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Arachidonic Acid Monooxygenase: Genetic and Biochemical Approaches to Physiological/Pathophysiological Relevance

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Abstract

Studies with rat genetic models of hypertension pointed to roles for the CYP2C and CYP4A arachidonic acid epoxygenases and ω -hydroxylases in tubular transport, hemodynamics, and blood pressure control. Further progress in defining their physiological functions and significance to human hypertension requires conclusive identifications of the relevant genes and proteins. Here we discuss unequivocal evidence of roles for the murine *Cyp4a14*, *Cyp4a10*, and *Cyp2c44* genes in the pathophysiology of hypertension by showing that: a) *Cyp4a14*(^{-/-}) mice develop sexually dimorphic hypertension associated with renal vasoconstriction, and up-regulated expression of *Cyp4a12a* and pro-hypertensive 20-hydroxyeicosatetraenoic acid (20-HETE) levels, and b) *Cyp4a10*(^{-/-}) and *Cyp2c44*(^{-/-}) mice develop salt sensitive hypertension linked to downregulation or lack of the *Cyp2c44* epoxygenase, reductions in anti-hypertensive epoxyeicosatrienoic acids (EETs), and increases in distal sodium reabsorption. Based on these studies, the human CYP4A11 and CYPs 2C8 and 2C9 genes and their products are identified as potential candidates for studies of the molecular basis of human hypertension.

Keywords

P450; arachidonic acid; epoxygenase; 20-HETE; EET; hypertension

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Jorge H. Capdevila: developed and characterized *Cyp4a* and *Cyp2c44* knockout mice lines, and wrote substantial portions of the manuscript.

Wen-Hui Wang: Identified and characterized roles of *Cyp4a* and *Cyp2c* and EETs in ENaC gating, and of high salt or high potassium diets in sodium excretion. Review the manuscript.

John R. Falck: Synthesized all P450 eicosanoids, EET analogs, and 20-HETE antagonist described in this work and/or published studies by the authors and collaborators. Review the manuscript.

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Cytochrome P450 (P450) hemeproteins belong to a complex gene superfamily expressed from bacteria to man, with approximately 57 genes identified in the human genome. The pharmacological and toxicological importance of mammalian P450s is well established, however, less is known regarding their physiological and/or pathophysiological relevance. The demonstration of roles for microsomal P450s in the metabolism of arachidonic acid (AA) and other polyunsaturated fatty acids suggested functional roles for the enzymes involved in these reactions. The subsequent characterization and chemical synthesis of most of the products generated from AA metabolism by the P450 enzymes led to the identification of the important biological activities associated with several of them (1-4). These discoveries opened new avenues for studies of the physiological/pathophysiological relevance of P450 and roles in diseases such as hypertension, cancer, and inflammation (5-7).

The P450 Monooxygenase metabolizes AA primarily to: a) four regioisomeric epoxyeicosatrienoic acids (EETs)(*Epoxygenase*), and/or b) 19- and 20-hydroxyeicosatetraenoic acids (19- and 20-HETE)(*$\omega/\omega-1$ hydroxylase*), with members of the *CYP2* and *CYP4* gene subfamilies identified as the predominant epoxygenases and ω -hydroxylases, respectively, in most rodent and human tissues (1) (Figure 1).

The enzymatic hydration of 8,9-, 11,12-, and 14,15-EET to dihydroxyeicosatrienoic acids (8,9-, 11,12-, and 8,9-DHET) was shown to be predominantly catalyzed by soluble (cytosolic) epoxide hydrolase (sEH) in 1983 (8). Subsequently, roles for sEH in the *in vivo* hydration of EET were proposed based on its stereoselectivity for the EET enantiomers found endogenously in organ tissues (9). Since then, extensive inhibitor studies characterized sEH as a key regulator of EET organ levels and functional responses, as well as target for drug development (reviewed in references 10 and 11).

The identification of EETs and 20-HETE as components of human and rodent organs, urine, and plasma established the epoxygenase and ω -hydroxylase branches of the AA Monooxygenase as formal metabolic pathways (Figure 1), and suggested that their metabolites were functionally relevant (1-7). While the EETs have been characterized as vasodilator and pro-angiogenic lipids and as mediators of peptide hormonal release and signaling, nociception, and distal sodium excretion (3-6); 20-HETE has been identified as inhibitor of Na/K-ATPase and proximal tubule transport, and as a potent vasoconstrictor (2,5,7). Nonetheless, the identification of the epoxygenase and ω -hydroxylase P450 isoforms responsible for the *in vivo* biosynthesis of bioactive metabolites has been complicated by a multiplicity of P450 isoforms that share extensive amino acid sequence homology, metabolize AA to similar products, and often show common immunological determinants. The identification of the functionally significant enzymes is urgently needed to define their physiological contributions, mechanism(s) of action, regulatory control, and genetic properties. Several lines of evidence indicated that members of the *CYP2C* gene subfamily could be responsible for the biosynthesis of functionally important EETs in renal and vascular tissues, including: a) the characterizations of rat *CYP2C23* and its murine homologue, *Cyp2c44*, as stereo selective epoxygenases and as the predominant epoxygenases in rat and mouse kidney, b) the identification of renal *CYP2C23* and *Cyp2c44* as dietary salt regulated epoxygenases, and c) the demonstration of reduced *CYP2C23*

expression and EET biosynthesis in the kidneys of hypertensive Dahl salt sensitive rats (2-6,12-15). Similarly, roles for rat CYP4A and mouse Cyp4a isoforms in the biosynthesis of functionally relevant 20-HETE were indicated by: a) the documentation of up-regulated renal CYP4A expression and 20-HETE biosynthesis during the onset of hypertension in the SHR/WKY rat model of spontaneous hypertension, b) differences in CYP4A2 expression and 20-HETE biosynthesis between salt resistant and sensitive Dahl rats (DR and DS genotypes, respectively), and c) antisense nucleotide inhibition of renal CYP4A1/CYP4A2 expression and normalization of the blood pressures of hypertensive SHR rats (2,5-7,16,17). Based on the above, as well as their tubular and vascular effects, anti- or pro-hypertensive properties were proposed for EETs and 20-HETE, and their corresponding CYP2C and CYP4A isoforms (16).

The availability of rat models of genetically determined hypertension opened the door to studies of gene-phenotype associations between products of the *CYP2C* and *CYP4A* genes and blood pressure control (2,5-7,16,17). However, the multi-genic and complex nature of the SHR/WKY and Dahl genetic models of hypertension precluded an unequivocal identification of roles for distinct P450s genes in blood pressure control. The advent of gene targeting techniques and the development of mouse models of monogenic dysfunction allows now studies of the physiological and pathophysiological significance of specific P450 isoforms. To date, mouse lines carrying disrupted copies of the genes coding for *Cyp2c44*, *Cyp2j5*, *Cyp4a10*, *Cyp4a14*, and *Cyp4f4* have been generated and characterized to variable extents (18-22). There is published evidence for the involvement of *Cyp2j5* in estrogen, but not AA metabolism (19), and for *Cyp4f4* in vitamin E catabolism (21). We summarize herein studies performed in wild type (WT) and in mice carrying globally disrupted copies of the *Cyp4a10* (*4a10 KO*), *Cyp4a14* (*4a14 KO*), or *Cyp2c44* (*2c44 KO*) genes (in isogenic *129SeV* backgrounds) that identify roles for these P450 genes on the control of blood pressures. Although not discussed, recent studies with *2c44 KO* mice also revealed that lack of the Cyp2c44 epoxygenase blunts tumor angiogenesis and growth (23).

A) Roles of the murine Cyp4a genes in blood pressure regulation

The mouse genome contains four *Cyp4a* genes: *Cyp4a10*, *Cyp4a12a*, *Cyp4a12b*, and *Cyp4a14* localized in chromosome 4. The *Cyp4a12a* and *Cyp4a12b* genes share extensive intron/exon sequence identity (24) and code for the only mouse Cyp4a proteins with significant 19- and 20-HETE synthase activity (Figure 1)(18,24). *Cyp4a12a* is expressed in male kidneys in an androgen sensitive manner (18,24). Two highly homologous *CYP4A* genes, *CYP4A11* and *CYP4A22*, are present in chromosome 1 of the human genome (Figure 1)(25). While *CYP4A11* metabolizes AA to mostly 20-HETE, an abnormal heme prosthetic group in *CYP4A22* renders it catalytically inactive (26).

a) *Cyp4a14*(-/-) mice, a model of androgen sensitive hypertension

A link between the *Cyp4a14* gene and blood pressure regulation was established by the demonstration that its disruption causes a type of hypertension that is sexually dimorphic, male specific, and associated with: a) increases in plasma androgens, b) up-regulated kidney expression of *Cyp4a12a*, and c) increased urinary excretion of 20-HETE (18). Castration lowers renal *Cyp4a12a* expression and normalizes the pressures of hypertensive *4a14 KO*

mice. Testosterone or dehydrotestosterone administration restores renal Cyp4a12a expression and the hypertensive phenotype of castrated *4a14 KO* mice (18). The pro-hypertensive role of Cyp4a12a and 20-HETE was confirmed when it was shown that overexpression of Cyp4a12a in transgenic mice raises renal 20-HETE levels and causes androgen-independent hypertension (27). The demonstration that hypertensive *4a14 KO* mice display increased renal vascular resistance and impaired microvascular auto-regulatory capacity, suggested that increased afferent arteriole resistance compromises the animal's tubular excretory capacity (18). Augmented proximal tubule fluid reabsorption was also implicated in the hypertensive phenotype of *4a14 KO* mice (28).

b) Cyp4a10(-/-) mice show salt sensitive hypertension

Male and female *4a10 KO* mice on low salt diets (0.05% NaCl) are normotensive but become hypertensive when fed diets containing normal (0.3%)(NS) or high salt (8% NaCl) (HS)(20). Compared to WT, *4a10 KO* mice show similar kidney Cyp4a12a expression and urinary 20-HETE levels but decreased renal Cyp2c44 epoxygenase expression and EET biosynthetic capacity (20). Hypertensive *4a10 KO* mice have a hyperactive collecting duct (CD) epithelial sodium channel (ENaC) and become normotensive when administered amiloride, an inhibitor of ENaC gating activity (20). Inasmuch as EETs, but not 20-HETE, inhibit ENaC gating (29), these results: a) pointed to roles for the *Cyp4a10* gene in the regulation of kidney Cyp2c44 expression and EET biosynthesis by dietary salt, and b) suggested that deficiencies in EET-mediated regulation of ENaC activity and distal Na reabsorption were responsible for the salt sensitive phenotype of *4a10 KO* animals (20). The transcriptional regulation of *CYP4A* and *CYP2C* gene expression by peroxisomal proliferator activated nuclear receptor alpha (PPAR α) ligands is established (23,30,31). Wyeth 14,643, a selective PPAR α ligand, increases renal Cyp2c44 expression and EET biosynthesis and normalizes the pressures of hypertensive *4a10 KO* mice (20).

Summarizing, the hypertensive phenotypes resulting from disruption of murine *Cyp4a14* and *Cyp4a10* pointed to pro-hypertensive and anti-hypertensive roles for products of these genes, respectively, and provided unequivocal evidence for their roles in the control of systemic blood pressures (18,20). Importantly, associations between hypertension and a variant of the human *CYP4A11* gene coding for a reduced 20-HETE synthase activity have been identified in different population studies (26,32-34) and linked to salt sensitive hypertension (35).

B) Roles of the murine Cyp2c44 gene in blood pressure regulation

The demonstration that EETs inhibited sodium reabsorption in dissected rabbit cortical CDs (5,6), that 11,12-EET is a powerful inhibitor of ENaC gating (29), and that the hypertensive phenotype of *4a10 KO* mice was associated with down-regulated expression of renal Cyp2c44 (20) implicated the Cyp2c44 epoxygenase in the biosynthesis of antihypertensive EETs. The murine *Cyp2c* gene subfamily is composed of a complex group of genes and pseudo-genes (non-coding sequences) localized in chromosome 19. Approximately five *Cyp2c* genes code for epoxygenases with variable degrees of catalytic turnover and/or regio- and stereoselectivities (36). Among these, Cyp2c44 is unique in that: a) it catalyzes the stereo-selective epoxidation of AA to 14,15-, 11,12-EET and minor amounts of 8,9-EET

(13), b) its amino acid sequence shows borderline homology to other Cyp2c proteins, and c) the gene is separated from the *Cyp2c* gene cluster by a 4.1 Mb interval, suggesting an structural basis for its unique responses to dietary salt and PPAR α ligands. Four *CYP2C* genes are present in chromosome 10 of the human genome: *CYP2C8*, *CYP2C9*, *CYP2C18* and *CYP2C19*, with only *CYP2C8* and *CYP2C9* coding for proteins with significant AA epoxygenase activity (Figure 1)(37). Rodent Cyp2c44 and 2C23 are catalytic homologues of the human CYP2C8 and 2C9 epoxygenases (12,13,37).

Cyp2c44(-/-) mice. A model of monogenic salt sensitive hypertension. EETs as natriuretic lipids

Key roles for the Cyp2c44 epoxygenase in the control of systemic blood pressure were unequivocally identified by the demonstration that lack of a functional *Cyp2c44* gene causes a type of hypertension that is, like most human hypertension, sensitive to dietary salt intake (22). As shown in Figure 2A, male WT and *2c44 KO* mice fed NS salt diets are normotensive (systolic pressures of 120 ± 3 and 124 ± 3 mm Hg, respectively)(22). In contrast, while WT mice on HS diets remain essentially normotensive, under similar conditions, *2c44 KO* animals become severely hypertensive (systolic pressures of 134 ± 2 and 168 ± 4 mm of Hg for WT and *2c44 KO* mice, respectively)(Figure 2A)(22).

Measurements of total urinary epoxygenase metabolites (EETs + DHETs), as estimates of *in vivo* kidney epoxygenase activity, showed that while WT mice on a HS diet increased the excretion of epoxygenase products by approximately 3 fold (from 0.8 ± 0.2 to 2.4 ± 0.3 ng/mg of creatinine) and remained normotensive, *2c44 KO* mice lacking a salt inducible Cyp2c44 epoxygenase were unable to do so and became hypertensive (*2c44 KO*: 0.9 ± 0.3 ng/mg of creatinine)(22). The dietary salt regulated nature of the Cyp2c44 epoxygenase is indicated by the fact that WT and *2c44 KO* mice on NS show similar levels of urinary epoxygenase metabolites (0.8 ± 0.2 and 0.7 ± 0.1 ng/mg of creatinine, respectively) suggesting that they are the product of alternate, non-salt sensitive, epoxygenases such as Cyp2c29 and/or Cyp2c38 (36). These studies revealed that the *Cyp2c44* genotype-dependent effects of HS on blood pressure were paralleled by changes in urine excretion of epoxygenases metabolites (22). The determining role played by EETs in the hypertensive phenotype of *2c44 KO* mice was confirmed when administration of a synthetic EET analog reversed the *2c44 KO* phenotype by increasing sodium excretion and normalizing the pressures of salt loaded *2c44 KO* mice (38).

As with *4a10 KO* mice (20), amiloride lowered the blood pressures of salt loaded *2c44 KO* mice to levels comparable to WT mice thus, uncovering the decisive impact of ENaC dysfunction upon the hypertensive phenotype of these animals (22). Electrophysiology studies in dissected CDs showed that disruption of the *Cyp2c44* gene caused a constitutive activation of ENaC and increased inward sodium currents (22); a phenotype similar to what it is seen in Liddle's Syndrome, a Mendelian form of human hypertension in which a hyperactive ENaC increases distal sodium reabsorption and blood pressure (39). As seen in Figure 3, AA inhibits ENaC activity in the CD of WT mice but it has no effects on *2c44 KO* mice channels. In contrast, 11,12-EET inhibits the *2c44 KO* channel, and reverses its constitutive activation (22). These results show that the effects of AA on ENaC activity

require its Cyp2c44 epoxygenase mediated conversion to EETs. Of note, 11,12-EET inhibits ENaC activity regardless of mouse *Cyp4a10* or *Cyp2c44* genotype confirming that disruption of these genes does not affect ENaC's intrinsic properties, but rather its EET-mediated regulation (20,22). An analysis of the regioisomeric composition of epoxygenase metabolites in the urine of WT and *2c44 KO* mice on HS diets showed that 11,12-epoxygenase derived metabolites were 5.6- fold lower in *2c44 KO* animals (22). In summary, these and published studies (5,6,20,22,29) identify: a) ENaC as a molecular target of the hypertensive phenotype resulting from lack of a functional, dietary salt regulated Cyp2c44 epoxygenase, and b) altered distal sodium transport as a causative agent of the *2c44 KO* hypertensive phenotype.

A recent characterization of mice with a CD specific deletion of the *Cyp2c44* gene (*CD-2c44KO*) showed that, at difference with global *2c44 KO*s, these animals become hypertensive when fed high potassium (HK) (2.5-5% KCl) but not HS diets (40). Real time quantitative PCR analysis of Cyp2c44 expression in nephron segments dissected from WT mice indicated that: a) HS diets up-regulated Cyp2c44 expression in the thick ascending limb and the distal convoluted and connecting tubule segments of the aldosterone sensitive nephron and had little or no effects on CDs, and b) the effects of HK diets were limited to proximal connecting tubules and CD segments (40). The fact that amiloride also normalizes the blood pressures of hypertensive *CD-2c44 KO* mice on HK suggests that lack of a HK sensitive Cyp2c44 epoxygenase in the CD enhances ENaC activity, increases sodium absorption, and raises blood pressure (40). Therefore, it appears that: a) up-regulated expression of Cyp2c44 in the distal convoluted and connecting tubules is sufficient to prevent excessive sodium absorption during a high sodium intake, b) conversely, when mice are fed a high potassium diet, Cyp2c44 is up-regulated in the CD and ENaC in that segment becomes a major player in maintaining sodium homeostasis (40,41), and c) the HS sensitive phenotype of mice carrying a globally disrupted *Cyp2c44* gene is associated with a hyperactive ENaC in the distal convoluted and/or connecting tubule portions of the aldosterone sensitive nephron (22,40,41). As expected from the discussed roles of HK diets on the CD expression of the Cyp2c44 epoxygenase and the inhibition of ENaC by its EET metabolites (22,29), *2c44 KO* mice lacking a Cyp2c44 epoxygenase along the extent of the aldosterone sensitive nephron, become hypertensive when fed HK diets but, under similar conditions, WT animals remain normotensive (40,41)(Figure 4)

Differential roles in sodium transport for ENaC along the aldosterone sensitive distal nephron have been reported (42), as well as the predominant roles played by this channel in the distal convoluted and connecting tubules during high sodium and low potassium intake, and for ENaC in the CD during a high potassium low salt intake (42). Studies with mice carrying a CD-selective disruption of the gene coding for the alpha subunit of ENaC showed that low sodium diets increased distal sodium reabsorption even in the absence of a functional ENaC in the animal's CDs (43). Of special relevance to this duality of functions in the aldosterone sensitive nephron is the suggestion that the amount of salt or potassium intake determines a segment selective up-regulation of Cyp2c44 and thus, its role in sodium transport and blood pressure regulation.

Several mechanisms have been identified as regulators of ENaC activity, including changes in channel proteolysis, translocation, membrane assembly, Nedd4-2 phosphorylation, ubiquitination and retrieval and degradation, as well as negative and positive effects by protein kinases including extracellular regulated kinase 1 and 2 (ERK1/2) among others (44-51). Added to that list is the identification of EETs, and 11,12-EET in particular, as a new class of *in vivo* regulators of ENaC activity (20,22,29). Furthermore, the demonstration that genetically determined or experimentally induced changes in the expression and/or activity of the Cyp2c44 epoxygenase causes salt sensitive hypertension identifies the EETs as a new class of endogenous natriuretic agents and as a compelling target for the development of new strategies for blood pressure management.

C) Mechanism(s) of EET-mediated regulation of ENaC activity and sodium excretion

Roles for epidermal growth factor (EGF) and mitogenic kinases in the regulation of ENaC activity by EETs were suggested by the conjunction of two apparently unrelated lines of studies. On the one hand, the EETs were shown to participate in EGF-mitogenic signaling and ERK1/2 activation (52,53) and, on the other hand, the demonstration that EGF inhibits ENaC activity by an ERK1/2 catalyzed threonine phosphorylation of the ENaC beta and gamma subunits (ENaC β and ENaC γ)(45,46,54,55). The *in vivo* relevance of these data was indicated by Western blot analyses showing that: a) salt loaded hypertensive *2c44 KO* mice show reduced kidney levels of anti-phospho-ERK1/2 and anti-threonine immunoreactive protein(s) with the electrophoretic mobility of ENaC γ (22), and b) compared to untreated controls, mice on HS and administered Cetuximab, an inhibitor of EGF receptor binding, show reductions in ENaC γ threonine phosphorylation (55). Importantly, a mechanistic commonality between the inhibitory effects of EGF and EETs on ENaC was further supported by the demonstration that inhibition of EGF receptor signaling (55) or disruption of the *Cyp2c44* gene and lack of the kidney Cyp2c44 epoxygenase (22) led to comparable HS mediated effects on ENaC activity, systemic BP (Figure 2A and 2B) and kidney levels of anti-phosphothreonine and -ENaC γ immunoreactive proteins (22,55). Based on these studies and similarities between EETs and EGF as inhibitors of ENaC activity and activators of mitogenic kinase pathways (22,29,45,46,52,54,55), it was proposed that: a) EGF and Cyp2c44 epoxygenase-derived EETs regulate ENaC activity by common ERK1/2-mediated mechanisms, b) the EETs mediate the effects of EGF on ENaC activity, and c) impairments in Cyp2c44 mediated ERK1/2 activation and reduced ENaC phosphorylation contribute to the hypertensive phenotype of *2c44 KO* mice. In summary, these results characterized functional roles for mitogen kinases and ERK members of mitogen activated kinase signaling cascades in renal sodium transport that appear distinct and independent of their more traditional roles on cell proliferation. The proposed participation of the Cyp2c44 epoxygenase in the *in vivo* regulation of mitogenic growth factor-ERK1/2-mediated signaling provides a common experimental platform that could explain the seemingly unrelated roles reported for the enzyme and its EET metabolites in channel activity, cell proliferation, and vasodilation (1-7,22,29,52,53). In this regard, in a xenograph model of tumorigenesis, *2c44 KO* mice show reductions in endothelial cell proliferation, tumor vascularization, and growth (23). Furthermore, in a mouse orthotopic model of human lung

cancer, peroxisomal proliferator activated receptor alpha (PPAR α) ligands down-regulate endothelial Cyp2c44 expression and reduce tumor vascularization, growth, and metastatic potential (56).

Summarizing the preceding studies, the blood pressure changes resulting from disruption of the *Cyp4a10*, *Cyp4a14* and *Cyp2c44* genes are mechanistically different and mediated by either indirect or direct gene-dependent effects:

- A. **Indirect:** these are associated with effects of the targeted gene on the expression of alternate genes. Examples of this are: 1) *Cyp4a10*(-/-) mice in which salt sensitive hypertensive is accompanied by down-regulated kidney expression of the anti-hypertensive Cyp2c44 epoxygenase, reduced urinary EET levels, and a hyperactive ENaC, and 2) *Cyp4a14*(-/-) mice in which sexually dimorphic hypertension is linked to androgen-mediated increases in the kidney expression of pro-hypertensive Cyp4a12a, increased 20-HETE excretion, and changes in renal hemodynamics and tubular excretion.
- B. **Direct:** resulting from a lack of the protein(s) encoded by the targeted gene. This is seen with salt sensitive hypertensive *Cyp2c44*(-/-) mice where lack of a functional kidney Cyp2c44 epoxygenase causes reductions in EET biosynthesis resulting in increases in ENaC activity and sodium reabsorption.

The mechanism(s) by which the *Cyp4a14* gene regulates plasma androgens and the expression of the Cyp4a12a 20-HETE synthase remains undetermined. However, sexual dimorphisms are seen in the SHR/WKY rat model of genetically determined hypertension (57), as well as in humans where hypertension is usually more prevalent and severe in males (58). It remains to be determined how the *Cyp4a10* or *Cyp4a14* genes, or for that matter HS, controls renal expression of the Cyp2c44 epoxygenase or Cyp4a12a 20-HETE synthase. In all these examples, changes in Cyp4a12a or Cyp2c44 expression are accompanied by changes in the corresponding mRNA levels (7,18,20,22,27,40) suggesting transcriptional mechanisms, however, changes in mRNA stability can be not be discarded as potential contributors. The transcriptional regulation of P450 genes, including the *CYP2C* and *CYP4A* subfamilies, by specific promoters has been reported (30,59). In this regard, rat CYP4A isoforms actively metabolize the 8,9-, 11,12-, and 14,15-EET to their corresponding 20-hydroxy-epoxides (Table 1), all of which bind and activate PPAR α with high affinity and efficiency (60). As mentioned, PPAR α ligands such as Wyeth14,643 induce the kidney expression of Cyp2c44 and normalize the pressures of hypertensive *4a10 KO* mice (20). An interesting possibility is that these epoxyalcohols could be involved in a PPAR α -mediated up-regulation of kidney Cyp2c44 by HS diets, and that the hypertensive phenotype of *4a10 KO* is due to deficits in Cyp4a10 catalyzed EET ω -hydroxylation.

Figure 5 summarizes potential interrelationships between the regulatory effects of the *Cyp4a14* and *Cyp4a10* genes on the kidney expression of pro-hypertensive Cyp4a12a and anti-hypertensive Cyp2c44 enzymes, the known effects of 20-HETE and 11,12-EET metabolites on renal vasoconstriction and sodium transport and, ultimately, on the control of kidney hemodynamics and/or tubular transport (2-7,18,20). The dashed arrow indicates the close interrelationship that exists between these endpoints in the regulation of systemic

blood pressures. The scheme is intended to suggest an explanation for the duality of functions attributed to the CYP4A enzymes as pro- and anti-hypertensive proteins (2,7). Thus, as suggested in Figure 5, their pro-hypertensive effects will be associated with up-regulated expression of rat CYP4A1/4A2 or mouse Cyp4a12a and increased biosynthesis of vasoconstrictor 20-HETE. Their anti-hypertensive responses will be associated with a *CYP4A/Cyp4a10* gene mediated upregulation of CYP2C23 or Cyp2c44 expression and EET biosynthesis in the thick ascending limb and aldosterone sensitive nephron segments. Rat CYP4A1, 4A2, 4A3, and 4A8 isoforms (Table 1)(60) as well as Cyp4a10 and Cyp4a14 proteins (unpublished data) are capable of generating putative PPAR α ligands by EET ω -hydroxylation. Of interest, the salt sensitive hypertensive phenotype of DS rats has been attributed to reductions in kidney CYP4A expression and 20-HETE formation (2), or to reduced CYP2C23 expression and EET biosynthesis (5,6,14). In both scenarios, PPAR α ligands ameliorate or reverse the hypertensive phenotypes of salt loaded DS rats and *4a10 KO* mice (2,20).

With few exceptions, our understanding of the molecular basis of prevalent forms of human hypertension remains limited. Consequently, its treatment and diagnosis continues to be challenging, particularly since its multifaceted etiology is the likely result of a multiplicity of environmental and genetic factors and coexisting conditions. A better understanding of the pathophysiological mechanisms and genetic basis of the human disease should contribute to improved clinical management and early diagnosis, and to ameliorate the devastating consequences of untreated chronic hypertension. The identification of roles for the Cyp2c44 epoxygenase in the regulation of ENaC activity, as well as the rapid nature of the inhibitory response elicited by its EET metabolites (22,29,40,41), introduces a new mechanistic paradigm to account for the regulation of this channel and distal sodium transport. Recognition that: a) the *Cyp4a14* gene and Cyp4a12a participate in the regulation of renal hemodynamics, b) the *Cyp4a10* and *Cyp2c44* genes influence tubular transport, and c) genetic or experimentally induced alterations in their function causes hypertension, offers fresh approaches for the understanding of mechanism(s) by which the kidney regulates renovascular tone, sodium excretion and, ultimately, blood pressure. Furthermore, the data discussed: a) provides an experimental foundation for future studies of the functional roles of the human CYP4A11 20-HETE synthase and CYP2C8/CYP2C9 epoxygenases in human hypertension, and b) suggest that maneuvers designed to target the expression of these genes, the biosynthesis of their metabolites, or to antagonize or mimic the functional properties of 20-HETE or 11,12-EET, respectively, could serve as a basis for the development of novel anti-hypertensive therapies. In support of these concepts, functional 20-HETE antagonists and EET analogs have been developed and their anti-hypertensive potential documented (7,38). The administration of a 20-HETE antagonist to hypertensive *4a14 KO* and androgen treated WT mice lowers their blood pressures, and prevents the onset of hypertension in doxycycline-treated Cyp4a12a transgenic mice (7,27). Similarly, orally available EET analogs lower blood pressures and increase natriuresis in salt loaded *2c44 KO* mice and ameliorate the onset of hypertension in SHR rats (38). In summary, it is expected that these studies will stimulate efforts to develop novel: a) CYP2C/EET and CYP4A/20-HETE based anti-hypertensive drugs, and b) strategies for the early detection and diagnosis of hypertension.

The studies summarized bring into consideration two important issues: *Firstly*, the implication of a role for EETs in tumor angiogenesis and growth raises pertinent questions regarding long term risks that might be associated with efforts to use inhibitors of EET metabolism as anti-hypertensive, anti-inflammatory drugs (61,62). Inhibition of enzymatic EET hydration could potentiate their mitogenic and tumorigenic activities; a possibility supported by tumorigenic studies with *2c44 KO* mice (23,56). *Secondly*, several drugs in current clinical use are metabolized by human CYP2C8 and/or CYP2C9 (63) with yet undetermined effects on the regulation and/or epoxygenase activity of these enzymes. It is therefore, advisable that current methods for drug evaluation take into consideration the potential physiological and/or pathophysiological consequences of interfering with the activity and/or expression of P450s involved in endogenous metabolic pathways.

D) Unresolved issues, perspective for the future

The current state of knowledge regarding this pathway of AA metabolism and bioactivation, and the increasing interest in its biomedical potential and/or clinical relevance, positions this area of research at the threshold of a new chapter in the studies of P450-eicosanoids and their relationship to human disease. This new chapter should include renewed efforts to identify physiologically relevant human CYP2 and CYP4 genes and enzymes, their tissue specific expression, regulatory control, signaling mechanism, and pathophysiological roles. Ultimately these efforts will serve as prelude to medicinal chemistry efforts to target human epoxygenases and ω -hydroxylases for the development of novel therapies for the treatment of cardiovascular disease, hypertension, renal injury, CNS disorders, and inflammation. Among unresolved issues we include:

A) A need for unequivocal identifications of the P450 genes and proteins responsible for the endogenous synthesis of bioactive EETs and 20-HETE

Abundant studies identified members of the CYP2C and CYP2J, and CYP4A and CYP4F gene subfamilies as functionally significant AA epoxygenase or ω -hydroxylases, respectively. However, most of these assignments are based on in vitro enzymatic activities, immunological and/or RNA based studies of expression levels, genetic associations, inhibitor studies, or functional responses to transgene induced changes in expression (2-7,41). It is expected that improvements in gene targeting and editing methods will hasten future developments of models of monogenic P450 dysfunction and lead to further progress in this important area. The identity of the human homologues of functionally relevant EET/20-HETE synthases is needed to define meaningful correlations between pathophysiological conditions and alterations in the genes coding for these enzymes and/or involved in their expression, and for the development of novel strategies for disease diagnosis and treatment.

B) The definition of EET signaling mechanism

After attempts by different groups (3,4), selective binding and trans-membrane signaling has been reported only for 14(R),15(S)-EET in guinea pig mononuclear or human U937 cells (64,65). While G-proteins, mitogenic kinases/phosphatases, cAMP-kinase A, prostanoids, PPAR nuclear receptors; as well as effects on ion channel activity have been identified as

potential mediators of EET responses (2-6), a membrane bound receptor, capable of selective EET binding and trans-membrane signaling, has yet to be identified. Exogenously added EETs readily cross cell membranes as evidenced by their rapid esterification into cellular glycerolipid pools (66); therefore, the possibility that their functional effects are mediated by extracellular or intracellular events needs to be considered.

As proposed in Figure 6: a) *extracellular signaling* would involve EET interactions with cognate receptors capable of trans-membrane signaling and amplification or, alternatively, by direct EET effects on the signaling properties of hormone receptors such the EGF-receptor (53); b) *Intracellular signaling* will require either EET crossing the cell membrane barrier by simple diffusion, carrier plasma lipoprotein uptake, or yet to be determined mechanisms. Otherwise, they could originate from phospholipase A_2 catalyzed AA release followed by P450-mediated epoxidation. Intracellular EETs could signal by direct: a) interaction with intracellular receptors such as mitogen activated protein kinases, protein phosphatases, adenylyl- and/or guanylylcyclase, G proteins, etc., b) effects on the activities of enzymatic pathways such as those catalyzed by mitogenic kinases, or c) effects on ion channel/transporter phosphorylation/de-phosphorylation. If documented, these type of intracellular actions will add EET to the list of known intracellular signal effector molecules such as cAMP and cGMP nucleotides, inositol-phosphates, NO and, in addition to a conceptual breakthrough, help rationalize many of their multiple and pleiotropic functional effects.

Less is known regarding mechanisms of 20-HETE signaling involving plasma membrane bound selective 20-HETE receptors and/or an intracellular signaling mechanism(s). As with EETs, 20-HETE shows pro-mitogenic and pro-angiogenic properties (2,7), however, more often these eicosanoids elicit opposing biological responses. For example, while EETs activate vascular smooth muscle calcium sensitive 256 pS maxi potassium channels or BK_{Ca} causing cell hyperpolarization and vasodilation, 20-HETE inhibits the BK_{Ca} channel causing cell depolarization and vasoconstriction (2,3,7). It is not understood how these two lipids regulate BK_{Ca} in opposing ways but, electrophysiology studies suggested G-protein mediation of EET responses (3). Whether unique intracellular mechanisms, G-proteins, or membrane bound receptors are involved in the channel responses to 20-HETE remains unknown.

C) The nature and source of the active EET-mediators

Although rarely recognized, between 90 to 95% of the EETs present in rodent and human tissues and plasma are found as esters of glycerophospholipids (1,4,66). These EET containing phospholipids, generated by stereoselective acylation of EET-CoA derivatives into lysolipids (66), raise important issues such as: a) their functional roles as a source of bioactive EETs made available independently of P450 catalyzed AA epoxidation, b) the existence of selective epoxy-phospholipid phospholipases A_2 responsible for EET release from distinct hormonally sensitive glycerophospholipid stores, c) whether the EET moiety confers unique functional properties to these phospholipids, d) whether epoxidation serves as a cellular mechanism to select functionally important phospholipids from merely bulk or structural pools, and e) are the EET-containing phospholipids or their di- or monoglyceride

metabolic products signaling molecules or simple reservoirs of bioactive EETs? In this regard, the 2-(11,12- and/or 14,15-epoxyeicosatrienoyl)-glycerols present in rat brain, kidney, and spleen bind and activate the CB1 and CB2 subtypes of the cannabinoid receptor (67).

D) The characterization of mechanisms by which sHE controls physiological responses

As a participant in cellular EET metabolism (1,4), roles for sHE in the pathophysiology of hypertension, renal injury, inflammation, and diabetes have been identified based mostly on inhibitor studies (10,11). However, studies with an *sEH*(-/-) mouse lacking the enzyme hydrolase and phosphatase activities yielded variable results with regards to the effects of gene disruption on EET levels, vasoactivity and blood pressures (68). Among issues in need of further study we emphasize: a) the accepted concept that EET hydration leads to functionally inactive DHETs (10,11), b) the bi-functional nature of sEH, and the coexistence of phosphatase and epoxide hydrolase domains at the protein N- and C-terminals, respectively (69), and c) the effects of sEH inhibitors on the phosphatase domain of the enzyme (68,69). Inasmuch as phosphatases play recognized physiological roles as modifiers of the functional properties of products of protein kinases, and/or phosphorylated lipid mediators this raises relevant questions regarding the contribution of the phosphatase domain to some of the functional roles described for sEH (70-72).

E) The characterization of mechanisms controlling the expression of selected AA epoxygenases and ω -hydroxylases in an organ/tissue/cell specific manner

The direct and indirect mechanisms by which P450 genes control functional phenotypes (18, 20) illustrates the importance of a gene-dependent regulation of different (and at times unrelated) genes in the development of functional phenotype(s). This added level of complexity could be of importance for the interpretation of associations between pathophysiological conditions and P450 gene variants. Furthermore, gene-mediated dysfunction can be masked or enhanced by co-existent morbidities (26,73) or be detectable under stress or non-stress conditions. Under a normal dietary salt intake *Cyp2c44*(-/-) mice show no overt blood pressure phenotypes; however, roles for this gene in blood pressure control or tumor angiogenesis are evidenced after the stress imposed by a high salt or potassium intake (22,40) or processes associated with tumorigenesis (23). At difference with *Cyp2c44*(-/-) mice, the hypertensive phenotypes of *Cyp4a10*(-/-) and *Cyp4a14*(-/-) mice are evident in the absence of dietary or other experimental manipulations (18,20).

It is of interest that the blood pressures of carriers of the *CYP4A11* 8590C allele are dietary salt sensitive (35) as is with *Cyp4a10*(-/-) mice (20), however, a relationship between this genotype, blood pressure and urinary 20-HETE excretion is yet to be established (73). Also, it remains to be determined: a) whether CYP2C8 and/or CYP2C9 derived EETs play a role in the hypertensive phenotype identified for carriers of the *CYP4A11* 8590C allele, and b) the prevalence and severity of hypertension in double carriers of the *CYP4A11* 8590C allele and *CYP2C8**3/*3 or *CYP2C9**2/*2 reduced EET synthase activity variants (74). A guanine to adenine (G/A) polymorphism in the human *CYP4F2* gene has been associated with increases in blood pressure and urinary 20-HETE excretion (7,75). However, the variant gene codes for a protein with reduced 20-HETE synthase activities due to a valine to

methionine substitution at residue 433 (75). This discrepancy between a reduced 20-HETE synthase activity and increased 20-HETE urinary excretion in carriers of the *CYP4F2* G/A variant is reminiscent of what has been reported for hypertensive *Cyp4a14*(-/-) mice (18), and suggests roles for the *CYP4F2* gene in the regulation of alternate pro- or antihypertensive gene(s).

Finally, there is a paucity of information regarding regulatory mechanisms that determines organ, tissue, and cell type selective expression of functionally significant P450 isoforms. This is relevant to their roles in kidney physiology/pathophysiology since the functional and biochemical segmentation along the length of the nephron is well known. Most of the data regarding zonal expression of CYP2C, CYP2J, CYP4A and CYP4F isoforms in rodent kidneys is based on either immunological, mRNA in-situ hybridization, or PCR amplification data (1-7). Although useful in distinguishing P450s from different gene subfamilies, these methods are limited when applied to P450s of the same gene subfamily since, in most cases, they share extensive open reading frame and amino acid sequence homology. Nevertheless, these limitations can be addressed by, for example, targeting mRNA untranslated segments, developing peptide antibodies against unique protein segments, performing differential analyses before and after gene deletion or antisense nucleotide inhibition or, when the methodology becomes more widely available, diagnostic peptide imaging of tissue sections by mass spectroscopy (23).

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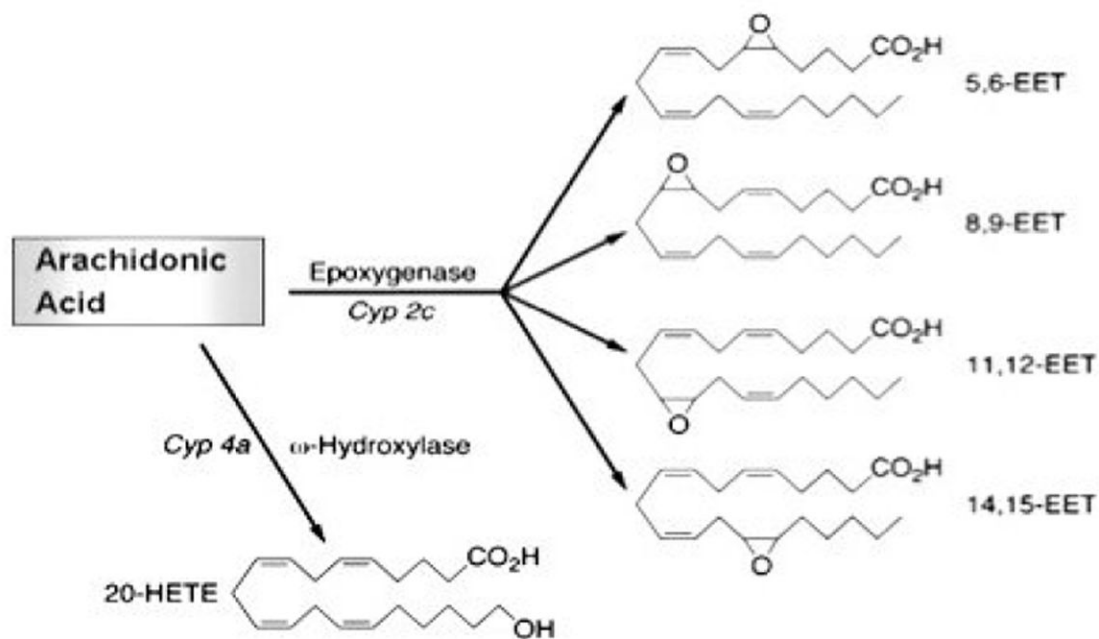
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Highlights

- A dysfunctional murine *Cyp4a14* gene causes androgen sensitive hypertension associated with increases in 20-HETE biosynthesis and renal vasoconstriction.
- A dysfunctional murine *Cyp4a10* or *Cyp2c44* gene causes salt sensitive hypertension associated with decreases in urinary EETs and a hyperactive epithelial sodium channel (ENaC).
- Roles for the *Cyp2c44* arachidonic acid epoxygenase and mitogen activated kinases in the EET-mediated regulation of ENaC activity are proposed.

The P450 Arachidonic acid Monooxygenase



CYP2C : Human **2C8, 2C9** Rodent **CYP2C23/2c44**

CYP4A : Human **4A11** Rodent **4A1, 4A2, 4A8/4a12a, 4a12a**

Figure 1. The Arachidonic Acid Monooxygenase and its Epoxygenase and ω-Hydroxylase Branches

The scheme emphasizes members of the murine Cyp2c and Cyp4a gene subfamilies as functionally relevant kidney epoxygenases and ω-Hydroxylases. Human and rodent CYP2C and CYP4A isoforms known to catalyze renal AA metabolism are indicated at the bottom of Figure 1.

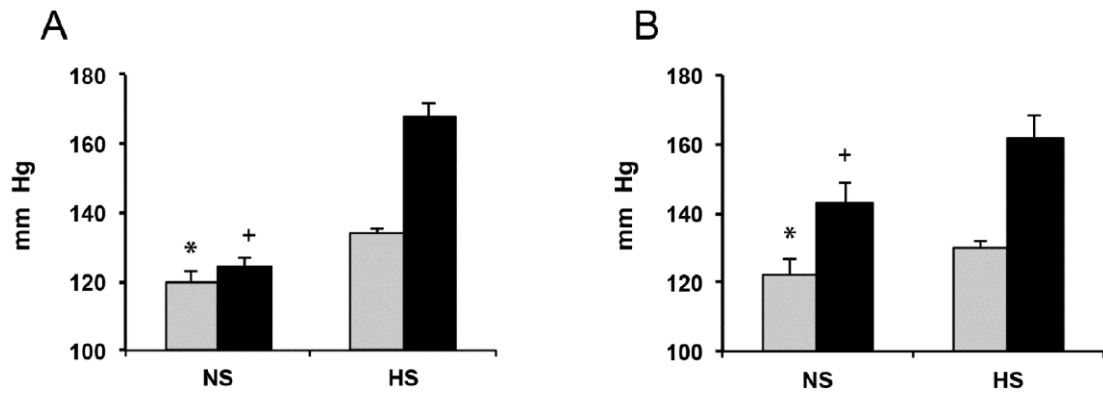


Figure 2. Global disruption of the *Cyp2c44* gene or inhibition of EGF-receptor signaling causes salt sensitive hypertension

The systolic pressures of adult male mice fed normal (NS) or high salt (HS) diets for 4 weeks were measured as described in references 20,22. Shown are averages \pm SE changes in blood pressure (in mm of Hg) for: **A**) WT and *2c44* KO mice (grey and black bars, respectively)(groups of 15 mice for WT in NS and HS and *2c44* KO on NS; and of 25 mice for *2c44* KO on HS)(25 measurements/animal), and **B**) WT mice on NS or HS were left untreated (5 and 6 mice respectively) (grey bars) or given injections of Cetuximab 8-10 days before blood pressure analyses (6 and 9 mice, respectively; every 48 hours, 12 mg/kg body weight)(black bars) as reported (55). **A**) Different from: * WT HS $p < 10^{-80}$, KO NS $p < 10^{-8}$; + KO HS $p < 10^{-270}$. The differences were between WT on NS and KO HS were non-significant ($p > 0.05$). **B**) Different from: * untreated on HS $p < 10^{-3}$, Cetuximab treated on NS or HS $p < 10^{-5}$ or $p < 10^{-6}$, respectively; + Cetuximab treated on HS $p < 10^{-4}$, untreated NS $p < 10^{-5}$.

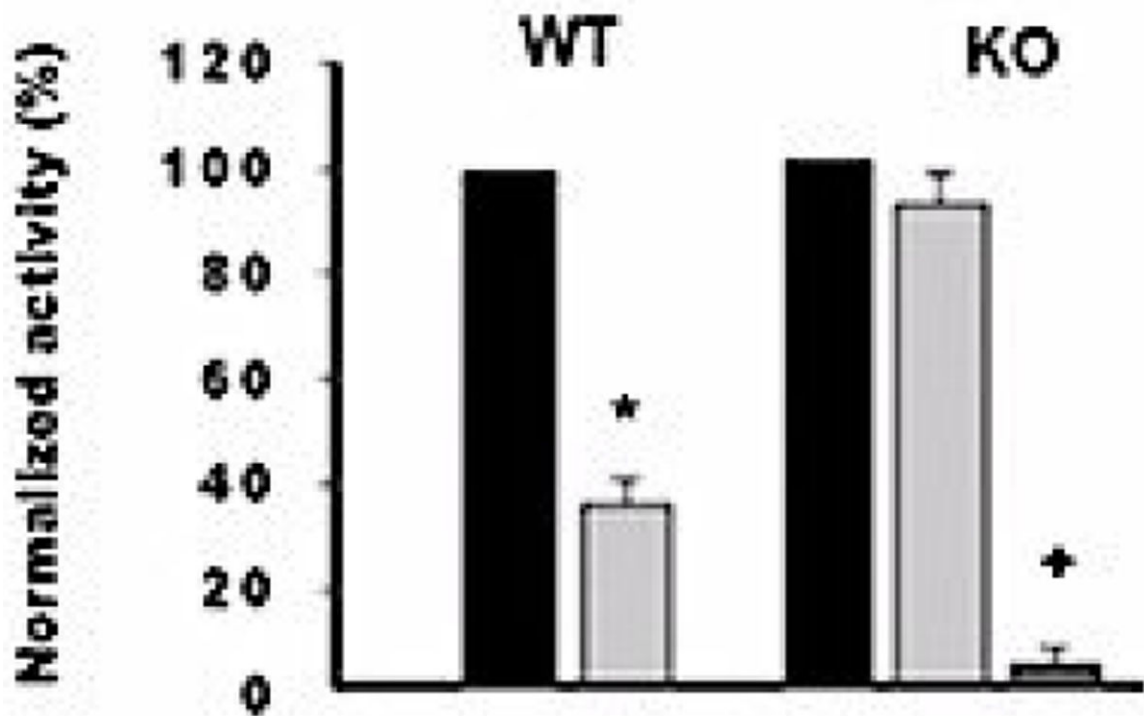


Figure 3. ENaC is hyperactive in the collecting ducts of global Cyp2c44 KO mice

ENaC gating activity was determined in patches of CDs dissected from WT and Cyp2c44 KO mice (20,22). Normalized ENaC activity (NPo) under control conditions (*black bars*), in the presence of 10 μM AA (*light gray bars*) or 0.2 μM 11,12-EET (*dark gray bar*). NPo, calculated from 60 s data samples during steady state according to: $Npo = \sum (t_1 + 2t_2 + \dots + it_i)$ (t_i = fractional open time at a given current level). Conductance was calculated from currents recorded at 3 holding potentials. Values (as a percentage of control activity) are averages \pm S.E. from 5 experiments. Different from controls: * $p < 0.01$; ... $p < 0.001$.

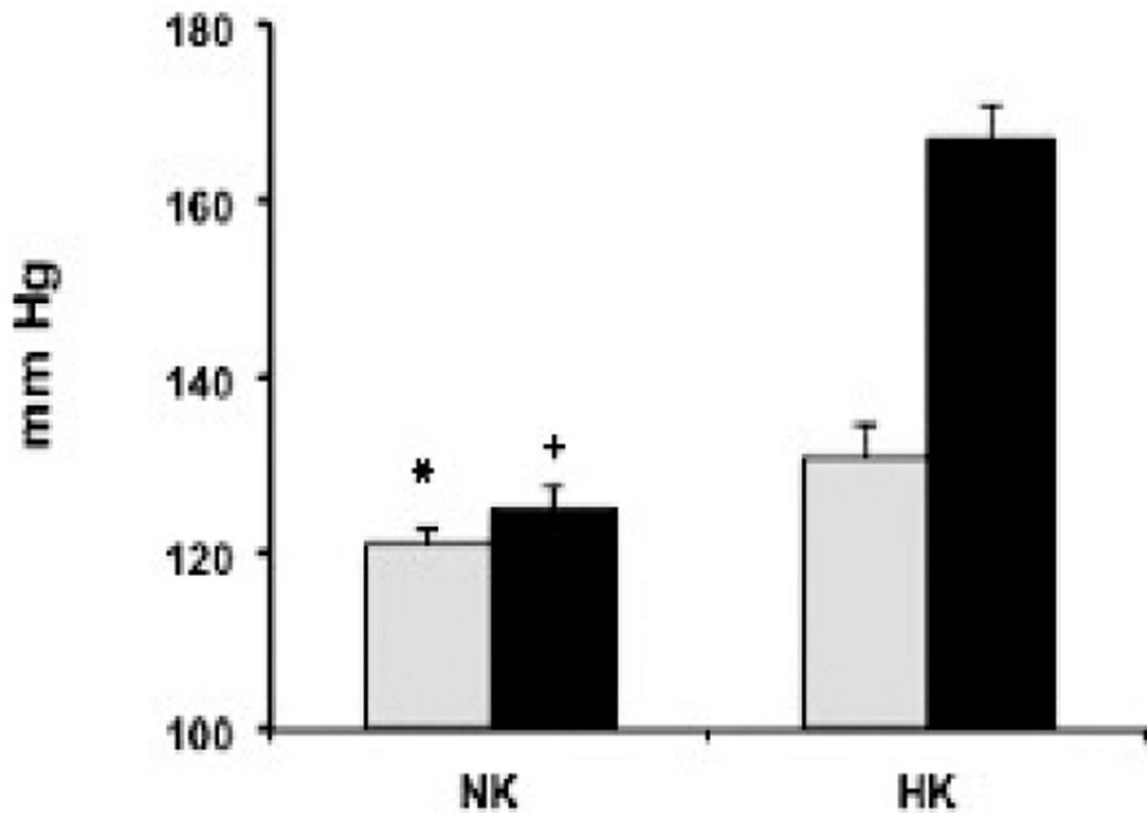


Figure 4. Global *Cyp2c44* KO mice are hypertensive when fed high potassium diets

The systolic pressures of WT and *Cyp2c44* KO male mice (grey and black bars, respectively)(8 mice each group) fed diets containing 0.3% NaCl and normal (NK) or high potassium (HK) (1.0 and 2.5% KCL, respectively)(40) for 2 weeks were measured as described (20,22). Shown are average blood pressures \pm SE (in mm of Hg) for WT and *2c44* KO mice (grey and black bars, respectively). Calculated from 30 measurements/animal. Different from: * KO on NK $p < 10^{-4}$, WT on HK $p < 10^{-20}$, KO on HK $p < 100^{-100}$; + WT or KO on HK $p < 10^{-22}$ or HK $p < 10^{-100}$.

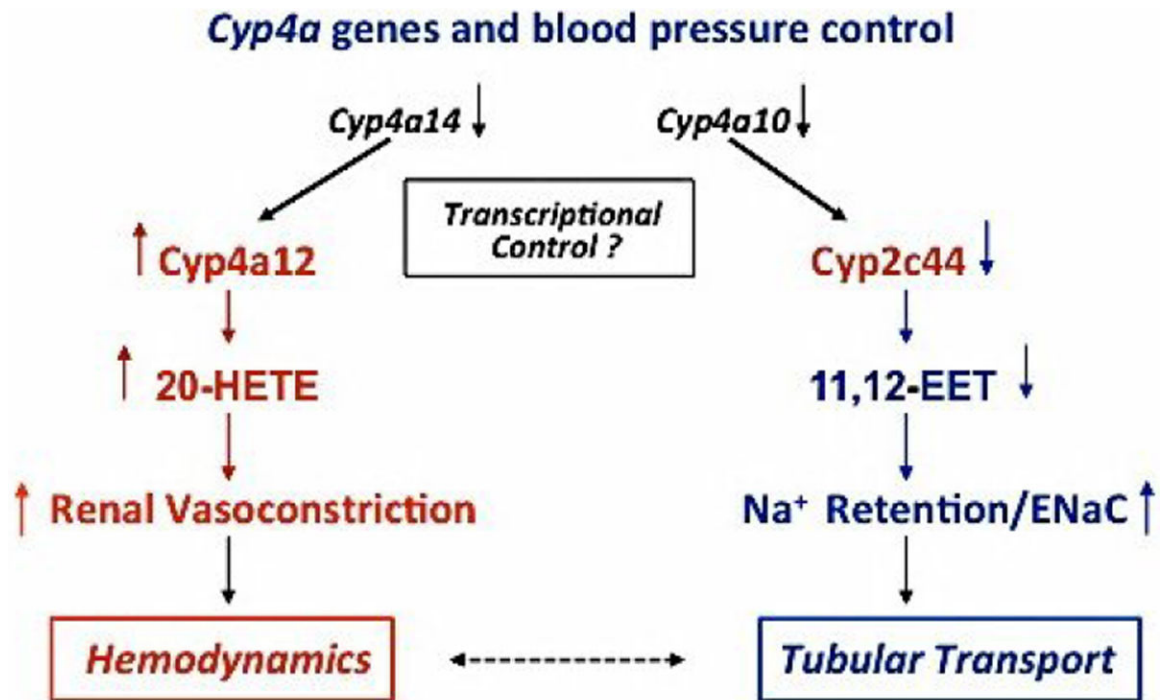
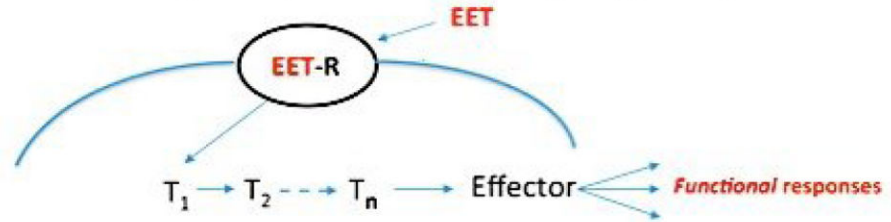


Figure 5. Schematic illustration of proposed mechanisms of blood pressure control by murine Cyp4a10 and Cyp4a14 genes

Shown are proposed hemodynamic and tubular transport responses resulting from disruption of the *Cyp4a10* and *Cyp4a14* genes and their effects on Cyp2c44 and Cyp4a12a expression, the biosynthesis of natriuretic 11,12-EET, vasoconstrictor 20-HETE, and blood pressure. Downward black arrows denote reductions or lack of gene product and/or function. Downward blue arrows indicate reductions in protein expression or product biosynthesis. The upward blue arrow signifies increases in ENaC activity and sodium retention. Upward red arrows indicate increases in protein and product formation, and renal vasoconstriction. A bidirectional dashed arrow is intended to illustrate the potential for pro- and anti-hypertensive effects of the Cyp4a isoforms as direct and/or indirect regulators renal hemodynamics and tubular transport.

Potential EET Signaling Mechanisms

A) Membrane Bound EET receptor i.e, transmembrane signaling = amplification cascade



B) Hormone/Intracellular Signaling pathways ex: cAMP, cGMP, IP₃, NO, etc

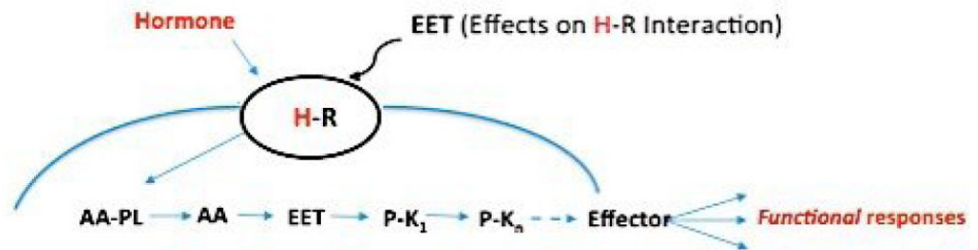


Figure 6. Potential mechanisms of EET signaling/action

Shown are: **A)** involvement of a membrane bound EET receptor capable of trans-membrane signaling/amplification, and **B)** roles for the EETs as intracellular signaling molecules capable of, for example, directly regulate protein kinase activity. Curved blue lines housing an EET (A) or hormone receptor (B) (black ovals) represent a plasma membrane barrier. **A)** T_1 to T_n symbolizes potential transducers leading to a final effector(s) and functional response. **B)** Hormone mediated intracellular release of AA and conversion to EETs capable of activating a cascade of protein kinases resulting in a functional response(s). Not shown is the possibility that EETs are released from endogenous EET containing phospholipids thus obviating a need for AA epoxidation.

Table 1
Rates of EET ω -hydroxylation by purified recombinant rat CYP4A proteins

Enzymatic activities were reconstituted in the presence of dilauroylphosphatidylcholine (50 mg/ml), by mixing each P450 enzyme with cytochrome b₅ and cytochrome P450 oxido-reductase in 1:1:10 molar ratio. Reactions were carried out at 35° in Tris-Cl buffer pH 7.4 containing 10 mM MgCl₂, 0.1 M KCl, and 1 mM NADPH. Organic soluble products were resolved by reversed phase HPLC and quantified by on-line β detection (60). Shown are average K_{cat} values \pm SE (in min⁻¹) calculated from time courses of product formation vs incubation time.

Protein	8,9-EET	11, 12-EET	14, 15-EET
CYP4A1	15.0 \pm 0.9	10.0 \pm 0.5	4.0 \pm 0.1
CYP4A2	4.0 \pm 0.2	7.0 \pm 0.4	3.0 \pm 0.3
CYP4A8	0.8 \pm 0.2	1.3 \pm 0.1	0.8 \pm 0.1

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