Salt-sensitive hypertension is associated with dysfunctional Cyp4a10 gene and kidney epithelial sodium channel

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Introduction

Prevalence, complexity, and multiple medical and socioeconomic consequences make hypertension a major health challenge for most of the Western world (1). While environmental factors and coexisting conditions play a role in the development and progression of hypertension, segregation and linkage analyses indicate that multiple genetic factors contribute to its complex etiology (2–7). Furthermore, clinical studies show that the cardiovascular and renal morbidity and mortality resulting from hypertension are markedly reduced by timely diagnosis and early clinical intervention (1). As the kidneys play a central role in the control of body salt and fluid balance, they are frequent targets for the treatment of hypertension, especially those forms sensitive to dietary salt (2–5). However, since the molecular basis of prevalent forms of the disease remains uncertain, its early diagnosis and treatment are largely symptomatic. It is expected that the identification of novel pathways/genes involved in blood pressure variations (3, 6, 7) will lead to new therapeutic targets and to improved diagnosis and treatment. Indeed, early detection and treatment are urgently needed to prevent the dangerous and profound consequences of untreated chronic hypertension.

The metabolism of endogenous arachidonic acid (AA) to epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE) by the epoxygenase and ω-hydroxylase branches of the cytochrome P450 (P450) AA monooxygenase is well established (8, 9). It is expected that studies could lead to new strategies for the early diagnosis and clinical management of this devastating disease.

Functional and biochemical data have suggested a role for the cytochrome P450 arachidonate monooxygenases in the pathophysiology of hypertension, a leading cause of cardiovascular, cerebral, and renal morbidity and mortality. We show here that disruption of the murine cytochrome P450, family 4, subfamily a, polypeptide 10 (Cyp4a10) gene causes a type of hypertension that is, like most human hypertension, dietary salt sensitive. Cyp4a10−/− mice fed low-salt diets were normotensive but became hypertensive when fed normal or high-salt diets. Hypertensive Cyp4a10−/− mice had a dysfunctional kidney epithelial sodium channel and became normotensive when administered amiloride, a selective inhibitor of this sodium channel. These studies (1) establish a physiological role for the arachidonate monooxygenases in renal sodium reabsorption and blood pressure regulation (2), demonstrate that a dysfunctional Cyp4a10 gene causes alterations in the gating activity of the kidney epithelial sodium channel, and (c) identify a conceptually novel approach for studies of the molecular basis of human hypertension.

Results

Disruption of the Cyp4a10 gene causes hypertension. Mating and selection of germ line chimeras carrying a mutant Cyp4a10 allele were used to generate homozygous Cyp4a10−/− and Cyp4a10+/− mice (from the progeny of an F2 cross of Cyp4a10−/− heterozygous mice) in isogenic 129/SvJ backgrounds. Cyp4a10−/− mice developed normally and lacked outward symptoms of disease or organ malfunction. Light microscopy analysis of paraffin-embedded kidney sections and analysis of renal function by measurement of plasma creatinine levels by HPLC (14) demonstrated that disruption of the Cyp4a10 gene had little or no effect on organ morphology (not shown) or renal function before or after animal salt loading (157 ± 7 and 168 ± 10; 161 ± 6 and 169 ± 9 μg

Nonstandard abbreviations used: AA, arachidonic acid; CD, collecting duct; Cyp4a10, cytochrome P450, family 4, subfamily a, polypeptide 10; DHET, dihydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; ENaC, epithelial sodium channel; Kiyoshi Nakagawa, Department of Medicine, Vanderbilt University, Nashville, Tennessee, USA. 2Department of Pharmacology, New York Medical College, Valhalla, New York, USA. 3Department of Biochemistry, Vanderbilt University, Nashville, Tennessee, USA. 4Veterans Affairs Hospital, Nashville, Tennessee, USA. 5Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas, USA.
Cyp4a10+/+ in the amount of sodium (43% ± 15%; 44% ± 7%; and 64% ± 5% of wild mice on low-salt, normal, and high-salt diets, respectively; < 0.001, significantly different from wild type.

Measurements of systolic blood pressure in adult Cyp4a10+/+ and Cyp4a10−/− mice provided decisive evidence of a role for Cyp4a10 in blood pressure control (Table 1). Compared with wild-type mice, male and female Cyp4a10−/− mice showed significantly increased systolic blood pressure when measured by either the tail cuff method (Table 1) or a carotid artery catheter (15) (average increases of 27 and 22 mmHg for the systolic blood pressure of male and female mice, respectively; n = 11; SEM ± 10% of the mean). Male and female Cyp4a10−/− mice were equally hypertensive (Table 1), and their plasma androgen levels and renal 20-HETE synthase activity were comparable to those of normotensive Cyp4a10+/+ mice (15).

Hypertension in Cyp4a10−/− mice is dietary salt sensitive. To explore the mechanism of the hypertensive phenotype, male Cyp4a10+/+ and Cyp4a10−/− mice were fed diets containing (w/w) either 0.05% (low salt), 0.3% (normal salt), or 8% NaCl (high salt) and, after 3 weeks, their systolic blood pressure, urine volume, and urine sodium and potassium concentrations were measured. While dietary salt had no significant effects on the blood pressure of wild-type animals (Figure 1A), Cyp4a10−/− mice on normal or high-salt diets were severely hypertensive and became normotensive when fed low-salt diets (Figure 1A). These salt-induced pressure changes were accompanied by reductions in urinary output during 16-hour collection periods (Figure 1B) and thus in the amount of sodium (43% ± 15%; 44% ± 7%; and 64% ± 5% of wild-type mice on low-salt, normal, and high-salt diets, respectively; n ≥ 14) and potassium (57% ± 0.6%; 52% ± 0.4%; and 79% ± 6% of wild-type mice on low-salt, normal, and high-salt diets, respectively; n ≥ 14) excreted during this time period. Furthermore, nuclear magnetic resonance measurements (16) showed that free fluid volumes in Cyp4a10−/− mice fed normal salt were higher than those of matched Cyp4a10+/+ controls (2.0% ± 0.1% and 1.5% ± 0.1% of body weight, respectively; wild type versus knockout, P < 0.002, n = 8). Taken together, these results show that a dysfunctional Cyp4a10 gene causes reduced sodium and potassium excretion, fluid retention, and hypertension.

Disruption of the Cyp4a10 gene does not alter renal 20-HETE synthase activity. Pro-and antihypertensive roles have been described for 20-HETE and its CYP4A2 synthase (9–11), and 20-HETE has been identified as an androgen-regulated prohypertensive 20-HETE synthase (15). Among the murine Cyp4a proteins (15, 17), only Cyp4a12 showed significant 20-HETE synthase activity (turnover: 6.7 ± 0.6, 1.0 ± 0.3, and ≤ 0.01 min⁻¹ for Cyp4a12, -a410, and -a414, respectively; n ≥ 6). Northern blots and enzymatic assays showed that disruption of the Cyp4a10 gene had only limited effects on kidney Cyp4a12 expression (not shown) or 20-HETE synthase activity (59 ± 3 and 54 ± 2 pmol/min/mg of microsomal protein for Cyp4a10+/+ and Cyp4a10−/− mice, respectively). These results indicated that hypertension in Cyp4a10−/− mice was unrelated to changes in kidney 20-HETE biosynthesis and its effects on renal hemodynamics (15).

Cyp4a10−/− hypertension is amiloride sensitive. Within the P450-derived metabolites of AA, the EETs have attracted special attention because of their multiple vasoactive, transport, and antihypertensive properties (8–11). Thus, for example, earlier studies demonstrated that renal EET biosynthesis was sensitive to dietary salt intake (18), that inhibition of renal epoxygenase caused salt-sensitive hypertension (19), and that hypertensive Dahl salt-sensitive rats showed reductions in kidney epoxygenase activity and urinary EET excretion (19). Moreover, studies with isolated rabbit collecting ducts (CDs) suggested a role for the EETs and the P450 epoxygenases in distal sodium excretion (8, 9, 20) and pointed to ENaC as their molecular target (21). Amiloride and its analogs promote sodium excretion and potassium sparing by selective inhibition of ENaC (22). To determine the contribution of ENaC dysfunction to the salt-sensitive Cyp4a10−/− hypertensive phenotype, we monitored the blood pressure of Cyp4a10+/+ and Cyp4a10−/− mice on normal salt diets daily, before and after the sequential administration in the drinking water of either amiloride, amiloride in 2% NaCl, or 2% NaCl alone. Within days of administration, amiloride normalized the blood pressure of hypertensive Cyp4a10−/− mice to levels comparable to those of wild-type mice (Figure 2). Cyp4a10−/− mice on amiloride remained normotensive, even when administered 2% salt water (Figure 2). Importantly, the effects of amiloride on Cyp4a10−/− mice were reversible since, upon its removal, the hypertensive phenotype of the Cyp4a10−/− animals was fully restored (Figure 2). In contrast, amiloride alone or in combination with 2% NaCl caused minor changes in the blood pressure of Cyp4a10−/− mice (Figure 2). These results indicate that ENaC dysfunction accounts for the Cyp4a10−/− hypertensive phenotype and suggest that products of the P450 AA monoxygenases could participate in the regulation of ENaC gating activity.

### Table 1
Male and female Cyp4a10−/− mice are hypertensive

<table>
<thead>
<tr>
<th></th>
<th>Cyp4a10+/+</th>
<th>Cyp4a10−/−</th>
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<tbody>
<tr>
<td><strong>Females</strong></td>
<td>103 ± 3</td>
<td>147 ± 3</td>
</tr>
<tr>
<td><strong>Males</strong></td>
<td>105 ± 2</td>
<td>151 ± 3</td>
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The systolic blood pressure (in mmHg) of conscious 12- to 14-week-old male and female mice fed solid diets containing 0.3% NaCl (w/w) was measured by the tail cuff method. Shown are averages ± SEM calculated from groups of 15 Cyp4a10−/− males, 10 Cyp4a10−/− females, 16 Cyp4a10−/− males, and 10 Cyp4a10−/− female mice. *P < 0.001, significantly different from wild type.

![Figure 1](https://example.com/figure1.png)

**Figure 1**
Hypertension in Cyp4a10−/− mice is salt sensitive. Groups of male Cyp4a10−/− (WT) (white bars) and Cyp4a10−/− (KO) (black bars) mice were fed low-salt, normal, or high-salt diets (containing 0.05, 0.3, or 8% NaCl w/w), respectively. After 3 weeks, their systolic blood pressure (in mmHg) (A) and their urine output during 16-hour collections (B) were measured. Values are averages ± SEM calculated from at least 14 (low salt), 15 (normal salt), and 24 (high salt) mice. *P < 0.05; **P < 0.001, significantly different from wild type. The blood pressure of Cyp4a10−/− and Cyp4a10−/− mice on low-salt diets was not significantly different.
Cy4a10<sup>−/−</sup> hypertension in mice is associated with changes in ENaC activity. A recent report (21) provided an explanation for the reported transport effects of the EETs in the distal nephron (8–11) by showing that in rat cortical CDs, (a) AA and 11,12-EET, an epoxygenase metabolite, inhibit ENaC activity; (b) AA-elicited inhibition of ENaC requires its conversion to EETs; (c) 11,12-EET is formed by CYP2C23 epoxygenase is present in this segment; and (d) ENaC inactivation by 11,12-EET is unique since indomethacin, a cyclooxygenase inhibitor, had no effect on the AA-induced responses, nor did 20-HETE mimic the EET response. We therefore studied ENaC requires its conversion to EETs; (c) 11,12-EET is formed by CYP2C23 epoxygenase is present in this segment; and (d) ENaC inactivation by 11,12-EET is unique since indomethacin, a cyclooxygenase inhibitor, had no effect on the AA-induced responses, nor did 20-HETE mimic the EET response. We therefore studied the effects of AA and of 11,12-EET on the ENaC activity of cortical CDs from Cy4a10<sup>−/−</sup> and Cy4a10<sup>+/+</sup> mice. Under control conditions, we observed significant differences in ENaC basal activity of human hypertension in which mutations in the Na<sup>+</sup>-coupled subunit of ENaC (ENaCα) are in the closed state are indicated by C. Short bars indicate that the CYP2C epoxygenase in regulating murine ENaC activity was indicated by the demonstration that 11,12-EET inhibits the channel, regardless of Cy4a10 genotype (Figure 3). In summary, the results shown in Figure 3 provide a mechanistic explanation for the salt-sensitive nature of the Cy4a10<sup>−/−</sup> hypertensive phenotype, extending the roles postulated for 11,12-EET in regulating sodi

\[ \text{Enalide}\]

Effects of AA and 11,12-EET on the ENaC activity of Cyp4a10<sup>−/−</sup> and Cyp4a10<sup>+/+</sup> mice. (A and B) ENaC sodium currents were determined using CD cell attached patches (21). Inward sodium currents when ENaCs are in the closed state are indicated by C. Short bars indicate channel current levels and dotted lines the current levels for a fully closed channel. (C) Normalized ENaC activities under control conditions (a) or in the presence of either 10 μM AA (b), 200 nM 11,12-EET (c), or a combination of AA and the epoxygenase inhibitor MS-PPOH [N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide] (21) (10 and 15 μM, respectively) (d). Values (as percentage of control activity) are averages ± SEM calculated from at least 5 different experiments. *P < 0.01, significantly different from controls.

Cy4a10<sup>−/−</sup> heterozygous in mice excreted into their urine during a 16-hour collection period (5.1 ± 0.7 and 3.2 ± 0.6 ng for wild-type and knockout mice, respectively; n = 4). These results, qualitatively similar to what has been reported for hypertensive Dahl salt-sensitive rats (19), suggested that a reduced renal EET biosynthetic capacity could be responsible for the ENaC dysfunction observed in hypertensive Cy4a10<sup>−/−</sup> mice (Figure 3).

It is now well established that the AA epoxygenases belong to the cytochrome P450 2 gene family (8) and that members of the Cyp2c and Cyp2j subfamily are expressed in the mouse kidney (25–27).

Enalide normalizes the blood pressure of hypertensive Cyp4a10<sup>−/−</sup> mice. The systolic blood pressure of male Cyp4a10<sup>−/−</sup> (discontinuous line) and Cyp4a10<sup>+/+</sup> (continuous line) mice (n = 5 each) on diets containing 0.3% NaCl (w/w) were monitored before (days 1 to 4) and after the sequential administration of drinking water containing amiloride (days 8 to 21), a mixture of amiloride and 2% NaCl (days 22 to 28), and finally 2% NaCl alone (days 29 to 34). *P < 0.001 Cyp4a10<sup>−/−</sup> versus amiloride-treated Cyp4a10<sup>−/−</sup> mice. **P < 0.001 Cyp4a10<sup>−/−</sup> versus Cyp4a10<sup>+/+</sup> mice for all additional amiloride treatment regimes. *P < 0.001 Cyp4a10<sup>−/−</sup> mice on water versus Cyp4a10<sup>−/−</sup> mice on 2% salt water.

study of the effects of dietary salt loading on kidney Cyp2c44 expression by Northern blot or real-time PCR quantification indicated that, while Cyp2c44 was the predominant Cyp2c form expressed in mouse kidney (not shown), salt loading caused variable and limited changes in its expression levels in the kidneys of Cyp4a10+/+ or Cyp4a10−/− mice. Furthermore, an extended Northern blot and/or real-time PCR study of the effects of salt loading on the levels of Cyp2c29, Cyp2c38, Cyp2c40, Cyp2j5, and Cyp2j6 transcripts, all identified as potential renal AA epoxygenases (25–27), yielded similar results. To determine whether the experimental manipulation of kidney Cyp4a10+/− expression and EET biosynthesis normalizes the blood pressure of Cyp4a10−/− mice and thus whether reductions in CD epoxygenase activity could be responsible for the hypertensive phenotype, Cyp4a10+/+ and Cyp4a10−/− mice were administered Wyeth 14643 (Wy), a selective PPARα ligand. Since Wy treatment raised the concentrations of 11,12-EET (Figure 3) and caused small but significant decreases in the animals’ blood pressure (Figure 4B), on the other hand, the PPARα ligand effectively normalized the blood pressure of hypertensive Cyp4a10−/− mice (Figure 4B) and raised their urinary excretion of epoxygenase metabolites and kidney Cyp2c44 mRNA levels to levels similar to or higher than those present in wild-type mice (Table 2) (Figure 4, A and C). This association between an increase in renal Cyp2c44 expression and activity and a reduction in systemic blood pressure supports the antihypertensive functions proposed for the EETs (8–11) and points to the EETs and the AA epoxygenase as endogenous regulators of ENaC activity (20, 21). The mechanism(s) by which the Wy regulates renal Cyp2c44 epoxygenase expression reported regioselectivity of the Cyp2c44 epoxygenase (27), treatment of Cyp4a10−/− mice with Wy also increased the urine levels of the 8,9- and 14,15-AA epoxygenases (2.3- and 1.6-fold increases, respectively) (Table 2). Finally, the levels of epoxygenase metabolites present in the urine provide an averaging measure of overall renal EET biosynthesis and are of limited value in predicting the EET biosynthetic capacity of individual nephron segments. However, studies in dissected rat kidney CDs have demonstrated EET biosynthesis and CYP2C23 epoxygenase expression in this segment (21). The detection sensitivity of current mass spectrometric methods precludes the analysis of EET biosynthesis in mouse nephron segments such as the CD, where ENaC activity is regulated by 11,12-EET (Figure 3). In parallel with the increases in epoxygenase metabolites shown in Table 2, Wy treatment raised the concentrations of Cyp2c44 mRNA transcripts present in the Cyp4a10−/− kidney (Figure 4A and C) and caused small but significant decreases in the animals’ blood pressure (Figure 4B). To determine whether the experimental manipulation of kidney Cyp2c44 epoxygenase levels and EET biosynthesis normalizes the blood pressure of Cyp4a10−/− mice and thus whether reductions in CD epoxygenase activity could be responsible for the hypertensive phenotype, Cyp4a10+/+ and Cyp4a10−/− mice were administered Wyeth 14643 (Wy), a selective PPARα ligand. 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### Table 2

Wy increases the urinary excretion of epoxygenase metabolites

<table>
<thead>
<tr>
<th>Urine source</th>
<th>Treatment</th>
<th>8,9-</th>
<th>11,12-</th>
<th>14,15-</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp4a10−/−</td>
<td>None</td>
<td>0.42±0.03</td>
<td>0.97±0.10</td>
<td>2.43</td>
<td></td>
</tr>
<tr>
<td>Cyp4a10−/−</td>
<td>Wy</td>
<td>1.30±0.13</td>
<td>3.31</td>
<td></td>
<td></td>
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Groups of adult Cyp4a10−/− and Cyp4a10−/− male mice were administered water or a solution containing Wy (0.02% w/v) as drinking water for 7–9 days. Urine collected from groups of 4–5 animals was pooled and mixed with synthetic [H2]−labeled 8,9-, 11,12-, and 14,15-EET (5 ng each) and [H2]−labeled 8,9-, 11,12-, and 14,15-DHET (5 ng each) as internal standards. EETs and DHETs present in the urine samples were extracted, purified, and quantified by LC/MS/MS as described in Methods. Values are averages ± SEM calculated from at least 4 different experiments. *P < 0.05; **P < 0.01, significantly different from untreated controls. No significant differences in levels of urinary 8,9- and 14,15-epoxygenase metabolites were observed between control and Wy-treated Cyp4a10−/− mice.

Figure 4

Wy normalizes blood pressure and Cyp2c44 expression in Cyp4a10−/− mice. Male Cyp4a10−/− (WT) and Cyp4a10−/− (KO) mice were administered water (white bars) or a 0.02% solution of Wy (black bars) as drinking water, and after 10 days, systolic pressure and kidney Cyp2c44 expression were measured. (A) Real-time PCR quantification of Cyp2c44 mRNA levels normalized using the β-actin mRNA as reference. Shown are averages ± SEM calculated from 6 experiments. *P < 0.005; **P < 0.003, significantly different from untreated controls. (B) Systolic blood pressure (in mmHg). Values are averages from groups of 5 mice each. #P < 0.01, significantly different from untreated knockout; #P < 0.01, significantly different from untreated wild type. (C) mRNAs from control (CT) and Wy-treated (Wy) wild-type and knockout (KO) mouse kidneys were analyzed by Northern blot using a Cyp2c44-specific probe. Loadings were normalized using a β-actin probe.
The CD contributes to the fine tuning of plasma sodium concentrations, and sodium reabsorption by the CD ENaC is a rate-limiting step for this physiologically vital function (2, 3, 23, 36–38). Disorders in which gain-of-function mutations in ENaC cause severe hypertension have illustrated the importance of this channel in the regulation of human blood pressure (2, 3, 23, 36). Like most ion channels, ENaC function can be regulated by changes in gating properties or membrane density, and the role of aldosterone in regulating apical channel expression in the CD is well established (2, 3, 23, 36–38). Nonetheless, the early responses to aldosterone occur prior to detectable changes in ENaC mRNA or protein levels and involve proteins thought to be responsible for ENaC trafficking and/or activity, including a serum- and glucocorticoid-inducible kinase (SGK), Nedd4-2, K-ras2, and channel-activating protease 1 (CAP-1) (36–40). Other than these hormone-mediated changes, little is known regarding factors governing intrinsic ENaC gating activity and sodium currents except that an inositol phospholipid–binding regulatory region in the γ subunit of ENaC was recently identified and characterized (41). The nearly instantaneous nature of the EET effects on ENaC activity (Figure 3, A and B) suggests a role for these eicosanoids in the real-time regulation of this channel and explains their reported effects on sodium transport (20, 21).

The phenotypic characterization of Cyp4a10−/− mice and that reported for Cyp4a14−/− mice (15) illustrate the key roles that these 2 genes have in the regulation of tubular function (Cyp4a10) and renal hemodynamics (Cyp4a14) (14) and provide a molecular basis for future studies of the pro- and antihypertensive effects associated with 20-HETE and EETs (9–11), respectively. It is of particular interest that both murine Cyp4a genes exert these renal and pressure effects by controlling the transcriptional activation of alternate P450 genes coding for either 20-HETE or EET synthases.

Based on the results discussed, we propose that under conditions of balanced salt intake and excretion, locally generated EETs participate in nonstimulated ENaC gating and the regulation of distal sodium reabsorption. Dietary-induced increases in plasma sodium cause compensatory volume changes and rapid and delayed epoxygenase-mediated responses (Figure 5). The rapid phase involves phospholipase-dependent AA release and metabolism to EETs. The delayed phase requires an upregulated AA epoxygenase expression and increased EET synthase activity in the CDs (Figure 5). Augmented EET levels cause an EET-mediated inactivation of ENaC, a reduction in inward sodium transport, and increased sodium excretion (Figure 5). Reductions in epoxygenase expression or activity increase ENaC-dependent sodium reabsorption and, to maintain plasma sodium levels within physiologically compatible levels, there is increased water retention and expansion of the plasma volume. These epoxygenase-mediated increases in ENaC-dependent sodium reabsorption and the attendant changes in plasma volume result in increased systemic blood pressure (Figure 5) and, ultimately, hypertension. The proposal in Figure 5 is consistent with the normotensive effects of amiloride and Wy on Cyp4a10−/− mice as well as the ENaC normalizing effect that 11,12-EET has on the CDs of these animals.

The demonstrations of altered tubular transport in salt-sensitive hypertensive Cyp4a10−/− mice offer new insights into the mechanisms by which the kidney regulates sodium excretion and suggest that interventions resulting in increased kidney epoxygenase expression (32) or EET concentrations (42, 43) could serve as the basis for the development of future antihypertensive therapies. Since an important subset of human hypertensives are salt sensitive (1–5) and this has been associated with alterations in sodium excretion (2–5), we
propose the human homologues of Cyp4a10 as candidate genes for studies of their role in hypertension. Recent clinical data indicating a role for 20-HETE in human salt-sensitive hypertension (44) and the identification of an association between a human CYP4A11 gene variant and essential hypertension (45, 46) lend further support to this proposal. Finally, synthetic PPARα ligands have been utilized as hypolipidemic agents with limited side effects. Based on the studies reported here and elsewhere (32–34), the evaluation of their potential anti hypertensive effects in humans seems warranted.

**Methods**

cDNA cloning, expression, and enzymatic analysis. The Cyp4a10, Aa12, Aa14 and 2c4+ Aa16 cDNAs were cloned and expressed in *E. coli* (14, 16, 47) (the Cyp4a10 gene sequence is available at www.ncbi.nih.gov; MGI:88611, 13117). Northern blot analyses of RNAs isolated from the kidneys of Cyp4a10+/- mice revealed the presence of transcripts recognized by the Cyp4a10 probe and that, compared with the Cyp4a10 mRNA, they contained several single nucleotide replacements, a 429 bp intronic insertion, and a 17 bp deletion at nucleotide 1255 (from the ΔTG translation initiator) that causes a frame shift and would generate a protein lacking a helix protrusive protein.

mRNA expression levels were estimated by Northern blot (15) or real-time quantitative PCR using a Bio-Rad iCycler iQ instrument (Bio-Rad) and the manufacturer’s reagents and software (Gene Expression Analysis for iCycler iQ Real Time PCR Detection System). Total RNAs were isolated from whole mouse kidneys using the TRIzol Reagent (Invitrogen Corp.) and, in some experiments, purified by oligo(dT) chromatography. For real-time quantitative PCR, kidney reverse transcriptase products were amplified using the following primer pairs: Cyp2b9, 2b10, and 2b19; 5′-TCGTTCCAGCAGACTATTGGA-3′ and 5′-CTTCTTTCCACTGGACACC-3′ and reverse primers (5′-CTTGAGGTTACGCTCTTGTT-3′) and sequence analysis showed that these RNA transcripts originated from a Cyp4a10 pseudogene and that, compared with the Cyp4a10 mRNA, they contained several single nucleotide replacements, a 429 bp intronic insertion, and a 17 bp deletion at nucleotide 1255 (from the ΔTG translation initiator) that causes a frame shift and would generate a protein lacking a helix protrusive protein.

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**Blood pressure measurements.** Systolic blood pressure of conscious 12- to 20-week-old mice was measured at an ambient temperature of 23°C using a tail cuff blood pressure analyzer (model 178, IITC Inc.). Prior to measurement, animals were exposed to the environment and instrument on a daily basis for at least 7 days. The blood pressure of trained mice was monitored for 30–40 minutes and final values obtained after 4 consecutive readings whose values were within ±5% of the mean. In some experiments, blood pressure was measured by means of a Micro-Renthane tapered catheter (300–500 μm OD) inserted into the right carotid artery as described (15). After phenotype confirmation with the Cyp4a10-/- ID12 clone, all subsequent studies were done using the Cyp4a10-/- and Cyp4a10+/- mice (at least 8 animals each), using a Bruker Minispec mq10 (Bruker BioSpin Corp.) nuclear magnetic resonance instrument. Urinary sodium and potassium were measured by flame photometry.

**Analysis and quantitation of urinary epoxygenase products.** Overnight urine samples were collected from groups of 4-5 adult mice in flasks containing triphenylphosphine (2-3 mg each) (30). After adding synthetic [20-HET]-labeled 8,9; 11,12- and 14,15-ETE (5 ng each) and [5,6-, 8,9-, 11,12-, and 14,15-HET]-labeled 8,9; 11,12-; and 14,15-DHET (5 ng each) as internal standards, the urine EETs and DHETs were extracted with acidified CHCl3/CH3OH (2:1) (24), purified by reverse phase HPLC as described (24), and methylated by reaction with diazomethane in ethyl ether (24). The resulting methyl-EETs and methyl-DHETs were quantified by LC/MS/MS using an Agilent Eclipse XDB-C18 column (2.1 × 150 mm; 5 μm) connected to a TQ-Quantum MS/MS spectrometer (Thermo Electron Corp.). EETs and DHETs were resolved using a linear solvent gradient that went from 80% CH3OH to 100% CH3OH in over 10 minutes and at a flow of 0.2 ml/min. For analysis and quantification, we utilized collision-induced fragmentation of the methyl-EET [107Ag adduct at m/z 441 and 444 (corresponding to the molecular ions of the biological sample and the internal standard, respectively) and methyl-DHET [107Ag adducts at m/z 459 and 467 (corresponding to the molecular ions of the biological and the internal standard, respectively). Diagnostic selective product ion analysis was done at m/z 261, 247, and 287 for [107Ag adducts of the methyl esters of 8,9-, 11,12-, and 14,15-EET, respectively; at m/z 317, 347, and 329 for the ion fragments generated by the [107Ag adducts of the methyl esters of 8,9-, 11,12-, and 14,15-DHET, respectively; and at m/z 323, 353, and 335, respectively, corresponding to the ion fragments originating from the corresponding [107Ag adducts of the deuterated internal standards. Quantiﬁcations were done using isotope ratios and ion intensities versus mass calibration curves (24).

**CD isolation and electrophysiology.** Male wild-type and Cyp4a10+/- mice were fed low-sodium diets for 5–7 days. Cortical CDIs were microdissected from kidney slices (1 mm thickness) were placed on an inverted microscope, superfused with HepES-buffered NaCl (21), and cut open to expose the apical membrane. Sodium currents were recorded and digitized as described (21).
Channel activity, defined as NPo, was calculated from data samples of 60 seconds during steady state as follows: NPo = Σ (ti + 2ti + ... + iti) / 20, where ti is the fractional open time at each observed current level. Channel conductance was calculated from the current intensities recorded at 3 or more holding potentials.

**Statistics.** Statistical analyses were performed using a 2-tailed Student’s t test. P < 0.05 was considered statistically significant.

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