Renin-Angiotensin-Aldosterone System

Antihypertensive and Renal Protective Effects of Renin-Angiotensin System Blockade in Uremic Rats Treated With Erythropoietin

Marcel Lebel, Marie-Eve Rodrigue, Mohsen Agharazii, and Richard Larivière

Background: Correcting anemia with recombinant human erythropoietin (rhEPO) in chronic renal failure has been associated with an increased blood pressure (BP), which may accelerate the decline in renal function. This has been attributed, in part, to the activation of the renin-angiotensin system. The present study was designed to investigate the protective effect of the angiotensin II-receptor blocker losartan compared with the angiotensin-converting enzyme inhibitor captopril and conventional triple therapy (TRx) in uremic rats receiving rhEPO therapy.

Methods: Renal failure was induced by renal mass ablation followed by a 3-week stabilization period. Uremic rats were then divided into five groups with similar systolic BP: vehicle; rhEPO (100 U/kg, subcutaneously, three times per week); rhEPO + losartan (20 mg/kg/d); rhEPO + captopril (20 mg/kg/d); and rhEPO + TRx (reserpine 5 mg/L, hydralazine 80 mg/L, hydrochlorothiazide 20 mg/L). Systolic BP as well as blood and renal parameters were assessed before and after a 3-week treatment period. Renal histology was evaluated at the end of the study.

Results: The uremic rats developed hypertension, anemia, proteinuria, and increased urinary endothelin-1 (ET-1) excretion. The rhEPO corrected the anemia but aggravated the hypertension (P<.01), glomerular sclerosis, tubular atrophy, and interstitial fibrosis. Treatment with losartan, captopril, and the TRx prevented the rhEPO-induced increased in systolic BP. The TRx was less effective in preventing histologic injuries despite similar systolic BP reduction.

Conclusions: Blockade of the renin-angiotensin system is highly effective in preventing both hypertension and renal histologic damage in rhEPO-treated uremic rats and this benefit seems to extend beyond the antihypertensive effect. Am J Hypertens 2006;19:1286 –1292 © 2006 American Journal of Hypertension, Ltd.

Key Words: Anemia, chronic renal failure, erythropoietin-induced hypertension, remnant kidney, renal protection, renin-angiotensin blockade, losartan.
Thromboxane A₂ synthesis inhibition and receptor blockade can prevent the progression of hypertension in renal failure rats treated with rhEPO. The effect of angiotensin AT1 receptor blockers has never been tested in this new iatrogenic form of hypertension.

The present study was designed to assess the antihypertensive and renal protective effect of the angiotensin receptor blocker losartan in rhEPO-induced hypertension in uremic rats compared to the angiotensin-converting enzyme inhibitor captopril and conventional triple therapy (TRx) consisting of reserpine, hydralazine, and hydrochlorothiazide.

Methods
Experimental Protocol

Chronic renal failure was induced in Wistar rats (Charles River, Saint-Constant, Quebec, Canada) by a two-stage 5/6 subtotal nephrectomy under isoflurane anesthesia as previously described. Through a 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 with light pressure. One week later, the right kidney was removed. Renal function was allowed to stabilize over the next 3 weeks to attain a state of chronic renal failure (doubling of serum creatinine concentrations and an increase of systolic BP to about 140 to 150 mm Hg). In addition, the animals develop all biochemical features observed in humans with end-stage renal disease. Therefore, this experimental model is suitable to study the effect of rhEPO on BP and to evaluate the effect of antihypertensive therapy.

Uremic animals were then divided into five groups with similar systolic BP (Table 1): group 1, vehicle; group 2, rhEPO; group 3, rhEPO + losartan; group 4, rhEPO + captopril; and group 5, rhEPO + TRx. The vehicle (saline 0.9%) and the rhEPO (100 U/kg) were administered subcutaneously three times per week for 3 weeks, as previously performed. Losartan (20 mg/kg/d), and TRx (reserpine 5 mg/L, hydralazine 80 mg/L, and hydrochlorothiazide 25 mg/L) were given in drinking water. The dosage of losartan, captopril, and TRx was selected according to previous studies by us and other investigators showing that this dose was the minimum required for each drug to obtain maximum systolic BP reduction in rats with reduced renal mass. Systolic BP was measured before treatment and at the end of the study by the tail–cuff method. Twenty-four-hour urine samples were collected before treatment and at the end of the study to assess urinary volume, protein, and immunoreactive (ir)ET-1 excretion. The animals were then anesthetized and exsanguinated by an abdominal aortic puncture. Blood samples were used to measure hematocrit and serum creatinine. The remnant kidney of uremic rats was dissected and used for histologic study.

Study Methods

Systolic BP was measured by the tail–cuff method after warming and with slight restriction using an I.I.T.C. Blood pressure system fitted with a model 29 pulsar sensor (I.I.T.C. Life Science, Woodland Hills, CA). Blood pressure 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 mm Hg. Serum creatinine and uric acid as well as proteinuria were determined with an autoanalyzer system (Ilab 1800, Lexington, MA). Reference values for serum creatinine in control Wistar rats of the same age and weight were 42.5 ± 1.3 Umol/L (mean ± SEM). Hematocrit was determined in duplicate in Pre-cal micro-hematocrit tubes (Becton-Dickinson, Parsippany, NJ) after centrifugation at 19,000 rpm for 2 min.

The irET-1 concentrations were measured in urine using a specific radioimmunoassay in C18 Sep-Pak extracted samples, as previously described. The recovery of the extraction procedure varied from 75% to 90%. The lower

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Systolic blood pressure (mm Hg)</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>End of the study</td>
<td>Before treatment</td>
</tr>
<tr>
<td>Nx + vehicle</td>
<td>380 ± 11</td>
<td>420 ± 11*</td>
<td>136 ± 5</td>
</tr>
<tr>
<td>Nx + rhEPO</td>
<td>366 ± 5</td>
<td>381 ± 19</td>
<td>146 ± 5</td>
</tr>
<tr>
<td>Nx + rhEPO + losartan</td>
<td>379 ± 6</td>
<td>413 ± 12*</td>
<td>145 ± 5</td>
</tr>
<tr>
<td>Nx + rhEPO + captopril</td>
<td>382 ± 7</td>
<td>426 ± 10*</td>
<td>147 ± 6</td>
</tr>
<tr>
<td>Nx + rhEPO + TRx</td>
<td>379 ± 8</td>
<td>358 ± 14‡</td>
<td>148 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SEM from 8 to 9 animals per group. TRx – triple therapy; Nx – 5/6th nephrectomy.

* P < .05 and † P < .01 v before the treatment period; ‡ P < .05 v uremic + vehicle and uremic + rhEPO + losartan or captopril; § P < .01 v uremic + rhEPO; || P < .05 and # P < .01 v uremic + vehicle.
ET-1 detection limit was 1 pg/tube with 50% tracer displacement around 10 pg/tube on the standard curve. The intra-assay and interassay coefficients of variation were 7% and 10%, respectively. Normal urinary irET-1 concentrations in age-matched control Wistar rats were 59 ± 9 ng/24 h.\(^{18}\)

Renal histologic assessment was performed at random on half of animals per group. Formalin-fixed kidneys were dehydrated and embedded in paraffin and 5-µm thick sections were mounted on glass slides. Renal tissue sections were stained with Masson-trichrome for the assessment of histologic injuries. Immunohistochemistry was performed to determine expression levels of α-smooth muscle actin using a specific rabbit anti-α-smooth muscle active antibody (Sigma, St. Louis, MO) and the avidin-biotin-peroxidase technique (Vectastain ABC-AP kit, Vector Laboratories, Burlingame, CA). Histologic analysis was performed in a blinded manner. Areas close to the scar zones were excluded from analysis. Glomerular sclerosis, vascular hypertrophy, tubular atrophy, and interstitial fibrosis were scored using the following semiquantitative scale from 0 to 3: 0 (not detectable), 1 (<25%), 2 (25% to 50%), and 3 (>50%). Vascular hypertrophy was scored from 0 to 3 as previously described by Pirani et al.\(^ {19}\) The score of each compartment was added to give a total score out of 12. The α-smooth muscle actin expression was quantified using ImageJ image analysis software (version 1.33u, NIH, Bethesda, MD). Briefly, excluding vascular regions, three to five images of each tissue were obtained at ×100 magnification. The percentage of pixels expressing the brown coloration of the α-smooth muscle actin were quantified.

### Statistical Analyses

Results are expressed as means ± SEM unless specified otherwise. Statistical comparisons were analyzed first by ANOVA followed by Student-Newman-Keul’s test for multiple comparisons. The total histologic score was examined by Kruskal-Wallis test followed by the comparison of each group to the overall median. Statistical significance was accepted at \(P < .05\).

### Results

#### Systolic BP

Before treatment systolic BP was similar in all five groups of uremic rats (Table 1). At the end of the study, systolic BP was increased in uremic rats receiving the vehicle and rhEPO compared with the pretreatment values (\(P < .01\)). This increase in BP was also significantly higher in uremic animals on rhEPO treatment versus those receiving the vehicle (\(P < .05\)). Treatment with losartan, captopril, and TRx prevented the increase in systolic BP usually seen with rhEPO therapy (Table 1).

#### Body Weight, Blood, and Urinary Parameters

Body weights of the animals in the five groups of rats were similar before treatment (Table 1). After treatment, body weights of treated animals were comparable to those receiving the vehicle, except for rats receiving TRx whose body weights were significantly lower than animals treated with losartan, captopril, and the vehicle (\(P < .05\)), as previously observed.\(^ {14}\) This may be related to the depressor effect of reserpine on the central nervous system.

All groups of uremic rats developed anemia (Table 1). As expected, hematocrit increased to a similar degree in animals receiving treatment with rhEPO (\(P < .01\)). Serum uric acid concentrations were similar in uremic animals receiving the vehicle and rhEPO (19 ± 1 µmol/L and 20 ± 4 µmol/L), and uricemia was not significantly affected by losartan (16 ± 4 µmol/L), captopril (28 ± 6 µmol/L), and the TRx (20 ± 4 µmol/L) at the end of the treatment period.

Urinary volumes (24 h) were similarly increased in all five groups of uremic rats ranging from 38 to 44 mL before treatment to 33 to 54 mL after the treatment period. Urinary volumes in normal control rats of the same weight were 18 ± 2 mL.\(^ {14}\) By design, serum creatinine was about twice the reference values before treatment (Table 2). Although serum creatinine concentrations and urinary protein excretion tended to be lower in treated animal groups, these changes were not statistically significant. Urinary irET-1 concentrations were significantly increased in un-

### Table 2. Serum creatinine and urine parameters assessed before treatment and at the end of the study

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum creatinine (µmol/L)</th>
<th>Urinary protein (mg/d)</th>
<th>Urinary irET-1 (pg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>End of the study</td>
<td>Before treatment</td>
</tr>
<tr>
<td>Nx + vehicle</td>
<td>84 ± 7</td>
<td>101 ± 7*</td>
<td>68 ± 13</td>
</tr>
<tr>
<td>Nx + rhEPO</td>
<td>77 ± 5</td>
<td>108 ± 11*</td>
<td>41 ± 11</td>
</tr>
<tr>
<td>Nx + rhEPO + losartan</td>
<td>79 ± 6</td>
<td>92 ± 11</td>
<td>72 ± 24</td>
</tr>
<tr>
<td>Nx + rhEPO + captopril</td>
<td>74 ± 5</td>
<td>88 ± 6</td>
<td>71 ± 20</td>
</tr>
<tr>
<td>Nx + rhEPO + TRx</td>
<td>88 ± 8</td>
<td>109 ± 11</td>
<td>71 ± 14</td>
</tr>
</tbody>
</table>

Values are means ± SEM from 8 to 9 animals per group. Abbreviations as in Table 1.

\* \(P < .01\) \(v\) before the treatment period; \† \(P < .05\) and \‡ \(P < .01\) v Nx + rhEPO.
treated uremic rats (rhEPO > vehicle). The antihypertensive treatments attenuated this increase in irET-1 (losartan > captopril > TRx; Table 2).

Renal Histology

The histologic damages in the kidneys of untreated uremic rats were moderate tubular atrophy, interstitial fibrosis, and vascular hypertrophy without any detectable degree of glomerulosclerosis (Fig. 1). These changes were clearly worsened in hypertensive uremic animals treated with rhEPO, where there were also lesions of myointimal proliferation ("onion-skin" lesions) and fibrinoid necrosis. Although TRx reduced the extent of these histologic findings, renin-angiotensin blockade (losartan and captopril) prevented most of the renal histologic damage induced by rhEPO therapy in uremic rats and, in general, the lesions were less severe than in control untreated uremic rats.

Table 3 provides a semiquantitative analysis of the histologic injuries (Masson-trichrome staining) in the different groups of uremic animals. Fig. 1 (right panel) shows immunohistochemistry for α-smooth muscle actin-positive myofibroblasts, the principal effector cells, that are responsible for the excess deposition of interstitial extracellular matrix leading to fibrosis. The brownish staining present in uremic rats who received the vehicle was clearly increased in the rhEPO-treated animals. In contrast to the TRx, losartan and captopril were highly effective in preventing the aggravation of interstitial fibrosis. Fig. 2 shows the quantitative expression of α-smooth muscle actin.

Discussion

During the 3 weeks after renal mass reduction, the animals developed renal failure with proteinuria, anemia, and hy-
pertension. Therapy with rhEPO corrected the anemia but aggravated the hypertension. To our knowledge, this is the first study that has been done to assess the effect of an angiotensin II receptor antagonist compared to an angiotensin-converting enzyme inhibitor and to TRx (hydralazine, reserpine, and hydrochlorothiazide) on rhEPO-induced hypertension and renal function in uremic rats. All three anti-hypertensive regimens were equally effective in reducing hypertension. However, the major finding of this study is that only those agents that blocked the renin-angiotensin system slowed down the progression of renal histologic damage. Despite the same reduction in BP, animals treated with the conventional TRx exhibited more severe glomerular, tubular, and vascular histologic alterations than animals receiving losartan or captopril.

Angiotensin II is known to play a crucial role in the progression of renal failure because this vasopressive peptide contributes to the intraglomerular capillary pressure and also directly stimulates the production of mesangial matrix proteins.20 Eggena11 and Barrett12 and their colleagues showed that rhEPO can modulate the renal and vascular tissue renin-angiotensin system as well as the expression of the angiotensin II receptor. Our results concurred with these findings and may account for the better outcome in renal histology with renin-angiotensin blockade as compared to the TRx.

Blockade of the renin-angiotensin system may also influence the production of other vasopressor systems such as ET-1. We demonstrated that the production of ET-1 is increased in rats with reduced renal mass17 and that administration of rhEPO further enhanced vascular ET-1 concentrations,9 as well as the expression of the ET-1 mRNA in the renal cortex.21 Endothelial cells express EPO receptors22 and these cells, when stimulated with rhEPO, release ET-1.23,24 Selective ET_{A} receptor antagonists have been effective in treating rhEPO-induced hypertension10,13 demonstrating that ET-1 plays a major role in this form of hypertension. Blocking the renin-angiotensin system with either an angiotensin II receptor antagonist or an angiotensin-converting enzyme inhibitor reduced production of ET-1 in blood vessels and glomeruli of rats with reduced renal mass15 and this effect is independent of BP.14 In the present study, urinary ET-1 concentrations were markedly increased in uremic rats receiving the vehicle or rhEPO and the renin-angiotensin blockade produced a significant reduction in renal ET-1 excretion. The TRx was less efficient in lowering urinary ET-1 concentrations despite similar systolic BP reductions. Therefore, the blunting effect of the renin-angiotensin blockade on ET-1 production may account, in part, for the renal protective effect of this class of antihypertensive drugs beyond their ability to reduce BP.

In contrast to TRx, treatment with losartan and captopril was more effective in diminishing the extent of tubular atrophy and interstitial fibrosis. To better demonstrate the renal interstitial fibrosis component we performed renal immunohistochemistry of {\(\alpha\)}-smooth muscle actin in the five experimental groups. Irrespective of the etiology, in the renal interstitial extracellular matrix,25,26 Ng et al27 provided morphologic and phenotypic evidence for the presence of this
contrast, rhEPO replacement therapy has been shown to cannot be accounted for the renal histologic changes. In addition, uricemia was unaffected by the antihypertensive treatments and, thus, previously reported by Vaziri et al. In addition, uricemia was not accounted for, at least in part, by the presence of more severe hypertension in rhEPO-treated rats. However, the group receiving rhEPO + TRx in the present study had significant renal damage despite adequate BP control. Therefore, the histologic changes and renal failure in rats receiving rhEPO cannot be entirely explained by the more severe hypertension. In the rat remnant kidney model, other mechanisms of renal damage have been postulated such as hyperuricemia. However, we found no difference in serum uric acid concentration in uremic animals receiving the vehicle or rhEPO as previously reported by Vaziri et al. In addition, uricemia was unaffected by the antihypertensive treatments and, thus, cannot be accounted for the renal histologic changes. In contrast, rhEPO replacement therapy has been shown to increase vascular ET-1 concentrations and preproET-1 mRNA expression in the renal cortex of uremic rats. In the present study, urinary ET-1 secretion was increased almost twofold with rhEPO therapy. The increased ET-1 production along with angiotensin II activation may trigger the cellular process of matrix protein production leading to renal sclerosis and reduced renal function. On the other hand, our results differ from the study by Bellizzi et al., who did not observe any effect on BP with rhEPO treatment and no progression of renal damage in the remnant kidney model. This discrepancy with the current study might be ascribed to the different species of rats used in their study (Sprague-Dawley) or a difference in experimental conditions such as the time of initiation of rhEPO treatment and the dosage. It is noteworthy that in vitro studies and animal models with acute renal failure have shown that EPO may have a renal protective effect as a result of its antiapoptotic effects in toxic and hypoxic conditions.

In chronic renal failure patients, protracted rhEPO treatment has been associated with either stabilization or a delayed decline in renal function. At present, most renal failure patients are treated for hypertension with either angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. These antihypertensive agents may, in addition, exert renal protection during rhEPO replacement therapy.

The hematocrit attained in rhEPO treated animals was higher than normal by an average of 22%, but similar in the four groups receiving rhEPO. This moderate overcorrection of anemia most likely had no effect on BP because we and other investigators did not observe any correlation between the level of hematocrit after rhEPO treatment and the increase in BP. Vaziri et al. showed that long-term administration of rhEPO resulted in a BP increase of equal magnitude in both iron-sufficient and iron-deficient chronic renal failure rats, although anemia was corrected in the former and persisted in the latter animals. Similarly, multiple small red blood cell transfusions used to simulate the action of rhEPO therapy on hematocrit in a group of vehicle-treated chronic renal failure rats did not alter BP.

In summary, rhEPO-induced hypertension in renal failure rats was adequately controlled with losartan and captopril, as well as conventional TRx (hydralazine, reserpine, and hydrochlorothiazide). The renin-angiotensin system blockade, but not the TRx, slowed down the progression of renal histologic damage and this effect was in addition to the benefit of BP control in uremic rats receiving rhEPO therapy. These findings may be useful in designing prospective studies aimed at identifying the most effective antihypertensive therapies for chronic renal failure patients receiving rhEPO replacement therapy.

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References