Differential Effects of Endothelin-1 Antagonists on Erythropoietin-Induced Hypertension in Renal Failure

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Abstract. Recently, it was reported that blood vessel immuno-reactive endothelin-1 (irET-1) content is increased in hypertensive uremic rats treated with recombinant human erythropoietin (rhEPO). The present study was designed to evaluate whether ET-1 receptor blockade can prevent the progression of hypertension in renal failure rats receiving rhEPO and, if so, whether selective ET<sub>A</sub> and nonselective ET<sub>A/B</sub> receptor antagonists are equally effective. Renal failure was induced by a two-stage 5/6 nephrectomy; the animals developed uremia, anemia, and hypertension. After a 4-wk stabilization period, the animals received either rhEPO (100 U/kg, subcutaneously, three times per week) or the vehicle for 4 wk. In protocol A, half of the rats in each group were simultaneously treated with the ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist bosentan (100 mg/kg per d). In protocol B, half of the rats in each group received the selective ET<sub>A</sub> receptor antagonist LU135252 (50 mg/kg per d). Systolic BP was recorded before and at 2 and 4 wk after the onset of treatment. Serum creatinine levels and hematocrit were measured before treatment and at the end of the study.

Recovery therapy with recombinant human erythropoietin (rhEPO) increases BP in anemic patients with renal insufficiency and may lead to the development of de novo hypertension or the exacerbation of existing arterial hypertension (1–4). This pressor effect appears to occur almost exclusively in uremic patients. Indeed, hypertension has not been reported in clinical trials in which rhEPO was administered in cases of nonrenal anemia (5–7) or in healthy volunteers (8). However, hypertension has been observed in normal rats treated with higher doses of rhEPO (9), or using the peritoneal route of injection (10). In renal failure (5/6 nephrectomy) rats, we have shown that uremia enhances the BP response to rhEPO (11). This pressor effect cannot be accounted for by the increase in hematocrit or red cell mass in uremic rats (12) and in renal failure patients (13). Other potential mechanisms include an inappropriate increase in peripheral vascular resistance (14), the direct vasopressor action of rhEPO (15), enhanced tissue renin-angiotensin activity (16), and nitric oxide resistance (12). More recent studies suggest possible links between rhEPO-associated hypertension and endothelium-derived vasoconstrictor autacoids probably because EPO receptors are present on the surface of vascular endothelial cells (17) and because these cells are capable of releasing endothelin-1 (ET-1) under the influence of rhEPO (18–20). We recently reported an increase in blood vessel ET-1 concentrations in hypertensive uremic rats treated with rhEPO (21).

The present study was designed to evaluate whether ET-1 receptor blockade can prevent the progression of hypertension in renal failure rats treated with rhEPO and, if so, to determine whether specific ET<sub>A</sub> and nonspecific ET<sub>A/B</sub> receptor antagonists are equally effective.

Materials and Methods

Animal Experiments

The research protocol and experimental manipulations were conducted in accordance with the guidelines of the Canadian Council of Protection of Animals and approved by the Animal Care Committee of Laval University. Male Wistar rats (200 to 220 g) were obtained from Charles River (St. Constant, Quebec, Canada) and allowed to acclimatize 1 wk in our animal facilities with temperature and humid-
ity control and a 12-h day/light cycle before any experimental intervention. Food (standard rat chow) and untreated tap water were available ad libitum. All animals were rendered uremic by a two-stage 5/6 nephrectomy procedure (11). Briefly, the rats were anesthetized with sodium pentobarbital (Somnotol, 50 mg/kg intraperitoneally; MTC Pharmaceuticals, Cambridge, Ontario, Canada) and via a left flank incision, approximately two-thirds of the left kidney was removed by excision of the upper and lower poles. Blood loss was minimized by the application of gelatin sponges (Gelfoam; Upjohn, Don Mills, Ontario, Canada). One week later, the right kidney was removed through a left lateral flank incision. Renal function was then allowed to stabilize over the next 4 wk to attain a state of chronic renal impairment. The animals were then treated subcutaneously with either rhEPO (Eprex, Janssen-Ortho, Inc., Don Mills, Ontario, Canada; 100 U/kg) or the vehicle (saline 0.9%) 3 times per week for 4 wk. Half of each group was allocated to one of two protocols. In protocol A, both groups of rats received either bosentan (Hoffman-LaRoche, Basel, Switzerland), a nonselective ETA/ETB antagonist (100 mg/kg per d) mixed with powdered standard rat chow or the same chow without medication. This oral dose has been demonstrated to block the action of pressor doses of intravenously injected ET-1 for more than 24 h (22). In protocol B, both groups of animals received either the selective ETA antagonist LU135252 (Knoll, Ludwigsafen, Germany) 50 mg/kg per d mixed with the powdered standard rat chow or the same chow without medication. This dosage of LU135252 was found to effectively lower BP in previous studies (23,24) and in our model of chronic renal failure (25). Serum creatinine levels and hematocrit were measured in jugular vein punctures in anesthetized animals (11) before treatment and at the end of the study. Systolic BP was recorded before treatment and at weeks 2 and 4. At the end of the study, the animals were held in metabolic cages to collect 24-h urine samples for creatinine clearance determination. Plasma ET-1 was measured at the time of sacrifice. The animals were anesthetized with pentobarbital (50 mg/kg intraperitoneally) and exsanguinated by abdominal aortic puncture. Blood samples for ET-1 determinations were collected in aprotinin (500 kallikrein inhibitory U/ml; Boehringer Mannheim, Mannheim, Germany) and ethylenediaminetra-acetic acid (1 mg/ml).

Methods

Systolic BP was measured by the tail-cuff method after warming and slight restriction using an I.I.T.C. BP system fitted with a model 29 pulsar sensor (I.I.T.C. Life Science, Woodland Hills, CA). BP was recorded using a computerized acquisition system (model MP100; Biopac System, Goleta, CA), and the average of three readings was used for analysis. Using this method, the systolic BP in control Wistar rats of the same age and weight was 119 ± 10 mmHg (mean ± SEM) (11). Hematocrit was determined in duplicate in Pre-Cal micro-hematocrit tubes (Becton-Dickinson, Parsippany, NJ) after centrifugation at 19,000 rpm for 2 min. Serum and urinary creatinine levels were measured by autoanalyzer (Ilab 1800; Lexington, MA). Reference values for control Wistar rats of the same age and weight were 42.5 ± 1.3 μmol/L (mean ± SEM) (11). Creatinine clearance was calculated as the product of urine concentration/plasma concentration and urine flow rate (ml/min). Reference values for control Wistar rats of the same age and weight were 2.5 ± 1 ml/min (mean ± SEM, n = 42). Plasma immunoreactive ET-1 (irET-1) was measured by specific RIA in C18 Sep-Pak extracted samples (Waters Associates, Milford, MA) (26). The recovery of the extraction procedure varied from 75 to 80% and was taken into account in the final calculations. The lower ET-1 detection limit was 1 pg/tube with the 50% tracer displacement around 10 pg/tube on the standard curve. The intra-assay and inter-assay coefficients of variation were 7 and 10% respectively.

Statistical Analyses

Results are expressed as mean ± SEM. Statistical comparisons within protocol A or B were analyzed first by ANOVA followed by Student-Newman-Keuls tests for multiple comparisons. Differences between groups of protocol A and B were performed using unpaired t test. Statistical significance was accepted at P < 0.05.

Results

Protocol A

At the end of the study, body weights were similar in the four groups of rats receiving rhEPO or the vehicle regardless of whether they were treated with bosentan (509 ± 10, 490 ± 14, 517 ± 12, and 490 ± 6 g, respectively). Table 1 shows hematocrit and serum creatinine values before and at the end of the treatment period, as well as creatinine clearance rates and plasma irET-1 values at the end of the treatment period. Before treatment, all animals were uremic with serum creatinine values of 1.5- to 1.8-fold above normal values (Table 1). As expected, the two groups of rats treated with rhEPO had significantly increased hematocrit. A further increase in serum

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit (%)</th>
<th>Serum Creatinine (μmol/L)</th>
<th>Creatinine Clearance (ml/min)</th>
<th>Plasma irET-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nx (n = 8)</td>
<td>42 ± 1</td>
<td>63 ± 3</td>
<td>1.8 ± 0.1</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Nx-rhEPO (n = 8)</td>
<td>38 ± 1b</td>
<td>74 ± 8</td>
<td>1.3 ± 0.2c</td>
<td>5.0 ± 1.3</td>
</tr>
<tr>
<td>Nx-Bo (n = 8)</td>
<td>38 ± 1b</td>
<td>69 ± 2</td>
<td>2.0 ± 0.1</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>Nx-Bo-rhEPO (n = 9)</td>
<td>39 ± 1</td>
<td>71 ± 4</td>
<td>1.5 ± 0.1f</td>
<td>6.1 ± 1.0</td>
</tr>
</tbody>
</table>

Table 1. Hematocrit, serum creatinine, creatinine clearance rate, and plasma immunoreactive endothelin-1 before and at the end of the treatment period.

a Nx, 5/6 nephrectomized rats; rhEPO, recombinant human erythropoietin; Bo, bosentan; irET-1, immunoreactive endothelin-1.

b P < 0.05 versus Nx.

c P < 0.05 versus Nx-Bo.

d P < 0.01 versus before treatment.

e P < 0.05 versus before treatment.

f P < 0.05 versus Nx-Bo.
creatinine was observed after 4 wk of treatment. The values were significantly different in the group cotreated with rhEPO and bosentan ($P < 0.05$). Creatinine clearance rates were lower in the two groups of rats receiving rhEPO compared to the animals treated with bosentan alone ($P < 0.01$ and $P < 0.05$). Plasma irET-1 concentrations were higher in rats treated with bosentan, but the difference did not reach statistical significance. Figure 1 illustrates systolic BP variations in the four groups of rats. Before the treatment period (week 0) and 4 wk after renal mass reduction, systolic BP was increased (range, $133 \pm 7$ to $134 \pm 4$ mmHg). In the group receiving the vehicle only (left panel), a further increase was observed at the end of the study ($158 \pm 7$ mmHg, $P < 0.05$). In contrast, the BP values of uremic rats receiving the vehicle plus bosentan were significantly decreased ($P < 0.05$) at week 2. The measurements obtained at the end of the protocol (week 4) were similar to those recorded before treatment ($137 \pm 6$ mmHg). In rats treated with rhEPO alone (Figure 1, right panel), BP increased progressively to $172 \pm 10$ mmHg ($P < 0.05$). A similar and progressive increase in BP was observed at week 2 ($155 \pm 8$ mmHg) and at week 4 ($168 \pm 9$ mmHg) in rhEPO-treated rats receiving bosentan.

**Protocol B**

LU135252-treated rats with or without rhEPO gained more body weight ($503 \pm 8$ and $505 \pm 7$ g, respectively) than untreated rats ($452 \pm 11$ and $475 \pm 7$ g, $P < 0.01$). Table 2 shows hematocrit and serum creatinine values before and at the end of the treatment period, as well as creatinine clearance rates and plasma irET-1 values at the end of the treatment period. Before the treatment, all rats were uremic with serum creatinine values of 1.7- to twofold above normal values. Mean serum creatinine values in control untreated rats before and at the end of the treatment were not significantly different from rats of protocol A under the same conditions. Mean creatinine clearance rate in control untreated rats was lower ($P < 0.05$) than control untreated rats of protocol A. As expected, the two groups of rats treated with rhEPO had significantly increased hematocrit ($P < 0.01$). At the end of the study, creatinine clearance rates were comparable in all four groups. Compared to untreated groups, plasma irET-1 concentrations were significantly higher ($P < 0.01$) in the two groups of rats treated with LU135252 regardless of rhEPO treatment. Figure 2 illustrates systolic BP variations in the four groups of rats. Before the treatment period (week 0) and 4 wk after renal mass reduction, systolic BP was increased (range, $149 \pm 3$ to $154 \pm 4$ mmHg). In the group receiving the vehicle only (left panel), a further increase was observed at the end of the study ($175 \pm 7$ mmHg, $P < 0.05$). In contrast, the BP of uremic rats treated with LU135252 remained at a similar level from week 0 to week 4 ($149 \pm 5$, $139 \pm 6$, and $158 \pm 5$ mmHg). In rats receiving rhEPO alone (Figure 2, right panel), BP increased progressively to $187 \pm 9$ mmHg ($P < 0.01$). In contrast, BP of rhEPO-treated rats receiving LU135252 remained at similar levels throughout the treatment period ($154 \pm 4$, $139 \pm 4$, and $160 \pm 7$ mmHg). The BP values at weeks 2 and 4 were significantly lower ($P < 0.01$ and $P < 0.05$) than the untreated rats at the same time point.

**Discussion**

In the present study, treatment with rhEPO was started 4 wk after 5/6 nephrectomy and development of renal failure, anemia, and hypertension. As observed earlier (11,21), the administration of rhEPO completely corrected the anemia and worsened the hypertension. Although plasma ET-1 is not altered by chronic rhEPO treatment (21,27), probably because 80% of ET-1 released by endothelial cells is directed abluminally and exerts local effects on vascular smooth muscle cells (28), we recently reported increased ET-1 content in blood vessels of uremic rats treated with rhEPO, suggesting a role for ET-1 in rhEPO-induced hypertension (21). To evaluate whether ET-1 receptor blockade could prevent the aggravation of hypertension in uremic rhEPO-treated rats, we treated rats with ET-1 receptor antagonists. Since the cardiovascular roles of ET$_A$ and
ET_b receptors in various models of hypertension are complex (29), we used both the nonselective ET_A/ET_B (bosentan) and the selective ET_A (LU135252) receptor antagonists. The results show that LU13522 was effective in preventing the aggravation of hypertension in rhEPO-treated rats. To our knowledge, this is the first report showing that ETA receptor blockade can reduce the progression of hypertension in uremic rats receiving chronic rhEPO replacement therapy. Our results are in keeping with the observation of Tojo et al. (30), who reported that the acute pressor effect of rhEPO in spontaneously hypertensive rats was completely blocked by the administration of BQ-123, an ETA receptor antagonist. In contrast, one of the major findings of the current study was that the nonselective ETA/ET_B blockade with bosentan did not prevent the worsening of hypertension in rhEPO-treated uremic rats. These results suggest a particular functional role of ET_B in rhEPO-induced hypertension as has been reported in some experimental models of hypertension. Indeed, Clozel and Breu (29) observed that the blockade of ET_B receptors with a selective antagonist induced a pressor effect in deoxycorticosterone acetate rats and spontaneously hypertensive rats, whereas such an effect was not observed in normotensive rats and rats rendered hypertensive by chronic nitric oxide synthase blockade with N^G-nitro-L-arginine methyl ester. A growing accumulation of recent literature points to an important autocrine role of vascular endothelial ETB receptors in mediating the agonistic vasorelaxing response through the release of potent vasodilators such as nitric oxide, prostacyclin, c-type natriuretic peptide, and adrenomedullin (31–33). It is tempting to speculate that in a state of excessive ET-1 release, as in renal failure animals treated with rhEPO, the maintenance of ETB receptor stimulation may be crucial to attain the full effect of an antihypertensive agent. Thus, the absence of response to bosentan in the present study may be due either to an increase of the basal level of endothelium-dependent vasodilation or to an upregulation of endothelial “vasodilating” ET_B receptors, which might play a role in counteracting the pressor influence of the previously reported ET-1 overproduction during rhEPO treatment. In keeping with this hypothesis, it has been shown that rhEPO-induced erythrocytosis resulted in a compensatory

Table 2. Hematocrit, serum creatinine, creatinine clearance rate, and plasma immunoreactive endothelin-1 before and at the end of the treatment period

<table>
<thead>
<tr>
<th>Group</th>
<th>Hematocrit(%)</th>
<th>Serum Creatinine(μmol/L)</th>
<th>Creatinine Clearance (ml/min)</th>
<th>Plasma irET-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before Treatment</td>
<td>End of the Study</td>
<td>Before Treatment</td>
<td>End of the Study</td>
</tr>
<tr>
<td>Nx (n = 13)</td>
<td>37 ± 1</td>
<td>36 ± 1</td>
<td>83 ± 4^b</td>
<td>88 ± 6</td>
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<tr>
<td>Nx-rhEPO (n = 12)</td>
<td>37 ± 1</td>
<td>47 ± 2^c,d,e</td>
<td>72 ± 2</td>
<td>92 ± 3^e</td>
</tr>
<tr>
<td>Nx-LU (n = 14)</td>
<td>36 ± 1</td>
<td>35 ± 3</td>
<td>85 ± 3^b</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>Nx-LU-rhEPO (n = 13)</td>
<td>36 ± 1</td>
<td>49 ± 3^c,d,e</td>
<td>86 ± 4</td>
<td>89 ± 10</td>
</tr>
</tbody>
</table>

* LU, LU135252. Other abbreviations as in Table 1.
^b P < 0.05 versus Nx-rhEPO.
^c P < 0.01 versus Nx.
^d P < 0.01 versus Nx-LU.
^e P < 0.01 versus before treatment.
^f P < 0.01 versus Nx-rhEPO.

Figure 2. Time course of systolic BP during the treatment period in 5/6 nephrectomized rats (Nx) receiving vehicle (Veh), rhEPO, or LU135252 (LU). *P < 0.05 and **P < 0.01 versus treated rats at the same time; †P < 0.05 and ‡P < 0.01 versus before treatment.
stimulation of nitric oxide production in normal rats (34–36). However, the effect of chronic rhEPO administration on L-arginine-nitric oxide system appears more complex in renal failure animals. Ni et al. (37) recently showed that uremic rats exhibit a downregulation of nitric oxide synthase expression and nitric oxide production. These data do not rule out an indirect upregulation of nitric oxide production (via increased pressure, shear stress, and ET-1) with the net effect depending on the relative predominance of these opposing influences.

It is interesting to note that, as previously observed (38–40), the present study shows that both selective ETA and nonselective ET_A/ET_B receptor antagonists are effective in reducing the progression of hypertension in uremic rats receiving the vehicle. It has been documented that in renal failure rats, the renal expression of the ET-1 gene is upregulated (41) and the production of ET-1 is increased in preglomerular arteries and glomeruli as well as in the thoracic aorta (42). The similar antihypertensive response to bosentan and LU135252 in uremia suggests a less predominant role of ET_B receptors in this experimental condition. It is noteworthy that although both ET-1 receptor antagonists attenuated the progression of hypertension in our uremic control rats, these antagonists did not normalize the BP as has been reported with the administration of angiotensin-converting enzyme inhibitors or with angiotensin II subtype 1 receptor antagonist (39,43). This phenomenon may be related to the dual action of the latter compounds, which act on both the renin-angiotensin and the ET-1 systems (43).

Renal function, as evaluated by serum creatinine levels and clearance rates, decreased progressively toward the end of the study, and the decline was similar in all groups regardless of ET-1 receptor antagonist treatment. These results differ from the report of Benigni et al. (38), who observed that ETA blockade prevented renal function deterioration in rats with reduced renal mass in the presence of a more severe degree of renal failure with serum creatinine twice as high. In keeping with this observation, we recently reported a reduction in the progression of renal failure in severely uremic 5/6 nephrectomized rats treated with a selective ETA receptor antagonist (25). Because the degree of renal failure is closely related to the overproduction of ET-1 in blood vessels and glomeruli (42), this may account for the effectiveness of ET-1 blockade on the prevention of glomerular injury in severe renal failure.

Plasma ET-1 concentrations tended to be higher in rats treated with bosentan as has been observed previously (44). This phenomenon could be accounted for by the occupancy of endothelial and smooth muscle cell receptors by the antagonist with a spillover of the unbound agonist into the circulation. Furthermore, Fukuroda et al. (45) reported that clearance of ET-1 may be mediated at least partly by the ET_B receptor. Because bosentan blocks both ETA and ET_B receptors, the clearance rate of ET-1 may be lower, resulting in increased plasma ET-1 levels. Along the same line, Löffler et al. (44) demonstrated in short-term experiments (240 min) that, contrary to the effect of bosentan, the selective ETA antagonists BQ-123 and FR-139317 had no effect on plasma ET-1 concentrations. These results, however, obtained under acute conditions, do not exclude the possibility that ETA-selective antagonists could also increase plasma ET-1 levels during chronic drug treatment by interacting with the ETA receptors on the smooth muscle cells, which are adjacent to the endothelial cell layer. Indeed, as is clearly indicated in the present study, the selective ETA receptor antagonist LU135252 produced a significant increase in plasma ET-1 concentration after 4 wk of treatment. Schiffrin et al. (46) also recently reported a significant increase in plasma ET-1 levels in one-kidney one-clip Goldblatt hypertensive rats treated with LU135252 for 4 wk at the same dosage.

In summary, the two ET-1 receptor antagonists bosentan and LU135252 were effective in attenuating the progression of hypertension in uremic rats receiving the vehicle. However, only selective ETA but not ETA/ET_B receptor blockade prevented the worsening of hypertension in renal failure rats receiving rhEPO therapy. These results suggest a differential role for ETA and ET_B receptors in the pathophysiology of rhEPO-induced hypertension in uremic rats.

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