Muller et al., Supplemental information

SUPPLEMENTARY MATERIALS AND METHODS

TaqMan analysis

TaqMan analysis for Cyp4a10, Cyp4a12a, Cyp4a12b, and Cyp4a14 were conducted according to the manufacturer's instructions using an Applied Biosystems 7700 Sequence detector (Applied Biosystems) and qPCR Mastermix Plus (Applied Biosystems). Each sample was measured in triplicate and expression level normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression using the standard curve method. The TaqMan primer sets were as follows:

Cyp4a10:

forward: TCT CTG CTC TAA GCC CAA CC

reverse: CGA GCA CAG AGG CCA CTT G

probe: 6-Fam TTT GCA GAC AGC CTC TCT GGC TTC CT-Tamra;

Cyp4a12a:

forward: GCC TTA TAC GGA AAT CAT GGC A

reverse: TGG AAT CCT GGC CAA CAA TC

probe: 6-Fam ACT CTG TTC GTG TAA TGC TGG ATA AAT GGG AA-Tamra;

Cyp4a12b:

forward: CCT TAT ACG GAA ATC ARG GCA GA

reverse: TGG AAT CCT GGC CAA CAA TC

probe: 6-Fam TCT GTT CAT GTC ATG CTG GAT AAA TGG GAA-Tamra

Cyp4a14:

forward: GAC CCT CCA GCA TTT CCC A

reverse: TCC TTG TCC TTC AGA TGG TGC

probe: 6-Fam CAT GCC TTC CCA CTG GCT TTG GG-Tamra

Production of recombinant baculoviruses and expression of CYP enzymes in Spodoptera frugiperda cells

The Bac-To-Bac System (GibcoBRL) was used to produce recombinant baculoviruses containing the cloned Cyp4a cDNAs under control of the polyhedrin promoter. All steps including bacmid and baculovirus preparation were performed according to the instructions of the manufacturer. Coexpression of the individual CYP isoforms with the human NADPH-CYP reductase (CPR) was performed as described previously [20]. Spodoptera frugiperda (Sf9) cells were grown in

ExCell 401 medium (JRH Biosciences) supplemented with 10% heat-inactivated fetal calf serum, 100 U penicillin, and 100 µg streptomycin/ml. After reaching a density of 2 x 10^6 cells/ml, the cultures were co-infected with the recombinant Cyp4a- and CPR-baculoviruses. The medium was supplemented with 100 µM riboflavin, 5 µM hemin chloride, and 30 µM methyrapone 24 h post-infection. Cells were harvested after 48 h, resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM EDTA, 0.5 mM DTT, and 100 µM phenylmethylsulfonyl fluoride, and lysed by brief sonication. Microsomes were prepared by differential centrifugation and resuspended in the same buffer without PMSF. The CYP:CPR ratio was adjusted by using different ratios of recombinant Cyp4a and CPR baculoviruses for infection.

Preparation of authentic standard compounds

LA metabolites: Authentic 12-hydroxy lauric acid (12-OH-LA) and dodecandioic acid (DDDA) were obtained from Merck. 11-OH-LA was chemically synthesized as described [25]. LA metabolites produced by the recombinant Cyp4a isoforms were treated with diazomethane and resolved by RP-HPLC as described in Materials and Methods (Analysis and quantification of metabolites). Radiolabeled products eluting at 33.6 (11-OH-LA), 35.0 (12-OH-LA) and 41.1 min (DDDA) were collected and further analyzed by gas chromatography-mass spectrometry (GC-MS). Electron impact (EI) spectra of the methylester or trimethylsilylether-methylester derivatives of metabolites and of the standard substances were recorded with a QP5050A (Shimadzu; 30-m/0.25-mm Optima-1 fused silica capillary column, 0.25 µm coating thickness, Macherey-Nagel). For the EI spectra, oven temperature was raised from 100° to 300°C (20°C/min) and held for 6 min at 300°C. The metabolite eluting at 33.6 min from RP-HPLC showed a retention time of 8.25 min in GC and fragmentation in MS identical to that of authentic 11-OH-LA. M+ was at m/z 304 for the metabolite originating from [1-^{14}C]-labeled LA and at m/z 302 for the unlabeled standard compound. Characteristic fragments occurred at m/z 260/258 and 117/117. The metabolite eluting from RP-HPLC at 35 min showed GC-MS properties identical to authentic 12-OH-LA: retention time 8.61 min, M+: 304/302, characteristic fragments at m/z 289/287 and 257/255. The metabolite eluting from RP-HPLC at 41.1 min was identical to authentic DDDA: retention time 8.34 min, M+:259/257, prominent fragment at m/z 227/225.

AA metabolites: Unlabeled standard compounds including 20-HETE and all regio-isomeric EETs were purchased from Cayman Chemicals. 16-, 17-, 18-, and 19-HETE were enzymatically synthesized using recombinant human CYP1A1 as described previously [26].

Radiolabeled 20-HETE was produced from [1-^{14}C]AA using recombinant Cyp4a12a and purified by RP- and NP-HPLC as described in Materials and Methods (Analysis and

quantification of metabolites). Authentic eicosatetraenedioic acid (20-COOH-AA) was prepared from 20-HETE using alcohol and aldehyde dehydrogenases as described [27]. 20-COOH-AA was converted to the corresponding methyl ester by treatment with diazomethane. This compound showed a retention time of 11.9 min in GC under the conditions described above and a mass peak at m/z at 362 (M+) as expected. The product of Cyp4a12a-catalyzed oxidation of 20-HETE showed identical GC retention time and MS fragmentation.

EPA metabolites: Authentic EPA epoxides were prepared by chemical oxidation, purified by RP- and NP-HPLC and identified by GC-MS as described previously [24]. The R,S- and S,R-enantiomers of 17,18-EETeTr were prepared by enzymatic epoxidation of EPA using recombinant human CYP1A1 [26] and bacterial P450BM3 [28], respectively.

SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1. Lauric acid metabolism by recombinant mouse Cyp4a enzymes. Representative RP-HPLC chromatograms showing the metabolites produced by Cyp4a10 **(A)**, Cypa12a **(B)**, Cyp4a12b **(C)**, and Cyp4a14 **(D)**. Note the different regioselectivities of the isoforms in catalyzing the hydroxylation of lauric acid (LA) to 11-hydroxy-lauric acid (11-OH-LA) and 12-hydroxy-lauric acid (12-OH-LA). The reactions were performed using 10 pmol Cyp in reaction mixtures of 100 µl, a substrate concentration of 30 µM and a reaction time of 5 min. The organic extracts were treated with diazomethane to convert the metabolites and the remaining substrate into the corresponding methylesters. The methylesters of the primary hydroxylation products eluted at 33.6 min (11-OH-LA) and 35 min (12-OH-LA), the methylester of dodecandioic acid (DDDA) originating from further oxidation of 12-OH-LA, at 41.1 min and the methylester of unconverted LA at 44.2 min. Minor products exclusively produced by Cyp4a12b and eluting between 12-OH-LA and DDDA (Fig. S1**C**) were not identified.

FIGURE S2. Arachidonic acid metabolism by recombinant mouse Cyp4a enzymes.

Representative RP-HPLC chromatograms showing the metabolites produced by Cyp4a10 **(A)**, Cypa12a **(B)**, Cyp4a12b **(C)**, and Cyp4a14 **(D)**. Note that 10- to 20-fold expanded scales were used to show the trace amounts of metabolites produced by Cyps 4a10 and 4a14 in comparison to the efficient product formation by Cyps 4a12a and 4a12b. The reactions were performed using 40 (Cyps 4a12a and 4a12b) or 200 pmol (Cyps 4a10 and 4a14) Cyp enzyme in reaction mixtures of 400 µl, a substrate concentration of 10 µM and a reaction time of 10 min (Cyps 4a12a and 4a12b) or 30 min (Cyps 4a10 and 4a14). The retention times of the products were 13.9 (20-COOH-AA), 15.5 (19/20-HETE), 16.4 (18-HETE) and 24.7 min (11,12-EET); AA eluted at 33 min. **(E)**, **(F)**, **(G)**: Analysis of the regioselectivities of Cyp4a12a and Cyp4a12b . The products eluting from

RP-HPLC at 15.5 min were collected from three or more parallel reaction samples and resolved into 19- and 20-HETE by NP-HPLC as described in Experimental Procedures (**E** and **F**). The additional minor product formed by Cyp4a12b (at 16.4 min in RP-HPLC, Fig. S2**C**) co-migrated with 18-HETE in NP-HPLC **(G)**. The mixture of 16-, 17- and 18-HETE used for comparison was synthesized with human CYP1A1. **(H)**: RP-HPLC chromatogram showing the conversion of 20-HETE to 20-COOH-AA. The reaction was performed under standard conditions using 10 pmol Cyp4a12a in a reaction volume of 100 µl, a 20-HETE concentration of 20 µM and a reaction time of 20 min. No product was formed in controls where either NADPH was omitted or microsomes containing only CPR but none of the mouse Cyp4a isoforms were used (not shown).

FIGURE S3. Eicosapentaenoic acid metabolism by recombinant mouse Cyp4a enzymes. Representative RP-HPLC chromatograms showing the metabolites produced by Cyp4a10 **(A)**, Cypa12a **(B)**, Cyp4a12b **(C)**, and Cyp4a14 **(D)**. Note that about 10-fold expanded scales were used to show the trace amounts of metabolites produced by Cyps 4a10 and 4a14 in comparison to the efficient product formation by Cyps 4a12a and 4a12b. Reaction conditions were as described in Fig. S2. The main hydroxylation products eluted from RP-HPLC at 14.2 min (19/20-HEPE), the epoxidation products at 19.6 (17,18-EETeTr) and 21 min (14,15-EETeTr or one of the remaining regioisomeric EETeTrs which all elute largely unresolved between 21 and 23 min), and unconverted EPA at 29.4 min. Minor products such as the triplet peak between 8 and 10 min with Cyp4a12a and at 15 min with all Cyps were produced in a NADPH- and Cyp-isoform-dependent manner but could not be identified. **(E)** and **(F)**: Analysis of the stereoselectivities of Cyp4a12a and Cyp4a12b in 17,18-epoxidation of EPA. The product peak eluting at 19.6 min from RP-HPLC was collected from three or more parallel reaction samples and further resolved into the R,S- and S,R-enatiomers using chiral-phase HPLC as described in Experimental procedures. **(G)** and **(H)**: Analysis of the regioselectivities of Cyp4a12a and Cyp4a12b in EPA hydroxylation. The products eluting from RP-HPLC at 14.2 min were collected from three or more parallel reaction samples and resolved into 19- and 20-HETE by NP-HPLC as described in Experimental Procedures. Additional minor products, which were particularly expressed with Cyp4a12b could not be identified.

Fig. S1A

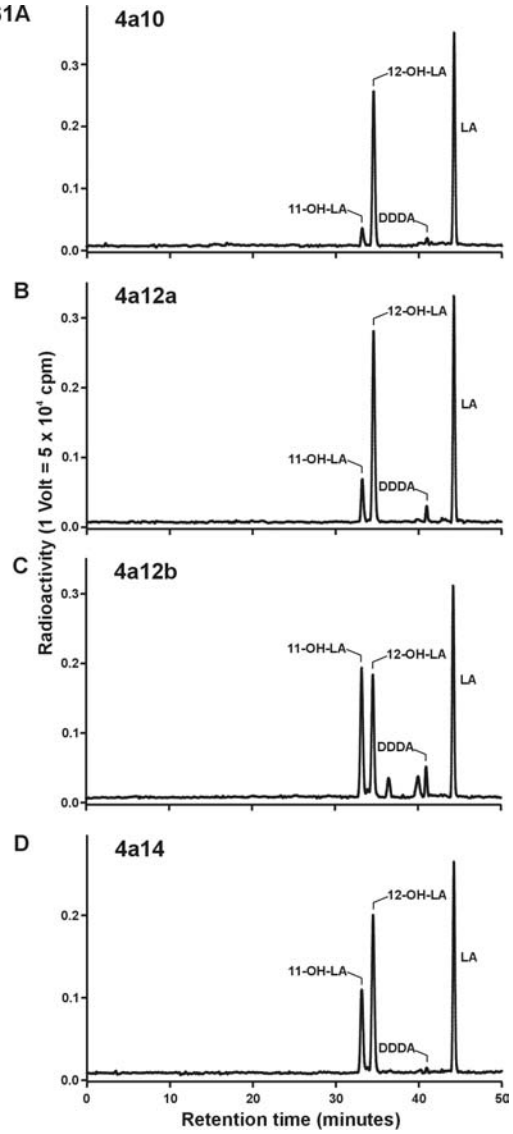

4a10
12-OH-LA
LA
11-OH-LA
DDDA
B
4a12a
12-OH-LA
LA
11-OH-LA
DDDA
C
4a12b
11-OH-LA
12-OH-LA
LA
DDDA
D
4a14
12-OH-LA
LA
11-OH-LA
DDDA
Radioactivity (1 Volt = 5 x 10⁴ cpm)
0.0
0.1
0.2
0.3
0
10
20
30
40
50
Retention time (minutes)

Fig. S2A

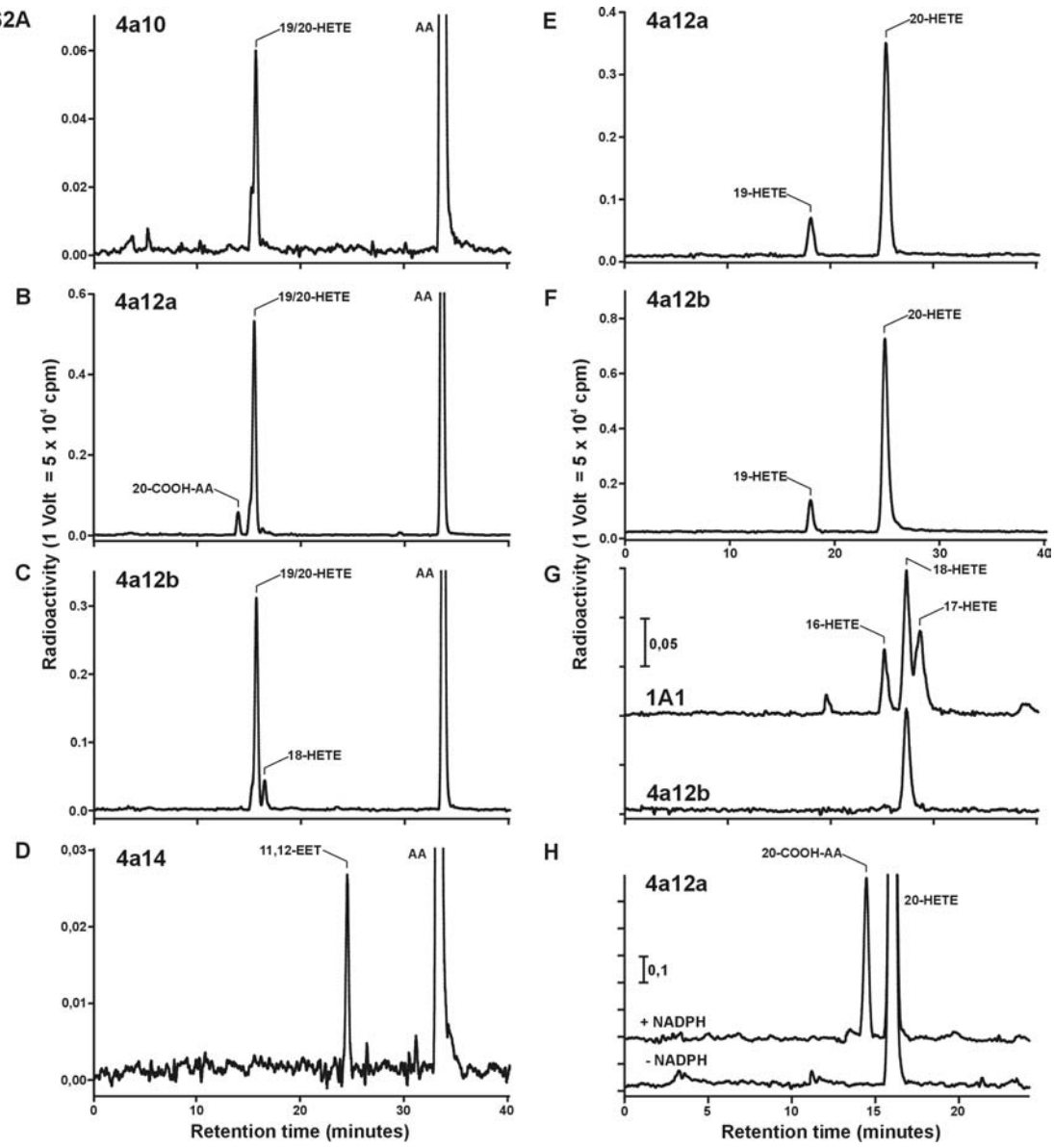
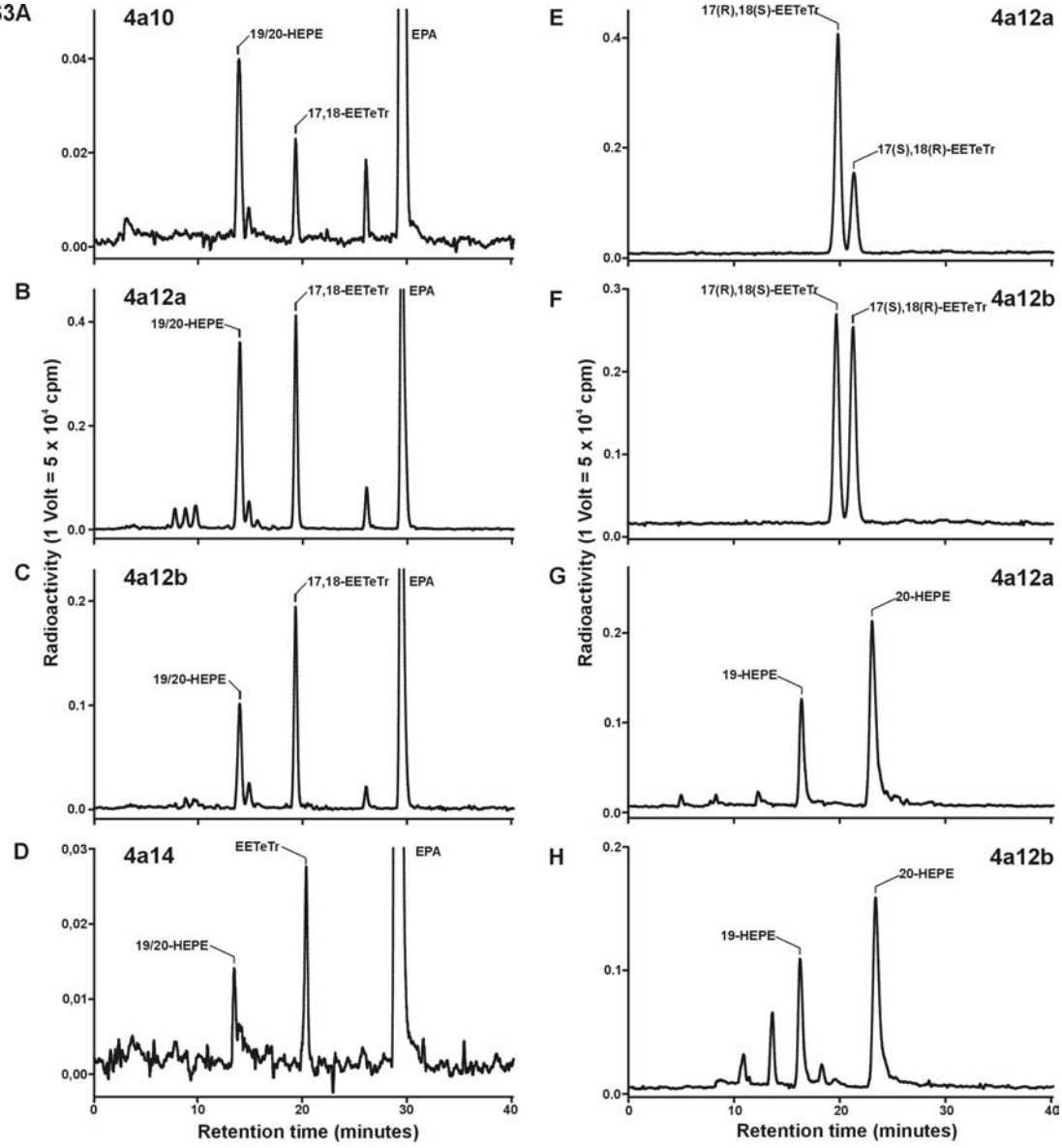

A
4a10
19/20-HETE
AA
B
4a12a
19/20-HETE
AA
20-COOH-AA
C
4a12b
19/20-HETE
AA
18-HETE
D
4a14
11,12-EET
AA
Radioactivity (1 Volt = 5 x 10⁴ cpm)
Retention time (minutes)
E
4a12a
20-HETE
19-HETE
F
4a12b
20-HETE
19-HETE
G
18-HETE
17-HETE
16-HETE
0,05
1A1
4a12b
H
20-COOH-AA
4a12a
20-HETE
0,1
+ NADPH
- NADPH
Retention time (minutes)

Fig. S3A


A
4a10
19/20-HEPE
17,18-EETeTr
EPA
B
4a12a
19/20-HEPE
17,18-EETeTr
EPA
C
4a12b
19/20-HEPE
17,18-EETeTr
EPA
D
4a14
19/20-HEPE
EETeTr
EPA
E
4a12a
17(R),18(S)-EETeTr
17(S),18(R)-EETeTr
F
4a12b
17(R),18(S)-EETeTr
17(S),18(R)-EETeTr
G
4a12a
19-HEPE
20-HEPE
H
4a12b
19-HEPE
20-HEPE
Radioactivity (1 Volt = 5 x 10⁴ cpm)
Retention time (minutes)