The Endothelin A Receptor Antagonists PD 156707 (CI-1020) and PD 180988 (CI-1034) Reverse the Hypoxic Pulmonary Vasoconstriction in the Perinatal Lamb

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ABSTRACT

Endothelin-1 (ET-1) is considered an intermediary in the constrictor response of the pulmonary vasculature to hypoxia and, by extension, is assigned a prime role in the pathogenesis of pulmonary hypertension. We report here the antihypertensive action in the conscious newborn lamb of two novel endothelin A receptor antagonists, sodium 2-benzo-[1,3]dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enolate (PD 156707) and 4-(7-ethyl-benzo[1,3]dioxol-5-yl)-1,1-dioxo-2-(2-trifluoromethyl-phenyl)-1,2-dihydro-116-benzo[e][1,2]thiazine-3-carboxylic acid potassium (PD 180988), differing in chemical properties and half-life within the body. PD 156707 and PD 180988, given in the right atrium as a bolus followed by infusion, had little or no effect on pulmonary and systemic hemodynamics under normoxia. Conversely, they both reversed the pulmonary hypertension due to alveolar hypoxia while producing minor changes, or no change at all, in systemic vascular resistance. Furthermore, their pulmonary vascular effect outlasted administration. Pulmonary hypertension being elicited by infusion of the thromboxane A2 analog, 9,11-epithio-11,12-methano-thromboxane A2 (ONO-11113) was instead not amenable to ETAR inhibition. Blood levels of ET-1, which rose with hypoxia but not ONO-11113 treatment, were not changed by either antagonist. Consistent with findings in vivo, when using isolated pulmonary resistance arteries from term fetal lamb, PD 156707 curtailed the hypoxia- but not the ONO-11113-induced constriction. We conclude that PD 156707 and PD 180988 are selective inhibitors of pulmonary vasoconstriction resulting from hypoxia. Our findings support the use of these or allied compounds in the management of pulmonary hypertension in the neonate.

Several lines of evidence implicate endothelin-1 (ET-1), acting via the ETAR receptor (ETAR) subtype, in the pathogenesis of pulmonary hypertension (Chen and Oparil, 2000). ET-1 constricts pulmonary resistance arteries and its action accords, for potency and pattern, with a mediator role in hypoxic vasoconstriction (Wang et al., 1995). Synthesis of the peptide and expression of ETAR also increase in response to hypoxic vasoconstriction (Wang et al., 1995). Synthesis of the peptide and expression of ETAR also increase in response to hypoxic vasoconstriction (Wang et al., 1995). Synthesis of the peptide and expression of ETAR also increase in response to hypoxic vasoconstriction (Wang et al., 1995).

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Perrault et al., 2001), experimental diaphragmatic hernia (lamb) (Thébaud et al., 2000), and increased blood flow (lamb) (Petrossian et al., 1999) and against the rebound hypertension consequent to abrupt discontinuation of NO inhalation (lamb) (McMullan et al., 2001).

A host of ET-1 antagonists have been developed lately differing in specificity for the ET-1 receptor subtypes, physicochemical properties, oral bioavailability, and rate of disposal by the body (Warner et al., 1994; Cheng et al., 1997). Among them, certain butenolide and benzothiazine analogs are of particular interest for high specificity against ETAR and effectiveness by both oral and parenteral routes (Doherty et al., 1995; Patt et al., 1997; Repine et al., 1998).

The present study was undertaken in the fetal and newborn lamb with the 2-fold objective of verifying and comparing the efficacy against hypoxic pulmonary vasoconstriction of two representative ET-1 antagonists, belonging to the

ABBREVIATIONS: ET-1, endothelin-1; ETAR, endothelin A receptor; ETBR, endothelin B receptor; Ao, aorta; BSA, bovine serum albumin; P, pressure; PA, pulmonary artery; PV, pulmonary vein; R, resistance, RA, right atrium; NO, nitric oxide; ONO-11113, 9,11-epithio-11,12-methano-thromboxane A2; PD 156707, (sodium 2-benzo-[1,3]dioxol-5-yl-4-(4-methoxy-phenyl)-4-oxo-3-(3,4,5-trimethoxy-benzyl)-but-2-enolate); PD 158312, 2-benzo[1,3]dioxol-5-yl-4-(4-methoxy-3-methylphenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enolic acid; PD 166793, (S)-2-(4-bromo-benzo[1,3]dioxol-5-yl)-4-(4-methoxy-3-methylphenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enolic acid; PD 166793, (S)-2-(4-bromo-benzo[1,3]dioxol-5-yl)-4-(4-methoxy-3-methylphenyl)-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enolic acid; PD 180988, 4-(7-ethyl-benzo[1,3]dioxol-5-yl)-1,1-dioxo-2-(2-trifluoromethyl-phenyl)-1,2-dihydro-116-benzo[e][1,2]thiazine-3-carboxylic acid potassium salt; pO2, partial pressure of O2; pCO2, partial pressure of CO2.
butenolidine (PD 156707) and benzo-thiazine (PD 180988) families. These antagonists share selectivity for ET₁R but differ in half-life within the host (Doherty et al., 1995; Patt et al., 1997; Repine et al., 1998). One of them, PD 156707, has already shown pulmonary antihypertensive properties in the adult rat (Haleen et al., 1998). Our ultimate aim, however, was to provide the experimental framework for a possible use of these, or allied compounds sharing the same versatility for pharmaceutical formulation, in the management of pulmonary hypertension in infants. An ET₂R rather than a dual ET₁R/ET₂R antagonist was chosen for investigation since animal data, obtained by us (Wang et al., 1995) and others (Ivy et al., 2000, 2001), assign to the ET₂R population a protective function against pulmonary hypertension. The choice of the sheep was motivated by the fact that in this species, as in humans, ET-1 exerts its pulmonary constrictor effect primarily, if not exclusively, via ET₁R (Fukuroda et al., 1994; Russell and Davenport, 1995; Wang et al., 1995). In addition, the same species lends itself well to an experimental approach combining suitable in vivo and in vitro preparations (Wang et al., 1995). A preliminary account of the results with PD 180988 has been reported (Coe et al., 2000).

Materials and Methods

In vivo work was carried out in newborn lambs of Suffolk/Rambouillet/Dorset crossbreed. Their average age at surgery was 4 days (range, 1–13 days), whereas the actual study was performed at 10–43 days of age (average, 19 days). Fetal lambs at term (gestation age, 136–199 days, term 145 days) of Southdown or Southdown/Dorset stock were used for in vitro work. Surgical procedures and experimental protocols were approved by the Animal Care Committee of our institutions.

Solutions and Drugs. Krebs medium for the isolated pulmonary resistance arteries had the following composition: 118 mM NaCl, 4.7 mM KCl, 1 mM KH₂PO₄, 0.9 mM MgSO₄, 2.5 mM CaCl₂, 11.1 mM glucose, and 25 mM NaHCO₃. The solution was bubbled with gas mixtures containing either no O₂ or 12.5% O₂ plus 5% CO₂ in N₂, and the resulting partial pressure of O₂ (pO₂) was, respectively, 7 ± 2 and 56 ± 1 mm Hg (pH 7.4). As shown previously, the O₂-containing mixture duplicated the neonatal condition, whereas the zero-O₂ mixture ensured a full-fledged hypoxic contraction (Wang et al., 1995). pO₂ was measured with a gas analyzer (model 1610; Instrumentation Laboratory, Lexington, MA). Sterile Hanks’ balanced salt solution (without CaCl₂, MgCl₂, MgSO₄, and phenol red) was obtained from ICN Biomedicals Inc. (Costa Mesa, CA) and was supplemented with antibiotic-antimycotic solution (100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml fungizone) (PSP from ICN Biomedicals Inc.) and a serine protease inhibitor (100 μM Pefabloc; Roche Molecular Biochemicals, Indianapolis, IN).

The following compounds were used: ET-1 (American Peptide Co., Inc., Sunnyvale, CA), ET-3 (Peptides International, Inc., Louis ville, KY), [¹²⁵I]-ET-1 and [¹²⁵I]-ET-3 (both from PerkinElmer Life Sciences, Beverly, MA), the thromboxane A₂ analog, ONO-11113 (courtesy of ONO Pharmaceutical, Osaka, Japan), bovine serum albumin (BSA) (Bayer Corp., Emeryville, CA), phosphoramidon (Sigma-Aldrich, St Louis, MO), leupeptin (Sigma-Aldrich), and pepstatin (Sigma-Aldrich). PD 156707 and PD 180988 were synthesized by the Chemistry Department at Pfizer (formerly Parke-Davis) Pharmaceutical Research. These compounds share selectivity for ET₁R over ET₂R (800- and 2500-fold difference in selectivity with, respectively, PD 156707 and PD 180988), but differ in their disposal by the body (half-life in the rat is 1 and 7.8 h, respectively, for PD 156707 and PD 180988). Doses of PD 156707 were derived from earlier work (Reynolds et al., 1995; Ignasiak et al., 1997), and their effectiveness was confirmed in preliminary experiments. Likewise, PD 180988 dosage was selected through trials. All other chemicals and solvents were of analytical grade purity.

ONO-11113 was dissolved in 5 mg/ml distilled ethanol, and aliquots (stored at −70°C) were diluted in Tris buffer (pH 7.4) (in vitro experiments) or with saline (in vivo experiments). Other substances dissolved readily in aqueous media.

Intact Animal. Newborn lambs (n = 30), with an average weight of 4.1 kg (range, 2.7–7.1), were chronically instrumented according to a published protocol (Wang et al., 1995). In brief, the animals were anesthetized and, through a left thoracotomy, had a flow probe (C and C Instruments, Culver City, CA or Transonic Systems Inc., Ithaca, NY) placed around the main pulmonary artery (PA). Catheters were positioned in both the left atrium and the right lower pulmonary vein (PV) and, concomitantly, the constricted ductus arteriosus was ligated. Four to 21 days after instrumentation (average, 8 days), at the age of 7 to 28 days (average, 12 days) and under anesthesia, indwelling vascular sheaths were placed in both carotid arteries and external jugular veins. Afterward, the lambs were allowed to recover for 1 to 29 days (average, 10 days) before being tested with either ET₁R antagonist. On the study day, using the vascular sheaths as a guide, the animals were fitted with additional catheters in the PA, the aortic root (Ao), and the right atrium (RA) (two or three distinct lines). Separate RA lines served for pressure (P) monitoring, for the delivery of the ET₁R antagonist, and when required, for administration of ONO-11113. A high-fidelity catheter (Millar Instruments, Houston, TX) was also positioned in the left ventricle for measuring dP/dt. Pressure lines were connected to strain gauge transducers (model 231D; Gould Instrument Systems Inc., Cleveland, OH), the flow probe to its own flowmeter (Gould model SP2202 or Transonic model T201D), and the Millar catheter to a Millar control unit (model TC-510). All hemodynamic signals including heart rate were displayed on a Gould recorder (model ES2000) and were also stored digitally on a microcomputer (CV-SOFT program; Odessa Computer Co., Calgary, AB, Canada) for offline analysis. Cardiac output, being derived from PA flow, was normalized for body weight (cardiac index). Pulmonary vascular resistance (PVR) was calculated by dividing the difference between mean PAP and mean left atrium pressure by cardiac index (expressed as mm Hg per milliter per minute per kilogram, i.e., units per kilogram). Systemic vascular resistance (AoR) was calculated by dividing the difference between mean AoP and mean RA pressure by cardiac index (expressed as units per kilogram). Arterial blood gases were measured periodically with a Nova Stat Profile 5 blood gas analyzer (Nova Biomedical Corp., Waltham, MA), and vascular lines were flushed intermittently with heparinized saline (4 units/ml). Blood samples were withdrawn from the PV for the assay of ET₁R antagonists and ET-1. Studies were performed in the conscious lamb breathing spontaneously either room air (normoxia) or a mixture of approximately 12% O₂ and 5% CO₂ in N₂ to elicit a steady-state hypoxic response (normocapnic hypoxia) over a 60-min period.

PD 156707 was dissolved in saline and was given into the RA as a bolus (0.1 mg/kg in 1-ml volume) followed by continuous infusion (0.15 mg/kg/h) with a pump (model 944; Harvard Apparatus, Inc., Holliston, MA) (20 ml/h). Two doses, henceforth called low and high, were used instead with PD 180988 (low dose, 20 μg/kg bolus with a 30 μg/kg/h infusion; high dose, 30 μg/kg bolus with a 50 μg/kg/h infusion); otherwise, vehicle and delivery procedure were the same as for PD 156707. Both ET₁R antagonists were tested under different conditions. In protocol 1, PD 156707 and PD 180988 (high dose) were given for 60 min in normoxia. A treatment of the same length (PD 156707 and PD 180988 at both doses) was used with sustained hypoxia (protocol 2), and hypoxic lambs not receiving an ET₁R antagonist served as reference. In protocol 3, the animals were also made hypoxic, but treatment with the ET₁R antagonists (PD 156707 and PD 180988 high dose) lasted 30 instead of 60 min. This allowed stopping the treatment through hypoxia. Protocol 4 was used only with PD 156707, and the antagonist was given to the normoxic
animal after raising pulmonary vascular tone with a continuous infusion of ONO-11113 (0.8 μg/kg/min for 60 min). PD 156707 administration began at 30 min through ONO-11113 infusion, once pulmonary hypertension had reached a steady value, and was continued up to the 60-min mark. The two antagonists were tested in different animals, and each animal was studied with one (n = 20), two (n = 9), or exceptionally three (n = 1) protocols. When using more than one protocol, the interval between successive experiments was 1 to 12 days (average, 5 days).

**Assay of ETₐR Antagonists and ET-1.** When studying the normoxic animal, PV blood samples were collected before ETₐR antagonist administration and at the end of the treatment (protocol 1). In hypoxia experiments (protocol 2), sampling was carried out first in normoxia and then during sustained hypoxia at the beginning and the end of the 60-min period (hypoxia control) or just before ETₐR antagonist administration (“zero time”) and at the end of the 60-min treatment (hypoxia treatment). With rebound evaluation experiments (protocol 3), blood samples were obtained in normoxia, during sustained hypoxia before and at the end of the 30-min treatment with the ETₐR antagonist, and again after a further 30-min hypoxia in the absence of treatment. In those instances in which pulmonary vascular tone was raised with ONO-11113 (protocol 4), blood was withdrawn first during the normoxic control, then after 30 min of ONO-11113 infusion, and last after a further 30-min period in which ONO-11113 infusion and ETₐR antagonist treatment were combined. In all cases, arterial blood (2 ml) was collected in heparinized tubes and centrifuged immediately in the cold to separate the plasma fraction. Plasma was then stored at −80°C for ETₐR antagonist assay. ET-1 was measured in the same samples in most experiments.

PD 156707 was assayed by liquid chromatography according to a published procedure (Rossi et al., 1996). In brief, plasma aliquots were collected together with an internal standard (PD 158312) [2-benzo[1,3]dioxol-5-y1-4-[4-methoxy-3-methylphenyl]-4-oxo-3-(3,4,5-trimethoxybenzyl)-but-2-enoic acid] to a C₁₈ Spherex column (particle size, 5 μm) (Phenomenex, Torrance, CA), and separation was conducted with a mobile phase of acetonitrile/ammonium phosphate (50 mM, pH 3.5) (44:56, v/v). Column effluent was monitored by electrospray ionization in negative ionization mode and with Mass Lynx version 3.1 operating software. Reference PD 180988 was measured by a liquid chromatographic/mass spectrometric procedure using PD 166793 structure as internal standard. The liquid chromatography system consisted of a Series-200 auto sampler/pump (PerkinElmer Instruments, Norwalk, CT) (flow rate, 0.2 ml/min) operating with a Betasil Phenyl analytical column (Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA) (particle size, 5 μm) and a mobile phase of acetonitrile/0.1% acetic acid (65:35, v/v). The mass spectrometer was a Quattro II tandem quadrupole model (Micromass, Manchester, UK) set to electrospray negative ionization mode and with Mass Lynx version 3.1 operating software. Reference PD 180988 was dissolved in methanol (range, 0.1–100 μg/ml) and solutions were subsequently diluted 10-fold in plasma to create a 10-point standard curve. PD 166793 was also dissolved in 5 μg/ml methanol. Each assay run included the 10-point standard curve, blank sample, blank sample with internal standard in singlet, and the analytical samples. Samples and standards were interspersed throughout the assay sequence. The procedure began by adding to the plastic tubes, in the order, 200 μl of the plasma sample (or blank plasma matrix for standards and blank samples), 20 μl of methanol in the case of analytical and blank samples (or 20 μl of the reference PD 180988 solution for the standard curve), and 25 μl of the PD 166793 stock solution. Each tube was then vortexed for 5 s and, after adding 0.5 ml of a KH₂PO₄ solution (0.5 M, pH 3) and 3 ml of diethyl ether, was shaken vigorously for 10 min. The resultant mixture was centrifuged (500g, 10 min), and the organic layer was separated and evaporated to dryness under a stream of N₂. The dry residue was dissolved in 400 μl of acetonitrile/water (50:50, v/v) and a 10-μl aliquot of this solution was used for the assay.

ET-1 was measured with a solid-phase enzyme-linked immunosorbent assay kit (Parameter; R & D Systems, Minneapolis, MN). In brief, samples were added to the mixture acetone/1 M HCl/H₂O (40:1.5, v/v) (1.5 ml) and centrifuged (1000g) for 15 min at 4°C. The resulting supernatant was separated and dried, and the residue was dissolved in assay buffer (250 μl) for subsequent quantitation of the peptide by optical density reading (Molecular Devices Corp., Sunnyvale, CA). Recovery through extraction was 36 ± 3%, whereas internal and intra-assay variations were, respectively, 5 and 4%. Limit of detection was 0.25 pg.

**Isolated Resistance Arteries.** Rings of pulmonary resistance arteries (sixth generation) were obtained from fetal lambs at term (n = 11) and were prepared for mechanical recording as previously reported (Wang and Coeanei, 1992). In brief, the vessel was suspended between two 25-μm tungsten wires inside a jacketed organ bath (capacity, 6.5 ml), and the solution was gassed with the O₂ mixture (i.e., 12.5% mimicking the neonatal condition. One of the wires was connected to an isometric force transducer (model DSC-6BE4–110; Kistler Morse, Redmond, WA). Once mounted, the vessel was stretched to about 30% of the expected transmural pressure in vivo and was equilibrated for at least 60 min. Afterward, this load was increased to a dimension at rest was measured (internal diameter, 172 ± 4 μm; length, 735 ± 38 μm; wall thickness, 33 ± 1 μm), and the vessel was stretched again in stepwise fashion until a functionally relevant tension, as predicted by the La Place equation, was achieved. The preparation was equilibrated for 30 min more, or until a stable baseline had been reached, before starting the actual experiment.

The study comprised two protocols and with a single exception, only one of them was used with each vessel. In protocol 1, the pO₂ of the medium was lowered from neonatal to hypoxic values (see above), and the resulting contraction was allowed to develop to a steady plateau. PD 156707 (0.1 μM) was then added to the bath, and recording continued for 60 min in the case of no response or as long as required for a full response. Conversely, protocol 2 was carried out in normoxia, and 0.1 μM PD 156707 was tested on the contraction to 0.1 μM ONO-11113. In either case, the response to spasmodgens was measured by the rise in tension over basal tension and was expressed in millinewtons per millimeter. Likewise, relaxant responses were expressed in absolute values, taking the baseline preceding the application as reference.

Compounds were injected into the organ bath in 65-μl volumes and saline vehicle had no effect on vessel tone. All concentrations are final in the bath.

**Endothelin Receptor Radioligand Binding Assay.** The main pulmonary artery was collected from term fetal lambs (n = 25) and was freed of loose connective tissue and fat. It was rinsed with sterile Hanks' balanced salt solution and was then transferred to a sterile plastic tube where it was frozen in liquid nitrogen for storage at −80°C. When required, individual specimens were pulverized in a freezer/mill (model 6700; Spex Industries, Edison, NJ), and the powdered tissue was resuspended in 10 ml of buffer containing 20 mM Tris, 2 mM EDTA, and 0.1% BSA (pH 7.4). The suspension was centrifuged at 5000g (5 min, 4°C), and the supernatant was filtered through cheesecloth. The filtrate was centrifuged again at 30,000g (20 min, 4°C), and the pellet was resuspended in buffer containing protease inhibitors (200 μM Pefabloc, 10 μM phosphoramidon, 10 μM leupeptin, and 1 μM pepstatin). The resulting tissue fraction (about 3 mg of protein/ml) was divided in 1-ml aliquots and frozen at −80°C for further work up.

To determine the relative proportion of ETₐR versus ETₐR, tissue aliquots were thawed and serially diluted over the range 1.10 to 1.1280. To each fraction, 30 pM of either [³²P]ET-1 (for ETₐR + ETₐR binding) or [¹²⁵I]ET-3 (for ETₐR binding) was added and incubated for 2 h at 37°C. Separate incubations were carried out with 100 nM of unlabeled ET-1 or ET-3 added to, respectively, [³²P]ET-1 and [¹²⁵I]ET-3 (both at 30 pM). In either case, the assay was terminated by filtration over GF/B filters (Whatman, Clifton, NJ) presoaked with 0.2% BSA in 50 mM Tris.

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Radioactivity being retained on filters was measured in a Betaplate counter (PerkinElmer Wallac, Gaithersburg, MD). Nonspecific binding was defined as binding in the presence of unlabeled peptide; specific binding was defined as total binding minus nonspecific binding. The proportion of ET<sub>R</sub> binding sites relative to the total number of ET<sub>A</sub> + ET<sub>R</sub> binding sites was estimated by the ratio of maximal [125<sup>I</sup>]-ET-3 to maximal [125<sup>I</sup>]-ET-1 binding. It was assumed that [125<sup>I</sup>]-ET-3 and [125<sup>I</sup>]-ET-1 had equal affinities for ET<sub>R</sub> and ET<sub>A</sub>

Competition binding assays were carried out between 30 pM [125<sup>I</sup>]-ET-1 and 0.01 to 10 nM ET-1, 0.01 to 320 nM PD 156707, or 0.16 to 250 nM PD 180988 using the same assay conditions as those described for saturation binding assays.

Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>PAP</th>
<th>PAR</th>
<th>AoP</th>
<th>AoR</th>
<th>HR</th>
<th>CI</th>
<th>LVP/dt</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Normoxia  (n = 4)</td>
<td>11 ± 1</td>
<td>62 ± 9</td>
<td>89 ± 3</td>
<td>504 ± 36</td>
<td>162 ± 11</td>
<td>179 ± 22</td>
<td>2829 ± 393</td>
</tr>
<tr>
<td>Hypoxia  (n = 4)</td>
<td>21 ± 2**</td>
<td>127 ± 13*</td>
<td>86 ± 3</td>
<td>568 ± 27</td>
<td>195 ± 7*</td>
<td>149 ± 8</td>
<td>3487 ± 532</td>
</tr>
<tr>
<td>Normalia (n = 7)</td>
<td>15 ± 0.5</td>
<td>78 ± 8</td>
<td>90 ± 4</td>
<td>679 ± 96</td>
<td>182 ± 20</td>
<td>145 ± 14</td>
<td>4921 ± 42</td>
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<tr>
<td>Hypoxia  (n = 6)</td>
<td>15 ± 0.7*</td>
<td>64 ± 10**</td>
<td>72 ± 5</td>
<td>368 ± 71</td>
<td>217 ± 11</td>
<td>238 ± 35</td>
<td>4410 ± 271</td>
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<td>PD 156707 treatment</td>
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<td></td>
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</tr>
<tr>
<td>Normoxia  (n = 3)</td>
<td>12 ± 0.7</td>
<td>46 ± 2</td>
<td>90 ± 5</td>
<td>331 ± 17</td>
<td>296 ± 5</td>
<td>268 ± 3</td>
<td>4688 ± 1000</td>
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<tr>
<td>Hypoxia  (n = 5)</td>
<td>15 ± 1*</td>
<td>110 ± 17</td>
<td>92 ± 3</td>
<td>673 ± 62</td>
<td>214 ± 10</td>
<td>140 ± 12</td>
<td>4448 ± 578</td>
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<tr>
<td>Hypoxia/low dose</td>
<td>14 ± 2*</td>
<td>58 ± 14</td>
<td>86 ± 5</td>
<td>332 ± 33</td>
<td>224 ± 13</td>
<td>254 ± 31</td>
<td>5930 ± 1861</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01 versus normoxia in the control group; † p < 0.05, †† p < 0.01 versus untreated animal under the same condition of oxygenation.

Results

Intact Animal. Chronically instrumented lambs were conscious at the time of the study and showed no sign of discomfort. In the absence of any treatment, blood gas (pO<sub>2</sub> 73 ± 3 mm Hg; pCO<sub>2</sub> 34 ± 3 mm Hg; pH 7.50 ± 0.01) and hemodynamic (Table 1) variables were within normal limits and remained stable throughout the period of observation. When breathing the low-O<sub>2</sub> gas mixture, the animals exhibited the expected fall in arterial pO<sub>2</sub> but no alterations in the acid/base status (pO<sub>2</sub> 44 ± 1 mm Hg; p < 0.01 versus control; pCO<sub>2</sub> 34 ± 2 mm Hg; pH 7.49 ± 0.01). Coincidentally, PAP and PAR rose and remained elevated for as long as hypoxia was maintained (Fig. 1A). Little or no change was noted instead in the other hemodynamic variables, specifically those pertaining to systemic vascular function (Fig. 1A; Table 1). Elevations in PAP and PAR, comparable in magnitude to those resulting from exposure to alveolar hypoxia, were also observed in the normoxic animal during intra-RA infusion of ONO-11113 (Fig. 2). Again, this constriction response, as for that elicited by hypoxia, appeared to be confined to the pulmonary vascular district and was sustained.

PD 156707 had no obvious effect on the acid/base and cardiovascular status (Table 1) of the normoxic lamb, the only evidence of the treatment being a transient, yet insignificant, fall in PAP, which could be followed by a rebound in the opposite direction. By contrast, when given to the hypoxic animal, the same antagonist reversed the pulmonary hypertension rapidly and completely, or nearly completely (Fig. 1B). The latter effect was sustained and in fact, outlasted the treatment period (Fig. 3A). Compared with the pulmonary vascular response, other changes induced by PD 156707 were marginal and altogether insignificant (Table 1). In particular, AoP and AoR showed a tendency to fall (Fig. 1B). On the other hand, even the pulmonary hypertension, if elicited by ONO-11113 rather than hypoxia, was not susceptible to PD 156707 inhibition and persisted unabated throughout the treatment (Fig. 2). Whether effective or not on the cardiovascular system, the antagonist produced sedation in the animals. The latter response manifested itself gradually and attained a maximum by 15 min after the start of drug administration.

Despite the lower dosage, PD 180988 essentially duplicated, with its hemodynamic and sedative effects, the results obtained with PD 156707. Complete reversal of the hypoxia-induced pulmonary vasoconstriction was observed with both doses of the compound (Fig. 4; Table 1). Likewise, normalization of pulmonary hemodynamics outlasted the treatment (Fig. 3B) and was not associated with any alteration in the acid/base equilibrium. However, at some variance with PD 156707, PD 180988 exerted a modest, but significant, systemic hypertensive effect. Specifically, when treated with the high dose in hypoxia, the animal showed a fall in AoP with a peak at about 40 min through administration (89 ± 6 versus 101 ± 3 mm Hg, p < 0.05) and subsequent partial reversal by 60 min.

During treatment with either ET<sub>R</sub> antagonist, there were no changes in left atrium pressure (8 ± 3 mm Hg) and RA pressure (1 ± 0.3 mm Hg), nor did the pressure gradient between left atrium and right PV depart from baseline values (0–3 mm Hg).

Blood ET-1. ET-1 levels were consistent among animals and increased when lowering blood pO<sub>2</sub> from normoxic to hypoxic values (Table 2). The latter effect developed rapidly, as one may infer from the presence of an upward trend as soon as pulmonary hypertension had reached the steady state (3.07 ± 0.5 pg/ml at zero time, n = 4). Conversely, no such increase was seen with the pulmonary hypertension...
secondary to ONO-11113 administration, and ET-1 values in this case (2.53 ± 0.33 pg/ml, n = 3) equaled those of the normotensive animal (Table 2). ET\(_\alpha\)R antagonists had no effect by themselves on ET-1, and regardless of the test condition, blood concentrations for the treatment groups overlapped with those of controls (Table 2).

**Blood PD 156707 and PD 180988.** ET\(_\alpha\)R antagonists were measurable in blood from all treated animals. Their levels, under any condition, were about 10-fold higher with PD 180988 than PD 156707 and were also more variable (Table 3). In addition, the values for PD 180988 showed no clear difference depending on the dosage, low versus high, used in the experiment. With either compound, however, blood levels attained after the 60-min treatment period did not vary significantly between normoxia and hypoxia (Table 3). Furthermore PD 156707 and PD 180988 behaved similarly in being more abundant at the 30- than the 60-min interval through the treatment in hypoxia (i.e., protocol 3, *see Materials and Methods*) (PD 156707, 158 ± 28 ng/ml, n = 3; PD 180988, 3097 ± 1715 ng/ml, n = 3). The latter finding implies that the initial bolus of the drug contributes in large measure to the observed values. Consistent with this conclusion is also the fact that concentrations being detected in blood 30 min after the cessation of a 30-min treatment (PD 156707, 35 ± 14 ng/ml, n = 4; PD 180988, 539 ± 396 ng/ml, n = 3) do not depart significantly from those found at the end of a 60-min period of uninterrupted treatment (see Table 3).

**Isolated Resistance Arteries.** Pulmonary resistance arteries contracted when the \(\text{pO}_2\) of the medium was lowered from neonatal to hypoxic values (Fig. 5). As expected from vessels with an intact endothelium (see Wang et al., 1995), this contraction started after some delay (average, 14 min, range, 4–21) and progressed slowly to a steady plateau (peak in 22 min, range, 18–26). In contrast, the contraction of the normoxic vessel to ONO-11113 was immediate in onset and development (peak in 15 min from application, range 13–19), and it also attained higher magnitude (Fig. 5). When tested on the sustained response to hypoxia, PD 156707 reversed gradually the contractile tone (average, 42 min for maximal effect) in all, but one, experiment (Fig. 5). This exceptional preparation, however, was characterized by an unusually...
strong hypoxic contraction (1.18 mN/mm before and during treatment) and was not included in the computation. No such effect of PD 156707 was seen when the tone of the vessel was raised with ONO-11113 (Fig. 5). In fact, there was a modest, albeit significant, further elevation in tone due to the antagonist (Fig. 5).

**Endothelin Receptor Radioligand Binding Study.** The relative specific binding of 30 pM of \[^{125}\text{I}]\text{ET-1} (\text{ET}_{\text{AR}} + \text{ET}_{\text{BR}} \text{ binding}) \text{ and } [^{125}\text{I}]\text{ET-3} (\text{ET}_{\text{BR}} \text{ binding}) \text{ to isolated fetal lamb PA tissue (radiolabeled ET-3/radiolabeled ET-1 binding) demonstrated a small proportion of ET}_{\text{AR}} (6.5\%) \text{ versus ET}_{\text{AR}} (93.5\%) \text{ receptor sites. Accordingly, in competition binding experiments, ET-1 inhibited } [^{125}\text{I}]\text{ET-1 binding to isolated fetal lamb PA tissue with an IC}_{50} \text{ of 0.24 nM, and this value was comparable to that of 0.3 nM for PD 156707 and 0.89 nM for PD 180988.**

**Discussion**

This study validates the concept that ET-1, acting via the \text{ET}_{\text{AR}} subtype, is a critical mediator for the contraction elicited by hypoxia in the pulmonary vasculature of the perinatal animal. By extension, it provides the experimental foundation for the use of \text{ET}_{\text{AR}} antagonists as pulmonary anti-
Different functions (i.e., ET<sub>AR</sub>, contraction; ET<sub>BR</sub>, relaxation) whereby the two receptor subtypes subserve generally a dichotomy in the pulmonary vascular ETR system of the lamb (Wang et al., 1995). Hence, we may conclude that there is a preference for testing ET<sub>AR</sub> antagonists with potential therapeutic use as pulmonary antihypertensive agents. Several findings support our conclusion: 1) blood levels of ET-1 are increased during the hypoxia- but not the ONO-11113-triggered pulmonary hypertension, 2) ET<sub>AR</sub> antagonists of diverse chemical structure reverse completely, or nearly completely, the hypoxic pulmonary response both in vitro and in vivo, and 3) the same compounds have, in contrast, no significant relaxant effect on the normoxic pulmonary vasculature whether in its resting state or during the contraction by ONO-11113. Furthermore, effective doses of the ET<sub>AR</sub> antagonists accorded with individual half-life values in the body, with the shorter acting among the compounds tested (PD 156707) requiring a higher dosage, and attendant blood levels were also within the expected range for an antihypertensive action (about 90 and 300 ng/ml for PD 156707 and PD 180988, respectively; S. J. Haleen and K. M. Welch, unpublished data). Normalization of pulmonary hemodynamics obtained with the ET<sub>AR</sub> antagonists during hypoxia implies the absence in the vasculature of an ET<sub>BR</sub> subtype mediating contraction. Our earlier work on isolated pulmonary resistance arteries, i.e., the putative prime target for the hypoxic stimulus, failed to show any ET<sub>BR</sub>-based response (Wang et al., 1995). This agrees with the results of the radioligand binding assay being reported here. An ET<sub><sub>B</sub></sub> set of receptors, on the other hand, was found in the small veins, but its activation resulted in relaxation of the pulmonary vasculature with the attendant lesser risk, on one hand, of blood being shunted locally and, on the other hand, of hypertension reoccurring as soon as administration of the drug is discontinued. In fact, rebound hypertension is a potentially troublesome complication of NO inhalation (Cueto et al., 1997), which we expect not to take place, at least in the same abrupt fashion, with any orally or parenterally administered ET<sub>AR</sub> antagonist. The latter assumption is based not only on the observation made here that the antihypertensive effect of...
the antagonists persists beyond the treatment period but also on the notion that a mechanism-based intervention may re
verse any hypertension-induced down-regulation of NO- and prostaglandin I2-linked pulmonary vasodilators systems (Shaull et al., 1997; Tuder et al., 1999). Hence, even in the case of a transient withdrawal from drug action, the pulmo
nary vasculature may be able to counter more effectively any drive toward excess contraction. Last, ETAR antagonists would prove particularly useful with any pathological condi
(especially, congenital diaphragmatic hernia) where the pulmonary hypertension fails to respond to NO (Neonatal In
haled Nitric Oxide Study Group, 1997).

Against all of these positive features, there are possible drawbacks. As mentioned above, ETAR antagonists may not remain selective in their action on the hypertensive pulmo
nary vascular district, and a significant systemic hypotension has complicated studies in the animal (Petrossian et al., 1999; McMullan et al., 2001) as well as the clinical trial of a dual ETAR/ETBR antagonist (i.e., bosentan) in the adult (Williamson et al., 2000). However, judging from our present work and the outcome of a recent clinical study (Channick et al., 2001), any such complication should be avoidable by adjusting the dosage. Another difficulty may derive from the involvement of alternative mechanisms, such as the interfe
rence with calcium-sensitive potassium channels, in the pathogenesis of pulmonary hypertension (Cornfield et al., 2000). Their contribution, should it become significant, could lessen the impact of any ETAR antagonist-based therapy.

ETAR, on the other hand, may modulate central nerve pathways involved in respiratory control (Kuwaki et al., 1996) and the response to stress (Kurihara et al., 2000), and this raises the question of an adverse effect of the ETAR antagonists on the brain. However, apart from the sedation, we could not detect any central action at doses effective on the pulmonary circulation. Significant in this context is also the fact that these compounds cross spuriously the blood-brain barrier, as one can infer from whole body autoradiography and the di
rect assay of cerebrospinal fluid (S. J. Haleen and K. M. Welch, unpublished data). A final, possible complication, also originating from experimental data (Coceani et al., 1999; Takizawa et al., 2000) is that the ETAR antagonist could reopen the ductus arteriosus in the young infant. Such an event, however, seems remote since closure of the vessel becomes irreversible beyond the immediate neonatal period.

In conclusion, then, it is safe to assume that the antici
pated benefits in using the ETAR antagonists for neonatal pulmonary hypertension outweigh any potential drawbacks. The new classes of antagonists, studied by us, lend them
selves well to this particular application, because they com
bine the required efficacy with versatility in both the route of administration and the time course of action. Conceivably, our data have implications not only for the management of the sick infant, but also for patients suffering from pulmo
nary hypertension in adult age.

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References


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