

## The response of the lamb ductus arteriosus to endothelin: developmental changes and influence of light

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### Abstract

Endothelin-1 (ET-1) is a putative messenger of oxygen in the ductus arteriosus. Since the ability of the vessel to contract to oxygen increases with gestation, we wished to ascertain whether ET-1 action is also developmentally regulated. A corollary objective was to assess whether any gestational variation in the ET-1 contraction is due to a change in the ET<sub>A</sub>-mediated action or to a shift in the balance between opposing, contractile (ET<sub>A</sub> - mediated) and relaxant (ET<sub>B</sub>-mediated), actions. Experiments were performed with isolated ductal strips from preterm (0.7 gestation) and near-term fetal lambs. ET-1 contracted the ductus dose-dependently ( $10^{-10}$ – $10^{-7}$  M) at both ages; however, the peak contraction was about double in magnitude at term. Regardless of age, ET-1 contraction was greater with preparations kept in the dark compared to those exposed to light. This effect of light was not seen after removing the endothelium or when treating the intact tissue with the ET<sub>B</sub> antagonist BQ788 (1  $\mu$ M). In the dark, however, BQ788 did not modify significantly the ET-1 response at either age. We conclude that ET-1 becomes a stronger ductus constrictor with fetal age, conceivably by acting on ET<sub>A</sub> receptors. Hence, the concept of ET-1 mediating the oxygen contraction is further validated. Peculiarly, the ET-1 contraction is curtailed by light through a hitherto undefined ET<sub>B</sub> receptor-linked process. © 2002 Elsevier Science Inc. All rights reserved.

*Keywords:* Ductus arteriosus; Endothelin; Oxygen; Light; Fetal development

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### Introduction

The ductus arteriosus is a special fetal shunt, connecting the pulmonary artery with the aorta and allowing blood to bypass the high-resistance vascular bed within the unexpanded lungs. After birth,

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the ductus closes coincidentally with the onset of lung ventilation and the attendant fall in pulmonary vascular resistance. Prostaglandin(PG) E<sub>2</sub> is primarily responsible for patency of the vessel in the fetus, while the rise in blood oxygen tension, marking the transition from intra- to extrauterine life, is viewed as a trigger for the constriction leading to closure [1–3]. Indeed, failure of the ductus to close, as often happens in the preterm newborn, is ascribed to the combination of two factors, the impact of relaxing mechanism(s) becoming operational early in gestation and the poor susceptibility to oxygen [1–3].

Several findings implicate endothelin-1 (ET-1) as a messenger in the constriction of the ductus to oxygen and, by extension, assign to the action of this peptide, in conjunction with the waning action of PGE<sub>2</sub> at birth, a role in the process of closure [4–8]. ET-1, acting via the ET<sub>A</sub> receptor subtype, is a singularly potent ductus constrictor. The peptide is also formed in the vessel wall, both within and without the endothelium, and its formation correlates positively with the oxygen tension. Interference with ET-1 synthesis or action, whether achieved pharmacologically or through genetic manipulation, curtails the oxygen effect. On the other hand, results with an ET<sub>A</sub> antagonist reportedly arguing against this concept [9] have been linked to a methodological problem [5]. All these data, however, have been obtained in the ductus from perinatal animals and, consequently, no information is available on the development of the ET-1-based mechanism vis-a-vis the increasing effectiveness of oxygen with gestation.

The purpose of our investigation was to ascertain whether, in accord with its postulated messenger function, ET-1 is a less effective constrictor on the preterm than the term ductus. A corollary aim was to examine whether any reduction in the ET<sub>A</sub>-mediated constrictor response is coupled with an enhancement of the ET<sub>B</sub>-mediated relaxant response. Theoretically, in the premature ET<sub>B</sub> receptors could override ET<sub>A</sub> receptors as a target for ET-1 being formed in response to oxygen. The existence in the vessel of a functional ET<sub>B</sub> receptor is supported by the observation, made at term, that ET-1 action conceals a relaxant component within the overriding contraction [5,10] and that oxygen, hence by inference ET-1, may exert a relaxant rather than a contractile effect when the ET<sub>A</sub> receptor is absent [5]. In the course of the investigation it was also realized that ET-1 action on the isolated ductus is modified by ambient illumination. This peculiar phenomenon had not been recognized in the past, and results obtained in the absence and presence of an overhead fluorescent lighting were pooled [10]. The latter observation entailed a re-evaluation of our previous study [10] and separate experiments in the light and the dark.

## Methods

Experiments were performed on preterm (103–107 days, 0.7 gestation) and near-term (133–139 days gestation; term, 145 days) pregnant sheep. Animals were mostly Southdown-Dorset crossbred, with Targhee and Suffolk-Finnish Landrace crossbred making the balance. Surgical procedures and experimental protocols were approved by the Animal Care Committee of our institution.

### *Solutions and drugs*

The Krebs solution had the following composition (mM): NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1, MgSO<sub>4</sub> 0.9, dextrose 11.1 and NaHCO<sub>3</sub> 25. 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 O<sub>2</sub> or O<sub>2</sub> at 2.5% plus 5% CO<sub>2</sub> in N<sub>2</sub>. Po<sub>2</sub> was measured with an Instrumentation Laboratory gas analyzer (model 1610, Lexington, MA) and was, respectively,  $9.8 \pm 0.3$  and  $18.8 \pm 0.5$  mmHg (pH  $7.403 \pm 0.004$ ). 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 (BSA)-supplemented saline. BQ123 and BQ788, which are selective antagonists for, respectively, the ET<sub>A</sub> [11] and ET<sub>B</sub> [12] subtype of the endothelin receptor (both courtesy of Merck Frosst, Montreal, Canada), were dissolved directly in saline. Doses of all compounds are given in molar concentrations and refer to their final concentration in the bath. Vehicle alone, without or with BSA (0.05%), had no effect on vessel tone.

### Experimental procedure

Anesthesia, cesarean delivery of fetuses, and isolation of the ductus arteriosus have been described in detail [13]. Throughout the procedure, care was taken not to expose the ductus to ambient air, and ice-cold Krebs solution gassed with the zero O<sub>2</sub> mixture was used for transportation and dissection.

As previously reported [13], the ductus arteriosus was opened and then cut perpendicularly to the main axis to yield one or two strips, depending on the age of the fetus and the attendant length of the vessel. Ductal strips were used intact or after removing the endothelium by rubbing filter paper (Whatman no. 41) over the intimal surface. Complete removal was confirmed in certain experiments by scanning electron microscopy [14]. Preparations were mounted in individual 10-ml baths 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 (term ductus,  $1.53 \pm 0.007$  g; 1 g weight = 9.8 mN) or a series of steps (preterm ductus,  $1.5 \pm 0.008$  g), and preparations were stretched by about 50% of the original length to obtain an optimal tension output [15]. Precautions were used at each step not to damage the endothelium when handling preparations of the whole ductus.

Tissues were equilibrated in Krebs solution gassed with the 2.5% O<sub>2</sub> mixture, and the same gas mixture was employed in the actual experiments to mimic the condition in utero [13]. Depending on the protocol, the room was either illuminated with fluorescent lamps located in the ceiling or was kept darkened.

The study included several protocols, but only one protocol was used with each vessel. In addition, to avoid possible interference from tachyphylaxis, ET-1 was tested once, and any change in its effect due to the ET<sub>A</sub> antagonist (BQ123), the ET<sub>B</sub> antagonist (BQ788), or room illumination was ascertained by comparing results in different vessels. In *protocol 1*, ET-1 was tested on the intact, untreated ductus from term and preterm animals. The peptide was added to the organ bath in cumulative doses (1 pM–0.1 μM), using 3- to 10-fold increments. Separate tests were done in the light and the dark. An equivalent experiment was carried out in *protocol 2*, using endothelium-denuded term preparations. In certain cases, BQ123 (1 μM) was included in the medium 60 min prior to the ET-1 test to ascertain whether contractile responses are ET<sub>A</sub>-mediated or result in part from activation of a subset of the ET<sub>B</sub> receptors (i.e. ET<sub>B2</sub>) [16]. In *protocol 3*, intact vessels (preterm and term) were tested with ET-1 after a 60-min pretreatment with BQ788 (1 μM), both in the absence and presence of light. The same protocol was used with few endothelium-denuded, term preparations.

### Analysis of data

Baseline contractile tension, which varied with the preparation (see Results), refers to the net active tension (i.e. total tension minus applied tension) developed by the preparation prior to any treatment. Responses to ET-1 and excess potassium are given as the fractional change from baseline.

Data are expressed as the mean  $\pm$  s.e. mean. Statistical comparison of two means was done by Student's *t* test for unpaired observations. Multiple comparisons were made with a two-way repeated measures analysis of variance (ANOVA) followed by an F-test for simple effect. Differences are considered significant for  $p \leq 0.05$ .

## Results

In agreement with previous work [13], intact ductal strips developed intrinsic tone during the initial period of equilibration, with tension values progressing first to a peak and then abating to a stable level. The ensuing baseline tone was higher for term than preterm preparations, but no obvious difference could be detected at either age depending on room illumination (Table 1). A similar contractile pattern was noted with term preparations lacking the endothelium, though their final tension output appeared to be greater in the dark (Table 1). Excess potassium elicited a stronger contraction in the term than the preterm ductus and, in the former case, its magnitude was slightly greater in the dark (Table 1).

### Effect of ET-1

ET-1 contracted the intact ductus dose-dependently over the range between  $10^{-10}$  and  $10^{-7}$  M (Figs. 1 and 2) and, in accord with previous results at term [10], this contraction was slow in development. While threshold concentration did not differ between the two fetal ages examined, peak contraction was about double in magnitude at term (compare Figs. 1 and 2). In comparison, the average response to excess potassium increased at most 65% over the same gestation period (Table 1). Regardless of age, however, the peptide proved to be more effective when preparations were kept in the dark compared to those exposed to light (Figs. 1 and 2). Conversely, no such change in ET-1 action depending on room

Table 1  
Contractile tension of term and preterm (0.7 gestation) ductus arteriosus

Preparation	Steady-state tension		Potassium contraction	
	dark	light	dark	light
intact ductus, term	0.75 $\pm$ 0.23 (14)	0.68 $\pm$ 0.17 (20)	6.5 $\pm$ 0.3 (10)	5.4 $\pm$ 0.3 (15)*
endothelium-denuded ductus, term	1.14 $\pm$ 0.47 (5)	0.38 $\pm$ 0.07 (17)*	–	6.2 $\pm$ 0.6 (4)
intact ductus, preterm	0.33 $\pm$ 0.09 (13)	0.2 $\pm$ 0.07 (12) <sup>+</sup>	3.9 $\pm$ 0.3 (9) <sup>++</sup>	4.1 $\pm$ 0.4 (6) <sup>+</sup>

Values (g) are mean  $\pm$  s.e. mean for the number of experiments given in parentheses. Note that values of steady-state tension (i.e. baseline tension) refer to the entire tension developed by the preparation (i.e. total tension minus applied tension), while responses to excess potassium are expressed as the increase of tension over baseline. \*vs. preparation kept in the dark at the same age,  $p < 0.05$  (Student's *t* test). <sup>+</sup> vs. term preparation kept in the light,  $p < 0.05$  (Student's *t* test). <sup>++</sup> vs. term preparation kept in the dark,  $p < 0.001$  (Student's *t* test).

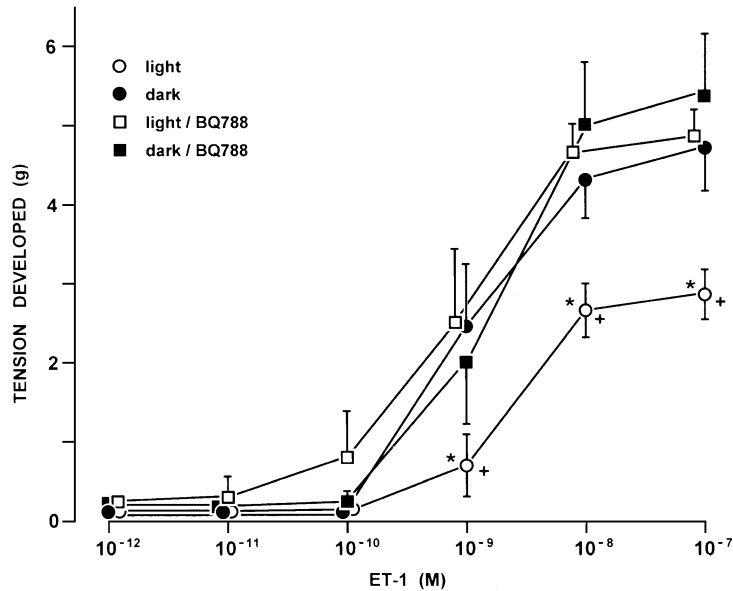


Fig. 1. Intact strip of ductus arteriosus from near-term fetal lamb. Concentration-response curves to ET-1 before (dark, n = 9; light, n = 15) and during (dark, n = 5; light n = 5) treatment with BQ788 (1  $\mu$ M). Note that the control curve in the light includes experiments from a previous series [10] (i.e. 14 of the original 16 experiments) where results were pooled regardless of room lighting. When necessary, in this and the following figures points have been offset to improve visibility. \*vs. control in the dark,  $p < 0.01$  (ANOVA). +vs. BQ788 treatment in the light,  $p < 0.01$  (ANOVA).

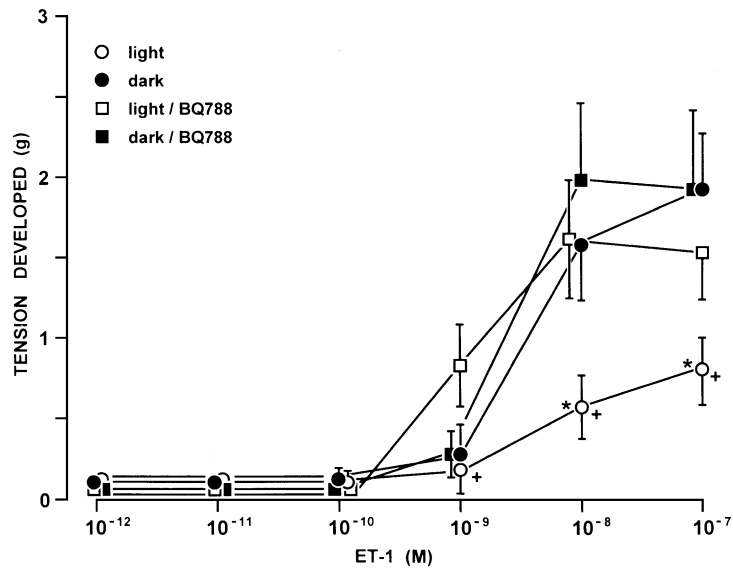


Fig. 2. Intact strip of ductus arteriosus from preterm (0.7 gestation) fetal lamb. Concentration-response curves to ET-1 before (dark, n = 8; light, n = 6) and during (dark, n = 5; light, n = 6) treatment with BQ788 (1  $\mu$ M). Note the different scale on the ordinate compared to Fig. 1. \*vs. control in the dark,  $p = 0.05$  (ANOVA). +vs. BQ788 treatment in the light,  $p < 0.05$  (ANOVA).

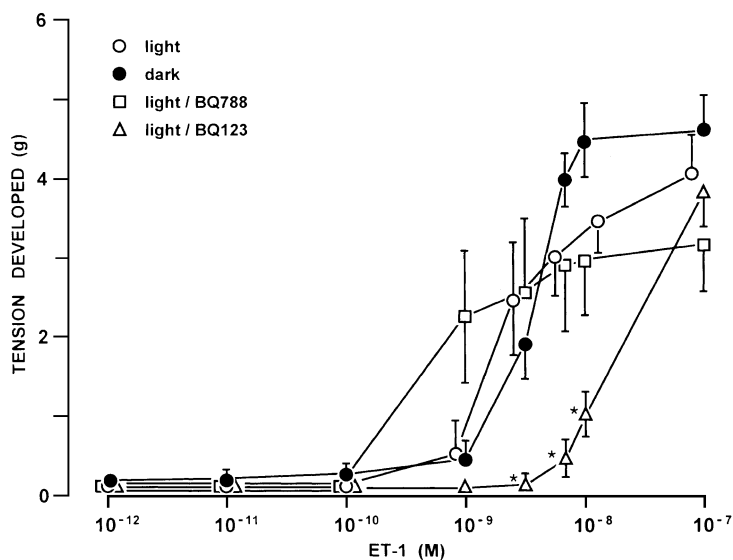


Fig. 3. Endothelium-denuded strips from near-term fetal lamb. Concentration-response curves to ET-1 before (dark,  $n = 5$ ; light,  $n = 6$ ) and during treatment with BQ788 ( $1 \mu\text{M}$ ) ( $n = 5$ ) or BQ123 ( $1 \mu\text{M}$ ) ( $n = 6$ ). \*vs. control in the light,  $p < 0.01$  (ANOVA).

illumination was noted, at least at term, after preparations had been freed of their endothelium (Fig. 3). In fact, there was no significant difference in responsiveness to ET-1 between the intact ductus being kept in the dark and the endothelium-denuded ductus exposed to light.

#### *Effect of BQ788 and BQ123 on the ET-1 response*

At both ages, any reduction in the ET-1 contraction due to light disappeared after adding BQ788 to the perfusion fluid, while the same treatment was without a significant effect in the dark (Figs. 1 and 2). Hence, response curves of BQ788-treated tissues to ET-1 overlapped over the entire dose-range (Figs. 1 and 2). Conversely, BQ788 did not cause any appreciable change in the contraction to excess potassium (4.7 and 4.4 g, respectively, before and after BQ788 in the light;  $n = 2$  for each group). Regardless of room illumination, BQ788-treated tissues still responded unequally to ET-1 depending on fetal age and, in that respect, did not differ from the untreated tissues (compare Figs. 1 and 2).

BQ123 curtailed the contraction of the endothelium-denuded ductus to ET-1, thus causing a rightward shift in the dose-response curve (Fig. 3). Under the same condition, BQ788 has instead no significant effect (Fig. 3).

## **Discussion**

The present study demonstrates that ET-1 mimics oxygen in becoming a more effective ductus constrictor with the progress of gestation. This developmental change is not caused by a relatively greater influence of  $\text{ET}_B$  receptors causing relaxation in the premature, but results instead from a varying

response to ET<sub>A</sub> receptor activation. Maturation of the contractile apparatus, on the other hand, could account only in part for our finding, because the potassium contraction is less affected than the ET-1 contraction by prematurity. Hence, the coincidence of effects between ET-1 and oxygen further validates the concept [4–8] of an oxygen messenger function for ET-1.

While the acquisition of new evidence in support of this mediator role for ET-1 is a significant result, the attenuation of ET-1 action by light is peculiar and warrants discussion. An unspecific effect of the light on muscle tone [17], making the vessel less responsive to ET-1, is unlikely. Photoinduced relaxation occurs in the ductus [18,19], as in other blood vessels [17], but in our experience this effect is small and generally short-lived. In fact, the relatively minor impact of light on ductal muscle is also evident here from the tension measurements made at rest and during treatment with excess potassium (see Table 1). Equally unfeasible, with the concentrations being used, is a structural modification of ET-1 by free radicals [20] generated upon exposure to light. The finding instead that light-induced reduction in the ET-1 contraction subsides after removing the endothelium or upon treating the tissue with BQ788 points to a selective involvement of the ET<sub>B</sub> receptors. Supporting our conclusion is also the lack of any light-induced change of ET-1 action in the endothelium-denuded ductus, hence in a preparation where only ET<sub>A</sub> receptors appear to be functional. The actual mechanism by which light alters ET<sub>B</sub> receptors and related messenger systems (eg. NO/endothelial NO synthase; prostanoid/cyclooxygenase-2) [21,22] remains speculative. A possibility, based on available data [23], is that light hinders the sequestration of NO into nitrosothiols, thus leading to a larger pool of free NO upon ET<sub>B</sub> activation. NO is a natural relaxant in the ductus of several species, including the lamb [24–26]. Any excess NO could, in turn, oppose the ET-1 contraction by exerting a direct relaxation or by interfering with the binding of the peptide to its ET<sub>A</sub> target [27]. Clearly, the two actions are not mutually exclusive and may well develop synergistically.

Regardless of whether or not NO is involved in this effect of light, our observation may have practical implications and may, in particular, explain the impact of ambient illumination on the development of a persistent ductus in prematurely born infants [28]. By extension, it also raises the prospect in such patients of employing an ET<sub>B</sub> antagonist as an adjuvant to the conventionally administered cyclooxygenase inhibitor [29,30].

In summary, our study demonstrates that, in constricting the ductus arteriosus, ET-1 shares with oxygen the increasing effectiveness with advancing gestation. This coincidence between the two agents is consistent with the concept of ET-1 being a messenger for oxygen. Peculiarly, the magnitude of the ET-1 contraction is curtailed by ambient light through a hitherto undefined ET<sub>B</sub> receptor-linked mechanism. It remains to be ascertained whether the latter phenomenon is specific for the ductus or extends to other blood vessels in the fetus and the adult.

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