Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ET_B receptors

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Departments of Medicine, Montreal Heart Institute, Montreal, Quebec H3T 1C8; Montreal General Hospital, Montreal, Quebec H3G 1A4; and Institut National de la Recherche Scientifique-Santé, Pointe-Claire, Quebec H9R 1G6, Canada

Dupuis, Jocelyn, Carl A. Goresky, and Alain Fournier. Pulmonary clearance of circulating endothelin-1 in dogs in vivo: exclusive role of ET_B receptors. J. Appl. Physiol. 81(4): 1510–1515, 1996.—The pulmonary circulation plays an important role in the removal of circulating endothelin-1 (ET-1). Plasma ET-1 levels are increased in pulmonary hypertensive states of various etiologies (e.g., idiopathic, heart failure, and congenital anomalies) in proportion to the severity of pulmonary hypertension. It is possible that reduced pulmonary clearance of this peptide contributes to the hyperendothelinaemia of those pathologies. The ET_A and ET_B receptors are abundant in lung tissues: on the vascular endothelium, the ET_B receptor is predominant and may contribute to ET-1 extraction through receptor-mediated endocytosis. We designed experiments to determine and quantify the importance of the ET_A and ET_B receptors in the pulmonary extraction of circulating ET-1 in anesthetized dogs. The single-pass cumulative tracer ET-1 extraction by the lung was measured with the indicator-dilution technique before and 5 min after intrapulmonary injection of the specific ETA antagonist BQ-123 (n = 5, 120–960 nmol) and the specific ET_B antagonist BQ-788 (n = 6, 1,000 nmol). The inhibitors had no significant effect on pulmonary and systemic hemodynamics. Mean cumulative pulmonary ET-1 extraction was not modified by BQ-123 [control (C): 36 ± 4%, antagonist (A): 34 ± 6%] but was completely abolished by BQ-788 (C: 34 ± 6%, A: 0 ± 2%, P < 0.001). The pulmonary rate constant (K) for ET-1 removal was also unaffected by BQ-123 (C: 0.050 ± 0.0085 s⁻¹, A: 0.047 ± 0.0012 s⁻¹) but significantly decreased and became close to zero after BQ-788 (C: 0.058 ± 0.014 s⁻¹, A: 0.009 ± 0.007 s⁻¹, P < 0.001). We conclude that the ET_B receptor is completely and exclusively responsible for pulmonary ET-1 removal in vivo. Future studies are needed to show whether desensitization or downregulation of the ET_B receptor may contribute to the increase in circulating ET-1 levels in conditions associated with pulmonary hypertension. This novel pulmonary endothelial cell function may play a protective role by modulating circulating ET-1 levels in the systemic circulation.

endothelin receptors; pulmonary metabolism; BQ-123; BQ-788

THE PULMONARY CIRCULATION effectively produces, modifies, and inactivates numerous circulating substances but, more specifically, vasoactive amines and peptides. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by vascular endothelial cells with a preferential abluminal secretion to effectively interact with the subjacent smooth muscle layer. ET-1 is nevertheless present in the circulation in small measurable amounts, and circulating ET-1 levels are increased up to fivefold in various pathological conditions (2, 15, 16). The fate of circulating ET-1 is incompletely understood, but it is rapidly removed by the pulmonary circulation of various species, suggesting that the lungs are an important site for ET-1 clearance (1, 5, 14, 18). The lung contains ET_A and ET_B receptors: ET_A receptors are particularly abundant in the blood vessels and bronchi, whereas the alveolar walls of the lung parenchyma are rich in ET_B receptors (11). The ET_B receptors are abundant on endothelial cells and possess equal affinity for the three isoforms of the endothelin family. Stimulation of the endothelial ET_B causes vasodilation through the release of nitric oxide and prostacyclin (9, 17). It was recently shown that ET_B receptor blockade reduces ET-1 removal by the lung, so the ET_B receptor could also act as a clearance receptor for ET-1 in the pulmonary circulation (6). In cultured human endothelial cells, the specific ET_B antagonist BQ-788 strongly inhibits labeled ET-1 binding to the cells and increases extracellular ET-1 levels, whereas the specific ET_A antagonist BQ-123 only weakly inhibits ET-1 binding and does not modulate extracellular ET-1 levels (12). We designed experiments to better define the role and importance of the ET_A and ET_B receptors in circulating ET-1 clearance by the lungs. We measured the single-pass pulmonary removal of radiolabeled ET-1 in anesthetized dogs before and after intrapulmonary injections of single doses of ET_A and ET_B receptor antigens.

MATERIALS AND METHODS

Surgical instrumentation. Eleven healthy mongrel dogs (26 ± 5.9 kg body wt) were consecutively studied. Anesthesia was induced with pentobarbital sodium (50 mg/kg), and the animals were intubated and mechanically ventilated using room air. Cutaneous electrocardiographic leads were installed, and a right arterial femoral catheter was inserted using the Seldinger technique for continuous blood pressure monitoring. The external right jugular vein was exposed, and a 7F multipurpose catheter was inserted and positioned in the pulmonary artery by use of fluoroscopic and pressure-tracing guidance. The left carotid artery was then dissected, and a modified 7F pig-tail catheter was advanced and positioned 2 cm above the aortic valve. To prevent clotting of the catheters, 3,000 U of heparin were administered intravenously.

Single-pass pulmonary ET-1 clearance. Pulmonary removal of ET-1 was measured with the single-bolus dual-label indicator-dilution technique, as previously described (5). The indicator-dilution curve was carried out by injection of a 2-ml bolus in the right ventricular outflow tract (just below the pulmonary valve) and simultaneous collection of subsequent fractions of blood from the root of the aorta with a Masterflex roller pump and a fraction collector. The injected bolus contained 4 µCi of ¹²⁵I-ET-1 (sp act 2,200 Ci/mmol), 1 ml of Evans blue dye-labeled albumin (5 mg/ml; this dye binds
tightly with albumin and is utilized as a vascular tracer that does not leave the vascular space within a single transit time, bovine serum albumin (4 g/100 ml), and unlabeled red blood cells and saline to match the hematocrit of the dog.

An aliquot of blood from each of the collected fractions was assayed in a gamma counter to determine $^{125}$I-ET-1 activity. Using this methodology, we assayed in a gamma counter to determine $^{125}$I-ET-1 activity. Using this methodology, we

The cumulative tracer ET-1 extraction was calculated as

$$\text{extraction} = 1 - \frac{\int_0^t C_{ET}(t) \, dt}{\int_0^t C_{Alb}(t) \, dt} \quad (4)$$

where the numerator and denominator represent the area under the fractional recovery curves for tracer ET-1 and tracer albumin, respectively. Using this methodology, we showed a mean cumulative tracer ET-1 extraction of $31 \pm 8\%$ in the anesthetized dog (5).

Effect of ET$_A$ and ET$_B$ receptor antagonists on pulmonary ET-1 clearance. For each animal, an indicator-dilution curve was performed at baseline and 5 min after intrapulmonary injection of a single bolus dose of an ET$_A$ or ET$_B$ antagonist. The highly specific ET$_A$ antagonist BQ-123 was injected in doses ranging from 120 to 960 nmol ($n = 5$). The effect of ET$_B$ blockade was verified by the injection of the highly specific ET$_B$ antagonist BO-788 ($n = 6, 1,000$ nmol). For each antagonist, one experiment was carried out with addition of the antagonist directly to the bolus injection mixture itself, rather than pretreatment of the animals by injection of the antagonists alone in the pulmonary artery; this manipulation was done to maximize the concentration of inhibitors at the level of the microcirculation at the time they may compete with labeled ET-1 in the removal process.

A typical set of indicator-dilution studies is depicted in Fig. 1. During control experiments, the tracer ET-1 recovery curve progressively separates from the albumin curve as ET-1 is being progressively extracted by the pulmonary microcirculation. Cumulative ET-1 extraction for the examples shown was 37 and 33% for the respective control experiments for BQ-123 and BO-788. After BQ-123 infusion (Fig. 1B) there was no variation in the relationships between tracer ET-1 and albumin, with an unchanged cumulative extraction of 38%. However, after BO-788 infusion, the tracer outflow profiles for ET-1 and albumin virtually superpose one another.

### Table 1. Hemodynamic parameters

<table>
<thead>
<tr>
<th></th>
<th>BQ-123 ($n = 5$)</th>
<th>BQ-788 ($n = 6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>I</td>
<td>C</td>
</tr>
</tbody>
</table>
| Heart rate, beats/min | $168 \pm 20$ | $169 \pm 24$ | $166 \pm 7$ | $167 \pm 5$
| SBP, mmHg | $202 \pm 33$ | $198 \pm 32$ | $176 \pm 17$ | $175 \pm 16$
| Diastolic | $132 \pm 18$ | $130 \pm 19$ | $134 \pm 21$ | $133 \pm 20$
| PAP, mmHg | $26 \pm 2$ | $24 \pm 3$ | $29 \pm 4$ | $29 \pm 4$
| Systolic | $8 \pm 5$ | $10 \pm 6$ | $17 \pm 3$ | $16 \pm 3$
| Blood flow, ml/s | $42 \pm 10$ | $41 \pm 9$ | $55 \pm 26$ | $51 \pm 23$

Values are means ± SD. Hemodynamic parameters were measured in control conditions (C) and 5 min after intrapulmonary infusion (I) of 2 different endothelin antagonists. Antagonists had no significant effects on systemic or pulmonary hemodynamics. SBP, systemic blood pressure; PAP, pulmonary arterial pressure. P is nonsignificant for infusion vs. control for all parameters.
onto the other, indicating that ET-1 pulmonary removal has been completely abolished by this antagonist (from 33 to 3%)

Data derived from indicator-dilution analysis are assembled in Table 2. The mean pulmonary transit times for ET-1 and albumin were unaffected by the two antagonists. The specific ETA receptor antagonist BQ-123 did not affect mean cumulative pulmonary ET-1 clearance (from 36.4 ± 4.2 to 34.0 ± 6.4%, P = 0.51). The specific ETB antagonist BQ-788, however, completely abolished pulmonary ET-1 removal (from 33.5 ± 6.6 to 20.3 ± 2.6%, P < 0.001).

Further analysis of the outflow profile relationships of the tracers helps characterize the ET-1 extraction process. Instantaneous ET-1 extraction increases linearly as a function of time over the whole of the primary dilution curve (before recirculation). A plot of the natural logarithm of the fractional recovery per milliliter of the vascular reference albumin over that of ET-1 as a function of time systematizes the relationship between the two tracers (Fig. 2). Such a plot depicts a characteristic linear increase, as previously described (5), suggesting that the underlying capillary transit times are inhomogeneously distributed: capillaries with

Table 2. Effect of ET<sub>A</sub> and ET<sub>B</sub> antagonists on pulmonary transit times and circulating ET-1 extraction

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Control ET-1 extraction</th>
<th>MTT Alb, s</th>
<th>MTT ET, s</th>
<th>K, s&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Antagonist ET-1 extraction</th>
<th>MTT Alb, s</th>
<th>MTT ET, s</th>
<th>K, s&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BQ-123</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.36</td>
<td>7.86</td>
<td>7.64</td>
<td>0.041</td>
<td>0.31</td>
<td>7.72</td>
<td>7.18</td>
<td>0.053</td>
</tr>
<tr>
<td>2</td>
<td>0.43</td>
<td>7.33</td>
<td>6.89</td>
<td>0.053</td>
<td>0.39</td>
<td>7.66</td>
<td>7.51</td>
<td>0.050</td>
</tr>
<tr>
<td>3</td>
<td>0.34</td>
<td>7.30</td>
<td>6.94</td>
<td>0.062</td>
<td>0.38</td>
<td>8.31</td>
<td>7.87</td>
<td>0.069</td>
</tr>
<tr>
<td>4</td>
<td>0.37</td>
<td>8.19</td>
<td>7.71</td>
<td>0.042</td>
<td>0.38</td>
<td>8.92</td>
<td>7.79</td>
<td>0.038</td>
</tr>
<tr>
<td>5†</td>
<td>0.32</td>
<td>9.34</td>
<td>8.87</td>
<td>0.051</td>
<td>0.24</td>
<td>10.15</td>
<td>9.62</td>
<td>0.046</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>0.36 ± 0.04</td>
<td>8.00 ± 0.75</td>
<td>7.61 ± 0.72</td>
<td>0.050 ± 0.008</td>
<td>0.34 ± 0.06</td>
<td>8.55 ± 0.92</td>
<td>7.99 ± 0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BQ-788</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.39</td>
<td>6.03</td>
<td>5.72</td>
<td>0.084</td>
<td>-0.01</td>
<td>4.23</td>
<td>4.37</td>
<td>0.006</td>
</tr>
<tr>
<td>7</td>
<td>0.35</td>
<td>7.08</td>
<td>6.63</td>
<td>0.055</td>
<td>0.0017</td>
<td>7.61</td>
<td>7.69</td>
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<tr>
<td>8</td>
<td>0.26</td>
<td>7.02</td>
<td>6.63</td>
<td>0.06</td>
<td>-0.005</td>
<td>9.25</td>
<td>9.02</td>
<td>0.009</td>
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<tr>
<td>9</td>
<td>0.26</td>
<td>8.26</td>
<td>7.96</td>
<td>0.056</td>
<td>0.01</td>
<td>8.79</td>
<td>8.64</td>
<td>0.021</td>
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<tr>
<td>10</td>
<td>0.42</td>
<td>10.58</td>
<td>10.34</td>
<td>0.048</td>
<td>-0.047</td>
<td>8.77</td>
<td>8.66</td>
<td>0.001</td>
</tr>
<tr>
<td>11†</td>
<td>0.33</td>
<td>9.53</td>
<td>9.43</td>
<td>0.042</td>
<td>0.03</td>
<td>9.12</td>
<td>9.6</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>0.34 ± 0.06</td>
<td>8.08 ± 1.57</td>
<td>7.79 ± 1.64</td>
<td>0.058 ± 0.014</td>
<td>-0.00 ± 0.02*</td>
<td>7.96 ± 1.75</td>
<td>8.00 ± 1.72</td>
</tr>
</tbody>
</table>

ET-1, endothelin-1; MTT Alb, mean transit time for albumin; MTT ET, mean transit time for endothelin; K, rate constant for pulmonary ET-1 removal. *P < 0.001 vs. control. †Experiments performed with addition of inhibitor directly to injection mixture of indicator-dilution study rather than pretreatment by injection into pulmonary artery 5 min before experiment.
The specific ET\(_B\) antagonist BQ-788 completely abolished ET-1 removal by the lung after a single injection of 1,000 nmol for all animals studied. The ET\(_A\) antagonist BQ-123 could not modify ET-1 extraction in equivalent doses. To greatly increase inhibitor concentration at the microcirculatory level, one experiment was performed with addition of \(\sim 1,000\) nmol of the inhibitors to the indicator-dilution injection mixture itself, rather than pretreatment of the animals by intrapulmonary injection of the inhibitors 5 min before the dilution study. Because the quantity of tracer ET-1 simultaneously injected was \(\sim 2\) pmol, this creates an antagonist-to-agonist ratio of \(5 \times 10^5:1\). Addition of the inhibitors to the bolus itself did not modify ET-1 extraction for BQ-123, whereas it still completely abolished extraction with BQ-788, as expected (Table 2).

**DISCUSSION**

We have shown that in vivo pulmonary removal of circulating ET-1 is completely and exclusively mediated by the ET\(_B\) receptors. The ET\(_A\) receptor is preponderant in the vascular smooth muscle, whereas the ET\(_B\) receptor is more abundant in the vascular endothelium (9). Stimulation of the ET\(_B\) receptor causes mixed responses depending on the species and vascular bed studied. In the piglet lung, an initial dose-dependent dilatation is followed by a dose-dependent vasoconstriction (13). A nomenclature is suggested to distinguish between ET\(_B_1\) (dilator response) and ET\(_B_2\) (constrictor response) receptors. Another potentially important role for the ET\(_B\) receptor was recently unveiled when Fukuroda et al. (6) demonstrated that the ET\(_B\) receptor contributes to pulmonary removal of circulating ET-1 in rat lungs. Using in vivo and isolated lung preparations, they demonstrated that the specific ET\(_B\) antagonist BQ-788 significantly decreased ET-1 removal by the pulmonary circulation whereas the ET\(_A\) antagonist BQ-123 had no significant effect. The major difference between the experiments of Fukuroda et al. and the present study is that BQ-788 incompletely prevented ET-1 removal by the lung, so other mechanisms for ET-1 removal could not be excluded.

The indicator-dilution technique is the most accurate method to study microcirculatory exchanges in vivo. Using this technique, we previously demonstrated that the canine pulmonary circulation extracts 31 ± 8% of circulating ET-1 during a single pulmonary transit time with a simultaneous equal release of ET-1 into the circulation, explaining the absence of arteriovenous difference in immunoreactive ET-1 levels across the pulmonary circulation (5). In the present study we not only confirm the role of the ET\(_B\) receptor in pulmonary removal of circulating ET-1 but, more specifically, establish its exclusivity, because ET-1 removal was completely abolished by the specific ET\(_B\) receptor antagonist BQ-788 (from 33.5 ± 6.6 to \(-0.3 \pm 2.6\%\)). The specific ET\(_A\) antagonist BQ-123 could not reduce ET-1 pulmonary removal. To ensure high enough concentration of BQ-123 in the microcirculation, we added the inhibitors directly to the indicator-dilution mixture to create an inhibitor-to-agonist ratio of \(\sim 1 \times 10^5:1\). This

![Fig. 2. Natural log ratio plot of fractional recovery per ml of tracer albumin over tracer ET-1 as a function of time (expt 10) before (○) and after (■) BQ-788 infusion. At baseline, log ratio increases linearly, and a rate constant \((k)\) characterizing ET-1 extraction can be estimated from slope of this relationship. ET-1 removal by lung is unidirectional without significant return of tracer to circulation during a single pulmonary transit time. After BQ-788, log ratio becomes a flat line and \(K\) is close to zero.

progressively longer transit times contribute to ET-1 extraction, explaining the linear increase in extraction with time. If a significant portion of the extracted ET-1 returned to the circulation, one would expect a flattening or decrease, later in time, of the log ratio curve. Such behavior is evident for a tracer like serotonin, which partly backdiffuses into the circulation after pulmonary extraction. Here, the constant linear increase in the log ratio therefore suggests that, over a single passage, the extracted endothelin is retained within endothelial cells and does not return to the circulation. The foregoing therefore suggests that the outflow relationship for tracer ET-1 can be described by

\[
C_{ET}(t) = C_{Alb}(t)e^{-k(t-t_0)}
\]

where \(K\) is an uptake rate constant and \(t_0\) is a common large vessel transit time. With the assumption of no variations in large vessel transit time (3), \(K\) can be estimated by computing the slope of the log ratio plots (Fig. 2). In the absence of correction for large vessels and catheter distortion, the value will be a minimum; it will be slightly smaller than the actual value. If formulated in terms of ordinary capillary modeling, \(K\) corresponds to \(PS/V_c\), the product of the vascular permeability (\(P\)) and the surface-to-volume ratio for the pulmonary capillaries (\(S_c/V_c\)). In the present set of experiments, where pulmonary blood flow and pressures remained constant, we may assume that \(S_c/V_c\) also remains constant and that \(K\) becomes an index of capillary permeability to circulating ET-1.

An example of a log ratio plot at baseline and after BQ-788 infusion is shown in Fig. 2 (expt 10), and the individual values for all experiments are assembled in Table 2. Control values for \(K\) are similar to the previously reported value of \(0.056 \pm 0.016 \text{ s}^{-1}\) (5) and do not significantly vary after BQ-123 (\(P = 0.73\)). Because the ET\(_B\) antagonist BQ-788 completely abolished ET-1 extraction, \(K\) decreased from 0.058 ± 0.009 to 0.009 ± 0.007 s\(^{-1}\) (\(P < 0.001\)) after that pharmacological intervention.
procedure did not modify the pulmonary ET-1 removal patterns: only BQ-788 completely abolished pulmonary ET-1 removal.

Although ET-1 removal was totally abolished, we did not detect a significant hemodynamic effect after BQ-788 infusion. We, however, only measured pressures 5 min after inhibitor injection, and this does not exclude later hemodynamic alterations. The clearance role of the ET<sub>B</sub> receptor may have important pathological and therapeutic implications. Desensitization or downregulation of this receptor may contribute to increased ET-1 levels in various disorders. Rats with monocrotaline pulmonary hypertension have decreased expression of ET<sub>B</sub> receptors (19). Because most pathologies associated with pulmonary hypertension have a hyperendotelinemia that is proportional to the severity of pulmonary hypertension (2, 10, 16, 20), it is possible that altered ET<sub>B</sub> function and/or expression contributes to the process by reducing pulmonary ET-1 clearance. Specific ET<sub>B</sub> antagonists and nonspecific ET<sub>A</sub> and ET<sub>B</sub> antagonists can increase circulating endothelin levels: in normal and hypertensive dogs, the nonspecific ET<sub>A</sub> and ET<sub>B</sub> antagonist bosentan causes a 30-fold increase in ET-1 levels (4). In humans with heart failure, a single dose of bosentan doubles the already high circulating ET-1 levels (7). In conscious rats, ETA antagonists can increase circulating endothelin levels (333:108–110, 1993). Studies done on cultured human endothelial cells confirm the predominance of the ET<sub>B</sub> receptor on the endothelium and its unique role in the modulation of extravascular ET-1 levels (12). Our data confirm this unique role of the ET<sub>B</sub> receptor in vivo. Like most pulmonary metabolic functions, the ET<sub>B</sub> receptor may have a protective role by modulating the systemic levels of a potent circulating vasoconstrictor. This new important role of the ET<sub>B</sub> receptor must be considered when experiments with endothelin receptor antagonists are designed and introduces the possibility of a “rebound phenomenon” secondary to the increased ET-1 levels after ET<sub>B</sub> receptor blockade.

The mechanism by which ET<sub>B</sub> receptors mediate ET-1 clearance remains to be determined but could be through receptor-mediated endocytosis. The fate of ET<sub>B</sub> receptor-bound ET-1 also remains unknown. Endothelial ET<sub>B</sub> receptors may clear circulating ET-1 and mediate its inactivation through later intracellular degradation or act as a transendothelial transporter that delivers ET-1 at the abluminal surface of the endothelium.

Pulmonary clearance of circulating ET-1 by the endothelial ET<sub>B</sub> receptor represents a novel endothelial cell function. The pulmonary circulation modifies and inactivates numerous vasoactive amines and peptides and may modulate the systemic levels of these substances. There is normally no net arteriovenous difference in circulating ET-1 levels across the lung (5, 16); the lung consequently produces an amount of ET-1 equal to the amount that has been extracted (5). Conditions associated with pulmonary endothelial cell dysfunction or pharmacological blockade of the ET<sub>B</sub> receptor may consequently alter this physiological balance and contribute to an increase in ET-1 levels.

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