Decreased Renal Cytochrome P450 2C Enzymes and Impaired Vasodilation Are Associated With Angiotensin Salt-Sensitive Hypertension

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Abstract—Excess dietary salt intake differentially modulates the activity of cytochrome (CYP) P450 enzymes in kidney cortex. Exactly how increased angiotensin (Ang) II levels and hypertension change the regulatory effect of high salt on CYP450 enzymes remains unclear. The present study investigated the effects of combined administration of Ang II and a high-salt diet on P450 epoxygenase and hydroxylase protein levels in kidney, as well as afferent arteriolar responses to acetylcholine and sodium nitroprusside. High dietary salt administration for 14 days resulted in increased renal cortical CYP2C11 protein levels, and a significant increase of CYP2C11 and CYP2C23 protein levels in renal microvessels. Administration of Ang II in combination with a high-salt diet prevented the upregulation of renal cortical CYP2C11 protein expression observed with high dietary salt alone, and significantly downregulated expression of CYP2C11, CYP2C23, and CYP2J protein in renal microvessels. A high-salt diet alone decreased CYP4A protein in kidney cortex, and renal cortical CYP4A protein level remained at a low level in Ang II–infused rats treated with a high-salt diet. Increases in blood pressure during Ang II infusion were greater in rats fed a high-salt diet. In addition, afferent arteriolar responsiveness to acetylcholine and sodium nitroprusside was significantly attenuated in Ang II–treated rats versus controls. This decrease was significantly enhanced in Ang II–treated rats given a high-salt diet. These results support the hypothesis that an inability to upregulate CYP2C and maintain CYP2J in the rat kidney and impaired afferent arteriolar vasodilation with chronic Ang II infusion contribute to salt-induced elevation of arterial pressure. (Hypertension. 2003;41[part 2]:709-714.)

Key Words: endothelium-derived factors ■ cytochrome P450 ■ epoxygenase ■ hydroxylase ■ kidney ■ microcirculation

Regio- and stereoisomeric epoxyeicosatrienoic acids (EETs) and 20-HETE are the major arachidonic acid products of cytochrome (CYP) P450 monoxygenase metabolism in the kidney. Epoxygenase metabolites, EETs, make important contributions to integrate kidney function by directly affecting tubular transport processes, vascular tone, and cellular proliferation.1–6 We have previously demonstrated that the renal microvessels dilate in response to 11,12-EET and 14,15-EET.7 In contrast, 20-HETE is a potent constrictor of renal arterioles. Furthermore, the action of 11,12-EET on the renal microcirculation, and its ability to activate vascular smooth muscle cell K+ channels, provides convincing evidence that this metabolite is an endothelium-derived hyperpolarizing factor (EDHF).1,6–10 Epoxidation of arachidonic acid has been attributed to members of the CYP2C and CYP2J families. Three CYP2C subfamily isoforms (CYP2C23, CYP2C24, and CYP2C11) are expressed in the rat kidney, with CYP2C23 being the predominate.11 Recent studies showed that CYP2J protein is expressed in rat kidney, and CYP2J protein expression is increased in spontaneously hypertensive rats.12,13 The main product of CYP2C23 isoform is 11,12-EET (57% of the products), although CYP2C11 epoxidizes the 11,12-olefins and 14,15-olefins with nearly equal efficiency.11 CYP2J isoforms preferentially catalyze epoxidation of arachidonic acid at the 14,15-position.12 Hydroxylation of arachidonic acid, to generate 20-HETE, is catalyzed by CYP4A and CYP4F isoforms. Four CYP4A isoforms (CYP4A1, CYP4A2, CYP4A3, and CYP4A8) are expressed in the kidney of rats, and these isoforms are able to catalyze the hydroxylation of fatty acids and produce 20-HETE.14 Salt sensitivity is the causative agent for an important subgroup of humans with essential hypertension.11 Considerable evidence suggests that endothelial dysfunction is linked to end-organ damage in human salt-sensitive hypertension. Studies have revealed that renal CYP450 ω-hydroxylase and epoxygenase activity are differentially modified by sodium chloride.15 High dietary salt downregulates the expression of...
CYP4A protein in the kidney and renal vasculature. In contrast, the expression of CYP2C11 and CYP2C23 and the formation of EETs in the kidney are increased by a high-salt diet. Although studies in Sprague-Dawley rats suggest that a salt-inducible renal epoxygenase has antihypertensive properties, kidney EET production is inappropriately low during the development of salt-sensitive hypertension. There are many CYP450 isoforms, expressed in the kidney, that produce EETs; however, little is known about which of these isoforms are regulated in the renal microvessels and about their involvement in the development of angiotensin salt-sensitive hypertension. The purpose of the present study was to determine if changes in CYP450 enzymes and afferent arteriolar dilation are associated with the salt sensitivity observed in angiotensin II–dependent hypertension.

Methods

Animals
Medical College of Georgia Animal Care and Use Committee approved the experimental procedures. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) weighing 200 to 225 g were divided into 4 experimental groups: The first group received a normal-salt diet (1% NaCl by weight), a second group was fed a high-salt diet (8% NaCl by weight), a third group was subjected to angiotensin II infusion, and the fourth group was treated with angiotensin II infusion and a high-salt diet for 2 weeks before the study. Angiotensin II was infused at a continuous rate via an osmotic pump between 9:00 AM and 12:00 PM.

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 12:00 PM.

Isolation of Renal Microvessels
Renal microvessels were isolated according to a method described previously. Briefly, the kidneys were infused with a physiological salt solution containing 1% Evans blue, and the renal microvessels were separated from the rest of the cortex with the aid of sequential sieving, a digestion period, and collection under a stereomicroscope. Renal microvessels collected from different groups were quickly frozen in liquid N2 and kept at −80°C in a freezer until assayed for protein levels.

Immunoblot Analysis of CYP4A, CYP2C23, CYP2C11, and CYP2J2 Protein
Kidney cortex and renal microvessels were harvested and processed as previously described. Samples were separated by electrophoresis on a 10% stacking Tris-glycine gel, and proteins were transferred electrophoretically to a nitrocellulose membrane. The primary antibodies used were goat anti-rat CYP4A1 polyclonal antibody (1:2000; Gentest), goat anti-rat CYP2C11 polyclonal antibody (1:500; Gentest), rabbit CYP2C23 polyclonal antibody (1:5000; from Dr Capdevila, Vanderbilt University, Nashville, Tenn), and rabbit anti-human CYP2J2 antibody (1:1000; from D.C.Z.). The CYP2J2 antibody was made against purified recombinant human CYP2J2 protein and cross-reacts with all CYP2J isoforms. The blots were then washed in PBS–0.1% Tween-20 and incubated with the secondary antibody (anti-goat 1: 30 000 for CYP4A and CYP2C11; anti-rabbit 1: 100 000 for CYP2C23 and CYP2J2) conjugated to horseradish peroxidase for 1 hour at room temperature and washed. Detection was accomplished by enhanced chemiluminescence Western blotting (ECL, Amersham Corp), and blots were exposed to X-ray film (Hyperfilm-ECL, Amersham Corp). Band intensity was measured densitometrically, and the values were factored for β-actin.

Renal Microvascular Responses
In vitro perfused juxtamedullary nephron preparation has been described previously. Briefly, after pentobarbital anesthesia (50 mg/kg IP) and midline laparotomy, the right renal artery was cannulated through the superior mesenteric artery, and the kidney was immediately perfused with a Tyrode’s solution containing 6% albumin and a mixture of L-amino acids. After the microdissection procedures were completed, the Tyrode’s solution was replaced by reconstituted red blood cell–containing solution, and renal artery perfusion pressure was set to 100 mm Hg. The tissue surface was continuously superfused with a Tyrode’s solution containing 1% albumin. After a 20-minute equilibration period, an afferent arteriole was chosen for study, and baseline diameter was measured. After the control period, the effects of 2 vasodilators, acetylcholine and sodium nitroprusside, were studied.

The afferent arteriole was exposed to increasing concentrations of acetylcholine and diameter changes were monitored for 3 minutes at each concentration. Afferent arteriolar responses to nitroprusside were assessed in the presence of the NO synthase inhibitor Nω-nitro-l-arginine (L-NNa, 100 μmol/L). Diameters were similar in all groups after administration of L-NNa and averaged 19±2 μm in control animals, 17±1 μm in angiotensin II–infused rats, 18±1 μm in high-salt diet–fed animals, and 17±1 μm in angiotensin II–infused animals fed a high-salt diet. L-NNa was administered via the superfusate for 15 minutes before and throughout the nitroprusside dose-response. Steady-state diameter to acetylcholine and nitroprusside was attained by the end of the second minute, and the average diameter at the third minute of each treatment period was used for statistical analysis.

Statistics
All data are presented as the mean±SEM. The significance of differences between groups for the afferent arteriolar diameter data were evaluated with an ANOVA for repeated measures followed by a Duncan multiple range post hoc test. An unpaired 2-tailed t test was applied to compare the CYP450 protein levels. A value of P<0.05 was considered statistically significant.

Results
Consistent with previous reports, systolic blood pressure was significantly increased in angiotensin II–infused rats. At 1 week, the increase in systolic blood pressure was significantly greater in angiotensin II rats given a high-salt diet (180±6 mm Hg, P<0.05, n=12) compared with rats given a normal-salt diet (113±3 mm Hg, n=8), a high-salt diet (117±6 mm Hg, n=8), or angiotensin II infusion (166±5 mm Hg, n=9). Systolic blood pressure remained elevated at 2 weeks in the angiotensin II and high salt group (188±5 mm Hg, n=12) compared with the angiotensin II–infused group (177±6 mm Hg, n=9).

Figure 1 presents representative Western blots and densitometric analysis of CYP2C11, CYP2C23, and CYP2J2 protein in the renal cortex 2 weeks after angiotensin II infusion or a high-salt diet. In rats given a normal-salt diet, angiotensin II administration did not change CYP2C11, CYP2C23, and CYP2J2 protein levels in kidney cortex. A high-salt diet alone increased CYP2C11 protein by 59%, and when angiotensin II treatment was combined with a high-salt diet, CYP2C11 protein was significantly decreased compared with that for a high-salt diet alone. Renal cortical CYP2C23 and CYP2J2 protein levels were similar among the normal-salt, high-salt, angiotensin II, and angiotensin II/high-salt groups.

The levels of CYP2C11, CYP2C23, and CYP2J2 protein in renal microvessels are shown in Figure 2. A high-salt diet significantly increased CYP2C11 and CYP2C23 protein levels in renal microvessels by 154% and 95%, respectively. The
increase in CYP2C23 protein level was prevented, and very little CYP2C11 was detected in renal microvessels in angiotensin II–infused rats given a high-salt diet. CYP2J protein is expressed in renal microvessels. Chronic angiotensin II infusion or a high-salt diet alone did not change CYP2J protein level. The combination of angiotensin II infusion and a high-salt diet significantly decreased CYP2J protein level compared with that for rats given a high-salt diet alone or angiotensin II infusion and normal-salt diet. These data suggest that chronic angiotensin II infusion with high dietary salt downregulated CYP450 epoxygenase levels.

CYP4A protein levels in kidney cortex and renal microvessels were also evaluated to compare the regulatory effects of angiotensin salt-hypertension on CYP450 epoxygenase and ω-hydroxylase enzymes. Figure 3 depicts the representative Western blot bands of CYP4A protein in kidney cortex and renal microvessels. Angiotensin II infusion did not alter renal cortical CYP4A protein expression, but renal cortical CYP4A protein levels were significantly decreased in rats given a high-salt diet (76% versus normal control). On the other hand, chronic angiotensin II infusion combined with a high-salt diet did not increase CYP4A protein level in kidney cortex compared with the level for a high-salt diet (63% versus normal control). In addition, angiotensin II infusion decreased CYP4A protein in renal microvessels, but CYP4A protein was not changed in rats given a high-salt diet with or without angiotensin II infusion.

The responsiveness of the afferent arteriole to acetylcholine and sodium nitroprusside was assessed to further investigate renal microvascular reactivity during angiotensin salt-hypertension. As shown in Figure 4, both acetylcholine and sodium nitroprusside (0.1, 1, and 10 μmol/L) produced a dose-dependent afferent arteriolar vasodilation. Afferent arteriolar responses to acetylcholine (10 μmol/L) and sodium nitroprusside (10 μmol/L) were significantly attenuated in the angiotensin II groups (15±2 and 20±3%, respectively) versus controls (26±4 and 30±3%, respectively), and this decrease was significantly greater in angiotensin II rats given a high-salt diet (7±1 and 13±1%, respectively) compared with the decrease observed in rats given only angiotensin II infusion. These data demonstrate that angiotensin II infusion combined with a high-salt diet severely impairs afferent arteriolar dilator responses in angiotensin II hypertension.
The development of hypertension after the long-term administration of initially subpressor doses of angiotensin II has many of the same renal and vascular changes that are associated with human essential hypertension. Likewise, angiotensin II–infused hypertensive rats demonstrate a further elevation in blood pressure when fed a high-salt diet, or "salt sensitivity." In the present study, the combination of a high-salt diet and angiotensin II infusion resulted in a greater increase in systolic blood pressure, which confirms salt sensitivity of angiotensin II–induced hypertension reported previously using radiotelemetry to measure arterial blood pressure. It is generally accepted that excess dietary salt intake differentially modulates the activity of CYP450 enzymes in kidney. Precisely how increased circulating angiotensin II levels change the regulatory effect of a high-salt diet on CYP450 enzymes remains unclear. Therefore, we performed experiments to determine the regulatory effects of combined administration of angiotensin II and high-salt diet on CYP450 epoxygenase and \( \omega \)-hydroxylase protein expression in the kidney. In this study, we found that a high-salt diet significantly increased CYP450 epoxygenase protein expression in renal microvessels, and angiotensin II infusion combined with a high-salt diet prevented the increase of CYP2C and reduced CYP2J protein levels in renal vasculature. Consistent with previous reports, a high-salt diet decreased CYP4A protein expression in kidney cortex. But, chronic angiotensin II infusion with a high-salt diet did not increase renal cortical CYP4A protein levels. Furthermore, impairment of endothelium-dependent and endothelium-independent dilations observed in angiotensin II–infused rats was greater in kidneys from rats given a combination of a high-salt diet and angiotensin II infusion.

In the present study, we confirmed previous observations that high salt stimulates CYP2C epoxygenase enzymes. CYP2C isoforms are considered the major renal arachidonic acid epoxygenases in the kidney. In particular, CYP2C23 is highly expressed in rat kidney, where it exerts an important role in regulating EET formation. The CYP2C11 isoform is expressed in the rat kidney at much lower levels than is CYP2C23. We have previously reported that angiotensin II infusion did not change microsomal CYP2C11 and CYP2C23 protein levels in kidney cortex. In the current study, dietary salt administration...
for 14 days did not significantly increase CYP2C11 and CYP2C23 protein in renal cortex, but CYP2C11 and CYP2C23 protein levels were significantly increased in renal microvessels isolated from high salt–treated rats. Furthermore, this increase in CYP2C11 and CYP2C23 protein levels was not present in chronically angiotensin II–infused animals fed a high-salt diet. Therefore, an inability to increase CYP2C protein level may contribute to the salt sensitivity of angiotensin-dependent hypertension.

CYP2J subfamily also contributes to the formation of EETs in human and animal 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. In addition, CYP2J3 biosynthesizes 19-HETE, which has been shown to inhibit voltage-gated calcium channels and is a potent vasodilator. Recent studies have shown that CYP2J immunoreactive protein is increased in the spontaneously hypertensive rat kidney and is associated with increased EET formation both in vitro and in vivo. Thus, several lines of evidence suggest that CYP2J protein plays a role in arachidonic acid CYP450 metabolism. In the present study, we found that renal cortical CYP2J protein level was similar among the normal-salt, high-salt, angiotensin II, and angiotensin II/high-salt groups. Our results are consistent with the report that CYP2J expression does not change with high-salt diet in rats.

In addition, one of the novel aspects of the report is the identification of CYP2J protein expression in renal microvessels. Findings demonstrate that CYP2J was expressed in renal microvessels, and a high-salt diet alone or long-term angiotensin II infusion did not change the CYP2J protein level in renal microvessels. Interestingly, the combination of angiotensin II infusion and a high-salt diet significantly decreased the renal microvascular CYP2J protein level. This finding suggests that renal microvascular CYP2J regulation may play an important role in the development of angiotensin II salt-sensitive hypertension.

In the present study, we confirmed previous observations that CYP4A protein expression is downregulated in the kidney of rats fed a high-salt diet. The combination of angiotensin II infusion and a high-salt diet did not increase renal cortical CYP4A protein levels compared with a high-salt diet alone. This finding is not consistent with a previous report that the downregulation of CYP4A can be prevented if circulating angiotensin II is maintained at normal levels by intravenous infusion. These apparent contradictory results can be explained by the differences in circulating angiotensin II levels and arterial blood pressure. Circulating angiotensin II levels are elevated 2- to 3-fold above normal, and hypertension developed in the current study. Nevertheless, angiotensin II infusion did not change renal cortical CYP4A level, and CYP4A protein in renal microvessels in angiotensin II–infused rats with or without a high-salt diet.

Previous studies show that the effects of salt loading on responses to acetylcholine are not consistent in peripheral vascular beds. Lenda et al reported that salt loading depressed acetylcholine-induced vasodilation of pial vessel, but Sifola et al reported no difference in the acetylcholine responses in perfused mesenteric vessels. Unlike peripheral vascular beds, the renal vasculature maintains dilator responses, and the epithelium responds with changes in tubular transport that favor sodium excretion. We observed afferent arteriolar vasodilations to acetylcholine and sodium nitroprusside that were similar in kidneys from high-salt and normal-salt rats. An unaltered afferent arteriolar responsiveness to vasodilator in response to a high-salt diet would not hamper the ability of the kidney to excrete sodium. Angiotensin II infusion and the resulting hypertension attenuated afferent arteriolar responses to acetylcholine and sodium nitroprusside. Furthermore, this impairment in afferent arteriolar vasodilation to acetylcholine and sodium nitroprusside was greater in angiotensin II–infused rats fed a high-salt diet.

Acetylcholine causes the relaxation of arteries through the release of several factors, including EDHF. In rats, an epoxygenase-derived EDHF mediates a large portion of the afferent arteriolar response to acetylcholine and bradykinin. Evidence suggests an increased role of EDHF in the relaxation that occurs in response to acetylcholine in animals fed a high-salt diet. In mesenteric vessels, the residual dilatation after Nω-nitro-L-arginine methyl ester in the low-salt rats and the much larger dilatation in high-salt animals were completely abolished by apamin and charybdotoxin. Thus, increasing EDHF is part of the normal physiological response to excess dietary sodium. Actions of 11,12-EET on the renal microcirculation and its ability to activate vascular smooth muscle cell K+ channels provides convincing evidence that this metabolite is an EDHF. Increased CYP450 epoxygenase protein level in kidneys, especially in renal microvessels, confirms the importance of EDHF in the maintenance of vasodilation to acetylcholine in salt-loaded animals. The impairment of afferent arteriolar responses to nitroprusside in angiotensin II–induced hypertension suggests alterations in vascular smooth muscle function. This finding is in agreement with previous studies demonstrating impaired NO/cyclic guanosine monophosphate signaling in spontaneously hypertensive rats fed a high-salt diet and the increase of the NO synthase inactivator Nω,Nω-dimethyl-arginine in the kidney of angiotensin II–infused rats fed a high-salt diet. Clearly, further investigation is required to delineate the role of EETs in the depressed vascular response to acetylcholine and sodium nitroprusside that develops in angiotensin II salt-sensitive hypertension.

**Perspectives**

The current study demonstrates that kidney CYP450 epoxygenase protein levels are elevated in salt-loaded animals, and this increase in CYP450 epoxygenase enzyme is abolished in angiotensin II–infused animals fed a high-salt diet. The combination of a high-salt diet and angiotensin II infusion resulted in a greater impairment in afferent arteriolar responses to acetylcholine and sodium nitroprusside. Although the involvement of epoxygenase enzymes in human salt-sensitive hypertension has not been characterized, these studies support the postulate that EET regulation is associated with the endothelial dysfunction observed in human salt-sensitive hypertension. Overall, the current report suggests...
that the inability to upregulate CYP2C and maintain CYP2J in rat renal microvessels, and impaired afferent arteriolar dilation in chronic angiotensin II infusion, may contribute to the salt-induced elevation in arterial pressure.

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