Mechanism of Endothelin-1–Induced Pulmonary Vasoconstriction

Michael J. Horgan, Joaquim M.B. Pinheiro, and Asrar B. Malik

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 ≥10^{-10} M, ET-1 produced dose-dependent increases in mean pulmonary artery pressure (EC_{50}~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 A_2 receptor antagonist SQ-29,548 (4 \times 10^{-9} M), or papaverine (10^{-5} 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 vasoconstrictor 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 vasoconstriction may involve calcium mobilization via dihydropyridine-sensitive calcium channels. (Circulation Research 1991;69:157–164)
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, CaCl$_2$ 1.8, MgCl$_2$ 1.05, KCl 2.68, NaHCO₃ 0.66, NaH$_2$PO$_4$ 0.130, Na$_2$HPO$_4$ 0.869, and dextrose 5.55; this solution was prebubbled with 95% O$_2$–5% CO$_2$. 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 H$_2$O. Because flow and left atrial pressure were held constant, changes in pulmonary arterial pressure ($P_{pa}$) reflected changes in the pulmonary vascular resistance (PVR). The lungs initially were inflated three times to 20 cm H$_2$O and then kept statically inflated throughout the experiment with 95% O$_2$–5% CO$_2$ at a pressure of 2 cm H$_2$O, with intermittent short-term increases in airway pressure to 20 cm H$_2$O.

**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 4×10$^4$ 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 $P_{pa}$ 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 ($P_{pc}$) was determined by the double-occlusion method, which was used to determine the arterial ($R_{pa}$) and venous ($R_{pv}$) resistances. The pulmonary capillary filtration coefficient ($K_{f,c}$) was measured during an isogravimetric period as described. $P_{pc}$ was elevated by raising the left atrial pressure for 5 minutes, and the $K_{f,c}$ 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. $K_{f,c}$ 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 $K_{f,c}$ 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 B$_2$ (TXB$_2$) and 6-ketoprostaglandin F$_3$ (6-keto-PGF$_{3alpha}$), the stable metabolites of thromboxane A$_2$ 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 walls (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 Hanks’ balanced salt solution (GIBCO) containing 0.5% bovine serum albumin (Sigma). The tracer $^{125}$I-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$^{-7.5}$ 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 $^{125}$I activity in the lower well samples was determined using a gamma-counter (Auto-Gamma 5000 series, Packard Instrument Co., Inc., Meriden, Conn.). The $^{125}$I-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 Ppa 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-oxacyclo[2,2,1]hept-2-yl-5-heptenoic acid (a gift of Dr. Martin Ogletree, 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 Ppa and lung weight during the study period. This dose of SQ-29,548 prevented pulmonary vasoconstriction response to the thromboxane mimic U46619 (10^-5 M) (The Upjohn Co., Kalamazoo, Mich.), administered 70 minutes after SQ-29,548; however, PAF (10^-10 M) produced the characteristic pulmonary vasoconstriction 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. 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 Ppa 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 vasoconstrictor on a molar basis than substance P,23 α-thrombin,20 and arachidonic acid (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^-8 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 TXB2 concentrations. A significant rise in TXB2 concentration is evident within 5 minutes after ET-1 injection. TXB2 concentration continued to increase further at 15 minutes (which
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 (4x10^{-6}) are shown. All three agents prevent pulmonary pressor response. Bars indicate ±SEM. *p<0.05 for each group, compared with control ET-1 group; †p<0.05, compared with time zero (baseline).

was associated with a secondary rise in PVR, and then it decreased toward baseline levels by 30 minutes, when PVR also decreased (although both TXB_{2} and PVR were elevated above baseline at this time). The increase in 6-keto-PGF_{2a} was significantly less (p<0.05) than the increase in TXB_{2} (from 0.6±0.4 ng/ml at baseline to 1.2±0.5 ng/ml at 5 minutes), and this level was maintained for the study period. Indomethacin inhibited TXB_{2} (Figure 4) and 6-keto-PGF_{2a} 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_{pp} increased in parallel with the development of pulmonary vasoconstriction after ET-1 (Table 1). The precapillary and postcapillary resistances (R_{pp} and R_{pv}) after the addition of ET-1 are shown in Table 1. R_{pp} increased progressively (p<0.01) and reached a peak value at 15 minutes. In contrast, R_{pv} 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_{pp} and R_{pv}, as well as the increase in P_{pc}. Papaverine (10^{-5} M) also prevented the ET-1-induced increases in R_{pp}, R_{pv}, and P_{pc}.

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_{pc}
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 $K_t$ 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 H$_2$O/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 $\alpha$-thrombin increased the 125I-albumin clearance (from baseline value of 0.150±0.20 to 0.355±0.15 ml/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 $10^{-9}$ 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 TXB$_2$, 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 D$_2$ and prostaglandin F$_2$ bind to the thromboxane receptor$^{24,25}$ and thereby may mediate pulmonary vasoconstriction. ET-1 is known to activate phospholipase A$_2$ 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 A$_2$.

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, dilation 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

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**Table 1. Pulmonary Hemodynamic Variables for All Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>Pulmonary arterial pressure (mm Hg)</th>
<th>Pulmonary venous resistance (cm H$_2$O/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 (10$^{-9}$ M, n=7)</td>
<td>0.142±0.086</td>
<td>0.150±0.20</td>
</tr>
<tr>
<td>ET-1 (10$^{-5}$ M, n=4)</td>
<td>0.092±0.016</td>
<td>0.075±0.011</td>
</tr>
<tr>
<td>ET-1 (10$^{-7}$ M, n=3)</td>
<td>0.075±0.018</td>
<td>0.075±0.018</td>
</tr>
</tbody>
</table>

* $p<0.001$, $\gamma<0.005$ from (baseline) time point.
The evidence that ET-1 binding to vascular smooth muscle receptors is prolonged and slowly reversible,27,28 Spinella et al29 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.19 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.30 ET-1 activates phospholipase C31,32 and stimulates phosphatidylinositol metabo-lism33,34; therefore, ET-1 can mobilize intracellular calcium stores.30,34 Calcium mobilization can activate calcium-sensitive potassium channels, explaining the transient membrane hyperpolarization observed with ET-1.35 Nonselective cation channels may be opened, resulting in a sustained membrane depolarization that activates voltage-gated calcium channels and sustained calcium influx.35 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-130,34,36 and the subsequent activation of nifedipine-sensitive calcium channels.30 The inhibition of the secondary pulmonary vasoconstriction with nifedipine agrees with the observation that the secondary calcium transient is dihydropyridine sensitive.30

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-

**Figure 5.** Pulmonary artery pressure response to 10⁻⁶ M endothelin (ET-1) in guinea pig lungs perfused at constant flow. Effects of 10⁻⁷ and 10⁻⁵ M nifedipine are shown. Note the comparable 1-minute responses but inhibition of the secondary rise in pulmonary artery pressure at both nifedipine concentrations. *p<0.01 compared with baseline; †p<0.05 compared with ET-1 only. Bars indicate ±SEM.

**Figure 6.** Lung weight changes after 10⁻⁶ M endothelin (ET-1) and the effect of papaverine (10⁻⁵ M), indomethacin (10⁻⁵ M), nifedipine (10⁻⁵ M), and SQ 29548 (4×10⁻⁶ M). All agents prevented progressive weight. *p<0.05; †p<0.01; compared with time zero.
tions of $10^{-7}$ and $10^{-5}$ M), because in all cases these agents prevented the ET-1–induced increase in $P_{ac}$. There was no evidence of an increase in vessel wall permeability, because $K_p$ 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 venoconstriction. Calcium influx mediated by dihydropyridine-type calcium channels may be involved in the venoconstriction, 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 venoconstriction 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.

Acknowledgments

The authors wish to thank Mr. John Everitt for his excellent technical assistance, Ms. Nancy Gertzberg for help with graphics, and Dr. Peter Del Vecchio for providing the endothelial cell cultures.

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Key Words • endothelin • pulmonary hypertension • cyclooxygenase inhibition • calcium • thromboxane • nifedipine • pulmonary vasoconstriction
Mechanism of endothelin-1-induced pulmonary vasoconstriction.
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doi: 10.1161/01.RES.69.1.157

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