Soluble Epoxide Hydrolase Inhibition Lowers Arterial Blood Pressure in Angiotensin II Hypertension

John D. Imig, Xueying Zhao, Jorge H. Capdevila, Christophe Morisseau, Bruce D. Hammock

Abstract—Epoxyeicosatrienoic acids (EETs) have antihypertensive properties and play a part in the maintenance of renal microvascular function. A novel approach to increase EET levels is to inhibit epoxide hydrolase enzymes that are responsible for conversion of biologically active EETs to dihydroxyeicosatrienoic acids (DHETs) that are void of effects on the preglomerular vasculature. We hypothesized that inhibition of soluble epoxide hydrolase (sEH) would lower blood pressure in angiotensin II (Ang II) hypertension. Rat renal cortical tissue was harvested and urine collected 2 weeks following implantation of an osmotic minipump containing Ang II (60 ng/min). Renal cortical sEH protein expression was significantly higher in Ang II hypertension compared with normotensive animals. Likewise, urinary 14,15-DHET levels were significantly increased in hypertensive compared with normotensive animals and averaged 8.1 ± 1.3 and 2.7 ± 1.1 ng/d, respectively. In additional experiments, the sEH inhibitor N-cyclohexyl-N-dodecyl urea (NCND; 3 mg/d) or vehicle (corn oil, 0.5 mL) was administered daily by intraperitoneal injection starting on day 10. Administration of NCND for 4 days lowered systolic blood pressure by 30 mm Hg in Ang II hypertensive animals, whereas the corn oil vehicle had no effect on blood pressure in normotensive or Ang II hypertensive animals. Measurement of blood pressure by indwelling arterial catheters in conscious animals with free movement in their cages confirmed that NCND had antihypertensive properties. Arterial blood pressure averaged 119 ± 5 mm Hg in normotensive, 170:± 3 mm Hg in hypertensive and 149 ± 10 mm Hg in NCND-treated, Ang II-infused animals. Administration of the potential metabolite of NCND, N-cyclohexylformamide to Ang II hypertensive rats did not lower the systolic blood pressure. These studies demonstrate that increased sEH expression in the Ang II hypertensive kidney leads to increased EET hydration. Moreover, sEH plays a role in the regulation of blood pressure, and inhibition of sEH during Ang II hypertension is antihypertensive. (Hypertension. 2002;39[part 2]:690-694.)

Key Words: renal blood flow ■ endothelium-derived factors ■ microcirculation ■ cytochrome P450 ■ kidney

The regulation of renal epoxyeicosatrienoic acid (EET) production has been intensively studied because the kidney has a relatively high epoxygenase activity and because significant alterations in renal EET production are observed in cardiovascular disease states.1,2 In general, epoxygenase metabolites have antihypertensive properties and contribute to the maintenance of renal hemodynamic function.3,4 EETs produced by the kidney act to increase renal blood flow and promote sodium excretion.4,5 Kidney EET production increases in response to high dietary salt in rats and is inappropriately low during the development of hypertension in some animal models.6–9 Once formed, renal EETs can be metabolized by epoxide hydrolase enzymes to dihydroxyeicosatrienoic acids (DHETs). Two recent reports indicate that epoxide hydrolase activity contributes to arterial blood pressure control.10,11 Currently, the contribution of epoxide hydrolase enzymes to the development of angiotensin II (Ang II) hypertension remains unknown.

The development of hypertension following the long-term administration of initially suppressor doses of Ang II has many of the same renal and vascular changes that are associated with human hypertension.12–14 We established that an elevated vascular resistance in the juxtaglomerular region contributes to the blunted renal pressure-natriuresis response in Ang II hypertension.15 An enhanced renal microvascular reactivity that was selective for Ang II during the early phases of hypertension has also been observed.15,16 We recently investigated the role of EETs in Ang II hypertension because CYP450 epoxygenase metabolites have antihypertensive properties, and 11,12-EET has been proposed to be an endothelium-derived hyperpolarizing factor (EDHF) in the renal microcirculation.5,16,17 These investigations revealed that acute elevation of 11,12-EET levels reversed the enhanced preglomerular reactivity to Ang II during hypertension.16 Although 11,12-EET dilates the renal microvasculature and ameliorated the enhanced reactivity to Ang II in...
hypertension, the epoxide hydrolase product, 11,12-DHET, was found to have no vasodilatory actions. Increased EET hydration to inactive DHETs could contribute to the increased preglomerular vascular reactivity and resistance present in Ang II hypertension. Therefore, the present experimental studies were performed to determine epoxide hydrolase regulation in Ang II hypertension and the ability of epoxide hydrolase inhibitors to lower arterial blood pressure in these animals.

Materials and Methods

Induction of Ang II-Infused Hypertension

Tulane University and Medical College of Georgia Animal Care and Use Committees approved the experimental procedures. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 180 to 200 g were divided into 3 experimental groups: 1 group received sham surgery, a second group was subjected to Ang II infusion, and the third group received Ang II and on day 10 was treated with N-cyclohexyl-N-dodecyl urea (NCND). Ang II was infused at a continuous rate via an osmotic minipump (60 ng/min) as previously described. NCND was added to corn oil (3 mg/500 μL) and warmed to 30°C, vigourously vortexed and sonicated until NCND was homogeneously suspended. The mEH inhibitor dodecylamine (3 mg/500 μL), or the potential metabolite of the sEH inhibitor N-cyclohexylforamide (3 mg/500 μL) were suspended in corn oil in the same manner. Following the measurement of blood pressure, the epoxide hydrolase inhibitors (3 mg/d IP) were administered once daily on days 10 to 13.

Measurement of Blood Pressure

Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography to monitor the progression of hypertension. In a separate series, indwelling femoral artery catheters were implanted to measure blood pressure in conscious animals as previously described. Blood pressure was measured between 9:00 AM and 12:00 PM before injection of epoxide hydrolase inhibitors or corn oil vehicle.

Kidney and Liver Epoxide Hydrolase Protein Levels in Ang II Hypertensive Rats

Kidney and liver samples were harvested and processed as previously described. Samples were separated by electrophoresis on a 3% to 8% stacking Tris-glycine gel, and proteins were transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane. The primary antibodies used were rabbit anti-mouse soluble epoxide hydrolase (sEH) antibody (1:2000; Drs Hammock, University of California, Davis) and rabbit anti-rat microsomal epoxide hydrolase (mEH) antibody (1:1000; Drs Oesch and Arand, University of California, Davis). The blots were then washed and incubated with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Detection was accomplished using enhanced chemiluminescence (ECL). The blots were then washed and incubated with the goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Detection was accomplished using enhanced chemiluminescence (ECL). Band intensity was measured densitometrically and the values were factored for β-actin.

Renal Urinary EET and DHET Levels in Ang II Hypertensive Rats

Animals were housed in separate metabolic cages (Nalgene Corp., Rochester, NY) that efficiently separated urine from food and feces. Urine was collected in a conical tube containing 5 mg triphenylphosphine and cooled by dry ice. Samples were stored at −80°C until assayed for EET and DHET levels. Urinary EET and DHET levels were determined as previously described. Because the labile 5,6-EET suffers extensive decomposition during sample extraction and purification 5,6-EET levels were not determined. Urine samples are mixed with an equimolar mixture of 1-C labeled (54 μCi/μmol) 8,9-, 11,12-, and 14,15-EET (20 to 30 ng each) or 1-C labeled (54 μCi/μmol) 8,9-, 11,12-, and 14,15-EET (20 to 30 ng each).

Figure 1. The top panel shows the comparison of systolic blood pressure in normotensive, Ang II-infused hypertensive and NCND-treated Ang II-infused hypertensive groups. The bottom panel shows the comparison of mean arterial blood pressures in normotensive, Ang II-infused hypertensive and NCND-treated, Ang II-infused hypertensive groups measured by indwelling catheters in conscious rats on day 14. Values are mean±SEM. * Significant difference between Ang II hypertensive and NCND-treated Ang II-infused hypertensive groups.

Statistical Analysis

All data are presented as mean±SEM. The significance of differences between groups for the blood pressure data were evaluated with an ANOVA for repeated measures followed by a Duncan’s multiple range post hoc test. An unpaired 2-tailed t-test was applied to compare the sEH and mEH protein levels and urinary EET and DHET levels. A P value of <0.05 was considered significant.

Results

Consistent with previous reports, Ang II-infused rats tended to gain less weight during the 2-week post-implantation period. Two weeks after the start of the infusion, normotensive animals weighed 325±8 g, Ang II-infused rats weighed 304±10 g, and Ang II-infused animals treated with NCND weighed 308±10 g.

The effect of NCND on systolic and arterial blood pressures in Ang II hypertensive rats is presented in Figure 1. Systolic blood pressure averaged 114±3 mm Hg before minipump implantation and increased significantly above control levels by day 10 to 187±6 mm Hg in animals receiving Ang II. Corn oil vehicle (0.5 mL, IP) treatment for 4 days did not change systolic blood pressure in Ang II hypertensive animals. NCND (3 mg/d, IP) decreased systolic blood pressure to 157±5 mm Hg on day 13 in animals receiving Ang II but did not alter blood pressure in normotensive animals. Measurement of blood pressure by indwelling arterial catheters in conscious animals with free move-
ment in their cages confirmed that NCND had antihypertensive properties when administered to Ang II-infused rats (Figure 1, bottom panel). Administration of the mEH inhibitor dodecylamine (3 mg/d, 189 ± 5 mm Hg, n = 4), or the potential metabolite of the sEH inhibitor N-cyclohexylforamide (3 mg/d, 192 ± 4 mm Hg, n = 4) to Ang II hypertensive animals for 4 days did not lower systolic blood pressure.

Figures 2 presents representative Western blots and densitometric analysis of epoxide hydrolase protein expression in the liver and kidney. Western blots and densitometric analysis demonstrate that kidney sEH protein expression was increased 2-fold 2 weeks after the start of Ang II infusion (Figure 2, top). The greater variability of sEH expression observed in the control Sprague-Dawley rats confirms previous findings that renal cortical sEH expression is low to barely detectable in Wistar-Kyoto (WKY) and Sprague-Dawley rats.11 Likewise, liver sEH protein expression was increased in Ang II hypertension, but this increase did not reach statistical significance (P = 0.55) and was not as robust as that observed in the kidney. In contrast, kidney and liver mEH protein expression was not different between hypertensive and normotensive animals (Figure 2, bottom).

Urinary EET and DHET levels were determined by NICI/GC/MS in normotensive and Ang II hypertensive rats. All 4 EET regioisomers were detected, but of the 4 DHET regioisomers only 14,15-DHET reached measurable levels. Urine was collected between days 11 and 12 for measurement of EET and DHET levels. Urinary excretion averaged 16.8 ± 1.6 mL/day in normotensive rats and was slightly elevated (22.9 ± 3.3 mL/day) in Ang II-infused rats. Administration of NCND to Ang II-infused rats resulted in a diuresis (34.1 ± 1.2 mL/day; P < 0.05) that corresponded with the decrease in systolic blood pressure on day 12. EET levels were decreased and 14,15-DHET levels increased 2 weeks following the start of Ang II infusion (Table 1 and Figure 3). Urinary EET levels in Ang II hypertension were significantly lower than those observed in normotensive animals, and averaged 5.7 ± 1.2 and 10.8 ± 1.9 ng/d (P < 0.05) respectively. A significant 40% increase in urinary EETs (8.0 ± 1.6; P < 0.05) occurred in Ang II hypertensive rats treated with NCND (Table 1). Likewise, 14,15-DHET excretion decreased and averaged 4.7 ± 1.3 ng/d in NCND-treated hypertensive animals (Figure 3).

**Discussion**

A role for the soluble form of the epoxide hydrolase enzymes in the long-term control of arterial blood pressure and the pathogenesis of experimental hypertension has recently been proposed.10,11 Therefore, we performed experiments to determine if an increased kidney sEH contributes to the elevated

**Urinary EET Excretion in Normotensive, Ang II Hypertensive and NCND Treated Ang II Hypertensive Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>8,9-EET (ng/d)</th>
<th>11,12-EET (ng/d)</th>
<th>14,15-EET (ng/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=6)</td>
<td>2.1 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>6.5 ± 1.3</td>
</tr>
<tr>
<td>Ang II Infused (n=6)</td>
<td>1.1 ± 0.7</td>
<td>1.0 ± 0.4</td>
<td>3.6 ± 1.2</td>
</tr>
<tr>
<td>Ang II Infused and NCND (n=6)</td>
<td>1.3 ± 0.4</td>
<td>1.6 ± 0.7</td>
<td>5.1 ± 1.4</td>
</tr>
</tbody>
</table>
arterial pressure in Ang II-dependent hypertension. The results of these experiments demonstrate that kidney sEH protein is increased in Ang II hypertension. In agreement with the increased sEH protein levels, urinary EET levels were decreased and 14,15-DHET levels increased 2 weeks after the start of Ang II infusion. Lastly, chronic administration of the highly selective sEH inhibitor NCND increased EET levels and decreased arterial blood pressure in Ang II hypertensive animals. These findings support the concept that kidney sEH plays a role in the regulation of arterial pressure during Ang II-dependent hypertension.

Epoxide hydrolase enzymes catalyze the conversion of epoxides to their corresponding diols by the addition of water. 14,15-EET appears to be a better substrate than 11,12-EET for endothelial sEH, although both are turned over rapidly. In the renal microcirculation the predominant vasodilatory EETs are 11,12-EET and 14,15-EET, and increased conversion of these epoxides to their corresponding diols by the addition of water, 14,15-EET appears to be a better substrate than 11,12-EET for endothelial sEH, although both are turned over rapidly. In the renal microcirculation the predominant vasodilatory EETs are 11,12-EET and 14,15-EET, and increased conversion of these epoxides to their corresponding diols could reduce this antihypertensive attribute. Genetic polymorphisms for the mEH and sEH enzymes have been described in the human population, and a mEH gene polymorphism has recently been associated with preeclampsia. Linkage of these epoxide hydrolase gene polymorphisms to other cardiovascular diseases is not yet known. Nonetheless, we found that kidney and liver mEH were not altered 2 weeks following the start of Ang II infusion. This finding is in agreement with a previous report that renal mEH protein expression is not different between spontaneously hypertensive (SHR) and white Wistar-Kyoto (WKY) rats. In support of the concept that kidney sEH contributes to the long-term regulation of arterial blood pressure, we observed much larger increases in renal cortical compared with liver sEH in Ang II hypertensive animals. The present findings demonstrate that sEH contributes significantly to the elevated blood pressure in Ang II-dependent hypertension. Besides elevating EET levels, sEH inhibition could lower blood pressure in other ways. sEH also converts the linoleic acid epoxides to their corresponding diols. Preliminary experiments have failed to reveal any significant influence of the linoleic acid epoxides, leukotoxin or iso-leukotoxin, on renal microvascular function (data not shown). These observations support the postulate that CYP450 epoxide metabolites derived from arachidonic acid are the primary epoxides involved in the regulation of renal hemodynamic function. Secondly, sEH inhibitors can alter incorporation of EETs into endothelial cell phospholipids, increase the conversion of 11,12-EET and 14,15-EET to chain shortened epoxy-fatty acids, and enhance the vasodilatory actions of EETs. The possible contribution of EET incorporation into endothelial cell membranes and chain shortened epoxy-fatty acids to the blood pressure lowering effects of NCND remain unknown. Nevertheless, the fact that NCND resulted in a diuresis, increased urinary EETs, and decreased urinary 14,15-DHET excretion rates supports the notion that elevation of renal vasodilatory and natriuretic EETs are partially responsible for the antihypertensive effect produced by sEH inhibition.

The ability of NCND to lower blood pressure in Ang II hypertension suggests that kidney sEH plays a central role in the development of hypertension. Two recent reports have provided evidence that sEH is important for the long-term regulation of arterial blood pressure. Sinal et al demonstrated that male sEH gene-disrupted mice have lower systolic blood pressures when compared with wild-type mice. Renal production of DHETs was decreased and EET formation increased in the sEH (−/−) mice, suggesting an important role for epoxide hydrolase metabolism in the regulation of blood pressure. A pivotal role for the sEH enzyme in the pathogenesis of experimental hypertension has also been demonstrated. Yu et al showed that kidneys of the SHR have increased expression of sEH and urinary DHET excretion. Moreover, administration of a single dose of N,N′-dicyclohexyl urea (DCU) decreased urinary DHET excretion and lowered blood pressure in the SHR. Blood pressure returned to a value similar to vehicle-injected SHR by 24 hours. In agreement with this finding, we did not observe a significant lowering of systolic blood pressure 24 hours after the administration of the first dose of NCND. In contrast, repeated administration of NCND resulted in a sustained antihypertensive effect. NCND administered once daily for up to 4 days lowered systolic blood pressure by 23 mm Hg by day 2, and this level was maintained to day 4. The sustained blood pressure lowering effect of NCND with time could be due to a build up of NCND or a bioactive metabolite of the drug with repeated administration. Further investigations will be needed to clearly define the mechanisms by which sEH inhibitors lower arterial blood pressure.

In summary, the current study demonstrated that kidney sEH protein levels were elevated in Ang II hypertension, and this was associated with increased levels of urinary 14,15-DHET. These studies demonstrate that increased renal sEH leads to increased EET hydration in Ang II hypertension. Administration of the selective sEH inhibitor, NCND, for 4 days lowered arterial blood pressure in Ang II hypertensive animals. Thus, the regulation of EETs and the sEH enzyme is a new target for therapeutic intervention in cardiovascular diseases.

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