO₂. Fetuses from normoxic dams served as reference. In either case, because of the small size of animals, blood was collected through a cut made at the apex of the heart. Hence, samples were a mixture of placental and venous blood. Blood PO₂ was also measured in the mother, using the descending aorta as the sampling site.

Fetuses were tracheotomized as previously described (20) and their respiratory rate was assessed at regular intervals throughout the 3-h observation period. Afterward, they were divided in two groups for morphological analysis and blood PO₂ measurement, respectively. Whereas animals in the first group were killed by cervical dislocation, those in the second group were anesthetized with halothane, and blood was collected by inserting a sharp-tipped glass micropipette into the abdominal aorta. Blood PO₂ was measured with an Instrumentation Laboratory gas analyzer (see Solutions and Drugs) or, in the case of small-volume samples (20–30 µl), with a Ciba-Corning gas analyzer (model 178, Tokyo, Japan).

EXPERIMENTS IN 129/SvEv MICE. Protocols were the same as in CD-1 mice (see EXPERIMENTS IN CD-1 MICE, In Vivo Studies). ETA−/− fetuses, however, could be kept alive only through a tracheotomy. Success rate was about 43%; failures were caused primarily by an inability to breathe. Less frequently, animals stopped breathing for no apparent reason before reaching the 3-h mark. In contrast, all 129/SvEv fetuses, heterozygous for the ETA gene mutation, and the CD-1 fetuses remained alive with or without a tracheotomy.

Processing of specimens. Mice, whether fetal or newborn, were processed according to Hörnblad and Larsson (16) with few modifications. Briefly, animals were placed with their side up in a Petri dish and were covered with an embedding medium (Tissue-Tek optimum cutting tempera-
ture compound (OCT); Sakura Finetek, Torrance, CA). The dish with the animal was quickly wrapped with aluminum foil and immersed in liquid nitrogen. Once frozen, specimens were stored at −20°C for at least 24 h before further workup. To prepare a block of tissue with the ductus, the carcass was fixed in the frozen state of its OCT embedding. With a razor blade, soft tissues covering the dorsum were removed, making certain that the exposed surface would be approximately parallel to the underlying descending aorta and hence perpendicular to the expected orientation of the ductus. With the use of this cut as a reference, a block of tissue was prepared and placed, with its dorsal surface down, onto a vinyl specimen mold (Tissue-Tek II Cryomold; Miles Laboratory, Elkhart, IN) to be embedded in OCT and frozen at −20°C for storage. Afterward, the OCT mold was removed and the tissue block was mounted in a Leitz freezing microtome (model 1720, Leitz, Germany). Serial sections (10-µm thick) were obtained along a plane perpendicular to the main axis of the ductus and were stained with 1% methylene blue.

Morphometric analysis. Images were digitized with Media Grabber v2.2 software (RasterOps, Waco, TX) and stored on a Macintosh iMac computer. Lumen area of the ductus was measured on the stored images with NIH Image 1.60 software (National Institutes of Health, Bethesda, MD), and the section with the smallest area was selected for computation.

Light and Electron Microscopy

Morphological examination was carried out in ductus specimens that had been prepared in the usual manner and pinned for fixation to a wax board. For routine histology, the vessel was fixed in neutral buffered Formalin and embedded in paraffin. Transverse sections were examined after staining with Movat pentachrome stain.

For transmission electron microscopy, specimens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, postfixed in 1% OsO4, and embedded in Epon Araldite. Ultrathin sections were contrasted with uranyl acetate and lead citrate before analysis in a Phillips instrument (model 201, Eindhoven, The Netherlands).

Analysis of Responses

Effects of contractile agents in vitro were measured by the fractional rise in tension over basal tension and were expressed in absolute values (mN/mm).

Data are means ± SE. Comparisons were made with the use of a Student’s t-test or ANOVA, followed when required by the Dunnett’s test. Differences were considered significant at P < 0.05.

RESULTS

In Vitro Studies

CD-1 mice. The isolated ductus arteriosus from wild-type CD-1 mice was consistent in size among experiments (Table 1) and, in responding to physiological and pharmacological stimuli, showed no major differences compared with other species (29). The ductus developed a variable degree of tension during equilibration at 2.5% O2 (0.14 ± 0.03 mN/mm in 60–90 min, n = 27) and, once stabilized, contracted to O2 in a concentration-dependent manner (Fig. 2). However, whereas four of these tissues showed a maximal, or near-maximal, response with O2 concentrations between 12.5 and 30%, the remainder required the 95% concentration to attain a peak. Regardless of its magnitude, the O2 contraction was sustained and often exhibited rapid, low-amplitude fluctuations (Fig. 2, inset). In no instance, on the other hand, did O2 cause a relaxation.

Indomethacin (2.8 µM) at 2.5% O2 also contracted the ductus, and the contraction equalled or even exceeded the response to O2 (maximum 0.8 ± 0.17 mN/mm, n = 4). This response had quick onset and a biphasic course, with a first peak at 10–20 min and a second peak at ~60 min. Conversely, the ETB antagonist BQ-788 (1 µM) had a marginal and often transient contractile effect (maximum 0.1 ± 0.07 mN/mm, n = 9).

ET-1 elicited a dual response, consisting of a modest relaxation at 0.1 nM and a progressively greater contraction with increasing concentrations up to a maximum at about 10 nM (Fig. 3A). No ET-1-induced relaxation was seen in ductus preparations pretreated with BQ-788, and the contraction developed unabated at all concentrations (Fig. 3B). This contraction, as evident from preliminary observations, could be inhibited by the ETA antagonist BQ-123 (1 µM) (Y.-A. Liu and F. Coceani, unpublished observations). The TXA2 analog ONO-11113 (0.1 µM) was also a constrictor agent and, at the chosen concentration, produced a greater contraction (0.84 ± 0.06 mN/mm, n = 29) than ET-1 (10 or 100 nM). With either spasmogen, responses were immediate in onset and developed rapidly to a plateau.

129/SvEv mice. The ductus of ETA−/− fetal mice did not differ in size from either that of the littermates, whether heterozygous or wild-type, or from that of the
CD-1 mice (Table 1). Despite the similarity, however, disruption of the ET_A gene caused marked changes in the contractile behavior of the vessel. ETA<sup>2</sup>/2 preparations did not generate any tone during the initial equilibration period and, in that respect, behaved differently from both the ETA<sup>1</sup>/2 (tension 0.14 ± 0.03 mN/mm, n = 9) and ET_A<sup>1</sup>/1 (tension 0.17 ± 0.03 mN/mm, n = 6) preparations. In addition, as expected, the ETA<sup>2</sup>/2 ductus, unlike the ductus from heterozygous and wild-type littermates, contracted marginally to ET-1 (100 nM) (Fig. 4A). All three preparations developed instead a contraction to ONO-11113 (0.1 µM) whose amplitude was relatively smaller with ETA<sup>2</sup>/2 (Fig. 4B). Most significant, however, was the fact that the tonic contraction to O<sub>2</sub>, although weaker in ET_A<sup>1</sup>129/SvEv mice than wild-type CD-1 mice (compare Fig. 5C with Fig. 2), was nearly absent in the ET_A null mutant (Fig. 5A). Also missing in the ET_A<sup>2</sup>/2 ductus were phasic contractions to O<sub>2</sub>, which in the ET_A<sup>+/+</sup> ductus were characteristically superimposed over the tonic contraction (Fig. 5D). These contractions were irregular or rhythmic, but in both cases their magnitude increased with gas concentration (0.02 ± 0.01, 0.05 ± 0.02, 0.11 ± 0.07, and 0.28 ± 0.05 mN/mm with, respectively, 5, 12.5, 30, and 95% O<sub>2</sub>; n = 6 for all values). Not only were contractile events, whether tonic or phasic, curtailed in the ET_A<sup>−/−</sup> ductus, but also a relaxation occurred in two of six preparations with each O<sub>2</sub> concentration. The latter response, which in the ET_A<sup>−/−</sup> ductus was also seen twice but only at 5% O<sub>2</sub> (mean loss of tension, 0.08 mN/mm), had variable magnitude (0.04 to 0.12 mN/mm) and subsided, in part or in full, with time.

In responding to O<sub>2</sub>, the ductus from mice heterozygous for the ET_A mutation combined traits of the homozygous<sup>−/−</sup> and the wild-type<sup>+/+</sup> genotypes. This points to an ET_A gene-dosage effect. As shown in Fig. 5B, O<sub>2</sub> had a contractile effect, but contrary to findings in the wild-type (Fig. 5C), it was barely visible and did not increase linearly with the O<sub>2</sub> concentration.
Coincidentally, phasic contractions were absent in half the preparations and, when present, had smaller amplitude (0.16 ± 0.04 mN/mm at 95% O₂, n = 5). In addition, O₂ caused occasionally a transient relaxation rather than a contraction.

**In Vivo Studies**

The ductus was patent in all fetuses, regardless of strain and ETA genotype (Figs. 6 and 7). The genotype, however, influenced the response of the vessel to O₂.

CD-1 mice. In intact, vaginally delivered mice, the ductus constricted rapidly during the first 3 h after birth and more slowly afterwards until complete closure was attained by 10–12 h (Fig. 6). No difference was noted between these animals and animals tracheotomized newborn (value at 3 h after cesarean delivery) (n = 4). Where necessary, points have been offset to improve visibility.

**Fig. 6.** Closure of ductus arteriosus in CD-1 mice in vivo. ○, Time course of postnatal closure of ductus. Zero point refers to full-term fetus in utero (n = 4–14). ●, Ductal constriction in utero after 3 h of maternal hyperoxia (value at time 0) (n = 10) and in tracheotomized newborn (value at 3 h after cesarean delivery) (n = 4). Where necessary, points have been offset to improve visibility.

**Fig. 7.** Postnatal closure of ductus arteriosus in 129/SvEv (ETA-deficient) mice. A: full-term fetus. B: newborn 3 h after birth. In both groups, no. of animals is given above each column. ETA −/− newborns were survived by making a tracheotomy (trach) immediately after cesarean delivery. Part of ETA +/+ newborns were tracheotomized and served as control. *P < 0.01 compared with fetus of same genotype.
Morphological Analysis

On visual examination, the ductus of ET\textsubscript{A} \textasciitilde \textasciitilde fetal mice (129/SvEv strain) could not be distinguished from that of littermates, whether wild or heterozygous, and the CD-1 mice. Likewise, no difference was noted among these vessels on both light and electron microscopy. As evident from a representative experiment (Fig. 9A), the wall of the ET\textsubscript{A} \textasciitilde \textasciitilde ductus presented a continuous endothelial lining and a well-developed media consisting of layers of smooth muscle cells with elastic laminae interposed. Ultrastructurally, endothelial cells appeared intact and were separated from the underlying muscle by an elastic lamina (Fig. 10). Equally consistent regardless of genotype were ductal changes in the newborn. As shown in Fig. 9B, pertaining again to data from the ET\textsubscript{A} \textasciitilde \textasciitilde group, the vessel was clearly constricted and its narrow lumen was occupied, in part, by heaped up endothelial cells.

**DISCUSSION**

Our results prove that it is feasible to set up an isolated preparation of fetal mouse ductus arteriosus. With this preparation and appropriate interventions to survive the ET\textsubscript{A} \textasciitilde \textasciitilde mouse postnatally and expose the same mutant antenatally to O\textsubscript{2}, we have introduced a new approach to study ductus regulation. The isolated ductus from wild-type mice appears functional in all respects. It is contracted strongly by indomethacin. Hence, from this finding and the reported effectiveness of the drug in vivo (22), we may assume that, in the mouse as in other species (3, 10, 29), prenatal patency can be maintained but from normal appearance of animals could be either (+/+) or (\textasciitilde \textasciitilde \textasciitilde). Arterial blood PO\textsubscript{2} of the hyperoxic dam, 76–83 kPa. *P < 0.05, compared with fetus of same genotype from normoxic dam. ND, not determined.

**Table 2. Blood PO\textsubscript{2} in fetal and neonatal 129/SvEv mice**

<table>
<thead>
<tr>
<th>ET\textsubscript{A} Genotype</th>
<th>Fetus Normoxic dam</th>
<th>Fetus Hyperoxic dam</th>
<th>Newborn</th>
</tr>
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<tbody>
<tr>
<td>\textasciitilde \textasciitilde</td>
<td>6.3 ± 0.7 (6)</td>
<td>9.8 ± 1.3 (6)</td>
<td>9.6 ± 0.5 (5)</td>
</tr>
<tr>
<td>+/-</td>
<td>6.4 ± 1.1 (6)</td>
<td>11.6 ± 1.5 (9)</td>
<td>10.9 ± 0.3 (5)</td>
</tr>
<tr>
<td>Unknown</td>
<td>6.6 ± 0.9 (5)</td>
<td>10.4 ± 0.8 (4)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values (kPa) are means ± SE for the no. of animals in parentheses and apply to samples collected from heart (fetus) or abdominal aorta (newborn) (for details, see methods). All newborns were tracheotomized. Note arterial blood PO\textsubscript{2} is slightly lower (P < 0.05) in ET\textsubscript{A} \textasciitilde \textasciitilde than ET\textsubscript{A} (+/+) newborns. Unknown genotype was not ascertained but from normal appearance of animals could be either (+/+) or (\textasciitilde \textasciitilde \textasciitilde). Arterial blood PO\textsubscript{2} of the hyperoxic dam, 76–83 kPa. *P < 0.05, compared with fetus of same genotype from normoxic dam. ND, not determined.

![Figure 8](image.png)

**Fig. 8.** Effect of maternal hyperoxia on ductus arteriosus in 129/SvEv (ET\textsubscript{A}-deficient) full-term fetal mice. Dams were made hyperoxic for 3 h, and each point applies to different fetus. Note that lumen size of ET\textsubscript{A}\textasciitilde\textasciitilde ductus is similar in hyperoxic and normoxic group (compare with Fig. 7). *P < 0.05 compared with ET\textsubscript{A} +/- mice. **P < 0.02 compared with ET\textsubscript{A} +/- mice.
mouse ductus, at least in comparison with the sheep and guinea pig ductus (Ref. 5; F. Coceani, unpublished data), is the effectiveness of ONO-11113 as a constrictor agent. In this respect, the mouse behaves as the rabbit (31), implying that there is a dichotomy across species over the susceptibility of ductal muscle to TxA2. It remains to be ascertained whether this observation has any bearing on the normal operation of the vessel.

A most novel finding in our study is that, both in vitro and in vivo, the constrictor effect of O2 on the fetal ductus is curtailed in the absence of the ET A receptor. Significantly, the loss of this response takes place without any obvious modification in the structure of the vessel. This validates a scheme implicating ET-1 as a critical link in the sequence of events being triggered by O2 and leading to contraction of smooth muscle cells (4, 8, 9). Coincidentally with this validation, the use of the ET A null mutant ductus unmasks the complexity of O2 action by showing a relaxant component under the normally overriding contraction. This transient relaxation denotes either stimulation of enzyme systems, such as those yielding PGE2 and NO, for which O2 is rate limiting or, in view of the accelerated formation of ET-1, activation of the ET B receptor. Equally important is the realization that in vivo the ET A null mutant, but not the ET A+/+, ductus is differentially affected by O2 before and after birth. The lack of prenatal constriction vis-à-vis normal postnatal closure in the mutant, although documenting the impact of ET A deletion under certain conditions, points to some other factor conditioning the
effectiveness of O₂. On the basis of available data and assuming that such data are applicable to the mouse, this factor is identified with PGE₂. It has been known for a long time that the influence of PGE₂ on the ductus abates at birth as blood levels of PGs fall and muscle cells become less responsive to this compound (3, 10, 29). The importance of the latter event to closure of the ductus has recently been highlighted by showing that the vessel closes only partially in newborn mice lacking the relevant PGE₂ receptor subtype (i.e., EP₄) (22). Significantly, partial constriction of the ductus in this EP₄⁻/⁻ mutant has been ascribed to ET-1 (22). When considering all these facts, it is reasonable to conclude that postnatal closure of the ductus relies on two overlapping and potentially interchangeable processes, i.e., withdrawal of PGE₂ relaxation and promotion of ET-1 contraction. This particular arrangement explains, in turn, how the ETₐ receptor may not respond to O₂ in utero and, yet, may close normally after birth. A functional PGE₂ mechanism would sustain patency in the former case, whereas its subsidence in the latter case would compensate for the lack of ET-1 and account for closure.

In apparent disagreement with our present results in the mouse and those reported earlier in the lamb (6) is a recent study (23) in which an ETₐ antagonist PD-156707 is ineffective on the contraction of the isolated lamb ductus to O₂ (Y.-A. Liu and F. Coceani, unpublished observations), albeit not as effectively as BQ-123 (6). In this connection, it should be noted that PD-156707 acquires two configurations in solution, an open form and the lactone (12), sharing the antagonist action on the ETₐ receptor but differing in solubility (the lactone is sparingly soluble and may precipitate on lowering pH; S. Haleen, personal communication). Hence, accessibility of this compound to its target within the tissue may not be optimal and may change with the local pH.

Our scheme for ductus regulation has far-reaching consequences. The two processes being implicated in postnatal closure of the vessel may be unevenly expressed across species and, in a given species, may have varying importance depending on physiological and pathophysiological conditions. The PGE₂-linked mechanism would appear to be more important than the ET-1-linked mechanism in the mouse ductus. Evidence supporting our view includes the impact of EP₄ deletion on ductus closure (22) and the coexistence in the isolated vessel of a brisk contraction to indomethacin with limited efficacy of O₂ over a physiological range of concentrations. An opposite situation is expected in the guinea pig ductus in which susceptibility to O₂ is greater (14, 24) and the intramural PGE₂ mechanism is peculiarly missing (11, 24). This dual control on ductus closure may also become evident under certain conditions. It is known that cyanotic infants are able to close their ductus, albeit more slowly than normally oxygenated infants. On the basis of our scheme, ductus closure in these patients can be ascribed to the removal of the
relaxing influence. Indeed, we have experimental evidence that the ET-1 system is not operational in the hypoxic ductus (8).

Two final points deserve a comment and they relate to the residual contraction to ET-1 in the ET\textsubscript{A}−/− ductus and the apparent conflict between our scheme assigning a mediator function to ET-1 in O\textsubscript{2} vasoconstriction and that of investigators (21, 32) implicating blockade of the K\textsuperscript{+} channels in the same response. It is known that ET\textsubscript{B} receptors may in certain cases mediate vasoconstriction rather than vasodilatation. In fact, two subsets of the ET\textsubscript{B} receptor, ET\textsubscript{B1} (relaxant) and ET\textsubscript{B2} (contractile), have been characterized pharmacologically to account for this dual response (13). Barring an unspecified effect, any ET-1 contraction in the ET\textsubscript{A}−/− ductus likely reflects the presence of the ET\textsubscript{B2} subtype. On the surface, it is more difficult to explain the coexistence of two processes in O\textsubscript{2} vasoconstriction, i.e., activation of the ET-1 system and inhibition of the K\textsuperscript{+} channels, seemingly distant operationally. In actual fact, however, there may not be incongruence in this finding because recent work has identified an inhibitory action of ET-1 on K\textsuperscript{+} channels (25, 26, 28). Hence, after considering all these facts, one could formulate a scheme with ET-1 acting as a messenger for the O\textsubscript{2} constriction both directly and through the inhibition of K\textsuperscript{+} channels. Further work would be required to verify this possibility.

In conclusion, our study demonstrates that ET-1, acting via the ET\textsubscript{A} receptor subtype, plays a critical role in the constrictor response of the ductus to O\textsubscript{2}. However, closure of this vessel in the normal condition in vivo entails not only activation of the ET-1 mechanism, but also removal of the relaxing influence of PGE\textsubscript{2}. These concepts have practical implications and introduce new possibilities for the management of infants requiring ductus patency for survival. An E-type PG is currently the treatment of choice; however, an ET\textsubscript{A} antagonist could become a useful adjunct, particularly if persistence of the shunt is necessary over an extended period of time.

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