Erythropoietin-Induced Antinatriuresis Mediated by Angiotensin II in Perfused Kidneys

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ABSTRACT

Erythropoietin (EPO)-induced hypertension is a common complication of EPO usage. The hypothesis that erythropoietin is antinatriuretic and that the sodium retention is mediated by intrarenal angiotensin II production was tested. Experiments were performed in Wistar rat kidneys perfused for 60 min in an isolated system. A dose-response curve was performed for EPO at 0, 10, 100, 1,000, and 10,000 mU/mL. EPO administration resulted in a dose-dependent decrease in sodium excretion to a maximum of 50% at the 1,000 mU/mL dose. In a second experiment, kidneys from five groups were perfused: controls, EPO (100 mU/mL), captopril (50 ng/mL), captopril (50 ng/mL) plus EPO (100 mU/mL), and the angiotensin receptor antagonist losartan (1 nM) plus EPO (100 mU/mL). The administration of EPO resulted in an immediate decrease in average sodium excretion (30%) with no change in GFR or other renal function parameters. Pretreatment with captopril or losartan blocked the effect of EPO. Captopril alone had no effect on renal function. A final experiment demonstrated the ability of losartan (10 nM) to block the pressor effects of angiotensin II (0.01, 0.1, and 1 nM). It was concluded that EPO acts within the kidney to cause the production of angiotensin II, which mediates the increased reabsorption of sodium.

Key Words: Sodium, losartan, captopril, rat

METHODS

Kidneys from male Wistar rats weighing 275 to 300 g were perfused in a recirculating isolated system that is described in detail elsewhere (19–21). Animals
were anesthetized with 500 mg/kg of pentobarbital sodium ip, and surgery was performed to remove the right kidney. The abdomen was opened, and the right ureter was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ). Sutures were placed around the right renal and superior mesenteric artery. The animal was injected with 1,000 U/kg of heparin iv. The right kidney was cannulated with a blunt 18-gauge steel needle inserted into the right renal artery through the superior mesenteric artery, and the sutures were tied. Simultaneous to entry into the renal artery, perfusate flow was begun from a washout reservoir containing 50 mL of the perfusion medium described below. After excision, the kidney was hung above a water-jacketed glass condenser where 95% O2-5% CO2 was supplied and the kidney’s temperature was maintained at 37°C. The perfusion medium was a Krebs-Henseleit buffer containing 5.5 g/dL of glucose, 45 mg/dL of inulin, [3H]inulin, and 20 amino acids (22). One hundred milliliters of the perfusion medium was used in the recirculating perfusion system. Perfusion flow was provided by the use of a nonpulsatile pump, and the perfusion medium was constantly filtered through an inline 0.45-μm-pore-size filter.

After isolation, kidneys were allowed to equilibrate to the system at a constant pressure for approximately 10 min and then the 60-min experiment was begun. Urine was collected in six 10-min intervals for the measurement of inulin, sodium, and potassium. Urine volume was determined gravimetrically. Perfusion volume loss due to urine formation was replaced by an equal volume of a 50/50 mixture of perfusion medium and saline. Perfusate samples were taken at the midpoint of the urine collection intervals and were not replaced. In the first set of experiments, we determined the sodium excretion dose-response curve for EPO. Three kidneys were perfused in one of five groups: 0, 10, 100, 1,000, and 10,000 mU/mL of EPO. EPO was administered to give the above concentrations at the beginning of the experiment in a volume of 100 mL. These experiments were performed as outlined above. Specifically, kidneys were perfused for 60 min and urine was collected in six 10-min intervals. Urine collection intervals 1 and 2 served as control periods.

In a third set of experiments, we demonstrated the ability of losartan to block the angiotensin II receptor in the isolated perfused kidney. Three kidneys in two groups were perfused for 60 min consisting of four 15-mm urine collection intervals. Group 1 was the control group, and Group 2 received losartan. The urine collection intervals were expanded to 15 min because the study design lent itself to the study of four periods (control and three doses of angiotensin II) and because the kidney has been shown to be stable over a period of 60 min. Losartan potassium (10 nM) was given at 0 min in the Group 2 perfusions. Urine collection interval 1 served as a control. Increasing doses of angiotensin II were given to a final concentration of 10−11, 10−10, and 10−9 M at 15, 30, and 45 min, respectively, in both groups.

In all experiments, perfusion pressure and flow were measured at the midpoint of the urine collection interval. The actual perfusion pressure was determined by subtracting the pressure created by the internal resistance of the perfusion apparatus from the total perfusion pressure at the observed flow rates. Renal vascular resistance was calculated by dividing the renal perfusion pressure by the renal perfusion flow. Urine and perfusate concentrations of sodium and potassium were determined with a Beckman Na/K analyzer (Electrolyte II, Beckman, Brea, CA). [3H]inulin was determined by scintillation counting and was used as a measure of GFR.

Three measures of renal function were performed. The GFR was determined as follows: GFR = urine inulin concentration × urine volume/urine inulin clearance. Each were assigned to one of five groups. Group 1 served as the control group. Group 2 was perfused with 100 mU/mL of α-EPO (Amgen, Thousand Oaks, CA) given 20 min into the perfusion. Group 3 was perfused with captopril given at 0 min to a final concentration of 50 ng/mL. Group 4 was pretreated with captopril given at 0 min to a final concentration of 50 ng/mL and was then treated with 100 mU/mL of EPO given at 20 min. Group 5 was pretreated with losartan potassium given at 0 min to a final concentration of 1 nM and was then treated with 100 mU/mL of EPO given at 20 min. The EPO concentration used in these experiments was chosen after the determination of trough EPO concentrations in patients receiving EPO three times a week in order to represent a physiologic concentration. The 100-mU/mL concentration used was closest to this trough concentration. These experiments were performed as outlined above. Specifically, kidneys were perfused for 60 min and urine was collected in six 10-mm intervals. Urine collection intervals 1 and 2 served as control periods.
A Friedman nonparametric statistical test for k-related samples was used to determine the effect of treatment for all measured variables in the experiments. This test allowed the comparison of results within each experiment and therefore eliminated the effect of interperfusion variability. The 20-min period before the addition of EPO served as a control period for each perfusion. A one-way analysis of variance with a Student Newman-Keuls posttest was performed on the dose-response urine sodium excretion data after a data transformation to decrease the effect of interexperiment variability. The data transformation consisted of dividing the urinary sodium excretion (UNaV) from periods 2 through 5 by the UNaV from period 1. The statistical analyses described were performed on the individual urine collection intervals numbered either 1 to 6 (experiments 1 and 2) or 1 to 4 (experiment 3). The data presented in the tables are the mean of urine collection intervals 1 and 2 (period 1) and the mean of urine collection intervals 4, 5, and 6 (period 2). The data in Tables 1 and 2 are shown as the mean of periods 1 and 2 for presentation purposes only. Further, the experimental design and statistical analysis were chosen apriori in order to minimize the effect of interexperiment variability. Specifically, we are able to investigate changes within experiments despite confounding variability between experiments.

Animal experimentation described in this article was approved by the University of Louisville Animal Care and Use Committee and was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

RESULTS
To investigate the sodium-retaining effect of EPO in the isolated perfused rat kidney, we constructed a dose-response curve as shown in Figure 1. EPO was given in doses between 0 and 10,000 mU/mL. The results are shown as the percent change in UNaV from the first 20 min of perfusion to the final 30 min of perfusion. Increased perfusate concentrations of EPO resulted in a dose-related decrease in UNaV. The maximum decrease of 50% occurred at 1,000 mU/mL. Further increases in EPO concentration did not further decrease sodium excretion. We performed a one-way analysis of variance with a Student Newman-Keuls posttest on the percent change in UNaV further decrease sodium excretion. We performed a Friedman nonparametric statistical test for k-related samples to determine the effect of treatment for all measured variables in the experiments. This test allowed the comparison of results within each experiment and therefore eliminated the effect of interperfusion variability. The 20-min period before the addition of EPO served as a control period for each perfusion. A one-way analysis of variance with a Student Newman-Keuls posttest was performed on the dose-response urine sodium excretion data after a data transformation to decrease the effect of interexperiment variability. The data transformation consisted of dividing the urinary sodium excretion (UNaV) from periods 2 through 5 by the UNaV from period 1. The statistical analyses described were performed on the individual urine collection intervals numbered either 1 to 6 (experiments 1 and 2) or 1 to 4 (experiment 3). The data presented in the tables are the mean of urine collection intervals 1 and 2 (period 1) and the mean of urine collection intervals 4, 5, and 6 (period 2). The data in Tables 1 and 2 are shown as the mean of periods 1 and 2 for presentation purposes only. Further, the experimental design and statistical analysis were chosen apriori in order to minimize the effect of interexperiment variability. Specifically, we are able to investigate changes within experiments despite confounding variability between experiments.

TABLE 1. Renal function parameters for isolated perfused kidneys receiving EPO in concentrations of 0, 10, 100, 1,000, and 10,000 mU/ml.

<table>
<thead>
<tr>
<th>Period</th>
<th>EPO Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>UV (mL/10 min)</td>
<td>0.23 (0.17)</td>
</tr>
<tr>
<td>Pressure (mm Hg)</td>
<td>62.5 (27.3)</td>
</tr>
<tr>
<td>Flow (mL/min)</td>
<td>25.0 (2.2)</td>
</tr>
<tr>
<td>RVR (mm Hg/mL per min)</td>
<td>3.62 (1.06)</td>
</tr>
<tr>
<td>GFR (mL/min)</td>
<td>1.56 (0.29)</td>
</tr>
<tr>
<td>FENa (%)</td>
<td>2.47 (0.80)</td>
</tr>
<tr>
<td>FEK (%)</td>
<td>2.92 (0.81)</td>
</tr>
<tr>
<td>UNaV (μEq/min)</td>
<td>21.2 (13.3)</td>
</tr>
</tbody>
</table>

* Period 1 represents the mean of urine collection intervals 1 and 2, and period 2 represents the mean of urine collection intervals 4, 5, and 6.
* Values are the mean of three determinations (standard deviation): UV, urine volume; Pressure, perfusion pressure; Flow, perfusion flow; RVR, renal vascular resistance; FEK, fractional potassium excretion.
* Different from period 1 by Friedman analysis of variance for related samples (P < 0.05).
TABLE 2. Renal function parameters for isolated perfused kidneys in control, EPO, captopril plus EPO, and losartan plus EPO perfusiona

<table>
<thead>
<tr>
<th>Period</th>
<th>Control</th>
<th>EPO</th>
<th>Captopril Plus EPO</th>
<th>Losartan Plus EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV (mL/10 min)</td>
<td>1</td>
<td>0.58 (0.43)</td>
<td>0.53 (0.37)</td>
<td>0.46 (0.27)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.60 (0.36)</td>
<td>0.44 (0.28)</td>
<td>0.66 (0.46)</td>
</tr>
<tr>
<td>Pressure (mm Hg)</td>
<td>1</td>
<td>86.1 (8.1)</td>
<td>93.8 (13.1)</td>
<td>93.0 (14.3)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>87.6 (9.7)</td>
<td>91.2 (14.4)</td>
<td>92.2 (13.5)</td>
</tr>
<tr>
<td>Flow (mL/min)</td>
<td>1</td>
<td>25.3 (4.1)</td>
<td>26.2 (4.2)</td>
<td>28.0 (5.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>24.2 (2.7)</td>
<td>26.3 (4.3)</td>
<td>27.3 (4.8)</td>
</tr>
<tr>
<td>RVR (mm Hg/mL per min)</td>
<td>1</td>
<td>3.54 (0.86)</td>
<td>3.69 (0.87)</td>
<td>2.99 (0.75)</td>
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<tr>
<td></td>
<td>2</td>
<td>3.71 (0.86)</td>
<td>3.58 (0.94)</td>
<td>3.04 (0.71)</td>
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<tr>
<td>GFR (mL/min)</td>
<td>1</td>
<td>1.01 (0.61)</td>
<td>0.59 (0.34)</td>
<td>0.43 (0.11)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.03 (0.72)</td>
<td>0.61 (0.35)</td>
<td>0.61 (0.20)</td>
</tr>
<tr>
<td>FENa (%)</td>
<td>1</td>
<td>3.21 (1.34)</td>
<td>7.78 (6.17)</td>
<td>7.20 (2.30)</td>
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<tr>
<td></td>
<td>2</td>
<td>2.97 (0.88)</td>
<td>4.26 (3.39)b</td>
<td>5.70 (1.43)</td>
</tr>
<tr>
<td>FEK (%)</td>
<td>1</td>
<td>24.3 (19.5)</td>
<td>52.1 (30.4)</td>
<td>34.8 (33.4)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25.0 (16.7)</td>
<td>53.5 (26.6)</td>
<td>36.0 (31.1)</td>
</tr>
<tr>
<td>UNaV (μEq/min)</td>
<td>1</td>
<td>52.6 (41.0)</td>
<td>50.4 (40.5)</td>
<td>44.3 (19.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>47.6 (29.3)</td>
<td>34.4 (29.8)b</td>
<td>52.1 (25.8)</td>
</tr>
</tbody>
</table>

a Period 1 represents the mean of urine collection interval 1 and 2 and period 2 represents the mean of urine collection intervals 4, 5, and 6. Values are the mean of five determinations (standard deviation); UV, urine volume; Pressure, perfusion pressure; Flow, perfusion flow; RVR, renal vascular resistance; FEK, fractional potassium excretion.

b Different from period 1 by Friedman analysis of variance for related samples (P < 0.005).

Figure 1. Dose-response curve for EPO given at 20 min into the perfusion. Values are the percent change in sodium excretion of the mean of the final 30 min to the initial 20 min of perfusion. Values are expressed as the mean of three experiments (standard deviation).

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The effects of control, EPO, EPO plus captopril, and EPO plus losartan perfusions on sodium excretion are shown in Figure 2. Sodium excretion remained constant throughout the 60 min of the control perfusion. In Group 2, UNaV was constant for the first 20 min and then showed a linear decrease in the following four urine collection periods after EPO administration (P < 0.005). Statistical analysis of the captopril and losartan data in Groups 4 and 5 showed that pretreatment of the kidney with either captopril or losartan 20 min before the addition of EPO blocked the sodium-retaining effects of EPO. Analysis of variance with a Student Newman-Keuls posttest showed that sodium excretion at 60 min was significantly less in the EPO group compared with that in the captopril and losartan groups (P < 0.05).

The renal function parameters for the experiments are shown in Table 2. Variability exists in the measures of renal function similar to that observed in Table 1. Statistical analysis of the renal function parameters between tables was not performed. The data are shown as the mean of the first 20 min (period 1) and the mean of the final 30 min (period 2). There were no significant changes in any of the measured parameters in the control, captopril, captopril plus EPO, and losartan potassium plus EPO perfusion when compared over time. EPO administration resulted in average decreases in FENa of 45% and in UNaV of 30%. Captopril given alone did not affect the measured renal function parameter, and UNaV did not vary with time (48.3 ± 26.2 [period 1] versus...
48.8 ± 22.2 [period 2] μEq/min. No other alterations in renal function were detected in any group except for the alterations in sodium excretion. Further, comparison of the UNaV from period 1 between all five groups did not show a significant difference, from which we can also conclude that none of the treatments were natriuretic.

To demonstrate the effectiveness of losartan potassium in blocking the angiotensin receptor, we gave angiotensin II and measured the pressor response. The results of this experiment are shown in Figure 3 and are corrected for the intrinsic pressure of the system. In the absence of losartan, angiotensin II administration resulted in a dose-dependent increase in perfusion pressure ($P < 0.05$). When losartan was added to the perfusate, the addition of angiotensin II did not increase the perfusion pressure. Losartan effectively blocked the pressor response to exogenously administered angiotensin II in the concentrations studied.

**DISCUSSION**

EPO administration corrects the anemia of chronic renal failure. However, a 30 to 35% incidence of EPO-induced hypertension has been reported in EPO-treated chronic renal failure patients (2-4,6,7). The mechanisms for EPO-induced increases in blood pressure are not clear. Possible explanations include an EPO-induced increase in systemic vascular resistance due to correction of hypoxic vasodilation (14,15) or an inadequate decrease in cardiac output concurrent with the increase in hematocrit and peripheral vascular resistance (14,15,23). In *vitro*, large, nonphysiologic concentrations of EPO cause vasoconstriction in isolated renal vessels (13). Dialysis patients with chronic renal failure given EPO have a decrease in PRA and plasma norepinephrine levels (14); however, blood volume has been reported not to increase (14,23). Rats administered EPO develop hypertension and reveal increases in renal and vascular renin substrate mRNA and renal renin mRNA (16).

Interpretation and analysis of the results of this study are complicated because of the variability associated with the measures of renal function. Stability and function of perfused kidneys are dependent on several factors, including the technical skill of the individual performing the perfusions. However, proper statistical design of the experiments can overcome much of the interexperiment variability. We have demonstrated the stability of the preparation for 60 min (Figure 2, control) in these experiments, and therefore, the intraexperiment variability is low. We designed our experiments to take advantage of the stability of the preparation (low intraexperiment variability) and incorporated an internal control in each experiment with which comparisons could be made. Further, it is our experience that poorly perfused kidneys waste sodium over time, which results in an increased UNaV and FENa. Because defects in the perfusion would support the null hypothesis, our conclusions that EPO is antinatriuretic may be more powerful than expected.

We demonstrated the antinatriuretic effect of EPO in an isolated system in the absence of neuronal and other circulating hormonal control. The effect of EPO on sodium excretion occurred rapidly, lasted for the duration of the experiment (40 min), and resulted in a 50% decrease in sodium excretion at the maximum doses. Although variability exists between groups, EPO administration invariably resulted in a decreased UNaV, regardless of initial values of UNaV, FENa, and GFR. We have recently demonstrated this
sodium-retaining effect of EPO in volunteers with normal renal function (24). EPO given iv at 150 U/kg resulted in a 4% decrease in UNaV in 24 h and an 8% decrease in UNaV over 4 days. This observation in humans supports the hypothesis that EPO is antinatriuretic. The difference in magnitude of the sodium retention between the two studies implies that, in subjects with normal renal function, other factors may compensate for alterations in sodium reabsorption. EPO administration also resulted in a decrease in FENa, shown in Table 2 but not in Table 1. The reason for this difference is unclear but could be because of the small number of observations per group in Table 1 and the variability of the measurements and could be confounded with a small but not statistically significant decrease in GFR seen in Table 1. We cannot determine from our observations if an EPO-induced antinatriuresis leads to an increased blood pressure, and sodium retention has not been observed in EPO-associated hypertension. However, patients with a GFR below 25 mL/min have a larger increase in blood pressure in response to sodium loading than do subjects with a GFR between 30 and 75 mL/min (17). Further experimentation will need to be performed to determine the role of EPO-induced sodium retention in hypertension.

The effect of EPO on sodium excretion was dose dependent and occurred at concentrations within the physiologic range (10 to 100 mU/mL). The peak antinatriuretic effect occurred between 100 and 1,000 mU/mL. This antinatriuretic effect was independent of an effect on the GFR and renal vascular resistance. Heidenreich et al. (13) demonstrated a direct vasoconstrictor effect of EPO on renal arterioles in vitro at concentrations of 10 to 200 U/mL. However, two other investigators were unable to show a vasoconstrictor effect of EPO at concentrations similar to those used in our study (25,26). The maximum concentration used in our experiment was 10 U/mL. Differences in the concentrations of EPO used may account for the differences in these studies. Also, the measurement of renal vascular resistance in the perfused kidney is a gross measure of what may be occurring in the renal vasculature and any changes in the renal microcirculation may not be detected in this model.

The antinatriuretic effect occurs rapidly and suggests that this effect was independent of new protein synthesis. Some other mechanism must be responsible for the EPO-induced sodium retention. EPO was thought to have renin-like activity before the introduction of recombinant EPO (27). However, the EPO extracts in these experiments may have been contaminated with renin. The renin-angiotensin system is an important regulator of sodium excretion and can act within the time frame of the above results. Previous experiments have shown that angiotensin II has a biphasic effect on sodium excretion (28–30). Angiotensin II given in low concentrations (10^{-11} M) results in an antinatriuresis, whereas higher concentrations result in a natriuresis. Further, it has been demonstrated that the rabbit proximal tubular cell is capable of synthesizing angiotensin II from renally produced angiotensinogen (31). Eggena et al. (16) provide further evidence of the involvement of the renin-angiotensin system. Those investigators have demonstrated that EPO causes hypertension in the rat and an increase in renin substrate mRNA in the kidney.

To determine if the EPO-induced antinatriuresis was mediated by angiotensin II, we used the two pharmacologic probes losartan and captopril. Losartan has been shown to be a potent and highly specific angiotensin II receptor antagonist that lacks the agonist properties of saralasin (32,33). Losartan effectively blocks the pressor effects of angiotensin II in this model. The addition of losartan to the perfusion medium before the addition of EPO blocked the EPO-induced antinatriuresis. This would suggest that the EPO-induced sodium retention is mediated through the angiotensin II receptor. The addition of the angiotensin-converting enzyme inhibitor captopril to the perfusion medium at an inhibitory concentration of 50 ng/mL also blocked the EPO-induced antinatriuresis. Kidneys perfused with captopril alone did not show a natriuresis. Further, comparison of the first 20 min of perfusion for the control, losartan, and captopril groups did not show a difference in UNaV. We would conclude from the use of both probes that the EPO-induced antinatriuresis is mediated by the intrarenal production of angiotensin II and by the subsequent binding of angiotensin II to the angiotensin II receptor. We also conclude that a captopril- or losartan-induced natriuresis did not take place. Our observations suggest that intrarenal production of angiotensin II is involved in the EPO-induced sodium retention. These observations are supported by Eggena et al. (16), who documented an EPO-induced increase in renal renin substrate mRNA. Tissue renin substrate levels have been shown to be a major factor in the rate of angiotensin II generation (34).

EPO administration to the isolated perfused kidney results in a 50% decrease in sodium excretion through an apparent production of intrarenal angiotensin II. We would assume that EPO acts by initially binding to an EPO receptor in the kidney. However, no renal EPO receptor has been identified. Detailed reviews of the EPO receptor in bone marrow progenitor cells have been published (35,36). Previous work on the EPO receptor has almost uniformly focused on erythroid cell lines and has not addressed the presence or absence of EPO receptors elsewhere. The lack of an identified renal EPO receptor does not preclude EPO from having a renal effect. The rat
kidney has been shown to incorporate radiolabeled EPO (37). EPO causes vasoconstriction of both isolated renal and mesenteric blood vessels (13), and Anagnostou et al. (38) have reported EPO receptors on endothelial cells. The data of Eggena et al. (16) have shown an EPO-induced increase in renal renin and renin substrate mRNA, which also supports the idea of an EPO receptor in the kidney. These results suggest that EPO could act intracellularly after incorporation into the cell or by binding to an unidentified receptor in the renal vasculature.

We conclude that the administration of EPO results in an increased reabsorption of sodium in the isolated perfused kidney. Further, the antinatriuretic effect of EPO is blocked by angiotensin-converting enzyme inhibition and angiotensin II receptor blockade. These findings point to the renin-angiotensin system as the mediator of the EPO-induced antinatriuresis. The antinatriuretic effect of EPO may be important in patients with chronic renal failure because it would exacerbate their already impaired ability to regulate sodium excretion. These alterations in sodium excretion could play a role in EPO-induced hypertension.

REFERENCES

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