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Research Article **Cardiology**

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

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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 (a) establish a physiological role for the arachidonate monooxygenases in renal sodium reabsorption and blood pressure regulation, (b) 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. It is expected that these results could lead to new strategies for the early diagnosis and clinical management of this devastating disease.

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 prevention. 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) as are the biological activities of these metabolites (9–11). Earlier studies identified members of the P450 *CYP2C* and *CYP4A* gene subfamilies as functionally relevant AA epoxygenases and ω-hydroxylases, respectively (8–11), and implicated them in the control of renal function and blood pressure (9–11). However, the unequivocal interpretation of these studies is complicated by the in vitro nature of the functional data available (8–11) and genetic complexity of the animal models studied (9–13). Consequently, notwithstanding their potential clinical importance, the physiological role of these enzymes in blood pressure regulation remains ambiguous and in need of experimental confirmation. We show here that a dysfunctional *cytochrome P450, family 4, subfamily a, polypeptide 10* (*Cyp4a10*) gene causes a type of hypertension that is dietary salt sensitive and associated with alterations in the activity of the renal epithelial sodium channel (ENaC).

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 malformation. 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, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; ENaC, epithelial sodium channel; 20-HETE, 20-hydroxyeicosatetraenoic acid; LC/MS/MS, liquid chromatography–tandem mass spectroscopy; NPo, channel number multiplied by the open probability; P450, cytochrome P450; Wy, Wyeth 14643.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1
Male and female *Cyp4a10*$^{-/-}$ mice are hypertensive

	Cyp4a10$^{+/+}$	*Cyp4a10*$^{-/-}$
Females	103 ± 3	147 ± 3^{A}
Males	105 ± 2	151 ± 3^{A}

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. $^{A}P < 0.001$, significantly different from wild type.

creatinine/dl for wild-type and *Cyp4a10*$^{-/-}$ mice fed normal or high-salt diets, respectively; $n \geq 4$).

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; $n \geq 14$) and potassium (57% ± 0.6%; 52% ± 0.4%; and 79% ± 0.6% of wild-type mice on low-salt, normal, and high-salt diets, respectively; $n \geq 14$) 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 characterized as a natriuretic eicosanoid and a renal vasoconstrictor (9–11). The phenotypic analysis of *Cyp4a14*$^{-/-}$ mice confirmed the hemodynamic properties of 20-HETE (15) and identified Cyp4a12 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, -4a10, and -4a14, respectively; $n \geq 6$). 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 monooxygenases could participate in the regulation of ENaC gating activity.

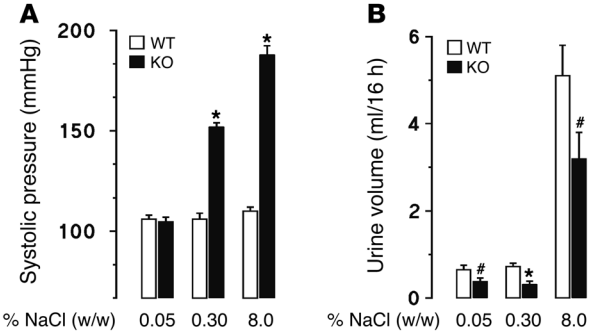

Figure 1
Hypertension in *Cyp4a10*$^{-/-}$ mice is salt sensitive. Groups of male *Cyp4a10*$^{+/+}$ (WT) (white bars) and *Cyp4a10*$^{-/-}$ (KO) (black bars) mice were fed low-salt, normal, or high-salt diets (containing 0.05, 0.3, or 8% NaCl w/w), respectively. After 3 weeks, their systolic blood pressure (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.

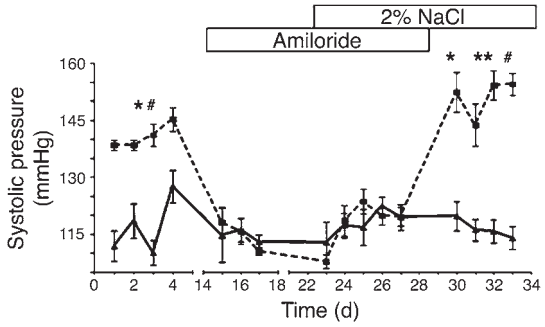

Figure 2
Amiloride normalizes the blood pressure of hypertensive *Cyp4a10*$^{-/-}$ mice. The systolic blood pressure of male *Cyp4a10*$^{-/-}$ (discontinuous line) and *Cyp4a10*$^{+/+}$ (continuous line) mice ($n = 5$ each) on diets containing 0.3% NaCl (w/w) were monitored before (days 1 to 4) and after 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*$^{-/-}$ versus amiloride-treated *Cyp4a10*$^{-/-}$ mice. #$P \leq 0.001$ *Cyp4a10*$^{-/-}$ versus *Cyp4a10*$^{+/+}$ mice for all additional amiloride treatment regimes. **$P < 0.001$ *Cyp4a10*$^{-/-}$ mice on water versus *Cyp4a10*$^{-/-}$ mice on 2% salt water.

Cy4a10$^{-/-}$ *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 CDs, and the 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 *Cyp4a10*$^{+/+}$ and *Cyp4a10*$^{-/-}$ mice. Under control conditions, we observed significant differences in ENaC basal activity between *Cyp4a10*$^{+/+}$ (channel number multiplied by the open probability [NPo] = 0.48) and *Cyp4a10*$^{-/-}$ (NPo = 1.25) mice (Figure 3, A and B). Thus, disruption of *Cyp4a10* caused constitutive ENaC activation and increased sodium reabsorption (Figure 3A). This is similar to what is observed in Liddle syndrome, a Mendelian form of human hypertension in which mutations in the β and γ subunits of ENaC cause gain of function and increased sodium currents (3–7, 23). Furthermore, while AA effectively inhibited ENaC gating in wild-type mice (Figure 3, B and C), it had only marginal effects on the knockout mice (Figure 3, A and C), suggesting that these animals had impaired epoxygenase activity. A role for the EETs and the AA epoxygenase in regulating murine ENaC activity was indicated by the demonstration that 11,12-EET inhibits the channel, regardless of *Cyp4a10* genotype (Figure 3). In summary, the results shown in Figure 3 provide a mechanistic explanation for the salt-sensitive nature of the *Cyp4a10*$^{-/-}$ hypertensive phenotype, extend the roles postulated for 11,12-EET in regulating sodium excretion to the murine nephron, and document a functional role for the AA monooxygenases in sodium plasma homeostasis. Thus, genetically determined (*Cyp4a10* disruption) reductions in EET biosynthesis or selective epoxygenase inhibition (Figure 3C) (21) equally abrogate the AA-mediated inactivation of ENaC and result in increased inward sodium flux.

Cy4a10$^{-/-}$ *hypertension in mice is associated with changes in renal EETs.* To explore the role of the EETs in the in vivo regulation of ENaC activity and in the *Cyp4a10*$^{-/-}$ hypertensive phenotype, we first measured by mass spectral methods (24) the EETs present endogenously in the kidneys or in urine collected from *Cyp4a10*$^{+/+}$ and *Cyp4a10*$^{-/-}$ mice. While no major differences were observed in EET organ levels, the *Cyp4a10*$^{-/-}$ mice showed significant reductions in the total amounts of epoxygenase products 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 *Cyp4a10*$^{-/-}$ 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 *Cy2c* and *Cyp2j* gene subfamily are expressed in the mouse kidney (25–27). Extensive chemical, biochemical, and immunological evidence show that CYP2C23 is (a) the predominant epoxygenase present in the rat kidney (28, 29), (b) the P450 isoform responsible for the regio- and stereochemical properties of the EETs generated by the kidney microsomal proteins (28, 29), (c) the renal epoxygenase induced in response to dietary salt loading (29), and (d) the CYP2C epoxygenase expressed in the rat CD (21). From a cDNA library, we cloned and expressed Cyp2c44, the mouse homologue of CYP2C23 (28) (88% amino acid sequence identity) and demonstrated that purified recombinant Cyp2c44 metabolizes AA to 11,12-EET as its major product (65% of total), with stereoselectivities similar to those previously reported for CYP2C23 (28, 29) and Cyp2c44 (27). However, a

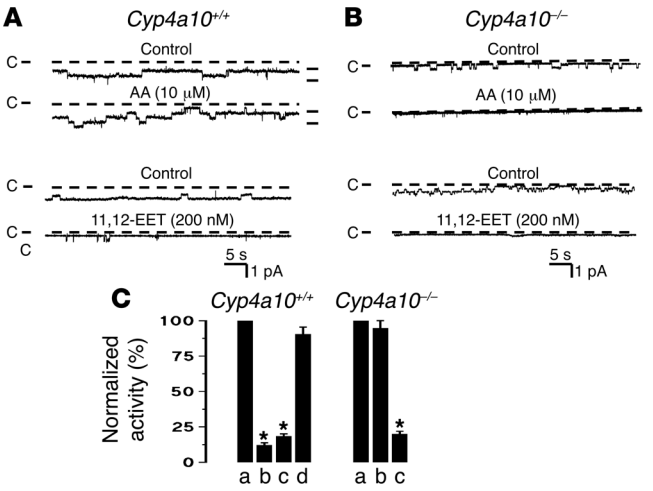

Figure 3
Effects of AA and 11,12-EET on the ENaC activity of *Cyp4a10*$^{-/-}$ and *Cyp4a10*$^{+/+}$ mice. (**A** and **B**) ENaC sodium currents were determined using CD cell attached patches (21). Inward sodium currents when ENaCs are in the closed state are indicated by C. Short bars indicate channel 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.

Table 2
Wy increases the urinary excretion of epoxygenase metabolites

		Epoxygenase metabolites (EETs and DHETs)[A]			
Urine source	**Treatment**	**8,9-**	**11,12-**	**14,15-**	**Total**
Cyp4a10$^{+/+}$	None	1.04 ± 0.10	0.42 ± 0.03	0.97 ± 0.10	2.43
Cyp4a10$^{+/+}$	Wy	1.25 ± 0.30	0.93 ± 0.36[B]	1.30 ± 0.28	3.48
Cyp4a10$^{-/-}$	None	0.42 ± 0.03	0.34 ± 0.07	0.84 ± 0.16	1.60
Cyp4a10$^{-/-}$	Wy	1.12 ± 0.22[C]	0.78 ± 0.09[C]	1.41 ± 0.11[D]	3.31

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 [$^2H_3$]–labeled 8,9-, 11,12-, and 14,15-EET (5 ng each) and [$^2H_8$]–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. [A]ng/mg of urine creatinine; [B]$P < 0.04$; [C]$P < 0.019$; [D]$P < 0.008$, 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.

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 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 (30, 31), and its effects on kidney epoxygenase metabolite urine excretion, Cyp2c44 mRNA levels, and blood pressure determined. Published studies have shown that PPARα ligands induce renal CYP2C23 epoxygenase expression (32) and that they reduce blood pressure in rat models of salt-sensitive hypertension (10, 32–34). To study the effects of Wy on the renal epoxygenase, we measured by liquid chromatography–tandem mass spectroscopy (LC/MS/MS) the levels of urinary EETs and dihydroxyeicosatrienoic acids (DHETs) before and after treating wild-type and *Cyp4a10* null mice with the PPARα ligand. Since DHETs result only from the hydration of precursor EETs, the combined concentrations of EETs and DHETs are a measure of renal epoxygenase biosynthetic capacity and urinary excretion. In *Cyp4a10*$^{+/+}$ mice, Wy treatment caused a 2.2-fold increase in the urinary excretion of the AA 11,12-epoxygenase metabolites (Table 2) and had a more limited impact on 8,9- and 14,15-epoxygenase metabolite excretion (Table 2). On the other hand, and while the overall activity of the renal AA epoxygenase of *Cyp4a10*$^{-/-}$ mice is lower than that of wild type (Table 2), Wy administration raised the overall excretion levels of epoxygenase metabolites by 2.0-fold and increased their concentrations to levels similar to those found in the urine of wild-type animals (Table 2). Furthermore, and as with wild-type mice, Wy was most effective in increasing the renal biosynthesis of 11,12-EET (Table 2), the EET regioisomer shown to be the most powerful regulator of ENaC gating activity (21). As shown in Table 2 and consistent with the 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 4, A and C) 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 urine epoxygenase metabolites and kidney *Cyp2c44* mRNAs 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

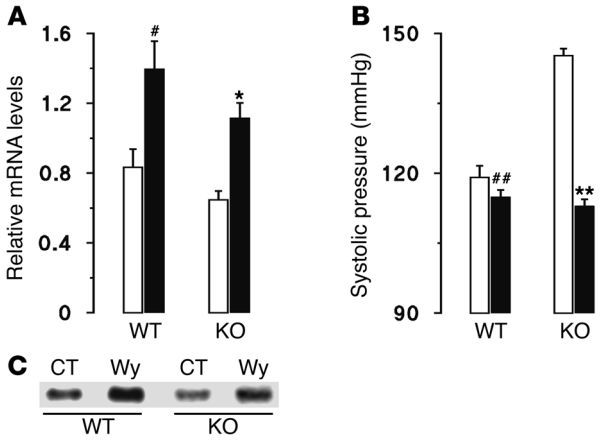

Figure 4
Wy normalizes blood pressure and *Cyp2c44* expression in *Cyp4a10*$^{-/-}$ mice. Male *Cyp4a10*$^{+/+}$ (WT) and *Cyp4a10*$^{-/-}$ (KO) mice were administered water (white bars) or a 0.02% solution of Wy (black bars) as drinking water, and after 10 days, systolic pressure and kidney Cyp2c44 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. *$P < 0.0005$; #$P < 0.003$, significantly different from untreated controls. (**B**) Systolic blood pressure (in mmHg). Values are averages from groups of 5 mice each. **$P < 0.001$, 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.

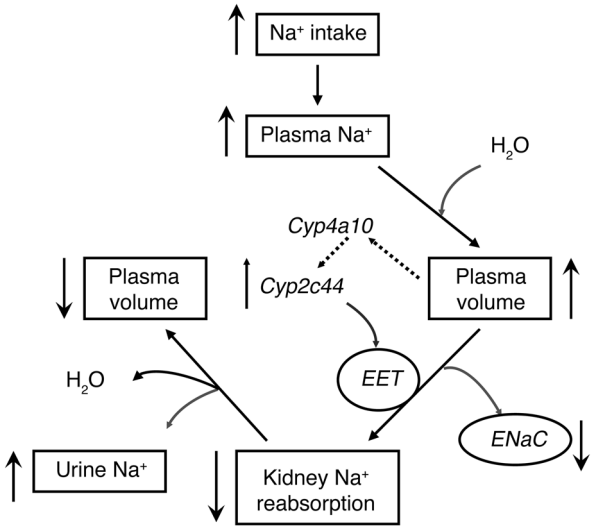

Figure 5
Roles proposed for the epoxygenase regulation of renal sodium reabsorption and plasma volume. Ascending and descending arrows denote increases or decreases in boxed parameters, respectively. Dashed arrows are used to indicate the participation of *Cyp4a10* gene product(s) in the regulation of kidney Cyp2c44 levels.

are under study. Nonetheless, the parallel upregulation by Wy of the kidney acyl-CoA oxygenase gene (not shown), a known PPARα target (30, 31, 35), suggests a role for this nuclear receptor in the process.

Discussion

The studies summarized above provide unequivocal evidence of a physiological role for the P450 AA monooxygenases and for the *Cyp4a10* gene in particular in the control of ENaC function, renal Na^+ transport, and, ultimately, systemic blood pressure. The Wy- and amiloride-sensitive nature of the *Cyp4a10*$^{-/-}$ hypertensive phenotype, and the documented association of the pressure effects of these compounds with an upregulated AA epoxygenase expression or ENaC inhibition, point to a cause-effect relationship among a Cyp4a10-mediated regulation of CD epoxygenase levels, EET biosynthesis, and ENaC activity. While the mechanism by which products of the *Cyp4a10* gene regulate renal epoxygenase expression remains to be defined, there is an established role for PPARα and its synthetic ligands in the control of P450 expression (30) and of fatty acid metabolism and lipolysis (31). Recent studies have identified several antihypertensive and cardiovascular protective effects resulting from PPARα activation and/or the administration of synthetic PPARα ligands (10, 32–34). Furthermore, it has been reported that some of the hypotensive effects of PPARα ligands are associated with changes in the renal expression of CYP4A (10) or CYP2C (32) isoforms, which are known targets for PPARα-dependent transcriptional control (30, 31), and concerted AA oxidation by CYP4A and CYP2C isoforms generates AA epoxy alcohols that are high-affinity PPARα ligands (35), suggesting that a regulatory loop involving these P450 isoforms may be involved in endogenous PPARα activation (35). As an extension of the studies discussed, we are proposing that a dysfunctional *Cyp4a10* gene alters PPARα ligand biosynthesis and that this impairs the PPARα-dependent expression of a CD Cyp2c epoxygenase and results in reduced CD EET synthesis and increased ENaC gating.

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 antihypertensive effects in humans seems warranted.

Methods

cDNA cloning, expression, and enzymatic analysis. The Cyp4a10, 4a12, 4a14 and 2c44 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 in these animals. RT-PCR cloning using Cyp4a10 forward (5′-TTGCAGACAGCCTCTCTGGCTTCC-3′) and reverse primers (5′-CTCGAGGTTACAGACTCTTCTC-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 ATG translation initiator) that causes a frame shift and would generate a protein lacking a heme prosthetic group.

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: Cyps 2b9, 2b10, and 2b19: 5′-TGCTCCACGAGACTACATTGA-3′ and 5′-GTACTTGAGCATGAGCAGGA-3′; Cyp2c29: 5′-TGTCCTGGAAGTCATCACAAA-3′ and 5′-TTGTTCAATGTGGTTTACCTGTT-3′; 2c37: 5′-CTGCTTTCCACACTGGACAC-3′ and 5′-CAGGTCCATATCATCACAGCA-3′; Cyp2c38: 5′-CCCCCAGGTTTTTATTCACC-3′ and 5′-GCATAAGGGCTCAGACAACTG-3′; 2c39: 5′-CCTCCTTTATTGCCTTCTGC-3′ and 5′-TGCATTTGTGCTATAACTCCAGA-3′; Cyp2c40: 5′-CATGGATATGACCCCCAAAC-3′ and 5′-TGGGACTGGGTTAAATGAGG-3′; Cyp2c44: 5′-TGCTACCTAGGCCAGAAACA-3′ and 5′-GATGAGGGCCTTGTGGATCT-3′; Cyp2j5: 5′-CCTGCTCACATGAGAAGTGATAG-3′ and 5′-GATGATAAGGTATGTCACTGGACAC-3′; Cyp2j6: 5′-GGTTGCTCACACGAGAGAAG-3′ and 5′-GCATGCCACTGAGCTCATAA-3′; β-actin: 5′-AGGTGACAGCATTGCTTCTG-3′ and 5′-AGGGAGACCAAAGCCTTCAT-3′. Averaged Cyp2c and β-actin fluorescence threshold crossing cycle calculated from a PCR baseline subtracted from curve fit (C_τ) values were utilized to calculate β-actin normalized mRNA expression levels.

Kidney microsomes were isolated and incubated with [1-^{14}C]AA (100 μM) and NADPH (1 mM) as described (48). Purified recombinant P450s (0.1 μM) were incubated with purified rat P450 reductase (1 μM) and dilauroylphosphatidylcholine (50 μg/ml) prior to the addition of AA (75 μM) and NADPH (1 mM). Cyp4a proteins were reconstituted in the presence of cytochrome b_5 (15, 48) (0.1 μM). Products were quantified as published (48). The creatinine concentrations of plasma samples obtained from *Cyp4a10*$^{+/+}$ and *Cyp4a10*$^{-/-}$ mice fed solid diets containing normal salt (0.3% NaCl w/w) or high salt (8% NaCl w/w for 14–16 weeks) were determined as reported (14).

Construction of the Cyp4a10-targeting vector. Overlapping clones encoding all *Cyp4a10* exons were isolated from a 129/SvJ mouse BAC genomic library (Invitrogen Corp.). A linearized pNTK targeting vector containing an *Nco*1 flanked *Cyp4a10* gene segment in which exon 3 and part of exon 4 were replaced by a neomycin cassette was electroporated into TL-1 129/SvEv Tac mouse ES cells. Recombinant ES cells were identified by Southern blots of *Acc*1 digests using a 1.0 kb *Cyp4a10* probe coding for exon 1 and segments of its flanking intron and 5′ upstream sequences. The neomycin replacement removed exon 3 of the *Cyp4a10* gene, interrupted in-frame translation at exon 4, and eliminated a diagnostic *Acc*I site, used for genotype analysis. Two ES clones carrying a *Cyp4a10* mutant allele (IE5 and ID12) were utilized for the generation of germ line chimeras. Mice were fed commercial solid diets containing 0.05, 0.3, or 8% NaCl (w/w) and allowed free access to water. Amiloride was administered in the drinking water and its concentrations adjusted to a daily dose of approximately 2 mg/kg of body weight. Wy was administered as a 0.02% w/v solution in the drinking water. Animal experiments were performed in accordance with NIH policies and approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

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*$^{-/-}$ IE5 line. Daily measurements of free fluid volume (16) were done at 10 am and at 3 pm for 3 consecutive days and in groups of age-matched male *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 quantification 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-$^2H_3$]–labeled 8,9-; 11,12-; and 14,15-EET (5 ng each) and [5,6-, 8,9-, 11,12-, and 14,15-$^2H_8$]–labeled 8,9-; 11,12-; and 14,15-DHET (5 ng each) as internal standards, the urine EETs and DHETs were extracted with acidified $CHCl_3$/CH_3OH (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-C_8 column (2.1 × 150 mm; 5 μm) connected to a TSQ-Quantum MS/MS spectrometer (Thermo Electron Corp.). EETs and DHETS were resolved using a linear solvent gradient that went from 80% CH_3OH, 20% H_2O containing 25 μM $AgBF_4$ to 100% CH_3OH containing 25 μM $AgBF_4$ 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 ^{107}Ag adducts at m/z 441 and 444 (corresponding to the molecular ions of the biological sample and the internal standard, respectively) and of the methyl-DHET ^{107}Ag 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 ^{107}Ag adducts of the methyl esters of 8,9-, 11,12-, and 14,15-EET, respectively; at m/z 317, 34,7 and 329 for the ion fragments generated by the ^{107}AG 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 ^{107}Ag 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 CDs 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 (t_1 + $2t_2$ + . . . + it_i) (20), where t_i is the fractional open time spent 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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