Original Research

Renal function and vasomotor activity in mice lacking the Cyp4a14 gene

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Abstract

The production of 20-hydroxyeicosatetraenoic acid (20-HETE) in the kidney is thought to be involved in the control of renal vascular tone and tubular sodium and chloride reabsorption. Cytochrome (Cyp) P-450 enzymes of the Cyp4a family in the mouse, namely 4a10, -12 and 14, are involved in 20-HETE synthesis. Recent advances in the molecular genetics of the mouse have produced mice in which Cyp4a isoforms have been disrupted and the consequence of such an approach is examined. This study evaluated the effect of deletion of the Cyp4a14 gene on blood pressure, renal vascular responses and tubular function. When compared with the wild-type (WT) litter mates, systolic blood pressure was greater in Cyp4a14 null (KO) mice as were renal vascular responses to angiotensin II or phenyephrine, G protein-coupled receptor (GPCR) agonists, but not KCl, a non-GPCR agonist. Renal vascular responses to guanosine 5′-O-(gamma-thio)triphosphate, a non-hydrolyzable GTP analog, or NaF_4, an activator of G-proteins, were also enhanced. However, vasodilation to bradykinin or apocynin but not sodium nitroprusside was blunted in Cyp4a14 null (KO) kidneys. These changes in KO mice were accompanied by increased 20-HETE synthesis, reduced renal production of nitric oxide (NO), increased lipid hydroperoxides and increased apocynin-inhibitable vascular NADPH oxidase activity that was prevented by administration of NO synthase (NOS) inhibitor, suggesting endothelial nitric oxide synthase (eNOS) uncoupling. Cyp4a14 KO mice also exhibited a diminished capacity to excrete an acute sodium load (0.9% NaCl, 2.5 mL/kg). These data suggest that deletion of the Cyp4a gene conferred a prohypertensive status via mechanisms involving increased 20-HETE synthesis and eNOS uncoupling leading to increased oxidative stress, enhanced vasoconstriction but diminished vasodilation as well as a defect in the renal excretory capacity in Cyp4a14 KO mice. These mechanisms suggest that the Cyp4a14-deficient mouse may be a useful model for evaluation of NO/20-HETE interactions.

Keywords: Cyp4a14, 20-HETE, nitric oxide, NADPH oxidase, eNOS, blood pressure, renal function

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Introduction

20-Hydroxyeicosatetraenoic acid (HETE), one of the primary eicosanoids produced in the microcirculation and the kidney, has been extensively studied in rats and humans. Its production occurs primarily via the actions of P-450 enzymes of the CYP4A and -4F families.[1] In the mouse, three isoforms of the CYP4A family have been cloned, namely Cyp4a10, -4a12 and -4a14 isoforms that are the homologs of CYP4A1, -4A2/3 and -4A8, respectively, in the rat.[2,3] In the mouse kidney, the Cyp4a12 is the primary isoform that is constitutively and differentially expressed in a gender-specific manner – with levels in males being much higher than those in females.[3] In the kidney, CYP4A proteins show distinct distribution along the vascular tree[4] and their suppression or overexpression in vascular tissues decreased or increased vascular reactivity and myogenic tone, respectively.[5–7] Thus, 20-HETE participates in the regulation of vascular tone by sensitizing smooth muscle cells to constrictor stimuli[8] and this appears to be the mechanism whereby it contributes to myogenic, mitogenic and angiogenic responses.[9–12] In the kidney, CYP4A proteins also show distinct distribution along the nephron[13] where 20-HETE produced participates in ion transport and tubular function.[1]

Nitric oxide (NO) and NO donors have long been known to inhibit the catalytic activities of CYP enzymes[14] and NO directly binds to the heme moiety of recombinant CYP4A[15] and inhibits the formation of 20-HETE.[16–18] *In vivo*, we and others have shown that chronic inhibition of NO synthesis increased expression of CYP4A protein and the synthesis of 20-HETE,[15,16] indicating the involvement of NO in the regulation of 20-HETE synthesis. In other studies, increased

CYP4A expression[19] or transduction with CYP4A2 cDNA[20] impaired acetylcholine-induced relaxation in renal arteries that was offset by inhibition of 20-HETE synthesis and reinstated by exogenous 20-HETE. This observation led to the conclusion that the NO-dependent component of acetylcholine-induced relaxation is suppressed by increased 20-HETE levels. A causative link between the CYP4A–20-HETE pathway and endothelial dysfunction was thus adduced by the observation that overexpression of CYP4A2 in rats caused hypertension, increased vascular CYP4A expression and 20-HETE production accompanied by endothelial dysfunction and increased levels of superoxide anion.[20] This was supported by human studies demonstrating correlations between elevated urinary 20-HETE and endothelial dysfunction[21] or reduced plasma F(2)-isoprostanes and urinary 20-HETE excretion.[22]

Based on the aforementioned interactions of NO and 20-HETE, including the observation that 20-HETE inhibited NO production and/or release,[19,20] it stands to reason that increased production of 20-HETE consequent to increased Cyp4a12 expression in Cyp4a14 KO mice[23] should result in vascular responses reminiscent of a phenotype characterized by decreased NO production. We, therefore, hypothesized that increased production of prohypertensive 20-HETE in Cyp4a14 null mice leads to decreased NO production via endothelial nitric oxide synthase (eNOS) uncoupling leading to increased production of free radicals and enhanced renal vasoconstriction but decreased vasodilation in Cyp4a14-deficient kidneys. We chose to test this hypothesis using the isolated perfused kidney because of (i) the abundance of NO and CYP4A/20-HETE system in the kidney; and (ii) the established role of the kidney in long-term regulation of blood pressure. We evaluated vascular responses to G protein-coupled receptor (GPCR) and non-GPCR agonists, a role for uncoupled eNOS in the responses and renal functional response in Cyp4a14-deficient mice. Our data indicate heightened renal vascular responses in Cyp4a14 KO mice that are consistent with endothelial dysfunction and eNOS uncoupling that may have arisen from increased formation of vasoconstrictor 20-HETE in Cyp4a14 KO mice. There was also evidence of diminished excretory capacity in Cyp4a14 KO mice that may contribute to the hypertensive phenotype.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless specified otherwise. Na tetrafluoride (NaF_4) was a gift from Dr Doug Eikenburg (Department of Pharmaceutical Sciences, University of Houston, Houston, TX, USA). 12,12-dibromododec-11-enoic acid (DBDD), a gift from Dr Camille Falck (University of Texas Southwestern Medical Center, Dallas, TX, USA), was stored in ethanol at $-70^{\circ}C$ until use.

Cyp4a14 knockout (KO) and the wild-type (WT) litter mates mice of both sexes used as breeder pairs were provided by Dr Jorge Capdevilla (Department of Biochemistry, Vanderbilt University Medical School, Nashville, TN, USA). The KO mice were generated from murine germline chimeras carrying a Cyp4a14 mutant allele. Mating to WT 129/SvJ mice and genetic selection led to generation of isogenic homozygous Cyp4a14 KO and WT mice. Genotype analysis indicated normal offsprings and normal developmental patterns for Cyp4a14 KO and WT mice.[23]

In view of the differential gender-dependent expression of Cyp4a14 and its phenotypic sequelae,[23] all experiments were conducted on male Cyp4a14 KO and WT mice (body weight 23–32 g) according to protocols approved by the Institutional Animal Care and Use Committee following the National Institutes of Health guidelines for laboratory animal use. The animals were placed in a room with lighting adjusted to produce a normal day/night cycle (illuminated from 8 to 20 h). They were maintained on a standard rat food (Purina Chow) and were allowed *ad libitum* access to water and food prior to the experiments.

Systolic blood pressure (SBP) and heart rate (HR) were determined in conscious animals by tail cuff plethysmography using the Blood Pressure Analysis System (Model SC-1000; Hatteras Instruments Inc, Cary, NC, USA). The mice were trained for blood pressure measurement by placing them in restrainers for 15 min two times daily for five consecutive days before determining their blood pressure.

Isolated perfused kidney

The preparation followed a modification of the isolated perfused kidney *in situ* for the mouse as we described elsewhere.[24] Briefly, following pentobarbital anesthesia (50 mg/kg, intraperitoneally), the left and right kidneys were exposed by a midline laparotomy. First, the left renal artery was cannulated (Butterfly needle, size 27 g; Becton-Dickinson, Franklin Lakes, NJ, USA) via the abdominal aorta to avoid interruption of blood flow and slowly perfused manually with 5–10 mL oxygenated (95% O_2/5% CO_2) Krebs–Henseleit buffer (pH 7.4). Next, the right kidney was cannulated via the portion of the abdominal aorta above the left kidney and slowly perfused manually with 5–10 mL oxygenated Krebs–Henseleit buffer. Each of the kidneys was then removed from the animals and perfused by means of a Masterflex cartridge pump (Masterflex C/L Model 77120-52; Cole Parmer Instrument Company, Vernon Hills, IL, USA) with oxygenated Krebs–Henseleit buffer (pH 7.4) at 37°C at a constant flow (0.4–0.6 mL/min) to obtain a basal perfusion pressure of 60–90 mmHg. Following a stabilization period of 10–15 min, responses to vasoconstrictor agonists were determined by administering random boluses of phenylephrine (PE, 3, 10 and 30 μg) or angiotensin II (AngII, 3, 10, 30 and 100 ng), GPCR agonists or KCl (3, 10 and 30 μmol), a non-G-protein-coupled receptor agonist, into the perfusate line proximal to the kidney. In view of the established role of Rho in GPCR signal transduction,[25] constrictor responses were also determined to GTPγS (guanosine 5′-O-(gamma-thio)triphosphate; 0.3, 1 and 3 μg), a non-hydrolyzable GTP analog and an activator of Rho-kinase, or NaF_4 (3, 10 and 30 μg), an activator of G-proteins. Similarly, vasodilator responses were determined for bradykinin (BKN; 30,100, 300 ng), an endothelium-dependent and GPCR vasodilator,

sodium nitroprusside (SNP; 1, 3, and 10 μg), an endothelium-independent, non-G-protein coupled receptor vasodilator or apocynin (30 and 100 μmol/L), an inhibitor of NADPH oxidase. For testing the effects of vasodilators, PE (1.0–5 μmol/L) was added to the perfusate to elevate renal vascular tone to 150–165 mmHg. The concentration of PE was adjusted to provide similar elevation of tone in kidneys from Cyp4a14 KO and WT mice. Thereafter, the vasodilator effects of BKN and SNP were evaluated. In some experiments, N^{ω}-L-nitro arginine methyl ester (L-NAME; 100 μmol/L) was added but the concentration of PE was adjusted to obtain comparable elevation of tone as in kidneys treated with PE alone. In additional experiments ($n = 4$), the role of increased 20-HETE in the renal vascular responses of Cyp4a14 KO and WT mice was determined by evaluating vasoconstriction induced by PE and AngII in kidneys treated with DBDD, an inhibitor of 20-HETE synthesis.[26] The contralateral kidney of the same mouse was used for time controls by evaluating responses to PE (3 and 10 μg) every hour for two hours followed by evaluation of the vasodilator response to BKN (30 and 100 ng) every 30 min for one hour. Apocynin (30 and 100 μmol/L) was then administered and responses were determined.

In every case, perfusion pressure was monitored constantly by means of a pressure transducer (model BPLR; World Precision Instruments, Sarasota, FL, USA) coupled to a transbridge (model TBM-4; World Precision Instruments) and a data acquisition system (model DI-712; DataQ Instruments, Akron, OH, USA). Because flow was maintained at a constant rate, changes in perfusion pressure were used as an index of changes in renal vascular resistance, with an increase in perfusion pressure indicating vasoconstriction. All responses were measured from the minimum of the pulse pressure and are the peak responses. Flow rate was determined manually. Drugs were kept on ice throughout the experiments and injected close-arterially in volumes not exceeding 25 μL.

Salt-loading

As 20-HETE has been reported to inhibit sodium transport and promote natriuresis,[27] we evaluated the capacity for sodium excretion in animals given an acute sodium load. In these experiments, Cyp4a14 KO ($n = 6$) and WT ($n = 7$) mice were used and five-hour urine volume (UV) and urinary excretion of sodium ($U_{Na}V$) were evaluated first under normal (basal) condition (Control) and then they were loaded with 0.9% NaCl (Salt; 2.5 mL/100 g body weight, intraperitoneally). As NO was demonstrated to play a role in the renal effects of CYP4A,[16,17] additional experiments were conducted in which the role of NO in the excretory ability of Cyp4a14 KO and WT kidneys was evaluated. In these experiments, a washout period of five days was allowed and urine voided (over 5 h) was collected in the same animals on the second day of treatment with L-NAME (50 mg/L drinking water for 2 d). Urine was also collected in the same animals given an acute sodium load (L-NAME/Salt) as above. For sodium loading experiments, animals were placed in metabolic cages for timed (5 h) collection of urine output. Urine Na^+ ($U_{Na}V$) was determined by a flame photometer (PFP7; Genway Ltd, Essex, UK).

Measurement of biochemical parameters

Protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as the standard.

Renal microsomal synthesis of 20-HETE

This was determined as ω-hydroxylase activity by a modification of the method we described previously.[16] Briefly, renal microsomes (0.3 mg protein) from Cyp4a14 KO and WT mice prepared by differential centrifugation techniques were incubated with [^{14}C]AA (0.2–0.4 μCi; 3 μmol/L) and cold AA (7 μmol/L) in 0.12 mol/L potassium phosphate buffer containing 5 mmol/L $MgCl_2$ in the presence of NADPH (1 mmol/L) and indomethacin (10 μmol/L) with or without DBDD (2 μmol/L), an inhibitor of 20-HETE synthesis[26] for 30 min at 37°C. 20-HETE synthesis was determined as renal microsomal conversion of ^{14}C-AA to ω-hydroxylase CYP metabolites. Metabolites were extracted with acidified ethyl acetate (pH 3–4) and subjected to reverse-phase high-performance liquid chromatography. The elution profile of the radioactive metabolites of AA was detected and counted using an on-line radioactive detector (Radiomatic Instruments, Tampa, FL, USA). CYP ω-hydroxylase activity was reported as the activity corresponding to the metabolite (i.e. 20-HETE) that elutes with a retention time of 7.2 min on the chromatogram. Enzyme activity, represented as specific activity, was calculated from percentage conversion of ^{14}C-AA in individual microsomal preparations relative to the mean total enzyme activity for each group of Cyp4a14 KO and WT mice.

Plasma lipid hydroperoxide

This was determined by a fluorometric kit from Cayman Chemicals (Ann Arbor, MI, USA). Urinary protein was determined by a colorimetric kit from Sigma-Aldrich.

Urinary excretion of NO ($U_{NOX}V$) measurement

Animals were placed in metabolic cages (Hatteras Instruments) with food and tap water provided *ad libitum.* Urine voided over a determined period was collected and measured by gravimetry and nitrite was determined using the Griess assay.

NAD(P)H oxidase enzyme activity assay

NAD(P)H oxidase activity was estimated by a modified enzyme activity assay[28] as we previously described.[29] Briefly, before sacrificing the animals, aortae were isolated, homogenized and centrifuged in HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) buffer (pH 7.4) and protein concentration was measured with Bradford reagent. Tetrazolium salt radical detector (Cayman Chemicals), dissolved in HEPES buffer (200 μL), was added to a 96-microwell plate. Equal volumes (20 μL) of protein samples were then added to separate wells and the reaction was initiated by addition of 100 μmol/L NADH and 100 μmol/L NADPH. Absorbance (at 450 nm) was measured

with a plate reader EL808IU (Bio-Tek Instruments Inc, Winoski, VT, USA) every 10 min for one hour at room temperature. Parallel experiments were carried out in the presence of apocynin (10 μmol/L), an inhibitor of NADPH oxidase. Changes in absorbance were then normalized to the protein concentration.

Statistical analysis

Data were expressed as absolute values with the exception of renal vascular response data that were expressed as changes (Δ) in perfusion pressure from preinjection values. All data were presented as mean $\pm$ SEM and compared between groups for significance using unpaired Student's *t*-test or the one-way analysis of variance as appropriate. In all cases, $P < 0.05$ was considered significant; n indicates the number of mice or kidneys.

Results

SBP in Cyp4a14 KO and WT mice

Figure 1 illustrates that SBP and HR in Cyp4a14 KO mice were 163 ± 17 mmHg and 469 ± 33 bpm, respectively. SBP was about 30% greater ($P < 0.05$) in KO mice ($n = 7$) than that in WT ($n = 9$) mice. However, there were no differences in HR.

Renal vascular tone in Cyp4a14 KO mice

Basal perfusion pressure was 72 ± 4 mmHg in kidneys from Cyp4a14 KO mice ($n = 7$), a value that was not different from that obtained in kidneys harvested from Cyp4a14 WT (76 ± 6 mmHg; $n = 8$) mice. The corresponding flow rates in these kidneys were 0.52 ± 0.04 and 0.50 ± 0.06 mL/min in WT and KO mice, respectively. Figure 2 illustrates that upon addition of PE (3 μmol/L) to Krebs buffer perfusing the kidneys, tone was elevated to a greater extent in Cyp4a14 KO kidneys ($\Delta = 106 \pm 6$ mmHg, $n = 6$) than in WT kidneys ($\Delta = 79 \pm 6$ mmHg; $n = 6$). This difference was abolished following inhibition of 20-HETE synthesis with DBDD (2.5 μmol/L): Cyp4a14 KO ($n = 4$), 44 ± 5 mmHg; WT ($n = 4$), 28 ± 4 mmHg. However, addition of L-NAME (100 μmol/L) hardly elevated tone in Cyp4a14 KO mice ($\Delta = 15 \pm 5$ mmHg; $n = 6$) but increased tone in Cyp4a14 WT mice ($\Delta = 58 \pm 5$ mmHg; $n = 5$), indicating a blunted NOS activity in Cyp4a14 KO mice.

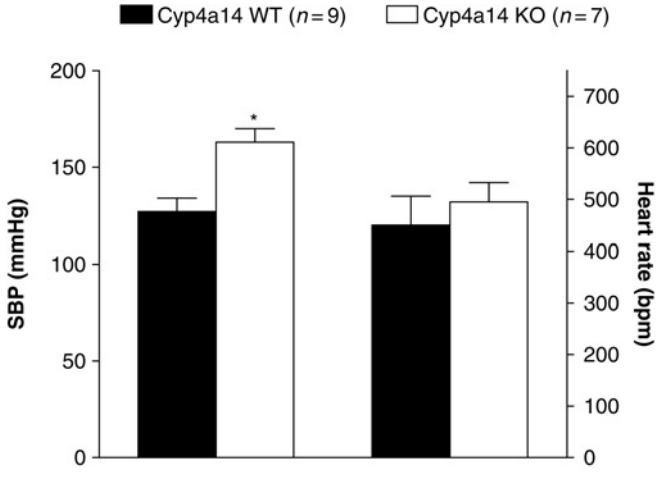

Figure 1 Systolic blood pressure (SBP) and heart rate in male Cyp4a14 knockout (KO) and wild-type (WT) mice. $^{*}P < 0.05$ Cyp4a14 WT versus KO mice

Renal vascular responses to constrictor agents

As constrictor responses are enhanced in 20-HETE overexpressing renal vessels,[6,7] we evaluated responses to PE and AngII, known GPCR agonists, or KCl, a non-GPCR agonist. Figures 3a and b illustrate that compared with WT mice, renal vasoconstriction elicited in response to PE and AngII were greater in Cyp4a14 KO mice ($P < 0.05$). However, vasoconstriction elicited by KCl, a non-GPCR-coupled agonist, was not different between Cyp4a14 KO and WT mice (data not shown). Thus, increases in perfusion pressure following injection of 3, 10 and 30 μmol KCl were 5 ± 4, 13 ± 3 and 78 ± 6 mmHg, respectively, in kidneys from Cyp4a14 KO mice; values that were not different from that obtained in Cyp4a14 WT mice, which were 4 ± 2, 13 ± 2 and 70 ± 17 mmHg, respectively. These data indicate that increased production of 20-HETE in Cyp4a14 KO mice conferred a selective effect on GPCR agonists.

As the responses of a number of agents involve guanine exchange factors that activate GDP/GTP exchange in the GTP-bound state,[25] renal vascular responses to GTPγS, a non-hydrolyzable GTP analog, or NaF_4, an activator of G-proteins, were also evaluated in kidneys of Cyp4a14 KO and WT mice. Figures 3c and d illustrate that GTPγS (0.3, 1 and 3 μg) and NaF_4 (3, 10 and 30 μg) elicited dose-dependent increases in renal perfusion pressure that were greater in Cyp4a14 KO mice than the WT controls ($P < 0.05$).

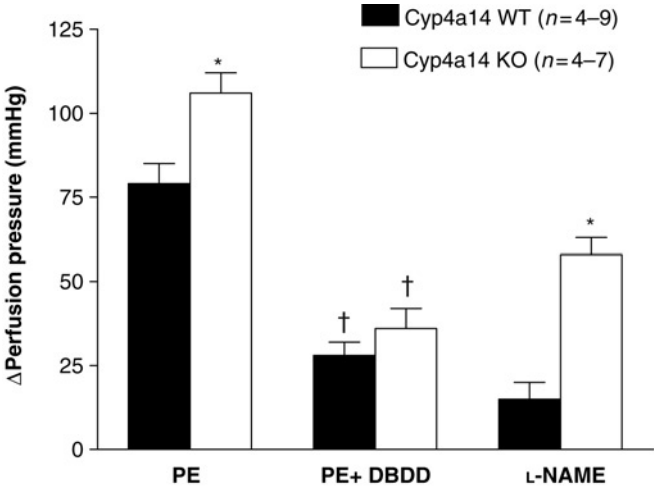

Figure 2 Changes in perfusion pressure in Krebs-perfused isolated kidneys from Cyp4a14 knockout (KO; $n = 4$–9) and wild-type (WT; $n = 4$–7) mice constricted with phenyephrine (PE, 3 μmol/L) in the presence or absence of DBDD (12,12-dibromododec-11-enoic acid; 2.5 μmol/L). Also presented are the changes in perfusion pressure in kidneys preconstricted with L-NAME (N^{ω}-L-nitro arginine methyl ester; 100 μmol/L). $^{*}P < 0.05$ Cyp4a14 WT versus KO mice; $^{\dagger}P < 0.05$ Cyp4a14 WT versus KO mice + DBDD

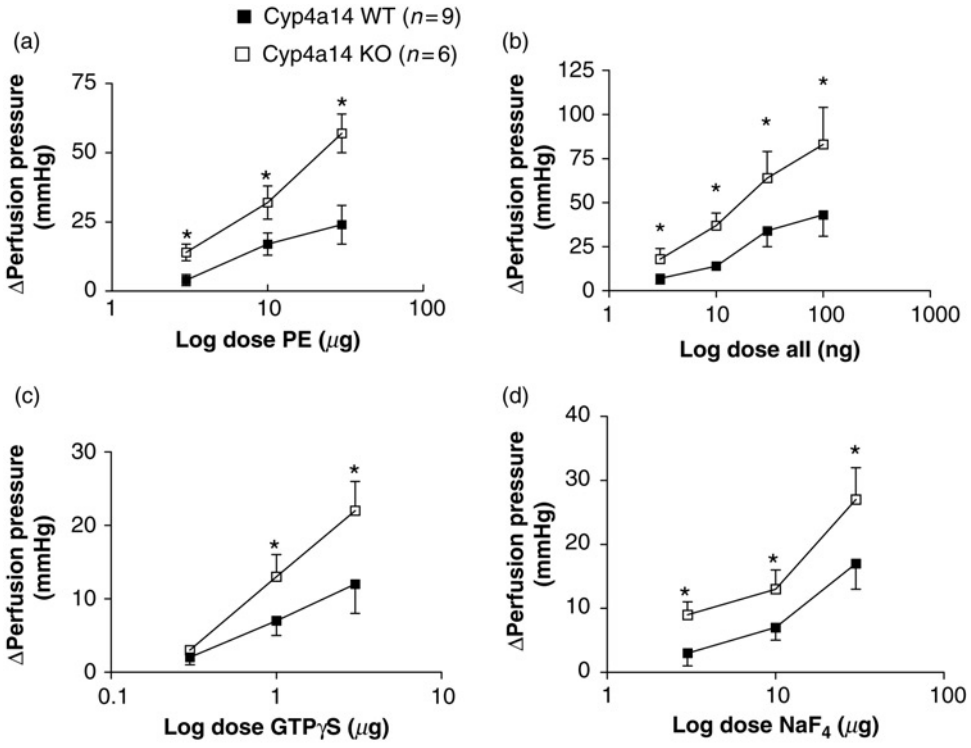

Figure 3 Changes in perfusion pressure in Krebs-perfused isolated kidneys from Cyp4a14 knockout (KO) and wild-type (WT) mice challenged with phenylephrine (PE, a), angiotensin II (AII, b), GTPγS (guanosine 5′-O-(gamma-thio)triphosphate) (c) or NaF_4 (d). $^*P < 0.05$ Cyp4a14 WT versus KO mice

Renal vascular responses to dilator agents

As dilator responses are diminished in hypertensive states and transduction with CYP4A2 cDNA[20] or increased CYP4A protein expression[19] evokes endothelial dysfunction, we evaluated if deletion of Cyp4a14 gene impairs endothelium-dependent responses by evaluating responses to BKN, an endothelium-dependent GPCR agonist, or SNP, an endothelium-independent, non-GPCR agonist. Figures 4a and b illustrate dose-dependent vasodilator responses to BKN (30, 100 and 300 μg) or SNP (1, 3 and 10 μg). The responses to BKN but not SNP were blunted in Cyp4a14 kidneys. Moreover, L-NAME (100 μmol/L) inhibited vasodilator response to BKN ($51 \pm 5\%$, $P < 0.05$) in WT but not KO kidneys ($11 \pm 3\%$, $P > 0.05$) (data not shown), implying endothelial dysfunction in Cyp4a14-deficient mice. Figure 4c illustrates data from additional experiments ($n = 4$) in which apocynin (30 and 100 mmol/L), an inhibitor of NADPH oxidase, elicited dose-related vasodilation in PE-preconstricted kidneys ($n = 4$) that was greater in Cyp4a14 KO mice ($58 \pm 6\%$, $P < 0.05$), implying greater production of NADPH-oxidase-derived free radicals in Cyp4a14 KO mice.

In time controls ($n = 4$), responses to PE (3 and 10 μg) or BKN (30 and 100 ng) were unchanged when evaluated up to three hours of perfusion with Krebs solution. Thus, in Cyp4a14 mice, responses to PE 3 and 10 μg were 7 ± 2 and 18 ± 3 mmHg at the beginning of the experiment, and two hours later the responses were 5 ± 1 and 15 ± 2 mmHg, respectively. Similarly, vasodilation elicited by BKN 30 and 100 μg were -20 ± 3 and -33 ± 4 mmHg on the first round of testing, and one hour later the values were unchanged, being -16 ± 3 and -28 ± 3 mmHg, respectively.

Renal microsomal synthesis of 20-HETE in Cyp4a14 WT and KO mice

Figure 5 illustrates that the specific activity of ω-hydroxylase as evaluated by renal microsomal conversion of [^{14}C]AA to HETE was higher ($72 \pm 6\%$; $P < 0.05$) in Cyp4a14 KO ($n = 4$) mice compared with Cyp4a14 WT ($n = 4$) mice (52.4 ± 4.5 versus 29.3 ± 3.2 pmol/mg protein/30 min). DBDD (2.5 μmol/L), the inhibitor of 20-HETE synthesis,[26] markedly inhibited ω-hydroxylase activity three- to four-fold ($P < 0.05$) in both groups of mice.

Urinary excretion of nitrite in Cyp4a14 WT and KO mice

As NO/20-HETE interactions have been shown to affect vascular tone,[1] we evaluated if the demonstrated increase in 20-HETE production in Cyp4a14 KO mice impact renal production of NO in these mice. Figure 6 illustrates that 24-h urinary excretion of nitrite was greater in Cyp4a14 WT ($n = 8$) mice than in the KO ($n = 6$) mice ($25 \pm 3\%$, $P < 0.05$), suggesting an inverse relationship between 20-HETE and NO production in Cyp4a14-deficient mice.

Lipid hydroperoxide generation in Cyp4a14 WT and KO mice

Since overexpression of CYP4A2 and increased 20-HETE synthesis were associated with increased free radical generation[30] which can be modulated by NO, we evaluated

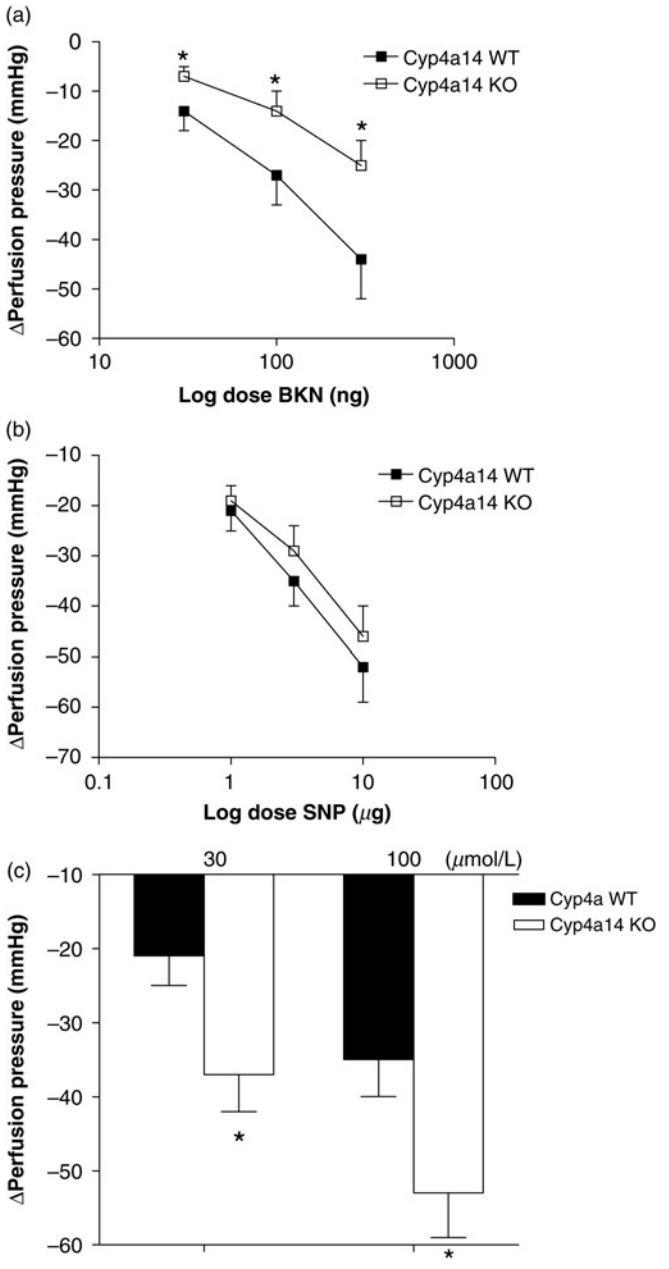

Figure 4 Changes in perfusion pressure in Krebs-perfused isolated kidneys from Cyp4a14 knockout (KO) and wild-type (WT) mice challenged with bradykinin (BKN, a), sodium nitroprusside (SNP, b) or apocynin (c). $^{*}P < 0.05$ Cyp4a14 WT versus KO mice

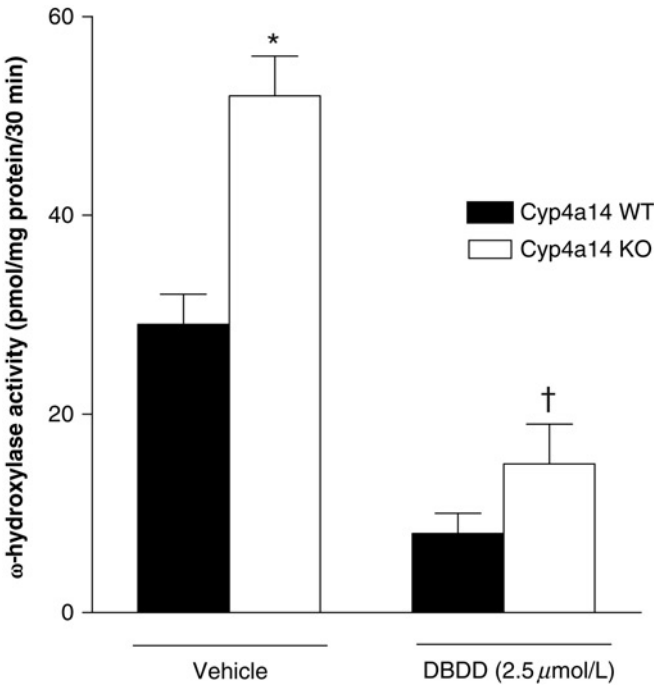

Figure 5 Microsomal conversion of ^{14}C-AA to 20-hydroxyeicosatetraenoic acid via ω-hydroxylase activity in Cyp4a14 knockout (KO) and wild-type (WT) kidneys in the presence of 12,12-dibromododec-11-enoic acid (DBDD; 2.5 μmol/L) or its vehicle. $^{*}P < 0.05$ Cyp4a14 WT versus KO mice; $^{\dagger}P < 0.05$ Cyp4a14 WT versus KO mice + DBDD

free radical generation in Cyp4a14 KO mice and compared it with that in the WT mice. Figure 6a illustrates that plasma lipid hydroperoxide production was $52 \pm 4\%$ ($P < 0.05$) greater in Cyp4a14 KO mice ($n = 6$), than that obtained in WT mice ($n = 6$). Figure 6b illustrates data from additional experiments using isolated aortae from these mice to evaluate the capacity for oxidative stress in Cyp4a14-deficient mice. There was a greater apocynin-inhibitable NAD(P)H oxidase activity ($50 \pm 6\%$, $P < 0.05$) in Cyp4a14 KO ($n = 6$) aortae compared with that from Cyp4a14 WT ($n = 6$) mice. In the presence of L-NAME (100 μmol/L), the increase in NADPH oxidase activity was prevented in Cyp4a14 KO mice such that there was no difference from Cyp4a14 WT mice indicating that eNOS uncoupling contributed to the increased oxidative stress in Cyp4a14 KO mice.

Sodium excretion, urinary output and nitrite excretion in salt-loaded Cyp4a14 WT and KO mice

In Cyp4a14 KO ($n = 6$–8) and WT ($n = 9$) mice placed on 0.9% NaCl (2.5 mL/100 g body weight intraperitoneally) and in which UV and $U_{Na}V$ were estimated five hours post-loading, Figures 7a and b illustrate that compared with controls, salt loading markedly increased UV (three-fold, $P < 0.05$) and $U_{Na}V$ ($46 \pm 10\%$, $P < 0.05$) in the WT but not in the Cyp4a14 KO mice. L-NAME blunted both basal (L-NAME)- and salt load (L-NAME/Salt)-induced increases in UV in WT ($56 \pm 6\%$, $P < 0.05$, $n = 9$) and $U_{Na}V$ in WT mice ($42 \pm 6\%$, $P < 0.05$) but not in KO ($n = 6$) mice. Under the same conditions, basal $U_{NOx}V$ was lower in Cyp4a14 KO compared with WT ($n = 9$) mice (9.2 ± 2.3 versus 21.1 ± 3.9 μmol/L/g kidney/24 h). However, following sodium loading, $U_{NOx}V$ (over 5 h) increased in WT (0.24 ± 0.03 to 0.44 ± 0.08 μmol/L/g kidney) but not in Cyp4a14 KO (0.18 ± 0.04 to 0.19 ± 0.03 μmol/L/g kidney) mice (data not shown).

Discussion

The role of the CYP4A/20-HETE pathway and its mechanisms in hypertension are well documented in both animal and human studies. Thus, elevation of 20-HETE production was reported in SHR,[6,31] DOCA/salt,[32] and AngII[33] hypertension in animals and in human hypertension.[34] Equally

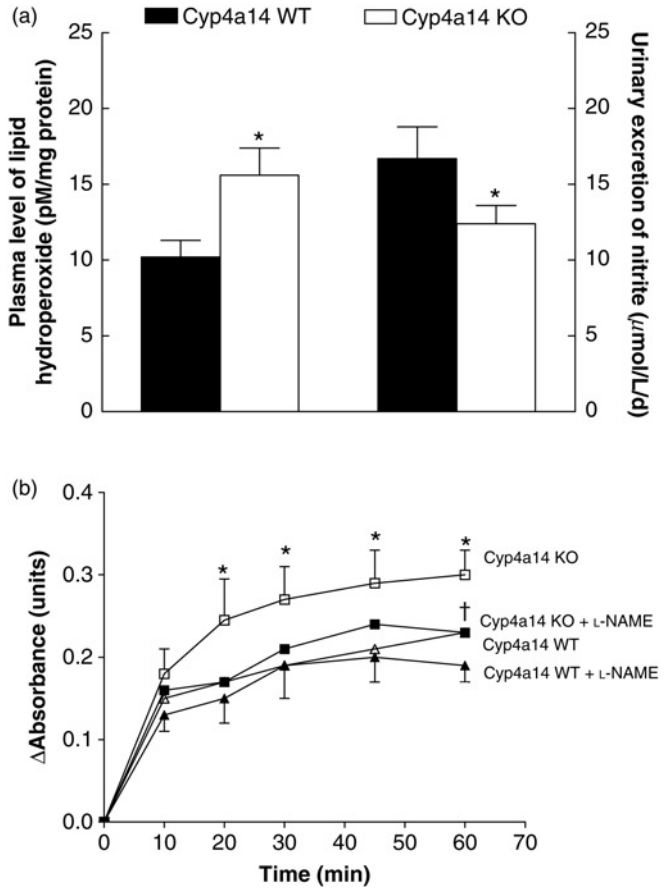

Figure 6 Differences in plasma lipid hydroperoxide concentration and urinary excretion of nitric oxide (a) or NADPH oxidase activity (in the presence or absence of N^{ω}-L-nitro arginine methyl ester [L-NAME; 100 μmol/L]); (b) in the aortae of Cyp4a14 knockout (KO, $n = 6$) and wild-type (WT, $n = 6$) mice. NADPH oxidase activity was measured by absorbance at 450 nm. $^{*}P < 0.05$ Cyp4a14 WT versus KO mice; $^{\dagger}P < 0.05$ Cyp4a14 KO versus Cyp4a14 + L-NAME

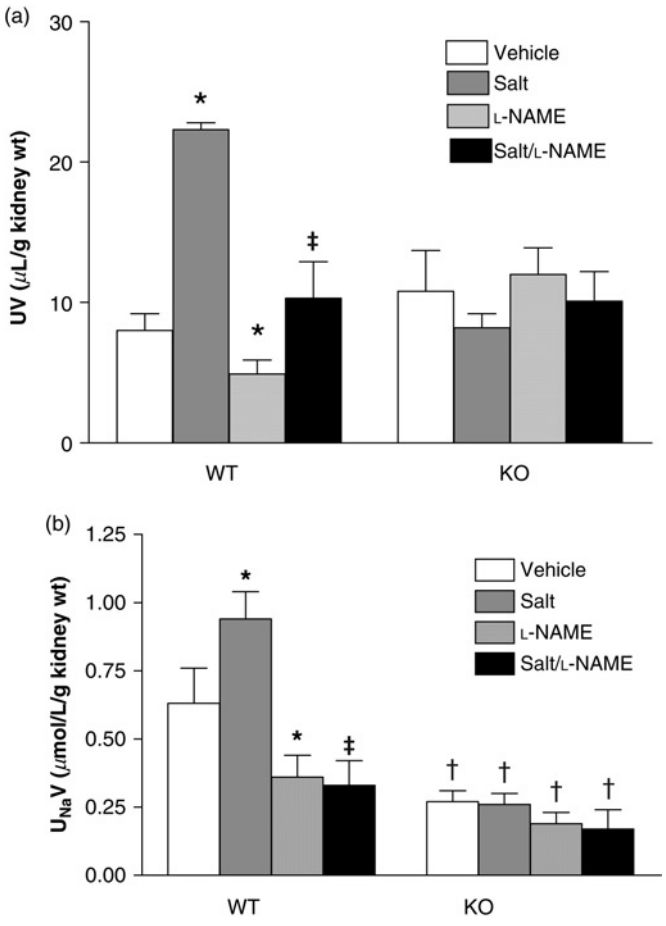

Figure 7 Urine output (UV, a) or sodium excretion ($U_{Na}V$, b) in Cyp4a14 knockout (KO) and wild-type (WT) mice under basal conditions (Control) or when challenged with acute sodium load alone (Salt), L-NAME (N^{ω}-L-nitro arginine methyl ester) (50 mg/L in drinking water for two days alone, L-NAME) or acute sodium load and L-NAME (Salt/L-NAME). $^{*}P < 0.05$ versus Control; $^{\dagger}P < 0.05$ versus Salt; $^{\ddagger}P < 0.05$ versus Cyp4a14 WT mice

well documented is the role of NO in hypertension and other cardiovascular diseases. Many studies including ours have evaluated NO/CYP4A (20-HETE) interactions and their effects on endothelial dysfunction using a number of models including the cyclosporine-induced[35] and androgen-mediated hypertension.[19] However, the Cyp4a14 null mouse presents a possible unique model because it is the result of genetic not drug-based manipulation.

Using the Cyp4a14 null mouse which has been shown to exhibit increased Cyp4a12 expression and 20-HETE production,[23] the results of the current study demonstrated increased renal microsomal 20-HETE synthesis, reduced NO production and increased oxidative stress in these mice. These metabolic changes were accompanied by increased vasoconstrictor responses to GPCR agonists but reduced vasodilator responses to endothelium-dependent GPCR agonists and increased dilator response to apocynin. In addition, there was a blunted renal excretory ability in these mice. Thus, the phenotypic expression in Cyp4a14 null mice is reminiscent of the changes seen in typical hypertensive states.

As a prohypertensive agent,[1] the mechanisms by which 20-HETE promotes hypertension have been suggested to be primarily linked to its ability to sensitize constrictor responsiveness and increase vascular resistance[36,37] and to cause activation of endothelial dysfunction through inhibition of NO-dependent vasorelaxation possibly via increased oxidative stress and diminished NO bioavailability.[20] The higher SBP and increased renal vasoconstriction in these experiments are consistent with the prohypertensive effect of 20-HETE and, therefore, the hypertension in Cyp4a14-deficient mice as demonstrated in the original study by Capdevilla's group.[23] In the latter study, there was also an increase in afferent arteriolar resistance in mice deficient in Cyp4a14 KO gene. Data from the current study showing enhanced agonist-induced vasoconstriction in Cyp4a14 KO mice are consistent with these observations and the enhanced vasoconstrictor responses to PE, AngII, NaF_4 and GTPγS but not to KCl suggest that genetic manipulation involving the Cyp4a14 gene confers a selective effect on agents that utilize the GPCR pathway for their activity. This notion is supported by the blunted vasodilator response to BKN but not to SNP. Such GPCR-selective agonist response may arise from subtle or profound changes in the components of a G protein machinery such as activation or sensitivity of guanine exchange

factors, activation of GDP/GTP exchange or conformational changes in the GTP-bound state or from a non-specific alteration in tissue sensitivity to GPCR agonists. Additional studies are clearly needed to address these mechanisms.

One wonders if the changes in blood pressure and vascular changes in Cyp4a14 KO mice in this study are a direct defect of NO/CYP4A(20-HETE) interactions in the kidney or a secondary consequence to elevation in systemic arterial pressure of Cyp4a14 KO mice that may perturbate this interaction in the kidney. Though we have no direct proof, our data do not appear to support that a defect in NO/20-HETE interactions was secondary to increase in blood pressure in as much as L-NAME administration did not increase perfusion pressure of the kidneys from Cyp4a14 KO mice. Moreover, in our previous studies[24] in which NO/20-HETE interaction was evaluated, a defect in NO production or increase in 20-HETE was demonstrated to be the stimulus for increase in blood pressure and not the other way round.

The reduced NO production in Cyp4a14 null mice that accompanies the increase in ω-hydroxylase activity, i.e. 20-HETE production in the present study, highlights the Cyp4a14-deficient mouse as a potentially useful model for evaluating NO/20-HETE(CYP4A) interactions and the mechanisms involved. It is clear from the results of many studies that NO is involved in the regulation of 20-HETE synthesis. Thus, addition of NO inhibited microsomal synthesis of 20-HETE and inhibition of NO increased CYP4A activity, 20-HETE synthesis and release.[15–17] The corollary is equally true: 20-HETE is involved in the release of NO from the endothelium. This is evident from the demonstration that increased CYP4A expression[19] or transduction with CYP4A2 cDNA[20] in the rat caused endothelial dysfunction manifested by impaired acetylcholine-induced relaxation by renal arteries that was offset by inhibition of 20-HETE synthesis and readily reinstated by exogenous addition of 20-HETE. The mechanisms involved in this interaction point to the fact that 20-HETE inhibits release of NO from the endothelium and interferes with its bioavailability *in vivo*.[20] The specific mechanisms that have been identified involve effects on the phosphorylation state of eNOS[38] and/or the uncoupling of eNOS activity via interference with HSP90 association[39] and/or rapid activation of reactive oxygen species generation, which, in turn, scavenge NO and reduce its bioavailability.[40] CYP4A–20-HETE may also exert a short-term effect on NO bioavailability, possibly via increased superoxide generation as suggested by Guo *et al.*[41] or a long-term effect via increased expression/protein levels of the NADPH oxidase system (see reference[30]). The demonstration that apocynin-induced vasodilation was greater in Cyp4a14 KO mice supports the increased oxidative stress in this strain.

Endothelial dysfunction is characterized by loss of NO bioavailability because of either reduced formation and/or accelerated degradation of NO. Recent studies provided a strong evidence for a mechanistic link between the vascular CYP4A/20-HETE pathway and endothelial dysfunction as increased 20-HETE was shown to impair NO production *in vitro* and of its function *in vivo*.[20,30] These novel findings implicate 20-HETE, a major eicosanoid of microcirculation, as a key regulator of eNOS and, thereby, endothelial function. The blunted endothelium-dependent vasodilator response in this study supports the presence of endothelial dysfunction in these mice. One of the implications of uncoupling eNOS activation is the production of free radicals and the demonstration of a direct correlation between 20-HETE levels and oxidative stress[22,41] validates this hypothesis. However, in order to demonstrate the applicability of *in vitro* observations of eNOS uncoupling/oxidative stress in the intact animal, we, therefore, evaluated the possibility of this mechanism in Cyp4a14 null mice. The greater increase in 20-HETE synthesis and lipid hydroperoxide production in Cyp4a14 null mice supports free radical-induced reduction in renal NO production/excretion, thus implying eNOS uncoupling in these mice. This observation was underscored by the demonstration that not only was there a greater vasodilator response to apocynin but there was also increased apocynin-inhibitable NADPH oxidase activity in vessels from Cyp4a14 null mice that was selectively impaired following treatment with NOS inhibitor. This observation suggests that uncoupled eNOS is probably the source of the increased NADPH oxidase activity or lipid peroxidation in Cyp4a14 null mice. Based on these observations, we opine that eNOS uncoupling probably accounts for the phenotypic expression of greater blood pressure, enhanced vasoconstriction and diminished vasodilation in these mice. In support of our hypothesis, we will expect that blockade of the function and/or synthesis of 20-HETE will attenuate 20-HETE-induced eNOS uncoupling in Cyp4a14 KO mice and thereby eliminate the differences in blood pressure and vascular responses between Cyp4a14 KO and WT mice.

As CYP4A expression/activity is expressed in both vascular and tubular sites within the kidney,[1] we evaluated for the first time, renal function in Cyp4a14 null mice. In the original study that characterized these mice, there was a blunted autoregulatory response,[23] implying a compromised excretory ability of Cyp4a14-deficient kidneys. In evaluating this untested notion, we determined sodium excretion and diuresis in acute sodium-loaded animals. Surprisingly, there was blunted diuresis and natriuresis in Cyp4a14 null mice following a sodium load. The additional observation that salt-stimulated NO production was also blunted in Cyp4a14-deficient mice supports our hypothesis that increased 20-HETE production in these mice was accompanied by diminished production of NO. The data showing a diminished excretory ability consequent to deletion of Cyp4a14 gene is consistent with the blunted autoregulatory response in Cyp4a14-deficient mice[23] that probably confers the hypertensive phenotype in these mice. At first glance, this observation negates the known natriuretic effect of 20-HETE[27] on account of its ability to inhibit ion transport in different segments of the nephron including the proximal tubule and the medullary thick ascending limb of Henle[1] where it is localized. Data from this study led us to posit that deletion of Cyp4a14 gene may confer a differential effect on the tubular versus vascular compartments in the kidney. Such a notion is consistent with the demonstration that hypertension resulted from increased 20-HETE production in renal vessels[1] as did a

defect in 20-HETE production in the medullary thick ascending limb of the loop of Henle accounting for hypertension in the Dahl salt-sensitive rat.[42] In the absence of Cyp4a14 expression data in these studies, we are unable to categorically define a differential Cyp4a14 expression/20-HETE production and its phenotype in tubular versus vascular compartments. However, we speculate that 20-HETE produced differential effects in the setting of these experiments: a prohypertensive effect in the vascular compartment versus an antihypertensive effect in the tubular compartment akin to the observation in the rat. Given the known effect of NO as an inhibitor of ion transport in different nephron segments and to promote diuresis,[43] an additional mechanism that appears to be operative in these mice could be reduction in renal tubular NO production (resulting from elevated 20-HETE) leading to a blunted diuresis/natriuresis. Future studies will propose to link changes in 20-HETE production in different nephron segments to specific ion transport mechanisms typical of those segments, e.g. Na^+K^+ATPase or Na^+H^+ exchanger (NHE) in the proximal tubule or $Na^+K^+2Cl^-$ (NKCC) transport in the thick ascending limb of the loop of Henle.

In conclusion, we have presented evidence to demonstrate that the Cyp4a14 KO mice exhibited a hypertensive phenotype which can be accounted for by 20-HETE-induced reduction in NO production via eNOS uncoupling that leads to increased oxidative stress, increased vasoconstriction but diminished vasodilation and blunted renal functional response to salt loading. Additional studies are clearly needed to address many molecular pathways that impact the renal vasculature and the tubules in an attempt to address a number of unresolved issues in this study.

Author contributions: AOO conceptualized and designed the study, did the analysis of the data and wrote the manuscript; PF conducted the experiments and did data analysis; LW, KT and MV conducted the experiments.

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