Cytochrome P-450 Arachidonate Metabolite Inhibition Improves Renal Function in Lyon Hypertensive Rats

Isabelle Messer-Létienne, Nicole Bernard, Richard J. Roman, Jean Sassard, and Daniel Benzoni

The present study evaluated the effects of miconazole, a selective inhibitor of epoxygenase activity, on renal hemodynamics and the pressure-natriuresis response of saline-drinking, uninephrectomized Lyon hypertensive (LH) and Lyon low blood pressure (LL) rats. Infusion of miconazole (final concentration, 1 µmol/L) into the renal artery had no effect on the renal function of LL rats over a range of renal perfusion pressures (RPP) from 100 to 140 mm Hg. In contrast, miconazole lowered renal vascular resistance (RVR, 17.9 ± 1.1 v 26.3 ± 1.5 mm Hg/mL/min/g, P < .01) and increased urinary sodium excretion (6.4 ± 1.2 v 4.2 ± 0.8 µmol/min/g, P < .05) in LH rats at a RPP of 140 mm Hg. To determine whether the effects of epoxyeicosatrienoic acids were dependent on activation of the thromboxane A2-prostaglandin H2 (TP) receptor, we studied the effects of a TP receptor antagonist, GR 32191B (0.1 mg/kg/min), on the renal response to an infusion of miconazole into the renal artery in LH rats. GR 32191B decreased basal RVR and prevented the dilation induced by miconazole. It did not, however, alter its natriuretic effect. The renal metabolism of arachidonic acid was also compared in LH and LL rats. The production of epoxygenase metabolites was 25% lower in microsomes prepared from the renal cortex of LH versus LL rats. Miconazole (1 µmol/L) reduced epoxygenase activity similarly, by approximately 60%, in both strains. These results suggest that endogenously formed P450 metabolites of arachidonic acid may serve as a substrate for the formation of vasconstrictor endoperoxides that interact with TP receptors in LH rats and contribute to the enhanced renal vascular tone but not the blunted pressure-natriuresis response. Am J Hypertens 1999;12:398-404 © 1999 American Journal of Hypertension, Ltd.

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From the Département de Physiologie et Pharmacologie Clinique, CNRS ESA 5014, Faculté de Pharmacie, Lyon Cedex, France (IM-L, NB, JS, DB); and Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin (RJR).

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Address correspondence and reprint requests to Isabelle Messer-Létienne, Faculté de Pharmacie, 8 avenue Rockefeller, 69373 Lyon Cedex 08, France; e-mail: letienne@rockefeller.univ-lyon1.fr

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renal metabolism of arachidonic acid (AA) is altered in LH rats. Recent studies have also indicated that AA is primarily metabolized via P450 pathways in the renal cortex to an array of vasoactive metabolites, i.e., 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienonic acids (EET), both of which can be metabolized by cyclooxygenase to vasoconstrictor endoperoxides. Thus, it is conceivable that differences in the renal metabolism of AA may contribute to the renal vasoconstriction in LH rats.

20-HETE is a potent renal vasoconstrictor that inhibits $Na^+$, $K^+$, 2$Cl^-$ transport in the thick ascending loop. It is also a potent inhibitor of Na$^+$-K$^+$ ATPase in the proximal tubule. EET have been reported to dilate arterioles in vitro and elevations in the production of these compounds are thought to promote sodium excretion. However, when infused intrarenally, 5,6- and 8,9-EET can be metabolized by cyclooxygenase to vasoconstrictor endoperoxides.

The role of alterations in renal epithelial activity in genetic models of hypertension remains to be determined. Makita et al. first reported that the production of EET was reduced in the kidneys of Dahl salt-sensitive rats fed a high-salt diet. However, this finding was not confirmed in a study by Ma et al. Other investigators have reported that the production of EET is lower in the kidneys of spontaneously hypertensive rats (SHR) than in Wistar Kyoto (WKY) controls. Because renal vascular resistance is elevated and pressure natriuresis is blunted in LH rats, compared with their LL controls, it is possible that alterations in the renal metabolism of AA by P450 may contribute to these changes. In the present study, the renal metabolism of AA was compared in microsomes prepared from the kidneys of LH and LL rats and the effects of miconazole on renal hemodynamics and the pressure-natriuresis response of both strains were determined.

**MATERIALS AND METHODS**

**Animals** Experiments were performed on 8-week-old LL and LH male rats weighing 250 to 280 g. They were housed under controlled conditions of temperature (21 ± 1°C), humidity (60 ± 10%), and lighting (8 h on, 20 h off). They were fed a standard rat chow containing 0.3% sodium by weight (Elevage UAR, Villemoisson sur Orge, France) and had free access to tap water. One week before the experiment the drinking water was replaced by 1% NaCl solution to elevate sodium intake and accentuate the differences in the pressure natriuretic response and renal hemodynamics between LH and LL rats. Systolic blood pressure (SBP) was measured by a plethysmographic method (Narco Biosystems, Houston, TX) in unrestrained conscious rats on the day before the experiment. All experiments have been conducted according to our institutional guidelines for animal care.

**Surgical Preparation** The left kidney was surgically prepared for study of the pressure-natriuresis response with neural and hormonal influences controlled as previously described. Seven to ten days before the experiment, the right kidney and adrenal gland were removed. On the day of the study, the rats were anesthetized with Inactin (100 mg/kg intraperitoneal [ip], Byk-Gulden, Constance, Germany) and placed on a heating blanket (model 50-6980, Harvard Apparatus, Edenbridge, Kent, UK) to maintain their body temperature at 37°C. After tracheotomy, the left jugular vein was cannulated for infusions. Catheters were placed into the left carotid artery and in the abdominal aorta via the femoral artery to sample blood and to record the mean arterial blood pressure using a pressure transducer (Model P23 ID, Statham Instrument Division, Gould, Inc., Cleveland, OH). The kidney was denervated by stripping all of the visible renal nerves and coating the renal artery with a 10% solution of phenol in ethanol (Merck, Darmstadt, Germany). The remaining adrenal gland was removed and the ureter was cannulated for urine collections. Two adjustable silastic balloon cuffs were placed around the aorta, above and below the left renal artery, and ligatures were placed around the superior mesenteric and celiac arteries so that renal perfusion pressure (RPP) could be manipulated to different levels. A 30-gauge needle was inserted into the abdominal aorta at the origin of the left renal artery for infusions. Finally, a flow probe (1RB) was placed around the left renal artery for measurement of renal blood flow (RBF) using a Transonic transit-time flowmeter (model T106, Transonic Systems, Ithaca, NY).

The animals received a bolus intravenous (iv) dose (250 mg/kg) of polyfructosan (Inutest, Laevosan, Linz, Austria) followed by a constant infusion at a rate of 4.2 mg/kg/min for measurement of glomerular filtration rate. A hormone cocktail, containing D-aldosterone (66 ng/kg/min), hydrocortisone (33 ng/kg/min), norepinephrine (333 ng/kg/min), and [Arg8] vasopressin acetate (0.17 ng/kg/min) was continuously infused at a rate of 0.33 mL/kg/min (pump model 2400-001 Harvard Apparatus, South Natick, MA) to minimize any possible differences in the plasma levels of these key sodium- and water-retain-
the proximal or the distal aortic cuff, respectively, were inflated. Renal vascular resistance (RVR, mm Hg/mL/min/g) was calculated as the ratio of RPP/RBF. Glomerular filtration rate (GFR, mL/min/g) was measured by the clearance of polyfructosan. Urine flow rate (µL/min/g) was determined gravimetrically. Sodium concentration was measured by flame photometry (Instrument Laboratories, model 243, Lexington, MA) and urinary sodium excretion (UNaV, µmol/min/g) and fractional sodium reabsorption (RNa, %) were calculated. All the renal function parameters were normalized per gram kidney weight.

Protocol 1: Effects of Miconazole on Renal Hemodynamics and the Pressure-natriuresis Relation

After surgery and a 1-h equilibration period, miconazole (400 µmol/L, Sigma Chemical), an inhibitor of renal epoxygenase activity, that can be given systemically, or vehicle (ethanol 0.02%), was infused directly into the renal artery of eight LH and eight LL rats. The rate of miconazole infusion was adjusted between 5 and 10 nmoles/min to the baseline RBF value to achieve a final concentration of 1 µmol/L in the blood entering the kidney. After 30 min of miconazole infusion, RPP was lowered to 100 mm Hg for 30 min, then progressively increased to 120 mm Hg for 25 min and finally to 140 mm Hg for 20 min. A 10-min equilibration was allowed after step changes in RPP before urine samples were collected. An arterial blood sample (300 µL) was also collected at the end of each period and replaced by an equal volume of 0.9% NaCl solution.

Protocol 2: Effects of Miconazole After Thromboxane A2-Prostaglandin H2 (TP) Receptor Blockade in LH Rats

The purpose of these experiments was to evaluate the possibility that P450 metabolites of arachidonic acid may serve as substrates for cyclooxygenase in LH rats and constrict the kidney through activation of TP receptors. In these experiments, the effects of miconazole on renal function were studied in LH rats pretreated with a TP receptor antagonist. The experimental protocol was identical to that described earlier except that GR 32191B (0.1 mg/kg/min) (Glaxo Group research, Ware, Hertfordshire, UK), a specific TP receptor antagonist, was added to the hormone cocktail and infused throughout the experiment. The dose of GR 32191B was chosen on the basis of previous work from our laboratory indicating that it completely blocks the vasoconstrictor response to a bolus iv injection of the thromboxane agonist U46619 at this dose.

During the experiments, pulsatile arterial pressure and RBF were continuously monitored using a computerized recording system (LabVIEW 4.0.1; Software, National Instruments, Austin, TX). Data were sampled every 2 ms and stored on CD-ROM. Average mean arterial pressure and RBF were computed offline.

Protocol 3: Renal Metabolism of AA

At the end of the in vivo experiments, the kidneys of LH and LL rats (two groups of six LH and six LL rats for each strain) receiving either miconazole or its vehicle were rapidly removed, frozen in liquid nitrogen, and kept at −80°C until assay.

Renal cortical tissue was homogenized in 10 mmol/L potassium phosphate buffer (pH 7.7) containing 250 mmol/L sucrose, 1 mmol/L EDTA, and 10 mmol/L MgCl2. The homogenate was centrifuged for 5 min at 3000 g to remove tissue chunks and at 11,000 g for 15 min to remove nuclei and mitochondria. Microsomal protein was isolated from the supernatant by centrifugation at 100,000 g for 60 min and resuspended in a 0.1 mol/L potassium phosphate buffer (pH 7.25) containing 1 mmol/L EDTA, 1 mmol/L DTT, 0.1 µmol/L PMSF, and 30% glycerol. Microsomes (0.5 mg protein) were incubated for 30 min at 37°C with excess substrate [1-14C]-arachidonic acid (0.1 µCi/ml, 10 µmol/L) in 1 mL of 100 mmol/L potassium phosphate buffer (pH 7.4) containing 5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L NADPH, and a NADPH-regenerating system consisting of 10 mmol/L isocitrate and 0.4 units/mL isocitrate dehydrogenase. As miconazole is a reversible inhibitor, it was added, at a concentration of 1 µmol/L, to some of the incubations in both strains to determine its effect on the renal metabolism of AA by P450. The reactions were terminated by acidification to pH 4.0 with 1 mol/L formic acid and the metabolites extracted twice with 3 mL ethylacetate. The ethylacetate was back-extracted with 1 mL water and the samples dried under nitrogen. Metabolites were separated using a 2 mm × 25 cm 18C-reverse phase HPLC column with a linear elution gradient ranging from acetonitrile:water:acetic acid (50/50/0.1) to acetonitrile:acetic acid (100/0.1) over 40 min. The radioactive products were monitored using a radioactive flow detector (Model 120, Radiomatic Instruments, Tampa, FL). Product formation was expressed as pmole/min/mg microsomal protein.

Additional experiments were performed in four rats to determine whether miconazole at the concentration used in our study inhibited thromboxane synthase. Platelet-rich plasma was prepared by low-speed centrifugation (120 g) for 10 min of rat blood mixed with sodium citrate (0.25%). Platelet-rich plasma (100 µL 20,000 platelets) was incubated with [1-14C]-arachidonic acid (1 µCi, 10 µmol/L) for 30 min at 37°C in 1 mL of a physiologic salt solution with and without the addition of miconazole (1 and 10 µmol/L). The products formed were extracted with ethyl acetate as described earlier and separated using a 2.5×460-mm 18C-reverse phase HPLC column with an isocratic elution (0.5 mL/min) with 30:70:0.1 acetonitrile:water:acetic acid for 30 min followed by a gradient to 100:0.1 acetonitrile:acetic acid over 70 min.
Statistics  Mean values ± SEM are expressed. The statistical significance of the differences between experimental groups was assessed using a two-way analysis of variance followed by a Fisher multiple range test. P < .05 was considered to be statistically significant.

RESULTS

Systolic blood pressure was significantly higher in LH than in LL rats and averaged 138 ± 1 vs 104 ± 1 mm Hg, respectively. The kidney weight/body weight ratios were similar in LH and LL rats and averaged approximately 0.60 g per 100 g body weight. Hematocrits were also not significantly different in LH and LL rats (43 ± 1% and 42 ± 1%) and they did not change over the course of the experiments.

Under control conditions, baseline RBF and GFR (measured at a RPP of 100 mm Hg) were significantly lower in LH than in LL rats (Figure 1). RBF was well autoregulated in both strains over a range of RPP from 100 to 140 mm Hg, whereas GFR increased as pressure was increased in both strains. The slope of the pressure-natriuresis relation was blunted in LH compared with LL control rats over the range of pressures from 100 to 140 mm Hg and urine flow rate and sodium excretion were significantly lower in LH than in LL rats at a RPP of 140 mm Hg (Figure 2). This was due to both an increase in the tubular reabsorption of sodium as well as to a diminished filtered load.

Protocol 1: Effects of Miconazole on Renal Hemodynamics and Pressure Natriuresis  Infusion of miconazole (1 μmol/L) into the renal artery had no effect on RBF, GFR, or the pressure-natriuresis response in LL rats. In contrast, it significantly increased RBF in LH rats by ~47% at a RPP of 140 mm Hg. After miconazole there was no significant difference in RBF in LH and LL rats (7.8 ± 0.5 vs 8.5 ± 0.6 mL/min/g). In addition, miconazole potentiated the pressure-natriuresis response in LH rats. Because miconazole had no effect on GFR in these rats, this effect was solely due to inhibition of tubular reabsorption of sodium.

Protocol 2: Effects of TP Receptor Blockade on the Renal Response to Miconazole  Administration of the TP receptor antagonist GR 32191B increased baseline RBF (from 5.7 ± 0.4 to 8.0 ± 0.6 mL/min/g, P < .01) and GFR (from 0.35 ± 0.03 to 0.66 ± 0.04 mL/min/g, P < .01) in LH rats. It also significantly blunted
the pressure-natriuresis response and increased tubular reabsorption of sodium when the kidneys were perfused at the highest level of RPP. After TP receptors were blocked by pretreating the LH rats with GR 32191B, miconazole had no effect on the RBF. However, it still decreased tubular reabsorption of sodium and increased sodium excretion at elevated levels of RPP (140 mm Hg).

Protocol 3: Renal Metabolism of Arachidonic Acid
Representative chromatograms illustrating the metabolites formed when microsomes prepared from the kidneys of LH and LL rats were incubated with AA are presented in Figure 3 (upper panel). The profiles of AA metabolites formed were similar in both strains. The major metabolites produced were 11,12- and 14,15-EET, 20-HETE, 11,12- and 14,15-DiHETE, and 20-hydroxy-11,12-EET, with retention times of 17, 16, 10, 9, 8, and 5 min, respectively. A comparison of the renal metabolism of arachidonic acid in LH and LL rats is presented in Figure 3 (lower panel, left). Total renal epoxygenase activity was 25% lower in LH rats than in LL rats studied under the present experimental conditions, whereas there was no significant difference in ω-hydroxylase activity between the strains. The addition of miconazole 1 μmol/L to the reactions selectively inhibited the formation of EET,
DiHETE, and 20-hydroxy 11,12-EET by 60% in both LH and LL rats. The production of 20-HETE was also reduced, by 26% and 36% in LH and LL rats, respectively.

In other experiments we found that platelet-rich rat plasma produced thromboxane B2 (TxB2, 2300 ± 514 pmol/h), 12-HETE (2232 ± 504 pmol/h), and DiHETE (1764 ± 416 pmol/hr) when incubated with arachidonic acid. Addition of a low concentration of miconazole (1 μmol/L) had no significant effect on the formation of TxB2 and 12-HETE but reduced the formation of DiHETE by about 50%. At a higher concentration (10 μmol/L), miconazole completely inhibited the formation of DiHETE and reduced the formation of TxB2 by 80%.

**DISCUSSION**

The present study compared the renal metabolism of AA in microsomes prepared from the renal cortex of LH and LL rats and the effects of the P450 inhibitor miconazole on renal hemodynamics and the pressure-natriuresis response in these two strains. We found that the production of epoxygenase metabolites was slightly but significantly lower in the kidneys of LH rats than in LL rats, whereas the production of 20-HETE was similar in the two strains. As EET have been previously reported to be potent renal vasodilators and natriuretic and diuretic substances, the present study examined the hypothesis that a deficiency in the production of EET may contribute to the renal vasoconstriction and blunted pressure-natriuresis response in LH rats. To this end, the effects of an infusion of miconazole into the renal artery on renal function of LH and LL rats were studied, because this compound has been reported to be a selective inhibitor of renal epoxygenase activity and it is one of the few P450 inhibitors available that can be given systematically. We also confirmed that miconazole 1 μmol/L reduced the formation of epoxygenase metabolites in microsomes prepared from kidney of both strains of rats by about 60%, but it had much less effect on the synthesis of 20-HETE. Infusion of miconazole into the renal artery had no significant effect on RBF, GFR, or the pressure-natriuresis relation of LL rats. However, it markedly increased RBF and potentiated the pressure-natriuresis response in LH rats. This finding was unexpected because total renal epoxygenase activity was lower in LH than in LL rats. If EET are vasodilators that promote sodium excretion, we expected to find that miconazole would reduce renal blood flow and lower sodium excretion to a greater extent in LL than LH rats. Thus, these findings do not support the view that a deficiency in the production of vasodilatory EET contributes to the renal vasoconstrictor and blunted pressure-natriuresis relation in LH rats.

One possible explanation for the present findings is that EET, in particular 5,6 EET, can be metabolized via the cyclooxygenase pathway to vasoconstrictor endoperoxides that interact with TP receptors and promote vasoconstriction. Thus, the lower production of EET in LH rats could reflect a greater metabolism of EET via the cyclooxygenase pathway to vasoconstrictor products.

To explore this possibility further, the effects of blocking TP receptors on the renal response to miconazole infusion were examined in LH rats. As previously reported, TP receptor blockade by GR 32191B markedly increased baseline RBF and GFR in LH rats, thus confirming that TxA2 predominantly increases preglomerular vascular resistance. GR 32191B also decreases urinary sodium excretion. As previous studies have indicated that thromboxane mimetics increase sodium transport in the loop of Henle, the mechanism responsible for the increase in tubular sodium reabsorption after administration of the TP receptor antagonist remains to be determined.

Pretreatment of rats with GR 32191B prevented the renal vasodilatory, but not the natriuretic effects of miconazole. Insofar as GR 32191B alone did not maximally increase the RBF (data not shown), these results indicate that endogenously formed P450 metabolites of arachidonic acid do not exert direct effects on the renal circulation that can account for the elevated vascular tone in the kidneys of LH rats. Rather, they appear to be metabolized via cyclooxygenase to vasoconstrictor endoperoxides in the kidney of LH rats and in this way contribute to the elevation in renal vascular resistance in this strain.

An alternative interpretation is that miconazole may have inhibited thromboxane synthease in LH rats, as miconazole has been reported to inhibit TxA2 synthesis and platelet aggregation with an IC50 of 10−5 mol/L. This hypothesis, however, seems unlikely because we found that infusion of miconazole into the renal artery at the concentration used in our study had no significant effect on the urinary excretion of TxB2 measured by enzyme immunoassay (data not shown). We also found that the low concentration (1 μmol/L) of miconazole used in our study had no significant effect on the formation of thromboxane by platelet-rich rat plasma. However, at the higher concentration of 10 μmol/L it did reduce thromboxane synthesis by about 80%. Moreover, in other experiments we found that miconazole had no significant effect on the renal vasoconstrictor response to bolus infusion of U46619 (20 μg/kg, ia), which argues against an effect of miconazole to block the thromboxane/endoperoxide receptor.

Another possibility that cannot be dismissed is that a part of the effects of miconazole may be due to inhibition of the production of 20-HETE, as miconazole...
reduced the formation of this substance by about 25% in the kidneys of LH rats. Blockade of the formation of 20-HETE would be expected to reduce renal vascular tone because it is a potent vasoconstrictor in the renal circulation.6 20-HETE can also be transformed by cyclooxygenase to vasoconstrictor endoperoxides,4 so inhibition of the formation of this substance also may have contributed to the portion of the vasodilator effects of miconazole that is dependent on activation of TP receptors.

In conclusion, the results of the present study indicate that the production of epoxygenase metabolites of arachidonic acid is lower in the kidneys of LH than in LL rats. However, a deficiency in the renal production of EET is not responsible for the renal vasoconstriction and blunted pressure-natriuretic response seen in LH rats. Rather, the present findings suggest that endogenously formed P450 metabolites of arachidonic acid (likely 5,6-EET, 20-HETE, or both) may serve as a substrate for the formation of vasoconstrictor endoperoxides that interact with TP receptors in LH rats and contribute to the enhanced renal vascular tone but not the blunted pressure-natriuresis response in this strain.

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