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# **Mouse Cyp4a Isoforms: Enzymatic Properties, Gender- and Strain-specific Expression, and Role in Renal 20-Hydroxyeicosatetraenoic Acid Formation**

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**Short title: Cyp4a and 20-HETE in mice**

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<sup>2</sup> Abbreviations used: CYP, cytochrome P450; AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; 20-COOH-AA, eicosatetraen-1,20-dioic acid; EPA, eicosapentaenoic acid; HEPE, hydroxyeicosapentaenoic acid; EETeTr, epoxyeicosatetraenoic acid; LA, lauric acid; OH-LA, hydroxy lauric acid; DDDA, dodecan-1,12-dioic acid; PUFA, polyunsaturated fatty acid; DOCA, desoxycorticosterone acetate; Ang, angiotensin; DHT, dihydrotestosterone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; PCR, polymerase chain reaction; CPR, NADPH-CYP reductase; cytb5, cytochrome b5; Sf9, *Spodoptera frugiperda*; IgG, immunoglobulin G; HPLC, high performance liquid chromatography; RP, reverse phase; NP, normal phase; GC, gas chromatography; MS, mass spectrometry; BK channel, calcium-activated potassium channel; mTALH, thick ascending limb of Henle.

## SYNOPSIS

Arachidonic acid (AA) hydroxylation to 20-hydroxyeicosatetraenoic acid (20-HETE) influences renal vascular and tubular function. To identify the cytochrome P450 (CYP) isoforms catalyzing this reaction in the mouse kidney, we analyzed the substrate specificity of Cyp4a10, 4a12a, 4a12b, and 4a14 and determined sex and strain-specific expressions. All recombinant enzymes showed high lauric acid hydroxylase activities. Cyp4a12a and Cyp4a12b efficiently hydroxylated AA to 20-HETE with  $V_{\max}$  values of about 10 nmol/nmol/min and  $K_m$  values of 20 to 40  $\mu$ M. 20-Carboxyeicosatetraenoic acid occurred as a secondary metabolite. AA hydroxylase activities were about 25-75-fold lower with Cyp4a10 and not detectable with Cyp4a14. Cyp4a12a and Cyp4a12b efficiently converted also eicosapentaenoic acid (EPA) to 19/20-OH- and 17,18-epoxy-EPA. In male mice, renal microsomal AA hydroxylase activities ranged between about 100 (NMRI), 45-55 (FVB/N, 129 Sv/J, BALB/c), and 25 pmol/min/mg (C57BL/6). The activities correlated with differences in Cyp4a12a protein and mRNA levels. Treatment with 5 $\alpha$ -dihydrotestosterone induced both 20-HETE production and Cyp4a12a expression more than 4-fold in male C57BL/6. All female mice showed low AA hydroxylase activities (15 to 25 pmol/min/mg) and very low Cyp4a12a mRNA and protein levels, but high Cyp4a10 and Cyp4a14 expression. Renal Cyp4a12b mRNA expression was almost undetectable in both sexes of all strains. Thus, Cyp4a12a is the predominant 20-HETE synthase in the mouse kidney. Cyp4a12a expression determines the sex and strain-specific differences in 20-HETE generation and may explain sex and strain differences in the susceptibility to hypertension and target organ damage.

**Keywords:** cytochrome P450, arachidonic acid, eicosapentaenoic acid, mouse, gender, kidney

## INTRODUCTION

Cytochrome P450 (CYP<sup>2</sup>) enzymes of the CYP4A subfamily are found in the liver, kidney, intestine, lung, heart and brain of various mammals including rat, rabbit, mouse, and man [1]. They conduct  $\omega$ -hydroxylation of fatty acids and related compounds. This reaction facilitates the degradation of long-chain fatty acids by initiating their conversion to dicarboxylic acids, that are efficiently oxidized by peroxisomal  $\beta$ -oxidation [2]. With arachidonic acid (AA; 20:4 n-6), CYP4A-catalyzed  $\omega$ -hydroxylation yields 20-hydroxyeicosatetraenoic acid (20-HETE). 20-HETE plays an important role in the regulation of renal vascular and tubular function. Altered renal CYP4A expression is associated with hypertension [3-5]. Urinary 20-HETE excretion is increased in patients with cirrhosis [4, 6] and endothelial dysfunction [7]. Moreover, a polymorphic CYP4A11 variant is associated with human hypertension [8]. The mechanisms linking 20-HETE to blood pressure regulation are only partially understood. Both increased and decreased 20-HETE levels can be associated with high blood pressure probably reflecting the dual role of 20-HETE to induce vasoconstriction (pro-hypertensive) and to promote natriuresis (anti-hypertensive) [5].

The CYP4A subfamily members expressed in mouse kidney include *Cyp4a10*, *Cyp4a12* and *Cyp4a14* [9-13]. Two *Cyp4a12* genes exist, *Cyp4a12a* and *Cyp4a12b*, that result from a tandem 100 kb duplication within the *Cyp4abx* cluster on chromosome 4 [14]. We showed earlier that renal 20-HETE production is decreased in desoxycorticosterone acetate (DOCA)-salt hypertensive mice [12]. Bezafibrate restored 20-HETE production and improved renal hemodynamics. Similarly, fenofibrate induced 20-HETE and prevented angiotensin (Ang) II-induced hypertension [15]. In both models, the 20-HETE producing CYP isoform(s) down-regulated in hypertension and induced by fibrates was not unequivocally identified. Targeted *Cyp4a14* gene disruption resulted in increased AA  $\omega$ -hydroxylase activities and caused hypertension in male mice [11]. The mechanism seems to involve increased plasma androgen levels in the *Cyp4a14* gene-disrupted mice followed by androgen-induced up-regulation of *Cyp4a12* [11]. *Cyp4a10* gene disruption results in salt-sensitive hypertension via affecting the regulation of the kidney epithelial sodium channel [16].

We cloned and expressed the *Cyp4a10*, *Cyp4a12a*, *Cyp4a12b*, and *Cyp4a14* cDNAs, determined the substrate specificity of the recombinant enzymes, and analyzed their strain- and gender-specific relationship to renal 20-HETE production. We tested whether or not the two *Cyp4a12* gene copies differ in terms of renal expression and enzymatic properties. For substrates, we relied on the CYP4A test substrate lauric acid (LA) to prove the successful reconstitution of enzymatic activities, AA to analyze the 20-HETE production capacity of the isoforms, and the fish oil omega-3 polyunsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA; 20:5 n-3), which can replace AA in CYP-dependent eicosanoid production. Secondary hypertension and susceptibility to target organ damage in mice is largely sex and strain dependent. For example, 129/Sv mice are more susceptible to DOCA-salt hypertension than

C57BL/6 mice [17]. Females are more resistant to Ang II-induced hypertension than males [18]. Since 20-HETE is involved in the pathophysiology of these hypertension forms [12, 15], we investigated renal CYP expression and AA metabolism in male and female mice of different mouse strains.

## **MATERIALS AND METHODS**

### **Animal strains and treatment**

12 to 15 week-old male and female mice of the following strains were used (n = 6 to 8 per gender and strain): NMRI, FVB/N, C57BL/6 (all three from Charles River Laboratories), 129 Sv/J, and BALB/c (from DIMED Schönewalde GmbH). The mice were allowed free access to standard chow (0.25 % sodium; SNIFF Spezialitäten GmbH) and drinking water ad libitum. In an additional set of experiments, male NMRI and C57BL/6 (n = 6 per group) were subcutaneously implanted for 10 days with release pellets containing 5 $\alpha$ -dihydrotestosterone (21-day pellet, 5mg DHT/day; Innovative Research of America, USA).

### **Preparation of RNA and TaqMan analysis**

Total RNA was isolated from liver, kidney, and renal arterioles using Qiashredder and RNeasy spin columns including chromosomal DNase digestion (Qiagen). For isolating preglomerular microvessels, the kidneys were cut in half along the cortico-papillary axis. The arterioles were prepared with the use of micro-scissors and forceps under an operating microscope and the surrounding tissue was carefully removed. TaqMan analysis of Cyp4a10, Cyp4a12a, Cyp4a12b, and Cyp4a14 mRNA expression was conducted using isoform-specific primers and probes as described in Supplemental online data.

### **Preparation of renal microsomes**

Renal microsomes were prepared using one and a half kidney per animal. The renal capsule and the fatty tissues were removed. Kidneys were minced and homogenized in five volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 150 mM potassium chloride, 2 mM EDTA, 2 mM DTT, 1  $\mu$ M FAD and FMN, and 0.25 mM PMSF in a motor-driven Teflon-glass Potter Elvehjem homogenizer. After differential centrifugation (10 min, 1,000 x g; 20 min, 10,000 x g; 90 min, 100,000 x g), the microsomes was suspended and homogenized in 50 mM Tris-HCl buffer, pH 7.7, containing 20 % glycerol, 5 mM EDTA, and 1 mM DTT. Aliquots were snap frozen in liquid nitrogen and stored at -80°C. The protein content was determined by the standard Lowry method.

### **Cloning of the Cyp4a-Isoforms**

The cDNAs for Cyps 4a10, 4a12a and 4a14 were generated by RT-PCR (Ready-To-Go RT-PCR kit, Amersham Pharmacia Biotech) using 300 ng of total renal RNA isolated from male NMRI as template. The Cyp4a12b cDNA was amplified and selected starting from total hepatic mRNA isolated from male C57BL/6. Reverse transcription was primed with oligo(dT) and PCR amplifications were done with the

following primer pairs (5'-3'): TCG GAA TTC GCA ATG AGT GTC TCT GCT CTA A / CCG CTC GAG CGG TCA GTG GTG GTG GTG GTG GAG CTT CTT GAG ATG TAG (forward/reverse primer for Cyp4a10 with 5'-added *Eco* RI- and *Xho* I-site, respectively), ACG CGT AAG CTG TTG TAT CAT GAG TGC / GCT AGC CAT TTG AGC TGT CTT GTT CTG (forward/reverse primer for Cyp4a12a and Cyp4a12b with 5'-added *Mlu* I- and *Nhe* I-site, respectively), and CGA CTC GAT CCA GAA ACT AC / ACA GGA CAC ATT GCA GAG AG (forward/reverse primer for Cyp4a14). The PCR products (about 1.6 kb) were cloned into the plasmid pCR2.1 (TOPO-TA-Cloning kit, Invitrogen) and at least three different clones per isoform were sequenced (Invitrogen GmbH). For cloning of Cyp4a12b, the RT-PCR product obtained with the Cyp4a12a/Cyp4a12b-specific primers was first digested with *Avr* II, which selectively cleaves the Cyp4a12a cDNA and thus excludes it from cloning.

### Expression of CYP enzymes

Coexpression of the individual CYP isoforms with the human NADPH-CYP reductase (CPR) was performed as described previously [19] using a baculovirus/Sf9 insect cell system (for the details see Supplemental online data). The CYP concentrations were estimated by means of CO-difference spectra using an extinction coefficient of  $91 \text{ mM}^{-1} \text{ cm}^{-1}$  [20]. CPR activities were assayed in 50 mM Tris-HCL buffer, pH 7.5, containing 0.1 mM EDTA, 0.05 mM cytochrome c, 0.1 mM NADPH, and 2.2 mM KCN at 25°C using an extinction coefficient of  $21 \text{ mM}^{-1} \text{ cm}^{-1}$  at 550 nm. The microsomes used for kinetic analysis had molar CYP:CPR ratios in the range of 1:0.9 to 1:1.2. For calculation, we assumed that 4.5 U ( $\mu\text{mol cytochrome c/min}$ ) correspond to 1 nmol CPR based on the specific activity of the purified 79 kDa enzyme (60 U/mg).

### Immunoblotting

Microsomal protein was separated on 10% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Life Sciences). An antibody raised in goat against purified rat CYP4A1 (Daiichi Pure Chemicals Co., Ltd.) and a peptide-specific antibody raised in rabbits against mouse Cyp4a12a were used as primary antibodies. The antigenic peptide (NH<sub>3</sub>-SRRIQLQDEEELEKLKKRR-COOH) corresponded to amino acids 266-286 of the Cyp4a12a protein (peptide synthesis by BIOSYNTAN GmbH, immunization by BioGenes GmbH). Anti-goat IgG and anti-rabbit IgG peroxidase conjugate (Sigma Chemical Co) were used as secondary antibodies. Blots were developed with the chemiluminescence substrate from Boehringer-Mannheim and evaluated with the Image Reader LAS-1000 (Fuji Photo Film Co). Ponceau staining was used for verifying equal protein loading.

### Fatty acid metabolism

[1-<sup>14</sup>C]LA (2.11 GBq/mmol, Amersham Bioscience), [1-<sup>14</sup>C]AA (2.07 GBq/mmol, Amersham Bioscience) and [1-<sup>14</sup>C]EPA (2.0 GBq/mmol, Perkin Elmer Inc.) were used as substrates. Standard reactions with the recombinant Cyp4a isoforms were performed in 100  $\mu\text{l}$  100 mM potassium phosphate

buffer, pH 7.2 containing 10 pmol Cyp and the substrate at a concentration of 10  $\mu\text{M}$  (AA and EPA) or 30  $\mu\text{M}$  (LA). Reactions were started with NADPH (1 mM final concentration) after preincubating the microsomes with the substrates for 10 min at 37 °C. Reactions were terminated adding citric acid (20 mM final concentration). Reaction products were extracted into ethyl acetate, evaporated under nitrogen, and resuspended in ethanol. Incubations with cytochrome b5 (cytb5; Calbiochem) were done in a molar Cyp:cytb5 ratio of 1:1. Microsomal enzymes and cytb5 were preincubated in a total volume of 15  $\mu\text{l}$  for 10 min on ice. For kinetic analysis, standard reactions were performed with seven different substrate concentrations between 1.25 and 50  $\mu\text{M}$  and the experiments were done in triplicate. Reactions were terminated after 1 min (LA) or 3 min (AA and EPA). For analysis of regio- and stereoselectivities, standard reactions were up-scaled (4- to 10-fold; incubation times up to 10 min). With mouse renal microsomes, the standard reaction conditions described above were modified. The 100 mM potassium phosphate buffer, pH 7.2 was supplemented with 1 mM EDTA and 50 nM FAD and FMN and reactions were performed using 800  $\mu\text{g/ml}$  renal microsomal protein with 40  $\mu\text{M}$  AA for 30 min or with 200  $\mu\text{M}$  LA for 3 min.

#### **Soluble epoxide hydrolase assay**

Soluble epoxide hydrolase activities were determined using the 10,000 x g supernatants obtained during the preparation of renal microsomes as described above. Reactions were performed at 37 °C for 10 min in 100  $\mu\text{l}$  100 mM potassium phosphate buffer, pH 7.2 containing 50  $\mu\text{M}$  [1- $^{14}\text{C}$ ]-14,15-epoxyeicosatetraenoic acid (EET). [1- $^{14}\text{C}$ ]-14,15-EET was prepared by chemical oxidation of radiolabeled AA according to [21]. The reactions were started by adding the 10,000 x g supernatant (5  $\mu\text{g}$  of protein), and extracted as described above.

#### **Analysis of metabolites**

Metabolites were analyzed using the HPLC system LC-10Avp from Shimadzu equipped with a radioactivity monitor (LB509, Berthold). Total metabolites were resolved by reversed-phase (RP)-HPLC on a Nucleosil 100-5C18 HD column (250 x 4 mm; Macherey-Nagel). LA metabolites were esterified with diazomethane and resolved in RP-HPLC with a linear gradient of acetonitrile/water/acetic acid (29.5/70.5/0.1, v/v/v) to acetonitrile/water/acetic acid (59.5/40.5/0.1, v/v/v) over 30 min followed by 15 min acetonitrile/acetic acid (100/0.1, v/v) at a flow rate of 1 ml/min. AA and EPA metabolites were resolved with a linear gradient of acetonitrile/water/acetic acid (50/50/0.1, v/v/v) to acetonitrile/acetic acid (100/0.1, v/v) over 40 min at a flow rate of 1 ml/min. 19- and 20-HETE as well as 19- and 20-hydroxyeicosapentaenoic acid (19/20-HEPE) were resolved by normal-phase (NP)-HPLC on a Nucleosil 100-5 column (250 x 4 mm; Macherey-Nagel) utilizing a linear gradient from hexane/2-propanol/acetic acid (99/1/0.1, v/v/v) to hexane/2-propanol/acetic acid (98.3/1.7/0.1, v/v/v) over 40 min at a flow rate of 1 ml/min as described [22]. 16-, 17-, and 18-HETE were resolved with hexane/2-propanol/ acetic acid

(100/0.4/0.1, v/v/v) at a flow rate of 1.5 ml/min according to [23]. 17,18-epoxyeicosatetraenoic acid (17,18-EETeTr) was treated with diazomethane and the methyl ester was then resolved into the R,S and S,R enantiomers on a Chiracel OB column (250 x 4.6 mm; Daicell) using a linear gradient from hexane/2-propanol (99.7/0.3, v/v) to hexane/2-propanol (98/2 v/v) over 40 min at a flow rate of 1 ml/min [24]. Preparation of authentic LA [25], AA [26, 27] and EPA [24, 26, 28] metabolites was performed as described in Supplemental online data.

### Statistical Analysis

All data derived from the animal experiments are presented as means $\pm$ SEM and were analyzed by ANOVA followed by the Tukey-Kramer multiple comparison test (InStat software; GraphPad Software Inc). The significance level was taken at  $P < 0.05$ . Apparent  $K_m$  and  $V_{max}$  values of the recombinant Cyp4a enzymes were calculated using the „Enzyme Kinetics Module“ of SigmaPlot 7 (SPSS).

## RESULTS

### Cloning of four different Cyp4a cDNAs

**Cyp4a10:** The Cyp4a10 cDNA cloned from NMRI showed 100 % identity to the corresponding mRNA (AK002528) and genomic sequences (contig NT\_039264) from C57BL/6 mice. However, it differed in two positions from the Cyp4a10 mRNA of FVB/N mice (NM\_010011), which has been used as the provisional NCBI reference sequence for Cyp4a10: 190A>C and 207A>C (counted from the start ATG), resulting in Q64K and Q69H substitutions (NMRI and C57BL/6 vs. FVB/N).

**Cyp4a12a:** The Cyp4a12-specific primer pair yielded cDNA clones from renal RNA of both NMRI and C57BL/6, whose sequences completely agreed with that of the MGC25972 gene (C57BL/6 contig NT\_039264). The encoded Cyp isoform was termed “Cyp4a10-like” or “similar to P450 4A8” on contig NT\_039264 but will be designated in the following as Cyp4a12a according to [14]. cDNA sequences were so far only available for FVB/N mice (BC014721, NM\_177406) and differ from that in NMRI and C57BL/6 by one nonsynonymous (391A>C; M131L) and one silent nucleotide substitution (1233C>G).

**Cyp4a12b:** The gene originally termed “Cyp4a12” in the C57BL/6 genome (contig NT\_039264) is located approximately 112 kbp downstream of Cyp4a12a. It will be designated here as Cyp4a12b according to [14]. The coding sequences of Cyp4a12a and Cyp4a12b differ at only 23 nucleotide positions resulting in 11 amino acid substitutions. The primer pair used in the present study allowed the amplification of both the Cyp4a12a and Cyp4a12b sequences. To enrich the Cyp4a12b cDNA, the RT-PCR product was digested with *Avr* II, which selectively cleaves the Cyp4a12a cDNA. In this way, clones were generated from C57BL/6 liver mRNA, which showed 100 % sequence identity to the predicted coding sequence of the Cyp4a12b gene (“Cyp4a12” on contig NT\_039264). However, compared to the



provisional NCBI reference sequence for the “Cyp4a12” mRNA (NM\_172306, strain not specified), there is a nonsynonymous 494G>A substitution (V165H in contig NT\_039264 and own cDNA sequence).

**Cyp4a14:** The Cyp4a14 cDNA cloned from NMRI mice showed with one exception (13T>G resulting in L5V) 100 % identity to the coding region of the mRNA reference sequence NM\_007822 (strain C57BL/6 x CBA) and to that predicted from the C57BL/6 genome (contig NT\_039264). This polymorphism has been also observed in other mouse strains (rs3022990).

#### **Metabolism of lauric acid by recombinant mouse Cyp4a isoforms**

Co-expression of the individual Cyp4a isoforms with CPR in Sf9 insect cells yielded highly active microsomal enzyme systems that hydroxylated LA with  $V_{\max}$  values between 40 and 76 nmol/nmol/min and apparent  $K_m$  values between 2 and 16  $\mu$ M (Table 1). Addition of cytb5 in a 1:1 molar ratio doubled the  $V_{\max}$  values of Cyp4a10, Cyp4a12a and Cyp4a12b. In contrast, the activity of Cyp4a14 was not significantly influenced by cytb5 (data not shown).

The primary products of Cyp4a-catalyzed LA hydroxylation were 11- and 12-OH-LA. The identity of these metabolites was confirmed by co-migration with the authentic standard compounds (for representative HPLC chromatograms see supplementary Fig. S1) and by GC-MS analysis as described in Supplemental online data. The 12-OH:11-OH ratio was isoform-specific (Table 1). The regioselectivity was very high with Cyp4a10 (94:6), moderate with Cyp4a12a (83:17) and Cyp4a14 (62:38) and almost not expressed with Cyp4a12b (48:52). Prolonged incubation or increased enzyme concentrations yielded substantial amounts of dodecan-1,12-dioic acid (DDDA) as a secondary product resulting from further oxidation of 12-OH-LA (data not shown).

#### **Metabolism of arachidonic acid by recombinant mouse Cyp4a isoforms**

AA was efficiently metabolized only by Cyp4a12a and Cyp4a12b (Table 2; for representative HPLC chromatograms see supplementary Fig. S2). Major primary metabolites were 20-HETE and 19-HETE which were produced by both Cyp4a12a and Cyp4a12b in a ratio of about 90:10. Cyp4a12b produced another primary metabolite that co-migrated with 18-HETE in NP-HPLC and represented about 12 % of the total product. Secondary product formation analogous to the oxidation of LA to DDDA was also observed with AA. The secondary metabolite co-migrated in RP-HPLC with authentic eicosatetraen-1,20-dioic acid (20-COOH-AA). The same metabolite was also directly produced from [ $1-^{14}$ C]20-HETE by the Cyp4a12 isoforms (supplementary Figs. S2B and S2H).

Michaelis-Menton kinetics were observed determining the AA conversion rates at different substrate concentrations. The apparent  $K_m$  values were 25 and 43  $\mu$ M for Cyp4a12a and Cyp4a12b, respectively. Both enzymes reached high  $V_{\max}$  values of about 10 nmol/min/nmol. The  $V_{\max}$  values were almost doubled in the presence of stoichiometric amounts of cytb5 (Table 2).

With Cyp4a10 and Cyp4a14, AA metabolites were clearly detectable only after increasing the enzyme concentration to 500 nM and prolonging the reaction time (Table 2 and supplementary Figs. S2A and S2D). The metabolites observed with Cyp4a10 co-migrated with 19/20-HETE. Cyp4a14 was almost completely inactive as an AA hydroxylase, but produced small amounts of 11,12-EET. At a substrate concentration of 10  $\mu$ M, Cyp4a10 reached an activity of 0.05 nmol/nmol/min (in the presence of cytb5) and was thus 70-fold less active than Cyp4a12a. At higher AA concentrations Cyp4a10 showed specific activities of 0.14 (40  $\mu$ M), 0.21 (100  $\mu$ M), and 0.63 nmol/nmol/min (200  $\mu$ M), without reaching substrate saturation. Due to these low conversion rates, a further detailed kinetic analysis was not possible.

#### **Metabolism of eicosapentaenoic acid by recombinant mouse Cyp4a isoforms**

EPA was converted by Cyp4a12a and Cyp4a12b with high catalytic efficiencies that even slightly exceeded those determined for AA (Tab. 3). In comparison, Cyp4a10 and Cyp4a14 showed only a weak capacity to convert EPA resembling their very low activities in AA conversion (Table 3; for representative HPLC chromatograms see supplementary Fig. S3).

Whereas Cyp4a12a and Cyp4a12b functioned solely as hydroxylases when converting AA, they catalyzed both a hydroxylation and an epoxidation reaction with EPA. The ratio of hydroxylase to epoxygenase activities was 1:1.3 with Cyp4a12a and 1:2 with Cyp4a12b. Accordingly, the epoxidation product represented the major EPA metabolite with both isoforms. The epoxidation product co-migrated in RP-HPLC with authentic 17,18-EETeTr. Chiral-phase HPLC resolved the 17,18-EETeTr peak collected from RP-HPLC into the respective R,S- and S,R-enantiomers. Cyp4a12a produced preferentially the R,S-enantiomer, whereas Cyp4a12b displayed no stereoselectivity (Table 3). The hydroxylase product consisted of 19- and 20-HEPE and both enzymes showed a regioselectivity in favor of producing the 20-OH metabolite (Table 3).

#### **Gender- and strain-specific renal expression of mouse Cyp4a isoform**

TaqMan-PCR analysis demonstrated that Cyp4a10 is highly expressed in the kidneys of both genders, whereas Cyp4a12a is predominantly a male- and Cyp4a14 a female-specific isoform (Fig. 1A-D).

Among the isoforms efficiently producing 20-HETE, only Cyp4a12a but not Cyp4a12b showed significant renal expression. Cyp4a12a levels in the males largely exceeded that in the females (45-fold for NMRI, 88-fold for FVB/N, 40-fold for 129 Sv/J, 7-fold for BALB/c and 48-fold for C57BL/6; Fig. 1B). Moreover, we observed marked differences in the renal Cyp4a12a mRNA levels between the five different male strains (Fig. 1B). The highest expression was observed in NMRI followed by FVB/N, BALB/c and 129 Sv/J. The renal Cyp4a12a expression level in male C57BL/6 reached only about 17 % of male NMRI and was statistically significantly lower compared to all other male strains.

In contrast, Cyp4a12b mRNA was almost undetectable in the kidneys of both gender of all strains tested. Reliable TaqMan-PCR amplification curves were only obtained with renal samples from female

129 Sv/J, and male and female C57BL/6 (Fig. 1C). Renal samples from all other strains yielded low unspecific signals not different from the non-template controls. However, Cyp4a12b mRNA was easily detectable under the same experimental conditions using lung instead of renal samples (data not shown) indicating that Cyp4a12b shows a different tissue specificity of expression.

We also analyzed the gender and strain differences in Cyp4a isoform expression at the protein level. The antibody raised against a Cyp4a12a-derived peptide efficiently recognized recombinant Cyp4a12a and Cyp4a12b, showed a very weak cross-reactivity with Cyp4a10 and did not react with Cyp4a14 (Fig. 1E). In contrast, the CYP4A1-antibody detected all four mouse Cyp4a isoforms (Fig. 1E). Western blot analysis of mouse renal microsomes confirmed the gender differences in Cyp4a12 isoform expression (Fig. 1F). The Cyp4a12-specific antibody yielded a single strong band with male and almost no reaction with female renal microsomes with exception of a very weak but reproducible Cyp4a12 protein expression in female 129 Sv/J and BALB/c (Fig. 1F).

#### **Gender- and strain-specific renal microsomal arachidonic acid hydroxylase activities**

Mouse renal microsomes of all strains tested showed both AA hydroxylase and AA epoxygenase activities (Fig. 2A). Renal microsomes of female mice had low AA hydroxylase activities ranging between 15 and 25 pmol/min/mg. Males showed 3 to 5-times higher activities than the respective female strain (Fig. 2B). The gender difference was most pronounced in NMRI, where males reached a renal AA hydroxylase activity of  $93 \pm 4$  compared to  $20 \pm 0.5$  pmol/min/mg in females. No significant gender-difference was observed in renal microsomal 20-HETE production of C57BL/6 ( $25 \pm 1$  vs.  $16 \pm 1$  pmol/min/mg, male vs. female).

Clear gender differences were also found in renal microsomal AA epoxygenase and in soluble epoxide hydrolase activities (Figs. 2C and 2D). Compared to the large differences in 20-HETE production among male strains (Fig. 2B), strain differences in epoxygenase and hydrolase activities were less pronounced.

#### **Induction of Cyp4a12a and of renal microsomal 20-HETE production by androgen treatment**

Androgen treatment induced total renal and renal arteriole Cyp4a12a mRNA expression more than 4-fold in C57BL/6 and about 1.4-fold in NMRI (Figs. 3A and 3B). Western blot analysis showed that the Cyp4a12 protein level was significantly higher in male NMRI compared to male C57BL/6 (Fig. 3C).  $5\alpha$ -DHT strongly increased the Cyp4a12 protein level in C57BL/6 (to 600 % of the untreated control) and to a lesser extent also in NMRI (to 200 %). Renal microsomal AA hydroxylase activities were  $23 \pm 4$  pmol/min/mg in untreated C57BL/6 and  $128 \pm 14$  pmol/min/mg in untreated NMRI (Fig. 3D).  $5\alpha$ -DHT-treatment increased the activity to  $90 \pm 7$  in C57BL/6 and to  $167 \pm 11$  pmol/min/mg in NMRI. These data show that  $5\alpha$ -DHT treatment significantly induced Cyp4a12a mRNA, protein and enzyme activity level in C57BL/6 leading to Cyp4a12a expression and 20-HETE production similar to NMRI already under basal conditions.

## DISCUSSION

In this investigation, we identified Cyp4a12a as the predominant AA  $\omega$ -hydroxylase in the mouse kidney and demonstrated that Cyp4a12a expression levels determine the sex and strain-specific differences in mouse renal 20-HETE production. We suggest the differences in 20-HETE we identified amongst the strains could account for resistance to target organ damage exhibited by some strains, but not others. Furthermore, the sex differences we identified, coupled with the androgen effects we observed, may explain the relative resistance of female mice to target organ damage compared to males within strains.

Recombinant Cyp4a12a showed a high regioselectivity in favor of producing 20-HETE from AA similar as observed before with mouse renal microsomes [12]. Cyp4a12a did not form any detectable amounts of AA epoxides. However with EPA, Cyp4a12a efficiently catalyzed the epoxidation of the 17,18-double bond that distinguishes this n-3 PUFA from AA. Thus, Cyp4a12a generates very different sets of metabolites when either AA or EPA becomes accessible as substrate. Such a shift in CYP-dependent eicosanoids may have physiological significance. 20-HETE is a powerful endogenous vasoconstrictor that functions via inhibition of BK channels in vascular smooth muscle cells [4]. In contrast, 17,18-EETeTr shows vasodilator properties and is a very potent activator of BK channels [22, 29]. BK channel activation is highly stereoselective [22] and we found that Cyp4a12a produces preferentially the active R,S-enantiomer. Alterations in cyclooxygenase- and lipoxygenase-dependent prostanoid and leukotriene biosynthesis have been generally assumed to contribute to the beneficial effects of fish oil n-3 PUFAs in cardiovascular diseases [30]. Considering the enzymatic properties of Cyp4a12a described above, it appears highly probable that also CYP-catalyzed 20-HETE production can be manipulated under in vivo conditions by n-3 PUFA-rich diets. How this state-of-affairs affects 20-HETE-mediated signaling pathways regulating vascular and renal function will be an important research topic.

Cyp4a12a did not only hydroxylate AA to 20-HETE but also catalyzed the subsequent oxidation steps necessary to convert 20-HETE to 20-COOH-AA. The ability to catalyze an oxidation cascade from a given fatty acid to the corresponding dicarboxylic acid was shown earlier for several CYP enzymes, examining other substrates than AA [31]. Alcohol dehydrogenase 4 may be the responsible enzyme in vascular smooth muscle and endothelial cells that converts 20-HETE to 20-COOH-AA [32]. This reaction may serve to overcome the vasoconstrictor action of 20-HETE [27, 33]. Moreover, 20-COOH-AA is known to inhibit the Na,K,2Cl-cotransporter in mTALH [4, 27] and to function as a dual activator of peroxisome proliferator-activated receptors  $\alpha$  and  $\gamma$  [34].

Our study indicates that both Cyp4a12 genes [14] are functional but differ significantly in the tissue specificity of expression. Considering the very high degree of homology between the two Cyp4a12 variants, the differences are remarkable that we observed comparing their enzymatic properties. In

contrast to Cyp4a12a, Cyp4a12b showed (i) no preference for  $\omega$ - over  $\omega$ -1 hydroxylation when converting LA, (ii) no stereoselectivity when epoxidizing the 17,18-double bond of EPA, and (iii) a higher EPA epoxygenase:EPA hydroxylase ratio. These findings indicate that the positioning of the substrate alkyl chain is probably less strict in the active site of Cyp4a12b compared to that in Cyp4a12a. Interestingly, there are also similar pairs of highly homologous CYP4A enzymes in man (CYP4A11 and CYP4A22; [8, 35]) and rat (CYP4A2 and CYP4A3; [36, 37]). However, the sequence differences in these CYP pairs concern other amino acid positions and have different functional consequences. Thus, the significance of the CYP4A gene duplications and of the subsequently evolved gene variants is not readily obvious and may differ in human, rat and mouse.

Cyp4a10 is the murine Cyp isoform showing the highest homology to CYP4A1 (92 % amino acid sequence identity), the most active rat AA hydroxylase [36]. However, our data show that Cyp4a10 displays only a very weak AA hydroxylase activity. Assuming that 10  $\mu$ M is a reasonable physiological AA concentration, the activity of Cyp4a10 would be about 70-fold lower than that of Cyp4a12a. Cyp4a10 gene disruption did not alter mouse renal AA hydroxylase activities confirming that the Cyp4a10 enzyme does not contribute to 20-HETE production [16]. Cyp4a12a and Cyp4a12b have 78 % sequence identity to CYP4A1 and are most closely related to CYP4A8 (87 %) which showed, however, the lowest AA hydroxylase activity among the rat CYP4A isoforms [36]. CYP4A8 shares with Cyp4a12a the inducibility by androgens [38]. Cyp4a14 is most similar to CYP4A2 (88 %) and CYP4A3 (89 %). The latter are highly expressed in the male rat kidney and display a significant 20-HETE synthase activity [36]. In contrast, Cyp4a14 shows a female specific expression and we could not detect any 20-HETE synthase activity with this isoform.

Our data on the sex specificity of renal Cyp4a gene expression in five different mouse strains show in agreement with previous studies [10, 11] that Cyp4a10 is highly expressed in both sexes, whereas Cyp4a12a is clearly a male and Cyp4a14 a female specific isoform. Moreover, we found that the expression of Cyp4a12a is correlated to the capacity of mouse renal microsomes to hydroxylate AA thus providing further evidence for the role of this isoform in 20-HETE production. Comparing the males of the different strains, NMRI mice had clearly the highest Cyp4a12a expression levels whereas C57BL/6 appears as a naturally occurring mouse model with a specific knock-down in Cyp4a12a/20-HETE expression. Our results and those of other authors [11] demonstrate that Cyp4a12 is inducible by androgens. The androgen effect was much more pronounced with C57BL/6 compared to NMRI. Providing a possible explanation, mouse strains have been long known to differ in blood testosterone levels and C57BL/6 is considered as being chronically testosterone deficient [39]. The androgen-dependent expression also explains the low of Cyp4a12a levels in the females of all strains. We noticed, however, that the gender differences in Cyp4a12a mRNA and protein expression levels were apparently

more pronounced than in the AA hydroxylase activities. Therefore, it remains to be analyzed whether the low but still significant renal microsomal AA hydroxylase activities in the females are in part due to different Cyp isoforms possibly including Cyp4F subfamily members [13, 40].

20-HETE plays an important role in the regulation of various cardiovascular processes. It acts as a second messenger in signaling pathways modulating vascular tone, salt excretion, cell proliferation and angiogenesis [4]. Since the intensity of these signaling pathways may depend on the extent of 20-HETE production, the question arises whether sex and strain-specific differences in the response to pro-hypertensive and pro-hypertrophic stimuli are partially due to differences in Cyp AA  $\omega$ -hydroxylase expression levels. Substantiating this possibility, androgen-induced overproduction of 20-HETE in mice [11] and rats [38] is associated with the development of hypertension. Moreover, rats transduced with an adenovirus for vascular overexpression of CYP4A2 developed endothelial dysfunction and hypertension [41]. Female mice, which have very low renal 20-HETE production, are more resistant to Ang II-induced hypertension [18] and to ischemia/reperfusion-induced kidney injury [42], compared with males. C57BL/6 males, which have lower renal 20-HETE production than the males of other strains, show reduced susceptibilities to the development of DOCA-salt hypertension [17], glomerulosclerosis [43] and injury-induced neointimal hyperplasia [44]. Recently, CYP-dependent AA metabolism has been also implicated in cardiac function and ischemia/reperfusion-induced myocardial injury [45]. Clearly, there are many other factors potentially involved in mediating gender-related differences in the manifestation of cardiovascular disease [46] and the relative importance of differences in CYP-dependent AA metabolism remains to be determined.

Our study provides comprehensive information on the expression and enzymatic properties of the Cyp4a subfamily members in the mouse kidney, identifies Cyp4a12a as the predominant 20-HETE synthase, and suggests that differences in Cyp4a12a/20-HETE expression may be involved in determining sex and strain-specific susceptibility differences to hypertension and other cardiovascular diseases. Transgenic and gene-deletion approaches to alter Cyp4a12a expression in selected tissues may help to directly prove the various physiological and pathophysiological functions attributed to 20-HETE in the regulation of renal, vascular, and cardiac function. In those studies, strain and sex selection will be a highly important aspect of study design.

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## FIGURE LEGENDS

### **FIGURE 1. Gender- and strain-specific renal expression of Cyp4a-isoforms**

**(A-D):** Renal mRNA expression of Cyp4a10, Cyp4a12a, Cyp4a12b, and Cyp4a14 in male and female NMRI, FVB/N, 129 Sv/J, BALB/c, and C57BL/6 mice. Data are mean $\pm$ SEM (n=5 in each group). Significant differences (p<0.05) between the groups are marked as follows: \* vs. other gender of the same strain; # vs. other males; + vs. other females; § vs. 129 Sv/J, BALB/c and C57BL/6; ^ vs. 129 Sv/J and C57BL/6; ° vs. NMRI. **(E):** Specificity of the antibodies used for Western blot analysis. Control - microsomal protein (8  $\mu$ g) isolated from Sf9 cells not expressing any of the Cyp isoforms; other lanes - individual recombinant Cyp4a isoforms (0.2 pmoles each); ab: 4A1 - antibody raised against rat CYP4A1; ab: 4a12 - antibody raised against a Cyp4a12a-specific peptide. **(F):** Western blot analysis of renal Cyp4a protein expression using the primary 4A1 and 4a12 antibodies; 7,5  $\mu$ g total microsomal protein were loaded per lane.

### **FIGURE 2. Gender- and strain-specific expression of enzymatic activities involved in mouse renal arachidonic acid metabolism.**

**(A):** Representative RP-HPLC chromatogram of AA metabolites produced by renal microsomes isolated from male and female NMRI mice. **(B-D):** Renal AA hydroxylase, AA epoxygenase, and soluble epoxide hydrolase activities of male and female NMRI, FVB/N, 129 Sv/J, BALB/c, and C57BL/6. Data are mean $\pm$ SEM (n=6 in each group). Significant differences (p<0.05) between the groups are marked as follows: \* vs. other gender of the same strain; # vs. other males; + vs. FVB/N and C57BL/6; § vs. BALB/c.

### **FIGURE 3. Effect of 5 $\alpha$ -dihydrotestosterone on Cyp4a12a expression and renal microsomal 20-HETE production in male NMRI and C57BL/6.**

**(A, B):** Cyp4a12a mRNA levels in total kidney and renal arterioles; **(C):** renal Cyp4a12 protein levels; **(D):** renal microsomal AA hydroxylase activities. Results are mean $\pm$ SEM (n=6 each). Significant differences (p<0.05) between the groups are marked as follows: \* vs. untreated C57BL/6; # vs. untreated NMRI; + vs. testosterone treated C57BL/6.

## TABLES

**TABLE 1.** Metabolism of lauric acid by recombinant Cyp4a enzymes

CYP	K <sub>m</sub> -value ( $\mu$ M)	V <sub>max</sub> -value (nmol/nmol/min)	Product distribution (%) <sup>a)</sup>	
			11-OH-LA	12-OH-LA
<b>4a10</b>	2 $\pm$ 0.4	55 $\pm$ 3	6	94
<b>4a12a</b>	16 $\pm$ 6	76 $\pm$ 17	17	83
<b>4a12b</b>	2 $\pm$ 0.6	40 $\pm$ 3	52	48
<b>4a14</b>	8 $\pm$ 2	40 $\pm$ 5	38	62

<sup>a)</sup> The products were resolved and quantified by RP-HPLC as shown in supplementary Fig. S1. The values are averages from experiments with at least three different microsomal preparations. The standard errors were less than 10 % of the mean. The product distribution was independent of the LA concentration in the range used for kinetic analysis (1 to 50  $\mu$ M).

**TABLE 2. Metabolism of arachidonic acid by recombinant Cyp4a enzymes**

CYP	K <sub>m</sub> -value (μM)		V <sub>max</sub> -value (nmol/nmol/min)		Product distribution (%) <sup>a)</sup>			
		+ cytb5		+ cytb5	18-HETE	19-HETE	20-HETE	11,12-EET
<b>4a10</b>	nd	nd	0.02 ± 0.01 <sup>b)</sup>	0.05 ± 0.01 <sup>b)</sup>	-	20 <sup>c)</sup>	80 <sup>c)</sup>	-
<b>4a12a</b>	25 ± 4	34 ± 6	8 ± 1	16 ± 2	-	13	87	-
<b>4a12b</b>	43 ± 12	72 ± 10	10 ± 2	19 ± 2	12	10	78	-
<b>4a14</b>	nd	nd	0.01 ± 0.003 <sup>b)</sup>	0.01 ± 0.001 <sup>b)</sup>	-	-	-	100

<sup>a)</sup> The metabolites were resolved and quantified by RP- and NP-HPLC as shown in supplementary Fig. S2. The values are averages from experiments with at least three different microsomal preparations. The standard errors were less than 10 % of the mean.

<sup>b)</sup> Activities of Cyp4a10 and Cyp4a14 are given for a substrate concentration of 10 μM since a detailed kinetic analysis was not possible due to the very weak activities of these isoforms towards AA. For comparison, under the same conditions, the corresponding values for Cyp4a12a were 2.3 and 3.7 (+ cytb5) and for Cyp4a12b 1.9 and 2.3 (+cytb5) nmol/nmol/min.

<sup>c)</sup> Roughly estimated from RP-HPLC analysis

nd - not determined

**TABLE 3. Metabolism of eicosapentaenoic acid by recombinant Cyp4a enzymes**

CYP	K <sub>m</sub> -value (μM)		V <sub>max</sub> -value (nmol/nmol/min)		Product distribution (%) <sup>a)</sup>				Stereoselectivity (%) <sup>a)</sup>	
		+ cytb5		+ cytb5	19-HEPE	20-HEPE	17,18-EETeTr	EETeTr	17(R),18(S)-EETeTr	17(S),18(R)-EETeTr
<b>4a10</b>	nd	nd	0.1 ± 0.01 <sup>b)</sup>	0.2 ± 0.03 <sup>b)</sup>	60 <sup>c)</sup>		40 <sup>c)</sup>	-	nd	nd
<b>4a12a</b>	29 ± 4	33 ± 2	14 ± 1	29 ± 1	12	32	56	-	73	27
<b>4a12b</b>	41 ± 5	62 ± 20	15 ± 1	30 ± 7	11	21	68	-	51	49
<b>4a14</b>	nd	nd	0.02 ± 0.01 <sup>b)</sup>	0.03 ± 0.01 <sup>b)</sup>	11 <sup>c)</sup>		-	89 <sup>c)</sup>	nd	nd

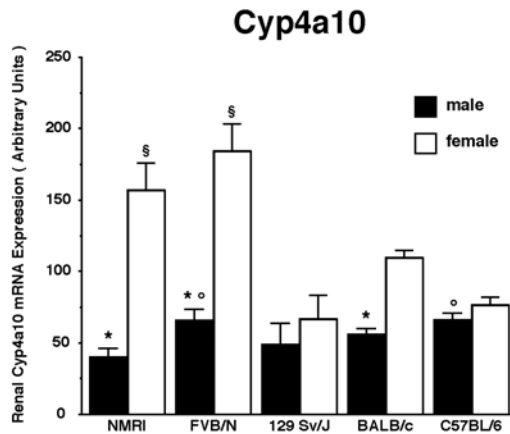
<sup>a)</sup> The metabolites were resolved and quantified by RP- followed by NP- or chiral-phase HPLC as shown in supplementary Fig. S3. The values are averages from experiments with at least three different microsomal preparations. The standard errors were less than 10 % of the mean.

<sup>b)</sup> Activities of Cyp4a10 and Cyp4a14 are given for a substrate concentration of 10 μM since a detailed kinetic analysis was not possible due to the very weak activities of these isoforms towards EPA. For comparison, under the same conditions, the corresponding values for Cyp4a12a were 3.6 and 6.7 (+ cytb5) and for Cyp4a12b 3.0 and 4.2 (+cytb5) nmol/nmol/min.

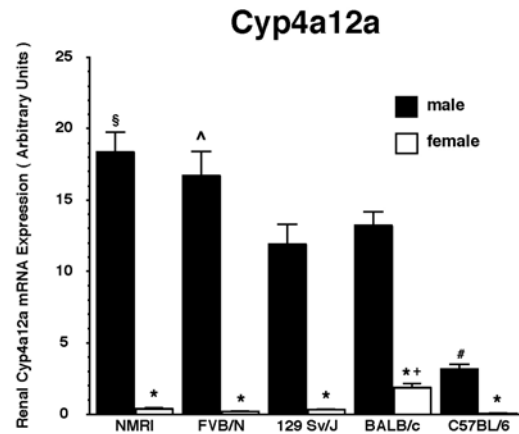
<sup>c)</sup> Estimation from RP-HPLC only

nd - not determined

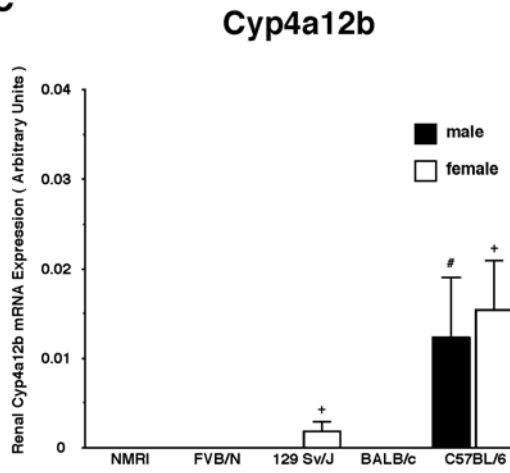
**Fig. 1A**



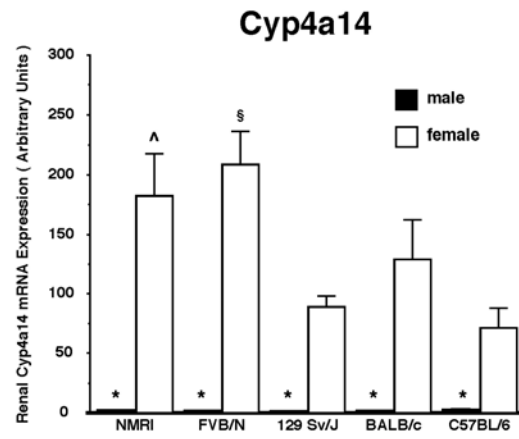
**B**



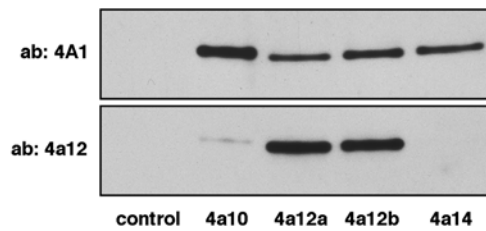
**C**



**D**



**E**



**F**

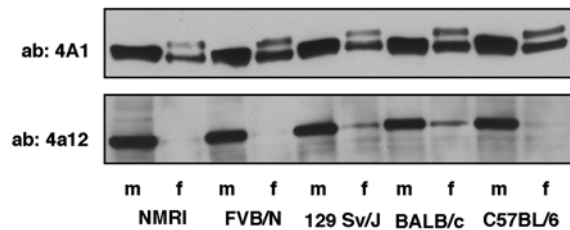
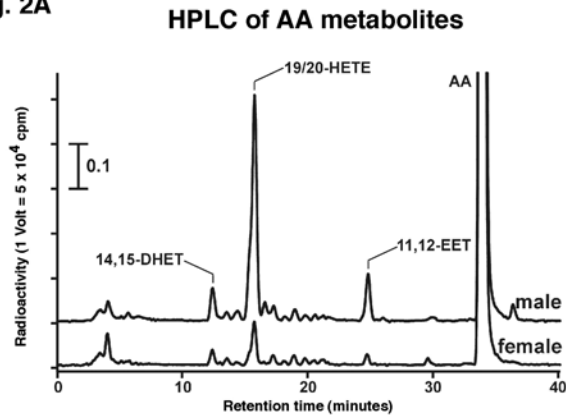
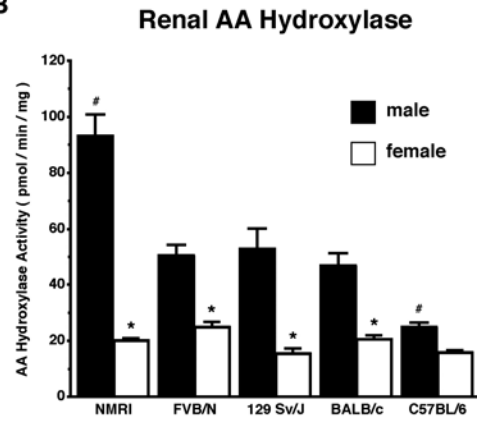


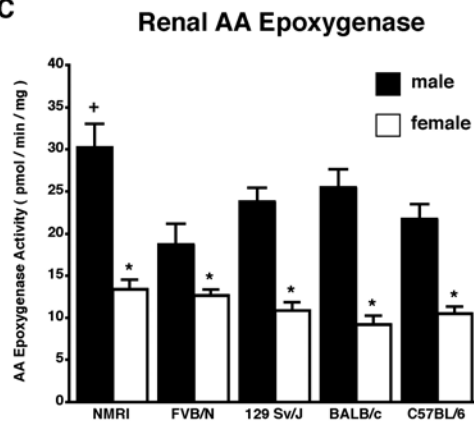
Fig. 2A



B



C



D

