Salt-Sensitive Hypertension After Exposure to Angiotensin Is Associated With Inability to Upregulate Renal Epoxygenases

Xueying Zhao, David M. Pollock, Darryl C. Zeldin, John D. Imig

Abstract—The current study was designed to determine whether angiotensin II infusion could lead to persistent salt-sensitive hypertension and to examine involvement of renal microvascular epoxygenases in this process. Six groups were studied: rats maintained on a normal salt diet for 4 weeks (NS); rats maintained on a high salt diet for 4 weeks (HS); and all other animals receiving angiotensin II (ANG) infusion and being fed a normal or high salt diet for 2 weeks; then the angiotensin II infusion was stopped and diets were either maintained or switched (ANG/NS-NS, ANG/NS-HS, ANG/HS-HS, ANG/HS-NS). Angiotensin II infusion resulted in a rise in blood pressure and an increase in urinary albumin excretion over the 2-week period. After angiotensin II withdrawal, blood pressure returned to normal in animals receiving a normal salt diet from weeks 2 to 4 (ANG/NS-NS and ANG/HS-NS groups). In contrast, blood pressure remained elevated in the group maintained on a high salt diet throughout the entire 4-week period (ANG/HS-HS group). Renal microvascular CYP2C11 and CYP2C23 protein levels were decreased by 50% to 60% in the ANG/HS-HS group compared with the NS group. Likewise, renal microvascular CYP2J protein was significantly decreased in the ANG/HS-HS group versus the NS group. Renal microvascular CYP2C11 and CYP2C23 mRNA levels were reduced in the ANG/HS-HS group compared with both the NS and HS groups. These results support the hypothesis that angiotensin II infusion induces persistent salt-sensitive hypertension after withdrawal of angiotensin II that may be due to downregulation of CYP2C and CYP2J epoxygenases in renal microvessels. (Hypertension. 2003;42[part 2]:775-780.)

Key Words: endothelium-derived factors ■ hypertension, sodium-dependent ■ angiotensin II ■ kidney ■ vessels

Epoxygenase metabolites, the epoxyeicosatrienoic acids (EETs), contribute to integrated kidney function by directly affecting tubular transport processes, vascular tone, and cellular proliferation.1–6 EETs are generally considered to be antihypertensive as the result of their vasodilatory and sodium transport properties.2,3,7–9 Epoxidation of arachidonic acid has been attributed to members of the CYP2C and CYP2J subfamilies.10–13 CYP2C subfamily isoforms are abundantly expressed in rat kidney, with CYP2C23 being the predominant isoform.11 Recent studies have shown that CYP2J protein is also expressed in rat kidney and that CYP2J protein expression is increased in spontaneously hypertensive rats.13

Salt sensitivity is an important characteristic of a subgroup of humans with essential hypertension and other forms of salt-dependent hypertension that occurs in blacks, diabetics, patients treated with cyclosporine, or with aging.14,15 Previous studies in animal models of hypertension demonstrate that salt-sensitive hypertension develops after short-term exposure to angiotensin II.16,17 The mechanisms by which transient angiotensin II–mediated blood pressure elevation can lead to persistent salt-sensitive hypertension have not been completely elucidated. Studies in rats suggest that a salt-inducible renal epoxygenase has antihypertensive properties, and kidney EET production is inappropriately low during the development of salt-sensitive hypertension.11,18–20 Our previous study also showed that P450 epoxygenase protein levels were significantly decreased in angiotensin II–infused animals fed a high-salt diet.21 The purpose of the present study was to determine if an inability to upregulate P450 epoxygenase enzymes is associated with persistent salt-sensitive hypertension after 2 weeks of exposure to angiotensin II.

Methods

Animals
The Medical College of Georgia Animal Care and Use Committee approved the experimental procedures. All procedures were carried out in accordance with institutional guidelines. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200 to 225 g were divided into experimental groups: rats maintained on a normal salt diet (0.8% NaCl by weight) for 4 weeks (NS); rats maintained on a high salt diet (8% NaCl by weight) for 4 weeks (HS); all other animals received angiotensin II (ANG) infusion.
infusion for 2 weeks and were fed a normal or high salt diet, and the angiotensin II infusion was stopped and diets were maintained or switched (ANG/NS-NS, ANG/NS-HS, ANG/HS-NS). Angiotensin II was infused at a constant rate by an osmotic minipump (65 ng/min) as previously described.22

Measurement of Blood Pressure
Systolic blood pressure was measured in conscious rats by tail-cuff plethysmography to monitor the progression of hypertension. Blood pressure was measured between 9:00 AM and noon. Mean arterial blood pressure was measured with the use of a radiotelemetry technique. Telemetry transmitters (Data Sciences) were implanted according to the manufacturer’s specifications into male Sprague-Dawley rats while under pentobarbital sodium anesthesia (65 mg/kg IP; Abbott Laboratories). After baseline measurements, rats were divided into 3 groups: HS, ANG/NS-NS, and ANG/HS-HS.

Urinary Albumin Levels in Angiotensin II–Hypertensive Rats
Animals were housed in separate metabolic cages (Nalgene Corp) 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 albumin. Urinary albumin levels were determined by an enzyme-linked immunosorbent assay (Exocell Inc).

Isolation of Renal Microvessels
Renal microvessels were isolated according to a method described previously.23 Briefly, the kidneys were infused with a physiological solution 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 protein or mRNA levels.

Immunoblot Analysis of CYP2C23, CYP2C11, and CYP2J2 Protein
Renal microvessels were harvested and microsomal fractions were prepared as previously described.23,24 Samples were separated by electrophoresis on 10% stacking Tris-glycine gels, and proteins were transferred electrothermally to nitrocellulose membranes. The primary antibodies used were goat anti-rat CYP2C11 polyclonal antibody (1:1,000; Gentest), rabbit anti-rat CYP2C23 polyclonal antibody (1:5,000; a generous gift from Dr Jorge Capdevila, Vanderbilt University), rabbit anti-human CYP2J2 polyclonal antibody (1:2,000), and goat anti-rat CYP4A1 polyclonal antibody (1:2,000; Gentest). The CYP2J2 antibody was made against a partially purified preparation of recombinant human CYP2J2 as described and has been shown to cross-react with all CYP2J isoforms.13 The blots were then washed in a PBS–0.1% Tween 20 solution and incubated with the secondary antibody (anti-goat IgG 1:3,000 for CYP2C11 and CYP4A; anti-rabbit IgG, 1:100 000 for CYP2C23 and CYP2J2) conjugated to horseradish peroxidase for 1 hour at room temperature and washed. Detection was accomplished with the use of enhanced chemiluminescence (ECL, Amersham Corp), and blots were exposed to x-ray film (Hyperfilm-ECL, Amersham Corp). Band intensity was measured densitometrically, and the values were normalized to expression of β-actin.

Reverse Transcription–Polymerase Chain Reaction
Total RNA was prepared from isolated renal microvessels with the use of an ultra-pure TRIzol reagent according to the procedure described by the manufacturer (Gibco-BRL). Random hexanucleotide primers were used for reverse transcription (RT) of equal amounts of RNA. Oligonucleotide primers were designed from the published cDNA sequences of CYP2C11, CYP2C23, and GAPDH. GAPDH was used as an internal standard. The sequences of the CYP2C11 primers are sense 5'-CAA TCC GCA GTC TGA GTT-3' and antisense 5'-TGC TGA GAA TGG CAT AAA-3'. The sequences of the CYP2C23 primers are sense 5'-TCA CTA CGC TTG CTC TTG-3' and antisense 5'-TAT CCC TTA TGG GTA TCT TC-3'. The sequences of the GAPDH primers are sense 5'-AAT GCA TCC TGC ACC ACC AA-3' and antisense 5'-GTA GCC ATA TTC ATT GTC ATA-3'. The expected sizes of the amplified CYP2C11, CYP2C23, and GAPDH polymerase chain reaction (PCR) products are 463, 325, and 515 base pairs, respectively. RT-PCR was performed as previously described.25 After amplification, 15 µL of each PCR reaction mixture was electrophoresed on a 1.5% agarose gel with ethidium bromide (0.5 µg/mL). The gel was scanned with ultraviolet illumination by means of Digital Imaging and Analysis (Alpha Innotech Corporation).

Statistics
All data are presented as mean±SEM. Physiological parameter comparisons between groups were made through the use of ANOVA combined with the Newman-Keuls multiple range test. An unpaired 2-tailed t test was applied to compare the P450 protein levels. A value of P<0.05 was considered statistically significant.

Results
Consistent with previous reports, blood pressures were markedly increased in angiotensin II–infused rats at days 10 to 14 (Table 1 and Figure 1). The increase in mean arterial blood pressure (MAP) was significantly greater in angiotensin-infused rats fed a high salt diet compared with rats fed a high salt diet alone and angiotensin-infused rats fed a normal salt diet. High salt diet alone caused a small, nonstatistically significant increase in MAP. After 2 weeks, the minipumps containing the angiotensin II were removed and blood pressure returned to normal in ANG/NS-NS and ANG/HS-NS groups (Table 1 and Figure 1). In contrast, systolic and mean arterial blood pressures were sustained at a significantly higher level 14 days after termination of angiotensin infusion

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### TABLE 1. Systolic Blood Pressure and Body Weights in Animal Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>SBP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS, n=6</td>
<td>260±3</td>
<td>109±3</td>
</tr>
<tr>
<td>HS, n=7</td>
<td>262±4</td>
<td>103±3</td>
</tr>
<tr>
<td>ANG/NS-NS, n=6</td>
<td>270±2</td>
<td>111±4</td>
</tr>
<tr>
<td>ANG/NS-HS, n=6</td>
<td>258±4</td>
<td>112±4</td>
</tr>
<tr>
<td>ANG/HS-NS, n=6</td>
<td>258±3</td>
<td>114±2</td>
</tr>
<tr>
<td>ANG/HS-HS, n=6</td>
<td>264±3</td>
<td>114±2</td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS, n=6</td>
<td>353±4</td>
<td>121±5</td>
</tr>
<tr>
<td>HS, n=7</td>
<td>342±5</td>
<td>110±7</td>
</tr>
<tr>
<td>ANG/NS-NS, n=6</td>
<td>332±8*</td>
<td>176±11*</td>
</tr>
<tr>
<td>ANG/NS-HS, n=6</td>
<td>314±15*</td>
<td>171±5*</td>
</tr>
<tr>
<td>ANG/HS-NS, n=6</td>
<td>241±17*</td>
<td>182±9*</td>
</tr>
<tr>
<td>ANG/HS-HS, n=6</td>
<td>220±9*</td>
<td>205±6*</td>
</tr>
<tr>
<td>Day 28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS, n=6</td>
<td>432±11</td>
<td>123±4</td>
</tr>
<tr>
<td>HS, n=7</td>
<td>381±12*</td>
<td>115±3</td>
</tr>
<tr>
<td>ANG/NS-NS, n=6</td>
<td>377±5*</td>
<td>125±4</td>
</tr>
<tr>
<td>ANG/NS-HS, n=6</td>
<td>355±11*</td>
<td>122±4</td>
</tr>
<tr>
<td>ANG/HS-NS, n=6</td>
<td>350±12*</td>
<td>126±2</td>
</tr>
<tr>
<td>ANG/HS-HS, n=6</td>
<td>334±11*</td>
<td>144±4*</td>
</tr>
</tbody>
</table>

NS indicates normal salt diet; HS, high salt diet; ANG, angiotensin II. *P<0.05 vs NS group.
in the ANG/HS-HS group (144±4 and 132±7 mm Hg, respectively) compared with the HS (115±3 and 120±5 mm Hg, respectively) and ANG/NS-NS (125±4 and 99±2 mm Hg, respectively) groups.

As shown in Table 2, infusion of angiotensin for 14 days resulted in hypertension in association with a marked increase in urine albumin excretion. The increase in urinary albumin level was significantly greater in the ANG/HS-HS group than in the NS group, HS group, and ANG/NS-NS group. These data suggest that the combination of angiotensin II and a high salt diet greatly exacerbates the albumin excretion.

Two weeks after angiotensin withdrawal, CYP2C23 and CYP2C11 protein levels were not different in the HS group (177±15 and 56±7, respectively) compared with the ANG/HS-HS group (203±22 and 62±18, respectively). In contrast, the levels of CYP2C23 and CYP2C11 protein were reduced in renal microvessels in ANG/HS-HS animals (64±2 and 21±4, respectively) compared with the NS animals (113±11 and 37±6, respectively). In addition, CYP2J protein level was also significantly reduced in the ANG/HS-HS group (73±6) compared with the NS group (95±6).

Figure 4 shows P450 epoxygenase mRNA levels in renal microvessels at the end of the 4-week period in the various groups. CYP2C23 and CYP2C11 renal microvessel mRNA levels were increased in HS animals. No significant differences were found in ANG/HS-NS animals compared with HS rats, but CYP2C23 and CYP2C11 mRNA were lower in the ANG/HS-HS group (69±19 and 43±5, respectively) compared with the HS and ANG/NS-NS groups.

Figure 2. Renal microvascular CYP2C11 and CYP2C23 protein expression in animals at day 28. Illustrated are representative Western blots showing 52-kDa CYP2C11 and 52-kDa CYP2C23 protein bands in normal salt–fed rats (NS, lane 1 to 2), high salt–treated animals (HS, lane 3 to 4), angiotensin II–infused rats maintained on a high salt diet for 2 weeks, after which the angiotensin II infusion was stopped and the rats were fed a normal salt diet (ANG/HS-NS, lanes 5 to 6) or continued on a high salt diet (ANG/HS-HS, lanes 7 to 8) for 2 more weeks.

Figure 1. MAP in conscious rats given a high-salt (HS) diet, angiotensin (ANG) II infusion with normal salt (NS) diet, or angiotensin II infusion with high salt diet. Rats began angiotensin II infusion with normal salt diet, or angiotensin II with high salt diet (ANG/HS-HS, lanes 7 to 8) for 2 more weeks. Two weeks after ending angiotensin infusion, the urinary albumin levels were not different during the 2-week period. Two weeks after ending angiotensin II infusion was stopped after 2 weeks of treatment (indicated by arrows). Values are mean±SEM for 12-hour periods.

Table 2. Urinary Albumin Excretion in Animal Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine Volume, mL/d</td>
<td>Albumin, mg/d</td>
</tr>
<tr>
<td>NS, n=6</td>
<td>15.9±1.2</td>
<td>0.70±0.15</td>
</tr>
<tr>
<td>HS, n=7</td>
<td>79.1±5.2</td>
<td>0.94±0.19</td>
</tr>
<tr>
<td>ANG/NS-NS, n=6</td>
<td>31.1±4.6</td>
<td>8.00±2.00*</td>
</tr>
<tr>
<td>ANG/NS-HS, n=6</td>
<td>39.6±6.1</td>
<td>8.15±3.95*</td>
</tr>
<tr>
<td>ANG/HS-NS, n=6</td>
<td>129.8±14.1</td>
<td>35.96±5.67*</td>
</tr>
<tr>
<td>ANG/HS-HS, n=6</td>
<td>163.5±29.7</td>
<td>41.20±8.18*</td>
</tr>
</tbody>
</table>

*P<0.05 vs NS group.
Methods section. PCR amplifications were then performed with murine leukemia virus reverse transcriptase as described in the

In addition, kidney-specific downregulation of P450 epoxy-

Comparison of renal P450 epoxygenase levels is essential for

inability to increase CYP2C protein levels is associated with

The current study showed that CYP2C11 and CYP2C23 pro-

In the past several years, multiple CYP2J enzymes have been isolated and characterized as arachidonic acid epoxygenases. 

Human CYP2J2, rat CYP2J3, and mouse CYP2J5 isoforms all

It is important to point out that the catalytic

CYP2C isoforms are considered the major renal arachidonic

CYP2C and CYP2J enzymes in renal vasculature.

P450 epoxygenase enzymes were associated with hypertension and end-organ damage in transgenic rats overexpressing both human renin and angiotensinogen genes (dTGR). We have previ-

CYP2C11 and CYP2C23, and CYP2J protein levels in renal microvessels are elevated in kidney to maintain fluid and electrolyte homeostasis in response to increased salt intake. Previous studies have shown an impaired ability to upregulate renal epoxygenase enzymes in animal models of salt-sensitive hypertension. In addition, kidney-specific downregulation of P450 epoxygenase enzymes is associated with hypertension and end-organ damage in transgenic rats overexpressing both human renin and angiotensinogen genes (dTGR). We have previ-

Figure 3. CYP2C11, CYP2C23, and CYP2J protein expression in renal microvessels from animals treated for 4 weeks. Illustrated are representative Western blots showing CYP2C11, CYP2C23, and CYP2J protein bands in renal microvessels isolated from normal salt–treated animals (NS, lanes 1 to 4) and angiotensin II–infused rats given a high salt diet, after which the angiotensin II infusion was stopped and rats were continued on a high salt diet (ANG/HS-HS, lanes 5 to 8) for 2 more weeks. Bar graphs depict densitometric values (arbitrary densitometry units) for renal microvascular protein levels (10 μg/lane; n=4 to 6/group). Values are mean±SEM. *Significant difference compared with normal salt control.

Discussion

Regulation of renal P450 epoxygenase levels is essential for

The current study extends these findings by examining whether angiotensin II infusion leads to persistent salt-sensitive hypertension even after removal of the angiotensin. This study also begins to examine the role of renal microvascular P450 epoxygenases in this process. Consistent with our previous study, the increase of mean arterial blood pressure was significantly greater in angiotensin II–infused animals fed a high salt diet compared with animals fed a normal salt diet. After removal of the angiotensin II, arterial blood pressures recovered to the normal levels in ANG/NS-NS, ANG/NS-HS, and ANG/HS-NS groups; however, blood pressure was sustained at a higher level in the ANG/HS-HS group. Renal microvascular CYP2C11, CYP2C23, and CYP2J protein levels were lowered in the ANG/HS-HS group compared with the NS group 2 weeks after withdrawing the angiotensin infusion. Therefore, persistent salt-sensitive hypertension after 2 weeks of angiotensin II administration and a high salt diet is associated with an inability to upregulate CYP2C and CYP2J enzymes in renal vasculature.

CYP2C isoforms are considered the major renal arachidonic acid epoxygenases in the kidney. In particular, CYP2C23 is highly expressed in rat kidney, where it is involved in regulating renal EET biosynthesis. The CYP2C11 isoform is expressed in rat kidney at much lower levels than CYP2C23. Increased expression of CYP2C enzymes and increased formation of EETs in the kidney occurs in response to increased salt intake. These salt-inducible renal P450 epoxygenases have antihypertensive properties and contribute importantly to the maintenance of body fluid and electrolyte composition. Our previous study confirmed that kidney P450 epoxygenase protein levels are elevated in salt-loaded animals; however, this increase in P450 epoxygenase enzymes was abolished in angiotensin II–infused animals fed a high-salt diet. These findings suggest that an inability to increase CYP2C protein levels is associated with the salt-sensitivity of angiotensin II–dependent hypertension. The current study showed that CYP2C11 and CYP2C23 protein levels were reduced in the ANG/HS-HS group 2 weeks after removal of angiotensin administration. Consistent with this data, renal microvascular CYP2C11 and CYP2C23 mRNA levels were increased in rats fed a high salt diet and decreased in ANG/HS-HS animals. The decreased levels of P450 epoxygenase enzymes were associated with a sustained hypertension after terminating angiotensin infusion. Taken together, these data suggest that inappropriate regulation of P450 epoxygenases plays a role in the development and maintenance of salt-sensitive hypertension.

The CYP2J subfamily also contributes to the formation of EETs in human and rodent kidneys. In the past several years, multiple CYP2J enzymes have been isolated and characterized as arachidonic acid epoxygenases. Human CYP2J2, rat CYP2J3, and mouse CYP2J5 isoforms all preferentially catalyze epoxidation of arachidonic acid at the 14,15 position. It is important to point out that the catalytic

Figure 4. Expression of CYP2C11 and CYP2C23 mRNA in renal microvessels. Total RNA was extracted from renal microvessels and reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase as described in the Methods section. PCR amplifications were then performed with CYP2C11, CYP2C23, or rat GAPDH sequence-specific oligonucleotide primers and AmpliTaq DNA polymerase as described. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide.
activity of the CYP2J isofoms is severalfold less than that of the CYP2C enzymes.28 In addition, CYP2J3 biosynthesizes 19-HETE, which has been shown to inhibit voltage-gated calcium channels and is a potent vasodilator.29 Recent studies have shown that CYP2J immunoreactive protein is increased in the spontaneously hypertensive rat kidney, and this is associated with increased EET formation both in vitro and in vivo.13 Thus, several lines of evidence suggest that CYP2J proteins may play a role in renal arachidonic acid metabolism and blood pressure regulation. Previous findings demonstrate that CYP2J enzymes are expressed in renal microvessels, and neither a high salt diet alone or chronic angiotensin II infusion alone changes the CYP2J protein level in renal cortex or microvessels.21 Interestingly, the combination of angiotensin II infusion and a high salt diet significantly decreased renal microvascular CYP2J protein levels. In our current study, 2 weeks after the removal of angiotensin II infusion, CYP2J protein levels remained lower in the ANG/HS-HS group compared with the NS group. This finding suggests that renal microvascular CYP2J downregulation may also play a role in the maintenance of salt-sensitive hypertension after angiotensin II infusion.

Overall, the current study demonstrates that kidney P450 epoxygenase mRNA and protein levels are decreased in ANG/HS-HS animals 2 weeks after stopping angiotensin II infusion and that this decrease in P450 epoxygenase enzymes is associated with a persistent salt-sensitive hypertension. These studies support the postulate that EET regulation is associated with the salt-sensitive hypertension that accompanies and follows angiotensin II infusion.

Perspectives
Studies conducted over the past decade have provided convincing evidence that the renal P450 epoxygenase enzymes play a critical role in the renal response to a chronic salt overload.10,11,18,19,21,30 Epoxygenase enzymes are upregulated in response to increased salt intake and the vascular and tubular actions of EETs appear essential for the proper maintenance of fluid and electrolyte homeostasis and arterial blood pressure in this setting. In the present study, we demonstrate an impaired regulation of renal microvascular CYP2C11, CYP2C23, and CYP2J enzymes and persistent salt-sensitive hypertension after a 2-week exposure to a high salt diet and angiotensin II infusion. These findings are consistent with other animal models of salt-sensitive hypertension that also lack proper regulation of renal epoxygenase enzymes. Obviously, the association of renal P450 epoxygenase regulation and salt-sensitive hypertension needs to be investigated in human subjects before this pathway can be deemed a viable therapeutic target for salt-sensitive hypertension.

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