Mechanism of Endothelin-1–Induced Pulmonary Vasoconstriction

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Endothelins are endothelial cell–derived peptides with potent vasoconstrictor properties. We investigated the actions of porcine/human endothelin-1 (ET-1) on the microvasculature of the guinea pig lung perfused at constant flow with Ringers-albumin. We measured the perfusion pressure, distribution of pulmonary vascular resistance (using the double occlusion method), lung weight change, and the pulmonary capillary filtration coefficient. At concentrations of \( \geq 10^{-10} \) M, ET-1 produced dose-dependent increases in mean pulmonary artery pressure (EC50, \( \sim 10^{-9.5} \) M), which were rapid in onset and biphasic (first phase peaking at 1–2 minutes; second phase peaking at 10–15 minutes) up to 60 minutes of the perfusion period. The vasoconstrictor response was sustained for the 60-minute perfusion period. The pulmonary vasoconstriction was inhibited by pretreatment with indomethacin (10^{-5} M), the thromboxane A2 receptor antagonist SQ-29,548 (4 \times 10^{-4} M), or papaverine (10^{-4} M). Nifedipine (10^{-6} or 10^{-7} M) had no effect on the first phase but prevented the second phase of the vasoconstriction. The vasoconstriction was primarily the result of a 10-fold increase in pulmonary venous resistance. Pulmonary edema developed after ET-1 challenge because of the vasoconstriction and the resultant pulmonary capillary hypertension. However, the pulmonary capillary filtration coefficient was unchanged, indicating that pulmonary vascular permeability did not increase. ET-1 also had no effect on transendothelial 125I-albumin flux. The results indicate that ET-1 is a potent thromboxane-dependent venuconstrictor in the guinea pig lung. ET-1 induces pulmonary edema because of its ability to increase pulmonary capillary hydrostatic pressure. The mechanism of the sustained pulmonary venoconstriction may involve calcium mobilization via dihydropyridine-sensitive calcium channels. (Circulation Research 1991;69:157–164)

Endothelins are endothelial-derived vasoconstrictor peptides isolated from medium conditioned by cultured endothelial cells.1–4 Endothelins are released in the circulation in the guinea pig lung in response to pulmonary vasoconstrictors such as thrombin.3 Studies indicate that the human/porcine endothelin, ET-1, is a potent constrictor of coronary,4 renal,5,6 mesenteric,7 and other peripheral4,7–9 vessels. ET-1 also constricts airways8,10,11 and intestinal7 smooth muscle. Under conditions of increased basal vascular tone, ET-1 induces vasodilation in the rat mesentery7,12 and other peripheral vessels7 and in the pulmonary circulation.13 ET-1 is a peripheral vasodilator but a mild pulmonary vasoconstrictor in the anesthetized cat.14 The pulmonary vasomotor actions of ET-1 and the mechanisms mediating the alterations are not well understood. Because of endothelin’s possible effect in increasing pulmonary capillary hydrostatic pressure and vascular permeability, ET-1 also may mediate pulmonary edema. In the present study, we used the guinea pig lung perfused at constant flow with Ringers-albumin to study the direct responses of ET-1 on pulmonary vasomotor tone and the roles of the cyclooxygenase pathway and extracellular calcium in mediating the pulmonary vasomotor responses.

Materials and Methods

Lung Perfusion

Thirty healthy male or female Hartley guinea pigs (Charles River Laboratories, Inc., Wilmington, Mass.) weighing 550±25 g (mean±SEM) were anesthetized with pentobarbital sodium (50 mg/kg i.p.; Abbott Laboratories, Chicago). The trachea was cannulated before the thorax was opened. Heparin sodium (700 units/kg) was administered by intracardiac injection, and the animals were bled by severing the abdominal aorta. The heart and lungs were removed carefully en bloc and suspended from one end of a beam balance. The lung perfusion system
has been detailed elsewhere. Briefly, catheters were placed in the pulmonary artery and left atrium. The lungs were covered with plastic wrap to reduce evaporative water loss. Perfusion of the system using a peristaltic pump (model 1215, Harvard Apparatus, Millis, Mass.) was begun within 5 minutes of pneumothorax with bubble traps included in the perfusion circuit. The perfusate was a phosphate buffered Ringer’s solution containing bovine albumin (0.5 g/100 ml) (fraction V, Sigma Chemical Co., St. Louis) and the following components (mM): NaCl 137, CaCl₂ 1.8, MgCl₂ 1.05, KCl 2.68, NaHCO₃ 0.66, NaH₂PO₄ 0.130, Na₂HPO₄ 0.869, and dextrose 5.55; this solution was prebubbled with 95% O₂-5% CO₂. The perfusion rate was set at 28 ml/min (0.05 l/kg/min) for all experiments. Recirculation of the perfusate was begun after the venous effluent was clear of visible blood. The final recirculating volume was 300 ml. The perfusate temperature was set at 37°C, and left atrial pressure was adjusted to 3 cm H₂O. Because flow and left atrial pressure were held constant, changes in pulmonary arterial pressure (Pₚₐ) reflected changes in the pulmonary vascular resistance (PVR). The lungs initially were inflated three times to 20 cm H₂O and then kept statically inflated throughout the experiment with 95% O₂-5% CO₂ at a pressure of 2 cm H₂O, with intermittent short-term increases in airway pressure to 20 cm H₂O.

Endothelial Cell Monolayer

Endothelial cells were grown to confluency on polycarbonate microporous filters (0.8-μm pore diameter) (Nucleopore Corp., Pleasanton, Calif.) and mounted onto the base of plastic wells as described. Bovine pulmonary microvessel endothelial cells were isolated and cultured according to the method of Folkman et al. Briefly, subpleural lung tissue was minced, treated with collagenase, and filtered through 160-μm nylon mesh, and the suspension was centrifuged. The pellet was washed three times and resuspended in RPMI-1640, 20% heparinized fetal bovine serum (Sigma) with retinal-derived growth factor, then plated on fibronectin-coated culture dishes. Endothelial cell colonies obtained after several days of incubation were isolated with a glass cloning cylinder and replated onto other fibronectin-coated dishes. The cells obtained were identified as endothelial cells by their morphology, absence of factor VIII–related antigen, incorporation of acetylated low density lipoprotein, and activity of angiotensin-converting enzyme. Dulbecco’s modified essential medium (DMEM) and 20% fetal calf serum (GIBCO, Grand Island, N.Y.) containing 4×10⁵ cells were seeded onto the gelatin-coated microporous filter. Studies were made 4 days after seeding, when monolayer confluency was verified by light microscopy.

Measurements

The lungs were suspended from a counterweighted beam balance for continuous weight monitoring. Left atrial pressure and Pₚₐ were measured continuously with pressure transducers (models P50 and P23, respectively, Gould Instruments, Cleveland, Ohio) and weight recordings were made on a three-channel recorder (model 2400S, Gould). Pulmonary capillary pressure (Pₚₑ) was determined by the double-occlusion method, which was used to determine the arterial (Rₑₐ) and venous (Rₑₐ) resistances. The pulmonary capillary filtration coefficient (Kₑₑ) was measured during an isogravimetric period as described. Pₑₑ was elevated by raising the left atrial pressure for 5 minutes, and the Kₑₑ was determined from the relation of rate of weight increase from minutes 2 through 5 when the increase in lung weight was assumed to be due to transvascular fluid filtration. Kₑₑ was measured (n = 7) after the addition of papaverine (0.4 mg/100 ml) (Sigma), which was used to paralyze the vasculature, thus abrogating any changes in vascular tone due to ET-1. In experiments using this protocol, papaverine independently did not alter the PVR, lung weight, or Kₑₑ values. At the end of each experiment, the lungs were dissected free of nonpulmonary tissue, weighed, and dried to a constant weight at 50°C.

In some experiments, left atrial effluent samples were collected and analyzed for thromboxane B₂ (TXB₂) and 6-ketoprostaglandin F₁α (6-keto-PGF₁α), the stable metabolites of thromboxane A₂ and prostacyclin, respectively, using a double-antibody radioimmunoassay.

The effect of ET-1 on endothelial monolayer permeability was determined using the permeability system described above. Briefly, the luminal wells (0.7-ml capacity), which consisted of the plastic cylinders with polycarbonate filters on which endothelial cells were grown, were suspended in larger abluminal compartments (25-ml capacity), the contents of which were stirred constantly and maintained at 37°C. Both compartments were filled with Hank’s balanced salt solution (GIBCO) containing 0.5% bovine serum albumin (Sigma). The tracer 1²⁵I-albumin was added to the upper well, and 400-μl samples were taken from the lower compartment every 5 minutes for 30 minutes before intervention and for 30 minutes thereafter. Intervention consisted of placing 25 μl of freshly prepared solutions of ET-1 in water (Peptides International Inc., Louisville, Ky.) into the luminal wells containing the endothelial monolayer to achieve final ET-1 concentrations from 10⁻⁹ to 10⁻⁷ M. Negative control intervention was 25 μl DMEM, and positive control was purified human α-thrombin (10⁻⁷ M) (courtesy of Dr. John W. Fenton, New York State Department of Health, Albany, N.Y.). The 1²⁵I activity in the lower well samples was determined using a gamma-counter (Auto-Gamma 5000 series, Packard Instrument, Co., Inc., Meriden, Conn.). The 1²⁵I-albumin clearance rate (a measure of endothelial permeability of albumin) was determined by least-squares linear regression for the 30-minute periods before and after ET-1. Each data point is the mean of three experiments for ET-1 and three for α-thrombin, and each experimental data point is the value obtained from a mean of eight wells.
Experimental Protocols

In a series of experiments (n=3–6, except n=2 for 10^{-8} M), the pulmonary vasomotor responses to varying ET-1 concentrations were determined. In other experiments (n=4), a dose–response curve for platelet activating factor (PAF) (Sigma) was obtained. Either ET-1 or PAF (10^{-8} to 10^{-12} M final concentration) was added to the circulating bath after baseline measurements. Changes in pulmonary vasomotor tone and weight gain were monitored continuously for 60 minutes after the addition of ET-1 or PAF.

We examined the role of the cyclooxygenase cascade on the ET-1–induced pulmonary vascular response by using indomethacin (10^{-5} M) (Sigma), a cyclooxygenase inhibitor (n=7). Indomethacin added to the perfusate 10 minutes before ET-1 administration did not alter baseline P_{Pa} and lung weight. We also examined the effects of the thromboxane receptor antagonist SQ-29,548,21 [1S-[1α,2β(5Z),3β,4α]]-7-[3-[(2-phenylamino)carbonyl]hydrazino][methyl]-7-oxabicyclo[2.2.1]hept-2-yl-5-heptenoic acid (a gift of Dr. Martin O’Gletree, Squibb Pharmaceutical, Princeton, N.J.), on the pulmonary vascular responses of ET-1. SQ-29,548 (4×10^{-6} M) was added to the perfusate (n=4) 10 minutes before the addition of ET-1. The drug solution (0.5 ml) contained 8% ethanol and 2% sodium carbonate. The addition of SQ-29,548 alone did not cause alterations in P_{Pa} and lung weight during the study period. This dose of SQ-29,548 prevented pulmonary vasomotor response to the thromboxane mimic U46619 (10^{-8} M) (The Upjohn Co., Kalamazoo, Mich.), administered 70 minutes after SQ-29,548; however, PAF (10^{-10} M) produced the characteristic pulmonary vasconstriction and increased weight gain in the SQ-29,548–treated lungs. The pulmonary vasculature remained responsive to KCl after treatment with indomethacin or SQ-29,548.

We examined the role of extracellular calcium using nifedipine, a dihydropyridine calcium channel blocker (Sigma), which was added to the circulating perfusate 10 minutes before the administration of ET-1 at a final concentration of either 10^{-5} M (n=3) or 10^{-7} M (n=4). Stock solutions of nifedipine in dimethyl sulfoxide were freshly prepared and 30 μl added to the 300 ml of perfusate.

Statistical Analysis

Intergroup comparisons and changes from baseline were made using the two-way analysis of variance. When differences were found, post hoc comparisons were made using a t test with a Bonferroni correction for multiple measurements.22 Significance was defined as p<0.05.

Results

Addition of ET-1 (10^{-12} to 10^{-8} M) to the Ringers-albumin–perfused lungs produced dose–dependent increases in PVR, with a threshold dose of 10^{-10} M (Figure 1). Because the lungs were perfused at constant flow, the changes in P_{Pa} directly reflect changes in PVR. PAF produced a greater increase in pulmonary vasomotor tone on a molar basis than ET-1 (Figure 1). The other points for Figure 1 are from previously obtained values in this laboratory using the same guinea pig preparation. ET-1 was a more potent pulmonary vasoconstricotor on a molar basis than substance P,23 α-thrombin,20 and arachidonic acid19 (Figure 1).

The addition of 10^{-9} M ET-1 produced biphasic increases in the pulmonary vasomotor tone (Figure 2). The increase occurring within 1–2 minutes after ET-1 challenge was threefold above baseline, followed by a decrease over the next 5 minutes and then a secondary increase of threefold above baseline 15 minutes after ET-1 challenge. Pulmonary vasomotor tone gradually returned toward baseline but remained 70% above baseline even at the end of the 60-minute study period.

Figure 3 shows the time course of the pulmonary vasoconstrictor response and the concomitant increase in lung weight in response to 10^{-9} M ET-1. The pulmonary vasoconstriction was biphasic (Figure 3B). The vasoconstriction was associated with a transient initial decrease in lung weight within 15 seconds, followed by a sharp increase within 1 minute and then a progressive increase over the next 16 minutes (Figure 3A). After this period, lung weight stabilized. Addition of a higher concentration of ET-1 (10^{-8} M) led to the rapid development of massive pulmonary edema within 20–30 minutes of the ET-1 administration; therefore, the time course studies were made using 10^{-9} M ET-1.

Figure 4 shows the effect of ET-1 on the pulmonary venous effluent TXB_{2} concentrations. A significant rise in TXB_{2} concentration is evident within 5 minutes after ET-1 injection. TXB_{2} concentration continued to increase further at 15 minutes (which
was associated with a secondary rise in PVR, and then it decreased toward baseline levels by 30 minutes, when PVR also decreased (although both TXB₂ and PVR were elevated above baseline at this time). The increase in 6-keto-PGF₂α was significantly less \((p<0.05)\) than the increase in TXB₂ (from \(0.6\pm0.4\) ng/ml at baseline to \(1.2\pm0.5\) ng/ml at 5 minutes), and this level was maintained for the study period. Indomethacin inhibited TXB₂ (Figure 4) and 6-keto-PGF₂α generation. Pretreatment with indomethacin or the thromboxane receptor antagonist SQ-29,548 prevented the initial and secondary increases in PVR (Figure 2).

The initial rise in PVR peaking at 1 minute was unchanged by \(10^{-7}\) M nifedipine but was decreased slightly by \(10^{-5}\) M nifedipine \((p<0.05\) compared with ET-1 control) (Figure 5). However, both nifedipine concentrations prevented the secondary rise in PVR.

\(P_{pa}\) increased in parallel with the development of pulmonary vasoconstriction after ET-1 (Table 1). The precapillary and postcapillary resistances \((R_{pa}, R_{pp})\) after the addition of ET-1 are shown in Table 1. \(R_{pa}\) increased progressively \((p<0.01)\) and reached a peak value at 15 minutes. In contrast, \(R_{pp}\) increased \((p<0.05)\) slightly and transiently from baseline. Pretreatment with indomethacin, SQ-29,548, and nifedipine inhibited the ET-1–induced increases in \(R_{pa}\) and \(R_{pp}\) as well as the increase in \(P_{pa}\). Papaverine \((10^{-5}\) M\) also prevented the ET-1–induced increases in \(R_{pa}, R_{pp}\), and \(P_{pa}\).

ET-1 resulted in a 48% increase in lung wet weight over the first 15 minutes, followed by its stabilization for the 60-minute perfusion period (Figure 6). \(P_{pa}\)

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**Figure 2.** Pulmonary artery pressure response to \(10^{-9}\) M endothelin (ET-1) in guinea pig lungs perfused at constant flow. Effects of papaverine \((10^{-5}\) M\), indomethacin \((10^{-5}\) M\), and SQ 29548 \((4\times10^{-8})\) are shown. All three agents prevent pulmonary pressor response. Bars indicate \(\pm SEM\). *\(p<0.05\) for each group, compared with control ET-1 group; †\(p<0.05\), compared with time zero (baseline).

**Figure 3.** Time course of changes in pulmonary weight and artery pressure \((P_{pa})\) after endothelin (ET-1) challenge \((10^{-9}\) M\) in guinea pig lung. Panel A: Note progressive rise in lung weight beginning 1 minute after ET-1; panel B: two phases of the rise in \(P_{pa}\) and marked increase in the pulmonary capillary pressure, indicative of pulmonary vasoconstriction. Asterisks indicate when double occlusion was made.

**Figure 4.** Thromboxane B₂ concentrations in guinea pig pulmonary venous effluent in response to endothelin (ET-1) and the effect of indomethacin \((10^{-5}\) M\) on thromboxane B₂ generation. *\(p<0.05\) vs. indomethacin group. Bars indicate \(\pm SEM\).
Table 1. Pulmonary Hemodynamic Variables for All Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Pulmonary artery pressure (cm H2O)</th>
<th>Pulmonary venous resistance (cm H2O/ml/min)</th>
<th>Pulmonary arterial resistance (cm H2O/ml/min)</th>
<th>Pulmonary capillary pressure (cm H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40 ± 10</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.05</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>ET-1 (10⁻⁸ M)</td>
<td>40 ± 10</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.05</td>
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Decreased toward baseline after 15 minutes, whereas lung weight continued to be elevated. Indomethacin, SQ-29,548, papaverine, and nifedipine prevented the development of pulmonary edema (Figure 6); this effect of nifedipine was observed at both concentrations of 10⁻⁵ and 10⁻⁷ M.

The Kₜₑ value was unchanged from baseline values after the administration of ET-1 (0.092±0.016, 0.075±0.011, 0.075±0.018, and 0.142±0.086 ml/min/cm H2O/g dry wt at baseline, 15, 30, and 45 minutes, respectively). ET-1 (10⁻⁹ to 10⁻⁷ M) also had no effect on the transendothelial 12⁵I-albumin clearance rates (Figure 7), whereas α-thrombin increased the 12⁵I-albumin clearance (from baseline value of 0.150±0.20 to 0.355±0.15 µl/min after thrombin).

Discussion

In the present study, we have demonstrated that ET-1 challenge of guinea pig lungs perfused with Ringers-albumin causes pulmonary vasoconstriction. The response was potent and concentration dependent, with a threshold ET-1 concentration of 10⁻¹⁰ M. The ET-1-mediated pulmonary vasoconstriction response was biphasic, with the initial rise in PVR peaking at 1 minute followed by a secondary increase peaking at 15 minutes. The increase in PVR was associated with thromboxane generation, because TXB₂, the primary metabolite of thromboxane, was released in the lung effluent after ET-1 challenge. In contrast, the increase in the effluent prostacyclin concentration was small. Thromboxane appears to be a critical mediator of the response, because pulmonary vasoconstriction was inhibited by the cyclooxygenase inhibitor indomethacin, as well as by the thromboxane receptor antagonist SQ-29,548. We cannot exclude the possibility that other arachidonic metabolites also are involved, because prostaglandin D₂ and prostaglandin F₂ bind to the thromboxane receptor 1,24 and thereby may mediate pulmonary vasoconstriction. ET-1 is known to activate phospholipase A₂ in vascular smooth muscle cells,25 and hence it may directly induce the release of arachidonic acid metabolites in lung tissue.7 Because the generation of thromboxane and the rise in PVR were sustained, the results suggest a persistent activation of phospholipase A₂.

The pulmonary vasoconstrictor response to ET-1 in the guinea pig lung is quite different from the pulmonary vasodilation observed in other studies.13 ET-1-induced pulmonary vasodilation appears to be dependent on the basal pulmonary vasomotor tone; that is, dilatation occurred in response to ET-1 in lungs with high tone, such as in the neonatal pulmonary circuit.26 The mechanisms of this effect are not clear, although cyclooxygenase-derived arachidonic acid metabolites do not appear to be involved,26 as is the case with ET-1-induced pulmonary vasoconstriction in the adult guinea pig lung.

The pulmonary vasoconstriction induced by ET-1 was long-lived, lasting the 60-minute duration of the perfusion period. This observation is consistent with
the evidence that ET-1 binding to vascular smooth muscle receptors is prolonged and slowly reversible. Spinella et al. have shown that prolonged binding of ET-1 to cell membranes is the result of the extreme hydrophobic nature of the C-terminus of the molecule. The high-affinity ET-1 binding may result in the long-lived generation of thromboxane and possibly other arachidonic acid metabolites, and hence may be responsible for the sustained nature of pulmonary vasoconstrictor response.

The primary site of ET-1 action in the guinea pig lung was in the pulmonary veins. The dominance of the pulmonary vasoconstriction may be explained by a "downstream" ET-1 action subsequent to the release of thromboxane, which is a well-known pulmonary venous constrictor. Alternatively, pulmonary vasoconstriction may be mediated by a greater "sensitivity" of pulmonary venous smooth muscle cells to ET-1 (e.g., greater receptor number or affinity).

ET-1 is known to induce biphasic vascular smooth muscle calcium transients, with only the second phase inhibited by diltiazem and nifedipine or by a calcium-free medium. ET-1 activates phospholipase C and stimulates phosphatidylinositol metabolism; therefore, ET-1 can mobilize intracellular calcium stores. Calcium mobilization can activate calcium-sensitive potassium channels, explaining the transient membrane hyperpolarization observed with ET-1. Nonselective cation channels may be opened, resulting in a sustained membrane depolarization that activates voltage-gated calcium channels and sustained calcium influx. Nifedipine (10⁻⁷ and 10⁻⁵ M) did not affect the ET-1-induced increase in PVR occurring at 1 minute but prevented the second phase of the response. This is consistent with the rapid mobilization of intracellular calcium stores induced by ET-1 and the subsequent activation of nifedipine-sensitive calcium channels.

The inhibition of the secondary pulmonary vasoconstriction with nifedipine agrees with the observation that the secondary calcium transient is dihydropyridine sensitive.

Pulmonary edema (a 48% increase in lung wet weight) developed in response to ET-1. The edema was the result of pulmonary vasoconstriction and the consequent rise in the capillary hydrostatic pressure. The pulmonary edema was prevented with indomethacin, SQ-29,548, and nifedipine (at both concentra-
sections of $10^{-7}$ and $10^{-5}$ M), because in all cases these agents prevented the ET-1–induced increase in P$_w$.

There was no evidence of an increase in vessel wall permeability, because $K_{pw}$ was unchanged. ET-1 also did not increase endothelial monolayer permeability to $^{125}$I-albumin. These results are consistent with other studies in which ET-1 had no effect on permeability to dextran in the hamster cheek pouch microvessels.37

In conclusion, the pulmonary venoconstrictor response to ET-1 in the guinea pig lung is biphasic. Thromboxane generation induced by ET-1 appears to be a critical signaling event responsible for the pulmonary vеноconstriction. Calcium influx mediated by dihydropyridine-type calcium channels may be involved in the vеноconstriction, because this response was inhibited by nifedipine. ET-1 also caused pulmonary edema as a result of the rise in pulmonary capillary hydrostatic pressure. In view of the potent and long-lived pulmonary vеноconstriction and edematogenic effects of ET-1, it is reasonable to assume that ET-1 release in situ will have profound effects in the pathogenesis of acute lung injury and other related disorders.

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