5,6-Epoxyeicosatrienoic Acid Mediates the Enhanced Renal Vasodilation to Arachidonic Acid in the SHR

Silvia I. Pomposiello, John Quilley, Mairead A. Carroll, John R. Falck, John C. McGiff

Abstract—We have shown a cytochrome P450–dependent renal vasodilator effect of arachidonic acid in response to inhibition of cyclooxygenase and elevation of perfusion pressure, which was enhanced in the spontaneously hypertensive rat (SHR) and linked to increased production of and/or responsiveness to epoxyeicosatrienoic acids (EETs). In the SHR, vasodilation elicited by low doses of arachidonic acid was attenuated by the nitric oxide synthase inhibitor Nω-nitro-L-arginine (50 μmol/L), whereas the responses to high doses were unaffected. Inhibition of epoxygenases with miconazole (0.3 μmol/L) in the presence of Nω-nitro-L-arginine greatly reduced the renal vasodilator response to all doses of arachidonic acid. Tetraethylammonium (10 mmol/L), a nonselective K+ channel blocker, abolished the nitric oxide–independent renal vasodilator effect of arachidonic acid as well as the vasodilator effect of 5,6-EET, confirming that EET-dependent vasodilation involves activation of K+ channels. Under conditions of elevated perfusion pressure (200 mm Hg) and cyclooxygenase inhibition, 5,6-EET, 8, 9-EET, and 11,12-EET caused renal vasodilatation in both SHR and Wistar-Kyoto rats (WKY), whereas 14,15-EET produced vasoconstriction. 5,6-EET was the most potent renal vasodilator of the EET regioisomers in the SHR by a factor of 4 or more. In the SHR, 5,6-EET– and 11,12-EET–induced renal vasodilatation was >2-fold greater than that registered in WKY. Thus, the augmented vasodilator responses to arachidonic acid in the SHR is through activation of K+ channels, and 5,6-EET is the most likely mediator. (Hypertension. 2003;42:548-554.)

Key Words: kidney rats spontaneously hypertensive cytochrome P450 arachidonic acids vasodilation

Cytochrome P450 (CYP)–dependent metabolism of arachidonic acid (AA) generates products that are vasoactive and that participate in the regulation of extracellular fluid volume.1–4 20-Hydroxyeicosatetraenoic acid (20-HETE) generated by the renal vasculature is considered prohypertensive because it is a potent vasoconstrictor agent5 that has been implicated in myogenic responsiveness,6 autoregulation of renal blood flow and glomerular filtration rate,7 and as a likely mediator.8,9 Moreover, there is evidence that 20-HETE is causally related to the development of hypertension in the spontaneously hypertensive rat (SHR), because interventions designed to reduce the generation of 20-HETE prevent the development of hypertension.10–12 In contrast to 20-HETE, the other major products of CYP-AA metabolism by the kidney, the epoxyeicosatrienoic acids (EETs), are considered antihypertensive because they elicit vasodilation13–15 and oppose the actions of 20-HETE via stimulation of K+ channels,16–18 as well as modulate the activity of angiotensin II.19 Moreover, the EETs have been proposed as endothelium-derived hyperpolarizing factors (EDHFs) that mediate the nitric oxide (NO)– and prostaglandin-independent vascular effects of acetylcholine (Ach) and bradykinin.20–23 Consequently, a deficit in EET-dependent mechanisms would be expected to predispose to hypertension, a contention supported by observations of increased conversion of EETs to inactive metabolites, dihydroxyeicosatrienoic acids (DHTs), by epoxide hydrolase in the SHR; inhibition of this enzyme lowers blood pressure,24,25 Moreover, in rats maintained on a high-salt diet, which itself does not increase blood pressure, inhibition of epoxygenase results in elevation of blood pressure, ie, renders the rat salt-sensitive.26

We have unmasked a CYP-dependent vasodilator effect of AA in the rat kidney that was demonstrated by inhibition of cyclooxygenase (COX), which abolished the prostaglandin endoperoxide-mediated vasoconstrictor effect and revealed a related CYP component to the AA response.27,28 The most likely mediators of the renal AA-induced vasodilator effect were EETs. A recent study that was designed to test the hypothesis that the CYP-dependent renal vasodilator effect of AA was impaired in the SHR revealed the reverse; ie, the renal vasodilator effect of AA was enhanced in SHR.29 This study also verified a role for CYP and further implicated EETs in the vasodilator effect of AA by using selective inhibitors of epoxygenase that attenuated the vasodilator...
response. The present investigation is an extension of the earlier study and was undertaken to further characterize the contribution of EETs to the enhanced NO- and prostaglandin-independent vasodilator effect of AA in the kidney of the SHR. First, the mechanism of the vasodilator effect of AA was determined in terms of dependence on epoxygenase activity and stimulation of K⁺ channels, the presumed target of EETs. Second, renal vascular responses to the EET regioisomers and their release in response to AA were compared in SHR and Wistar-Kyoto rats (WKY). We confirmed a role for epoxygenase in the renal vasodilator effect of AA that was also shown to depend on K⁺ channel activity. In the SHR kidney, vasodilator responses to 5,6-EET and 11,12-EET were increased compared with WKY; 5,6-EET exhibited the greatest vasodilator activity.

**Methods**

**Materials**

Phenylephrine, N⁴-nitro-l-arginine (l-NA), and inorganic salts purchased from Sigma were dissolved in water. Indomethacin and miconazole (Sigma) were dissolved in 4.2% NaHCO₃ and ethanol, respectively. Sodium arachidonate (NuChek) was dissolved in distilled water, divided into 1 mg/mL aliquots, and stored under N₂ at −70°C.

**Animals**

Male SHR and WKY (Charles River Laboratory, Wilmington, Mass.) 13 to 15 weeks of age, were used for these studies. The animals were maintained in an air-conditioned room with a 12-hour dark/light cycle and given standard rat chow (Ralston Purina Co) and free access to tap water. They were allowed 3 to 5 days to adjust to the new environment before the experiments were started. Blood pressure was measured in conscious animals by the tail-cuff method and in the presence of indomethacin and miconazole (Sigma) were dissolved in 4.2% NaHCO₃ and ethanol, respectively. Sodium arachidonate (NuChek) was dissolved in distilled water, divided into 1 mg/mL aliquots, and stored under N₂ at −70°C.

**Perfused Kidney In Situ**

The perfused kidney in situ has been described previously. In brief, after pentobarbital anesthesia (60 mg/kg IP) was induced and midline laparotomy performed, the right renal artery was cannulated via the mesenteric artery with a 19-gauge needle and perfused in situ at constant flow with a pump (model 50SS, Watson Marlow) with warmed (37°C) and gassed (95% O₂–5% CO₂) Krebs-Henseleit solution of the following composition (in mmol/L): NaCl 118, KCl 4.7, KH₂PO₄ 1.19, MgSO₄ 1.19, CaCl₂ 1.9, NaHCO₃ 25, and glucose 5.5. Flow rate was adjusted to 7 to 10 ml/min to achieve a basal perfusion pressure of 70 to 90 mm Hg, which was measured with a pressure transducer (Harvard Apparatus) and recorded on a chart recorder (model 1244, Soltex). In all experiments, perfusion pressure was elevated to ~200 mm Hg with phenylephrine (0.5 to 1×10⁻⁶ M). AA (0.25 to 10 μg) and EETs (5.6, 8.9, 11.12-, or 14.15-EET; 10 μg) were administered as bolus injections into the renal artery to test vascular reactivity. Indomethacin (10 μmol/L) was added to the perfusate in all experiments to inhibit COX. l-NA, miconazole, and tetraethylammonium (TEA) to inhibit NO synthase (NOS), epoxygenase, and K⁺ channels, respectively, were added to the perfusate 30 minutes before the elevation of perfusion pressure with phenylephrine. In those preparations from which the renal effluent was collected, the kidney was removed after arterial cannulation and suspended in a water-jacketed organ bath at 37°C.

**Protocol 1: Mechanism of AA-Induced Vasodilatation in SHR**

All experiments were performed at ~200 mm Hg perfusion pressure and in the presence of indomethacin (10 μmol/L). First, we studied the effects of l-NA (50 μmol/L) to inhibit NO synthesis on the renal vascular responses to AA in the SHR. In the presence of l-NA, we tested the effect of the selective epoxygenase inhibitor miconazole (0.3 μmol/L) and TEA (10 mmol/L), a nonselective inhibitor of K⁺ channels, on the renal vasodilator responses to AA. Dose-response curves to AA (0.25 to 10 μg) were determined and compared with those from vehicle-treated kidneys. Responses to Ach (100 ng), an endothelium-dependent vasodilator agent that exhibits dependence on CYP and K⁺ channel activity as well as NO, were used to assess the effectiveness of miconazole and TEA, whereas responses to sodium nitroprusside (1 μg) were used to assess the effects of miconazole and TEA independent of their effects on epoxide synthesis and K⁺ channel activity, respectively, ie, to determine unwanted effects of either on intrinsic vascular smooth muscle activity.

**Protocol 2: Renal Vascular Effect of EETs**

Experiments were performed at ~200 mm Hg perfusion pressure and in the presence of indomethacin (10 μmol/L). Phenylephrine (0.5 to 1×10⁻⁶ mol/L) was added to the perfusate to increase perfusion pressure to ~200 mm Hg. Vascular responses to the individual EET regioisomers (5.6-, 8.9-, 11.12-, or 14.15-EETs) were measured in the absence and presence of TEA (10 mmol/L) to verify the role of K⁺ channels.

**Protocol 3: Release of CYP Metabolites**

The release of EETs into the perfusate (venous and ureteral) of SHR and WKY kidneys that had been treated with indomethacin and constricted with phenylephrine was measured as described. In brief, the effluent was collected for 5 minutes before and after a bolus injection of AA (5 μg). To measured volumes of renal perfusates, 7.5 ng of a mix of EET-d₅ (8.9-, 11.12-, and 14.15-EETs) was added as internal standards. The eicosanoids were extracted and separated by high-performance liquid chromatography. Fractions containing CYP metabolites were further purified to separate individual EETs (8.9-, 11.12-, and 14.15-EETs) on a silica column and an isocratic flow of hexane, isopropanol, and acetic acid (99.5%/0.4%/0.1%, vol/vol/vol). 8.9-EET and 11.12-EET did not separate completely, and therefore, these fractions were combined for joint determination. The fractions were evaporated to dryness, derivatized, and quantified by negative chemical ionization gas chromatography–mass spectrometry. Measurement of 5,6-EET was performed as described in a recent publication. In brief, after addition of 2 ng D₆-5,6-lactone as an internal standard, eicosanoids were extracted. 5,6-EET and 5,6-DHT were converted to 5,6-d-lactone by incubation with dried chloroform and 25 μg camphorsulfonic acid at room temperature for 40 minutes. Samples were purified by reverse-phase high-performance liquid chromatography. The fraction that eluted with the retention time of lactone was dried, subjected to hydrolysis (methanol/water/triethylamine) to form 5,6-DHT, derivatized, and quantified by gas chromatography–mass spectrometry.

**Statistical Analysis**

Results are expressed as mean±SEM. A Student 2-sample t test was used to analyze differences between groups. A probability value of <0.05 was considered significant.

**Results**

**Protocol 1: Mechanism of AA-Induced Vasodilatation in SHR**

In phenylephrine-preconstricted kidneys pretreated with the COX inhibitor indomethacin, the NOS inhibitor l-NA (50 μmol/L) had no effect on vasodilator responses to the higher doses of AA (5 and 10 μg; Figure 1), whereas responses to 0.25, 1, and 2.5 μg AA were reduced, suggesting a contribution of NO. However, responses to Ach were not affected by
Inhibition of NOS, indicating that mediators other than NO can maintain responsiveness to Ach. Responses to sodium nitroprusside were increased when NO synthesis was inhibited, an expected consequence of removal of endogenous NO (Table). In the presence of L-NA, selective inhibition of eicosanogenases with miconazole (0.3 μmol/L) further attenuated the renal vasodilator response to AA (Figure 1). Miconazole also reduced the vasodilator effect of Ach, supporting a role for EETs in the NO-independent vasodilator effect of this agent. Miconazole reduced responses to sodium nitroprusside relative to the L-NA–treated group; responses to sodium nitroprusside, however, did not differ between control and L-NA–treated group (Table). In the presence of L-NA and indomethacin (to isolate the NO-and COX-independent components of the AA renal vasodilator effect), inhibition of K⁺ channels with TEA significantly diminished the vasodilator response to AA in the SHR (Figure 2), indicating that the eicosanoid mediator of the renal vasodilator response to AA activates K⁺ channels. Similarly, the NO-independent vasodilator response to Ach was reduced by TEA, consistent with a contribution of an EDHF (Table). In contrast, vasodilator responses to sodium nitroprusside were insignificantly reduced in the presence of TEA.

**Protocol 2: Renal Vascular Effect of EETs**

Vascular responses to the 4 EET regioisomers were tested in kidneys treated with indomethacin to inhibit COX and phenylephrine to elevate perfusion pressure to ~200 mm Hg. Under these conditions, 5,6-, 8,9-, and 11,12-EETs caused renal vasodilatation, whereas 14,15-EET caused constriction in both SHR and WKY (Figure 3). The decrease in perfusion pressure elicited by 5,6-EET was enhanced 2-fold in the SHR compared with the WKY (Figure 3) and exceeded the vasodilator effects of 8,9- and 11,12-EETs by several-fold. The renal vasodilator effect of 8,9-EET and the vasoconstrictor effect of 14,15-EET were similar in the SHR and WKY (Figure 3), whereas 11,12-EET produced a greater vasodilator response in the SHR. Furthermore, the vasodilator response to 5,6-EET in the SHR was as much as 6-fold greater than those to 8,9- and 11,12-EETs.

In kidneys from the SHR in which COX was inhibited with indomethacin and perfusion pressure was elevated with phenylephrine, 5,6-EET produced dose-dependent vasodilatation (Figure 4). In the presence of TEA (10 mmol/L) to inhibit K⁺ channels, 5,6-EET produced a greater vasodilator response in both SHR and WKY, whereas 8,9- and 11,12-EETs were less effective in producing vasodilation. Under these conditions, 14,15-EET caused vasoconstriction in both SHR and WKY (Figure 4).

### Effect of L-NA, L-NA + Miconazole and TEA on Renal Vasodilator Responses to Ach and SNP in SHR

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ach</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-97±9</td>
<td>-61±12</td>
</tr>
<tr>
<td>L-NA, 50 μmol/L</td>
<td>-100±11</td>
<td>-99±20</td>
</tr>
<tr>
<td>L-NA + Miconazole, 0.3 μmol/L</td>
<td>-39±12 *</td>
<td>-50±15</td>
</tr>
<tr>
<td>Control</td>
<td>-83±4</td>
<td>-65±9</td>
</tr>
<tr>
<td>TEA, 10 mmol/L</td>
<td>-42±16*</td>
<td>-41±11</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM. SNP indicates sodium nitroprusside. *P<0.05 vs control.
channels, the vasodilator activity of 5,6-EET was abolished, and at the 2 higher doses, reversed to a constrictor response.

**Protocol 3: Release of CYP Metabolites**

We measured the release of EETs into the renal effluent before and after challenge with AA (5 μg) in phenylephrine-preconstricted kidneys pretreated with the COX inhibitor indomethacin. Levels of EETs in the perfusates from SHR and WKY kidneys were not different except for 14,15-EET, which was increased in the SHR. After challenge with AA, there was a tendency for increased release of EETs from SHR and WKY kidneys. Of note, AA increased 5,6-EET release from the SHR kidney by 0.36±0.04 ng/min (P<0.05) but did not increase 5,6-EET release from WKY kidneys (Figure 5). In contrast, renal release of 14,15-EET was selectively increased in WKY.

**Discussion**

We have shown in the isolated, perfused rat kidney that a vasodilator response to AA can be uncovered when COX is inhibited and renal perfusion pressure is elevated. This response is dependent on the CYP-related metabolism of AA by epoxygenase activity, which was based on the use of inhibitors and interventions that modified CYP activity.28,33 The most likely mediators of AA-induced vasodilation are EETs that activate K⁺ channels to cause hyperpolarization and relaxation of vascular smooth muscle.20–22

A recent study in the SHR29 revealed an enhanced renal vasodilator effect of AA that could be accounted for by several different mechanisms, including increased CYP-dependent metabolism of AA to EETs, the putative mediators, and/or increased renal vascular sensitivity to EETs in the SHR, possibly arising from greater expression/activity of K⁺ channels, the target of the EETs. The present study addressed the mechanism of AA-induced vasodilation in the SHR in terms of dependence on epoxygenase and activation of K⁺ channels and compared the release and vasodilator activity of EETs in the SHR and WKY as potential mechanisms for the enhanced renal vasodilator effect of AA in the SHR.

The results of this study confirmed a role for CYP in the vasodilator effect of AA in the SHR, because miconazole, a selective inhibitor of epoxygenase at the concentration used,34 greatly reduced the response to AA. Similarly,
miconazole reduced the NO-independent renal vasodilator effect of Ach, supporting an earlier study in the rat kidney that suggested the participation of a CYP-dependent EDHF in the renal vascular response to Ach. However, some caution in the interpretation of the results with miconazole is necessary, because this agent in the presence of L-NA also reduced the vasodilator effect of sodium nitroprusside when compared with treatment with L-NA alone. This study further showed that activation of K⁺ channels is involved in the vasodilator response to AA, providing support for EETs as mediators, in view of their ability to activate K⁺ channels. Thus, TEA, a nonselective inhibitor of K⁺ channels, markedly reduced the vasodilator effect of AA in the SHR kidney. Moreover, TEA abolished the vasodilator effect of 5,6-EET in SHR kidneys, thereby establishing the link between AA, EETs, and K⁺ channels. That TEA was effective in inhibiting K⁺ channels is supported by the reduced NO-independent vasodilator effect of Ach, which has been attributed to the generation of an EDHF, and agrees with the results of our earlier study. Also in agreement with the earlier study, we found that TEA reduced, albeit insignificantly, the renal vasodilator effect of sodium nitroprusside, which invites caution in interpretation, because we cannot exclude the possibility of a “nonselective” effect of TEA on vasodilator mechanisms. However, it should be noted that NO has been shown to activate K⁺ channels, which probably explains the inhibitory effect of TEA on the renal vasodilator response to sodium nitroprusside.

There are 3 major findings of this study: (1) confirmation of our previous study that increased renal perfusion pressure, when COX was inhibited, uncovered a renal vasodilator system served by one or more EETs; (2) the SHR kidney exhibits increased vasodilator responses to EETs, particularly 5,6-EET; and (3) the renal vasodilator effect of 5,6-EET was inhibited by blockade of K⁺ channels. Because vasodilator responses to EETs were increased in the SHR, it is not necessary to invoke an increase in synthesis to account for the enhanced vasodilator effect of AA. Interestingly, EET formation from AA by renal cortical microsomes from SHR was increased 2-fold compared with that in WKY and corresponded to increased urinary excretion, consistent with the increased expression of CYP2J2 in the SHR kidney. The present study indicates that total basal EET release from the SHR kidney was not significantly increased compared with that from WKY. On challenge with AA, total EET release from kidneys of both SHR and WKY was similar, approximately a 2-fold increase, and therefore, we cannot invoke an increase in total EET formation to account for the increased renal vasodilator effect of AA in the SHR kidney. However, in light of studies that have shown an increased activity of epoxide hydrolase in the SHR kidney, measurements of DHTs would also be required to make any definitive statements regarding epoxygenase activity. In the WKY kidney, AA failed to increase the release of 5,6-EET, whereas in the SHR kidney, AA stimulated increases in 5,6-EET. The increased release of 5,6-EET from the SHR kidney in response to AA, as well as the greater response to this regioisomer, likely contributed to the enhanced vasodilator effect of AA in the SHR. There is a possibility that a DHT metabolite of 1 or more EETs contributes to the enhanced vasodilator effect of AA in the SHR kidney, because some of these have been reported to possess potent vasodilator activity in the dog coronary artery, although most studies indicate that DHTs lack vasoactivity. Moreover, epoxide hydrolase activity is increased in the SHR, and therefore, formation of DHTs should be increased, especially when epoxygenase activity is also increased. We can exclude a contribution of 14,15-EET to the vasodilator activity of AA in both SHR and WKY kidneys, because this regioisomer causes vasoconstriction in this preparation, which also eliminates it from consideration as an EDHF in the rat kidney.

The enhanced renal vasodilator effect of 5,6-EET in the SHR kidney cannot be attributed to a generalized increase in vasodilator responses, because the responses to 8,9-EET were not different in SHR and WKY kidneys. This would also tend to exclude a change in K⁺ channels in the SHR if both 5,6-EET and 8,9-EET induced vasodilation via activation of K⁺ channels. K⁺ channel expression and/or activity is reportedly increased in the SHR, manifestations that should increase responses to K⁺ channel activators, including EETs. The renal vasodilator response to bradykinin is also enhanced in the SHR. Because the NO-independent component of the response to bradykinin is also mediated through an EET that presumably acts on K⁺ channels, it would seem that vasodilator mechanisms that require epoxygenase and K⁺ channel activity are upregulated in the SHR kidney.

Although the results of this study support a role for EETs in the renal vasodilator effect of AA in both SHR and WKY kidneys, it is difficult to reconcile the small increases in release (nanograms per minute) with the amounts needed (microgram quantities) to produce a vasodilator effect. There are several possible explanations for this apparent discrepancy. First, EETs are rapidly acylated, and therefore, the amount that reaches the active site, ie, the K⁺ channels of vascular smooth muscle of preglomerular vessels, might be much less. Second, epoxide hydrolase might rapidly convert administered EETs to the corresponding DHTs. Third, EETs released from the endothelium in response to a vasoactive hormone or AA might be directed abluminally toward the vascular smooth muscle, resulting in very high concentrations in the subendothelial space. Consequently, exogenous administration would require quantities sufficient to achieve comparable concentrations in the perfusate to produce an effect. This situation is analogous to the administration of high concentrations of exogenous NO to elicit a response in perfused organ systems. Preferential abluminal release of EETs, coupled with rapid acylation, would also explain the low concentrations of EETs measured in the renal perfusate. Fourth, the EET regioisomers that were used in these experiments were racemic mixtures, whereas endogenous enzymatic synthesis results in the predominance of a specific enantiomer. Thus, administration of racemic mixtures might mask the activity of stereoisomers; eg, the
11R,12S-EET isomer is a far more potent vasodilator than is 11S,12R-EET, which exhibits minimal activity.\textsuperscript{40} The results of this study confirmed increased renal CYP-dependent vasodilator responses to AA in the SHR and suggest that the enhanced response is related primarily to increased responses to EETs. We propose that these changes represent expression of an antihypertensive mechanism designed to counter the elevation of blood pressure in the SHR. This mechanism might be rapidly expressed when blood pressure is elevated.\textsuperscript{29} The putative EET mediators of this antipressor mechanism—5,6-EET and 11,12-EET—possess properties that would oppose elevations of blood pressure: vasodilatation, promotion of Na\textsuperscript{+} excretion, and antagonism of pressor mediators.\textsuperscript{41} Furthermore, EETs have been shown to serve vasodilator mechanisms in humans.\textsuperscript{42}

**Perspectives**

Inhibition of epoxygenase activity renders rats hypertensive when challenged with salt loading. In a classic study, Makita and colleagues\textsuperscript{26} identified 5,6-EET as the epoxide most affected by increased dietary salt. One or more EETs have been proposed to serve as EDHFs.\textsuperscript{5,6} 5,6-EET has been shown to serve vasodilator mechanisms of blood pressure: vasodilatation, promotion of Na\textsuperscript{+} excretion, and antagonism of pressor mediators.\textsuperscript{41} Furthermore, EETs have been shown to serve vasodilator mechanisms in humans.\textsuperscript{42}

**References**

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