Effects of converting enzyme inhibitors on renal P-450 metabolism of arachidonic acid

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Ito, Osamu, Ken Omata, Sadayoshi Ito, Kimberly M. Hoagland, and Richard J. Roman. Effects of converting enzyme inhibitors on renal P-450 metabolism of arachidonic acid. Am J Physiol Regulatory Integrative Comp Physiol 280: R822–R830, 2001.—The effects of blockade of the renin-angiotensin system on the renal metabolism of arachidonic acid (AA) were examined. Male Sprague-Dawley rats were treated with vehicle, captopril (25 mg·kg⁻¹·day⁻¹), enalapril (10 mg·kg⁻¹·day⁻¹), or candesartan (1 mg·kg⁻¹·day⁻¹) for 1 wk. The production of 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) by renal cortical microsomes increased in rats treated with captopril by 59 and 24% and by 90 and 58% in rats treated with enalapril. Captopril and enalapril increased 20-HETE production in the outer medulla by 100 and 143%, respectively. In contrast, blockade of ANG II type 1 receptors with candesartan had no effect on the renal metabolism of AA. Captopril and enalapril increased cytochrome P-450 (CYP450) reductase protein levels in the renal cortex and outer medulla and the expression of CYP450 4A protein in the outer medulla. The effects of captopril on the renal metabolism of AA were prevented by the bradykinin-receptor antagonist, HOE-140, or the nitric oxide (NO) synthase inhibitor, N⁶-nitro-L-arginine methyl ester. These results suggest that angiotensin-converting enzyme inhibitors may increase the formation of 20-HETE and EETs secondary to increases in the intrarenal levels of kinins and NO.

20-hydroxyeicosatetraenoic acid; epoxyeicosatrienoic acids; cytochrome P-450; P-450 reductase; kinins; nitric oxide; angiotensin-converting enzyme

Recent studies indicate that 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) are the primary metabolites of arachidonic acid (AA) produced in the kidney (5, 6, 19, 24, 35) and that these substances play important roles in the regulation of both renal tubular and vascular function. The proximal tubule (28), renal microvessels (19, 23), and glomerulus (21) produce 20-HETE and EETs when incubated with AA, whereas the thick ascending limb of Henle’s loop (TALH) produces 20-HETE (6, 10, 20, 22). 20-HETE constricts rat renal arterioles in vitro (19, 23), whereas EETs dilate these vessels (17, 41). Inhibiting 20-HETE production impairs autoregulation of renal blood flow (18, 42) and blocks tubuloglomerular feedback responses in vivo (43). 20-HETE inhibits sodium transport in both the proximal tubule (29, 30, 34) and the TALH (10, 12, 13, 22). EETs also inhibit sodium transport in the proximal tubule (31, 32) and the cortical collecting duct (CCD) (33) and oppose the hydroosmotic effect of vasopressin in the CCD (14).

Despite the importance of cytochrome P-450 (CYP450) metabolites of AA in the regulation of renal function, very little is known about the factors that regulate the production of 20-HETE and EETs in the kidney. There are studies indicating that the renal production of EETs increases in Sprague-Dawley (5, 15, 25, 27) and Dahl salt-resistant (Dahl R) rats (25) fed a high-salt diet. On the other hand, renal production of 20-HETE and/or EETs has been reported to fall in Sprague-Dawley (27), spontaneously hypertensive (38), Brown Norway (38), Dahl salt-sensitive (Dahl S), and Dahl R rats (24) fed a high-salt diet. Recently, we found that the expression of CYP450 4A proteins decreased in the kidney of Sprague-Dawley rats fed a high-salt diet and that this effect was blocked by fixing circulating ANG II at normal levels by intravenous infusion (3). These studies suggest that elevations in intrarenal and/or circulating levels of ANG II may stimulate the formation of CYP450 metabolites of AA in the kidney. To test this hypothesis further, in the present study we examined the effects of blocking the renin-angiotensin system on the renal metabolism of AA. The results indicate that the renal production of CYP450 metabolites of AA increases in rats treated with angiotensin-converting enzyme (ACE) inhibitors but not in rats receiving an ANG II type 1 (AT₁) receptor antagonist. Furthermore, our findings suggest that ACE inhibitors enhance the levels of CYP450 4A and P-450 reductase proteins in the kidney by increasing intrarenal levels of kinins and nitric oxide (NO) rather than blocking the formation of ANG II.

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METHODS

General. Experiments were performed on 96 9-wk-old male Sprague-Dawley rats purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN). The rats were housed in an animal care facility at the Medical College of Wisconsin that is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols involving animals were reviewed and received approval from the Animal Welfare Committee at the Medical College of Wisconsin.

Rats were maintained on a normal salt diet (1% NaCl by weight) and had free access to food and water throughout the study. The rats were treated with vehicle (n = 12), captopril (25 mg/kg per day, n = 6), enalapril (10 mg/kg per day, n = 6), or the AT1-receptor antagonist candesartan (1 mg/kg per day, n = 6) in drinking water for 1 wk to block the renin-angiotensin system chronically. Other rats were studied to determine whether the effects of the ACE inhibitors on the renal metabolism of AA might be secondary to changes in the levels or actions of mineralocorticoids. In these experiments, the renal metabolism of AA was studied in rats fed a normal salt diet, 7 days after a sustained release pellet (Innovative Research of America, Sarasota, FL) containing spironolactone (50 mg, n = 12) or DOCA (25 mg, n = 6) was implanted subcutaneously. Finally, experiments were performed to determine whether the effects of the ACE inhibitors on the renal metabolism of AA are dependent on elevations in the intrarenal levels of kinins and NO. In these experiments, rats that were fed a normal salt diet and given captopril (25 mg•kg\(^{-1}\)•day\(^{-1}\)) or vehicle in drinking water were treated with the bradykinin type 2 (B\(_2\)) antagonist (HOE-140; d-Arg\([Hyp3, Thi5, D-Tic7, Oic8]\)-bradykinin; 70 \(\mu\)g•kg\(^{-1}\)•day\(^{-1}\), n = 6) or the NO synthase inhibitor N\(^{\mathrm{N}}\)-nitro-L-arginine methyl ester (L-NAME); 100 mg•kg\(^{-1}\)•day\(^{-1}\), n = 6) for 1 wk.

Preparation of renal microsomes. Rats were anesthetized with pentobarbital sodium (50 mg•kg\(^{-1}\)), and the kidneys and liver were rapidly removed. The renal cortex, outer medulla, and liver were homogenized in 3 ml of a 10 mM potassium buffer (pH 7.4) containing (in mM) 250 sucrose, 1 EDTA, and 0.1 phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 3,000 \(g\) for 15 min, and the supernatant was centrifuged at 9,000 \(g\) for 15 min followed by 100,000 \(g\) for 1 h. Microsomal pellets were resuspended in 100 mM potassium buffer (pH 7.25) containing 30% glycerol, 1 mM dithiothreitol, and 0.1 mM PMSF. The protein concentration of the samples was measured using the Bradford method (4) with bovine \(\gamma\)-globulin (Bio-Rad Laboratories, Hercules, CA) as a standard.

Renal metabolism of AA. The metabolism of AA by CYP450 enzymes was measured by incubating microsomes (0.25 mg protein) with \(^{14}\)C]arachidonic acid (0.2 \(\mu\)Ci/ml, 10 \(\mu\)M, Amersham Life Science, Arlington Heights, IL) in 1 ml of a 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM Mg\(^{2+}\), 1 mM EDTA, 1 mM NADPH, and a NADPH-regenerating system (10 mM isocitrate and 0.4 U/ml isocitrate dehydrogenase) at 37°C for 30 min. The reactions were terminated by acidification to pH 3.5 with 0.1 M formic acid. The metabolites were separated by HPLC on a 2.1 \(\times\) 250-mm C\(_{18}\) reverse-phase column (catalog no. 57935, Sulphelo, Belfonte, PA) using a linear elution gradient ranging from acetonitrile-water-acetic acid (50:50:0.1 vol/vol/vol) to acetonitrile-acetic acid (100:0.1 vol/vol) over a 40-min period. Products were monitored with a radioactive flow detector. The production rate for each metabolite was calculated and expressed as picomoles formed per minute per milligram of protein.

Immunoblot analysis. Proteins were separated by electrophoresis on a 10 \(\times\) 20-cm, 7.5% SDS polyacrylamide gel for 1.5 h at 150 V. The proteins were transferred to a nitrocellulose membrane at 100 V for 1 h at 4°C. The membrane was blocked overnight at 4°C in a buffer (TBST-20) containing 10 mM Tris-HCl, 150 mM NaCl, 0.08% Tween 20, and 10% nonfat dry milk (Bio-Rad). The membrane was incubated for 2 h with a 1:2,000 dilution of a goat polyclonal antibody raised against rat CYP450 4A1 that recognizes other CYP450 4A isoforms (Daichi Pure Chemical, Tokyo, Japan) (20, 21) or a 1:2,000 dilution of a goat polyclonal antibody raised against rat CYP450 4E1 (Daichi Pure Chemical). The membrane was rinsed several times with TBST-20 buffer and then incubated with a 1:4,000 dilution of a horseradish peroxidase-coupled, anti-goat secondary antibody (Santa Cruz Biologlaboratory, Santa Cruz, CA) for 1 h. Blots were washed four times for 5 min in TBST-20 and developed using an enhanced chemiluminescence kit (ECL, Amersham Life Science). The relative intensities of the bands in the 50- to 52-kDa range for CYP450 4A isoforms or at 85 kD for P-450 reductase were determined using a densitometer.

Statistical analysis. Mean values \(\pm\) SE are presented. The significance of difference in mean values was evaluated using an ANOVA and the Duncan’s multiple-range test (37). A P value <0.05 was considered to be significant.

RESULTS

Effects of ACE inhibitors and AT\(_1\)-receptor antagonist on the renal metabolism of AA. The effects of the ACE inhibitors, captopril and enalapril, or the AT1 antagonist, candesartan, on the renal metabolism of AA were studied in microsomes prepared from the renal cortex of rats treated with vehicle, captopril, or enalapril (Fig. 3). In contrast, the levels of CYP450 metabolites of AA are presented in Fig. 1. The production of 20-HETE and EETs by renal cortical microsomes increased by 59 and 24%, respectively, in rats treated with captopril. In rats treated with enalapril, cortical 20-HETE and EETs production rose by 90 and 58%, respectively.

In outer medullary microsomes, the production of 20-HETE increased by 100% in rats treated with captopril and by 143% in rats treated with enalapril. There was no significant production of EETs and dihydroxyecisatrienoic acids by outer medullary microsomes in rats treated with vehicle, captopril, or enalapril. Blockade of AT\(_1\) receptors with candesartan had no significant effect on the production of 20-HETE and EETs by renal cortical or outer medullary microsomes.

To determine if the effects of the ACE inhibitors on increasing production of 20-HETE and EETs are specific to the kidney, the effects of these drugs were also studied in microsomes prepared from the liver. The results of these experiments are presented in Fig. 2. Chronic treatment of the rats with the ACE inhibitors did not significantly alter the production of 20-HETE and EETs in the liver.

Effects of ACE inhibitors on the expression of CYP450 4A and CYP450 reductase proteins. The effect of ACE inhibitors on the expression of CYP450 4A and CYP450 reductase proteins in microsomes prepared from the renal cortex is presented in Figs. 3 and 4. The proteins were transferred to a nitrocellulose micro-
some of rats treated with captopril and by 45% in rats treated with enalapril (Fig. 4).

The effects of the ACE inhibitors on the expression of CYP450 4A and CYP450 reductase proteins in the outer medulla of the kidney are presented in Figs. 5 and 6. The levels of CYP450 4A protein increased significantly by 44% in the outer medulla of rats treated with captopril and by 100% in rats treated with enalapril (Fig. 5). The levels of CYP450 reductase protein rose by 29% in the outer medulla of rats treated with captopril and by 49% in those treated with enalapril (Fig. 6).

**Influence of mineralocorticoids on the renal metabolism of AA.** A comparison of the production of 20-HETE and EETs in the renal cortex and outer medulla of rats fed a normal salt diet that were chronically treated with vehicle, spironolactone, or DOCA is presented in Fig. 7. Chronic treatment of the rats with spironolactone or DOCA had no significant effect on the production of 20-HETE in microsomes prepared from either the renal cortex or outer medulla. However, both drugs increased epoxygenase activity in the renal cortex.

**Effects of a B2-receptor antagonist or an NO synthase inhibitor on the renal response to captopril.** The results of experiments to elucidate a role for bradykinin and NO in mediating the effects of captopril on renal CYP450 4A activity are presented in Fig. 8. Chronic treatment of the rats with HOE-140 or L-NAME alone for 1 wk had no significant effect on the production of 20-HETE in either the renal cortex or outer medulla. Chronic treatment of the rats with captopril increased the formation of 20-HETE in the cortex and outer medulla, and this effect was completely blocked in rats treated with HOE-140 or L-NAME.

**DISCUSSION**

Recent reports that the renal production of 20-HETE falls in rats fed a high-salt diet suggest that a decrease in the intrarenal and/or circulating levels of ANG II may reduce the renal metabolism of AA by CYP450 enzymes (3, 21, 24, 27). To explore this hypothesis further, in the present study we examined the effects of blocking the formation and/or
actions of ANG II on the metabolism of AA in the kidney of rats. The present results indicate that the production of 20-HETE increases in the renal cortex and outer medulla of rats treated with ACE inhibitors, but not in rats treated with an AT1-receptor blocker. These findings suggest that the ability of ACE inhibitors to induce the renal formation of CYP450 metabolites of AA is probably not related to blockade of the formation and/or actions of ANG II. Indeed, the ACE inhibitors and the AT1-receptor blocker should have identical actions to reduce, rather than increase, the formation of 20-HETE if ANG II promotes the expression of CYP450 4A enzymes. Nor is it likely that the effects of the ACE inhibitors are due to the antihypertensive properties of these agents, because we and others have found that ACE inhibitors and AT1-receptor blockers have little or no effect on blood pressure in Sprague-Dawley rats fed a normal salt (1.0% NaCl) diet. They also do not chronically elevate sodium excretion or alter sodium balance. These observations led us to explore alternative hypotheses for the mechanism by
which ACE inhibitors increase the renal formation of 20-HETE.

We first examined whether the effects of the ACE inhibitors might be secondary to a fall in the release or actions of aldosterone. This does not appear to be the case, because chronic administration of deoxycorticosterone or spironolactone had no significant effect on the formation of 20-HETE in the kidney. This finding was unexpected, because the urinary excretion of 20-HETE is elevated in DOCA-salt hypertensive rats (26). The difference may be related to the influence of hypertension on the renal metabolism of AA or the fact that DOCA-salt hypertensive rats are uninephrectomized and fed a high-salt diet, whereas the rats in the present study were maintained on a normal salt diet and were not uninephrectomized.

DOCA and spironolactone did significantly enhance the formation of EETs in the renal cortex. This was somewhat perplexing because DOCA is a mineralocorticoid receptor agonist, whereas spironolactone is a weaker agonist that serves as a receptor blocker. The reason for the similar effects of these agents on the renal metabolism of AA is unknown. It may be related to the fact that both compounds bind equally well to

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**Fig. 5.** Effects of captopril (A) and enalapril (B) on the expression of CYP450 4A protein in microsomes prepared from the renal outer medulla of rats. Lanes 1–4 were loaded with renal microsomes (50 μg) prepared from rats treated with vehicle, lanes 6–9 were loaded with renal microsomes (50 μg) prepared from rats treated with either captopril or enalapril, and lane 5 was loaded with microsomes (25 μg) that were prepared from the liver of a rat and that express all 3 CYP450 4A isoforms. Values are means ± SE from 4 rats. *Significant difference from the corresponding value in rats treated with vehicle.

**Fig. 6.** Effects of captopril (A) and enalapril (B) on the expression of CYP450 reductase protein in microsomes prepared from the renal outer medulla of rats. Lanes 1–4 were loaded with renal microsomes (50 μg) prepared from rats treated with vehicle, lanes 6–9 were loaded with renal microsomes (50 μg) prepared from rats treated with either captopril or enalapril, and lane 5 was loaded with microsomes (25 μg) prepared from the liver of a rat. Values are means ± SE from 4 rats. *Significant difference from the corresponding value in rats treated with vehicle.
the mineralocorticoid receptor or that they both are steroids that have some activity on sex hormone or glucocorticoid receptors.

The present study also explored whether the increase in renal formation of 20-HETE after administration of the ACE inhibitors might be secondary to an increase in the intrarenal levels of kinins and stimulation of the formation of NO. These experiments revealed that chronic treatment of rats with the NO synthase inhibitor, L-NAME, or the B2-receptor blocker, HOE-140, blocked the ability of captopril to increase the formation of 20-HETE in the renal cortex and outer medulla. Because HOE-140 and L-NAME both have been reported to increase blood pressure in rats, it is possible that they altered the renal metabolism of AA by raising blood pressure in the captopril-treated rats. However, neither drug had any effect on the renal formation of 20-HETE or EETs when given alone, so it seems unlikely that elevations in blood pressure per se alter the renal metabolism of AA. Rather, the present results are more consistent with the view that the ACE inhibitors increase the formation of 20-HETE secondary to elevations in the intrarenal levels of kinins that are known to increase formation of NO.

Recent studies demonstrated that NO directly binds to the heme of CYP450 enzymes and inhibits the formation of EETs and 20-HETE in the kidney (2, 27, 39).
Thus it is difficult to understand why chronic exposure of the kidney to elevated levels of NO increases the formation of 20-HETE. The answer is probably related to the recent finding of Sewer et al. (36) that chronic elevations in NO production markedly enhance the expression of CYP450 4A mRNA and protein in the liver and kidney of rats. Because the inhibitory actions of NO on P-450 4A activity are reversible, the upregulation of CYP450 4A protein expression in rats exposed to elevated levels of NO should increase 20-HETE formation measured in vitro, because the assay is performed in the absence of substrate and cofactors needed to make NO. Overall, the results of the present study indicate that there are complex interactions among the kallikrein-kinin system, NO pathway, and the formation of CYP450 metabolites of AA in the kidney. Each of these compounds can alter the production and activity of other systems, and CYP450 metabolites of AA are now known to contribute to some of the actions of both kinins (13, 16) and NO (2, 39) on renal function.

The results of our immunoblot experiments indicate that the ACE inhibitors enhanced formation of 20-HETE in the renal cortex without altering the expression of CYP450 4A protein. Rather, they enhanced the expression of P-450 reductase protein. These findings suggest that the increase in 20-HETE formation after treatment of rats with captopril is probably due to the elevation in P-450 reductase protein. This mechanism is consistent with a recent observation that the formation of 20-HETE by recombinantly expressed CYP450 4A enzymes is highly dependent on the amount of CYP450 reductase protein added to the reaction (40).

The ACE inhibitors also increased the formation of EETs in renal cortical microsomes. Numerous CYP450 isoforms have been identified as renal epoxygenases, including members of the CYP450 2C8, 2C9, 2C11, 2C23, 2C24, and 2F families (31). Unfortunately, at the present time the antibodies to many of these enzymes are not readily available so we were not able to determine whether chronic treatment of rats with ACE inhibitors increases the expression of one or more of these isoforms. Alternatively, it should also be noted that the elevation in epoxygenase activity in the renal cortex could be completely due to the increase in CYP450 reductase protein levels that was seen in the present study.

In the present study, we found that the ACE inhibitors increased the expression of CYP450 4A and P-450 reductase proteins in the renal outer medulla much more than in the cortex. They had no significant effect on the expression of these proteins or the formation of 20-HETE in the liver. This may be due to the lack of B2 receptors in the liver (9) or the fact that NO synthase enzymes are more highly expressed in the thick ascending limb and collecting duct in the renal medulla than in the liver (11). Thus blocking the degradation of kinins with ACE inhibitors would be expected to increase kinin levels and the formation of NO more in the renal medulla of the kidney than in the liver, and this may lead to a greater induction of the expression of CYP450 4A protein.

Previous immunohistochemical studies have indicated that B2 receptors are expressed in proximal straight tubules, TALH, connecting tubules, CCDs, and afferent arterioles (9, 11). However, no staining could be found in the proximal convoluted tubules, glomeruli, or the macula densa cells. We recently demonstrated that the expression of CYP450 4A mRNA and CYP450 4A proteins is highly expressed in many of the same locations as B2 receptors, specifically in the proximal tubules, TALH, glomeruli, macula densa, and renal microvessels (20). 20-HETE and EETs also mediate some of the renal actions of bradykinin. For example, bradykinin increases cytosolic calcium levels via the B2 receptors in the proximal tubule (1), and inhibitors of the formation of 20-HETE have been reported to block the inhibitory actions of bradykinin (13) on sodium transport in the TALH. In addition, EETs released by the endothelium have been reported to contribute to the vasodilator actions of bradykinin on renal arterioles (16). Thus the results of the present study suggest that a positive feedback loop may exist such that chronic blockade of the breakdown of kinins leads to an upregulation of the formation of CYP450 metabolites of AA, and this may contribute to the diuretic and natriuretic actions of kinins in the kidney.

Investigators have yet to determine the potential role that upregulation of CYP450 enzyme expression and changes in the renal metabolism of AA by P-450 enzymes play in mediating the antihypertensive actions of ACE inhibitors. It has long been recognized that ACE inhibitors lower renal vascular resistance and promote sodium excretion, thereby shifting the relationship between sodium excretion and arterial pressure to lower pressures (7, 8). 20-HETE and EETs are natriuretic and inhibit sodium and water transport in the proximal tubules (29-32, 34), TALH (10, 13, 22), and CCDs (14, 33). EETs released by the endothelium also contribute to the vasodilator actions of bradykinin on the afferent arteriole (16). Thus it is possible that upregulation of the formation of CYP450 metabolites of AA may contribute to the antihypertensive actions of ACE inhibitors by inhibiting sodium transport and resetting the pressure-natriuresis relationship to lower levels of arterial pressure.

Perspectives

The results of the present study suggest that ACE inhibitors induce expression of CYP450 4A and P-450 reductase proteins and increase formation of 20-HETE and EETs in the kidney secondary to elevations in the intrarenal levels of kinins and NO. Because 20-HETE and EETs are natriuretic, stimulation of the renal formation of P-450 metabolites of AA may contribute to the natriuretic and antihypertensive properties of ACE inhibitors.

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