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LC–MS/MS analysis of epoxyalcohols and epoxides of arachidonic acid and their oxygenation by recombinant CYP4F8 and CYP4F22

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ABSTRACT

CYP4F22 and CYP4F8 are expressed in epidermis, and mutations of CYP4F22 are associated with lamellar ichthyosis. Epoxyalcohols (HEETs) and epoxides (EETs) of 20:4*n*−6 appear to be important for the water permeability barrier of skin. Our aim was to study the MS/MS spectra and fragmentation of these compounds and to determine whether they were oxidized by CYP4F22 or CYP4F8 expressed in yeast. HEETs were prepared from 15-hydroperoxyeicosatetraenoic acid (15-HPETE), 12-HPETE, and their [$^2H_8$]labeled isotopomers, and separated by normal phase-HPLC with MS/MS analysis. CYP4F22 oxygenated 20:4*n*−6 at C-18, whereas metabolites of HEETs could not be identified. CYP4F8 formed ω3 hydroxy metabolites of HEETs derived from 12*R*-HPETE with 11,12-epoxy-10-hydroxy configuration, but not HEETs derived from 15*S*-HPETE. 8,9-EET and 11,12-EET were also subject to ω3 hydroxylation by CYP4F8. We conclude that CYP4F8 and CYP4F22 oxidize 20:4*n*−6 and that CYP4F8 selectively oxidizes 8,9-EET, 11,12-EET, and 10,11*R*,12*R*-HEET at the ω3 position.

Cytochromes P450 (CYPs)[1] are of paramount importance for metabolism of endogenous and exogenous compounds. In humans, 57 CYP enzymes have been identified so far [1], and grouped into families and subfamilies based on amino acid similarities. Members of the CYP4 family oxidize fatty acids [2], leukotrienes [3,4], and prostaglandins [5], but the physiological function has not been established for several of them (e.g., CYP4F22, CYP4X1, and CYP4Z1 [6]). It seems plausible that some of these orphan CYPs may be involved in metabolism of lipids formed from arachidonic acid (20:4*n*−6) by any of three oxidative systems: cyclooxygenase (COX), lipoxygenase (LOX), and CYP. The presence of an oxidative pathway and expression of a specific orphan CYP in a tissue could generate a hypothesis on the catalytic function of this CYP isoform.

Human epidermis contains 15*S*-LOX type 1, 12*S*-LOX, and 12*R*-LOX [7]. Skin also contains CYPs involved in the transformation of vitamin A and vitamin D, and some members of the CYP4 family with unknown epidermal function [8,9]. Of these enzymes, 12*R*-LOX has lately attracted particular medical interest. 12*R*-LOX is only expressed in epidermis and in tonsils [7,10], and is up regulated in psoriatic lesions [11]. It transforms 20:4*n*−6 to 12*R*-hydroperoxyeicosatetraenoic acid (12*R*-HPETE), which is important for the development of the water permeability barrier [12–14]. Recently, mutations in the gene for 12*R*-LOX were linked to a rare skin disease, lamellar ichthyosis [15,16]. 12*R*-HPETE is further stereospecifically transformed to an epoxyalcohol, 11(*R*),12(*R*)-*trans*-epoxy-8(*R*)-hydroxyeicosa-5(*Z*),9(*E*),14(*Z*)-trienoic acid (8*R*,11*R*,-12*R*-HEET) (Fig. 1) by the enzyme epidermal LOX-3 (eLOX3) [17]. Mutational studies have also associated eLOX3 with lamellar ichthyosis [15,16,18–20], and recently the gene for CYP4F22 was linked to this disease [9]. Lefévre et al. have proposed that CYP4F22 takes part in bio activation of products derived from 12*R*-HPETE and 8*R*,11*R*,12*R*-HEET [9,21], but this hypothesis has not yet been investigated likely reflecting difficulties in expression of recombinant CYP4F22.

Epidermal 12*S*-HPETE is formed by platelet-type 12*S*-LOX in humans. HEETs derived from 12*S*-HPETE are found in many tissues and hepoxilin A_3 may influence the secretion of insulin and the release of serotonin [22,23], but their function in skin is unknown.

20:4*n*−6 can be oxidized by epoxygenases of the CYP1-3 subfamilies to four regio isomeric *cis* epoxides (5,6-epoxyeicosa-8(*Z*),11(*Z*),14(*Z*)-trienoic acid (5,6-EET), 8,9-epoxyeicosa-5(*Z*),11(*Z*),14(*Z*)-trienoic acid (8,9-EET), 11,12-epoxyeicosa-5(*Z*),8(*Z*),14(*Z*)-trienoic acid (11,12-EET), and 14,15-epoxyeicosa-5(*Z*),8(*Z*),11(*Z*)-trienoic acid (14,15-EET)[24]. EETs affect the water permeability barrier in mouse [25], and both

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[1] *Abbreviations used:* CP, chiral phase; CYP, cytochrome P450; EET, epoxyeicosatrienoic acid; HEET, epoxy-hydroxyeicosatrienoic acid; eLOX, epidermal lipoxygenase; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; LOX, lipoxygenase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; NP, normal phase; PCR, polymerase chain reaction; PGH, prostaglandin H; RP, reverse phase; TIC, total ion current.

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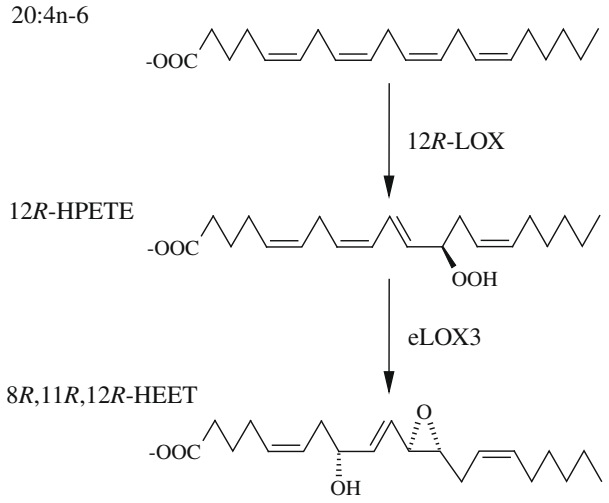

Fig. 1. 20:4*n*−6 is metabolized to 12*R*-HPETE by 12*R*-LOX, and isomerized to 8*R*,11*R*,12*R*-HEET by eLOX3.

ω-hydroxy-EETs and 8*R*,11*R*,12*R*-HEET activate PPARα [26,27], an important regulatory element of inflammation.

CYP4F8 and CYP4F22 are both highly expressed in epidermis, and in seminal vesicles and testis, respectively [5,8,9,28]. CYP4F8 is known to catalyze 19-hydroxylation of PGH_1 and PGH_2 formed by COX-2 of seminal vesicles, a key step in biosynthesis of the two main prostaglandins of human seminal fluid, 19-hydroxy-PGE_1 and -PGE_2 [5]. Since 19-hydroxy-PGs are not found in epidermis, an alternative function for CYP4F8 in skin is likely. Recombinant CYP4F8 oxidizes 20:4*n*−6 and other long chain fatty acids [5,29], and could be involved in the oxygenation of epoxyalcohols in the skin.

In an attempt to find the function of CYP4F8 and CYP4F22 in epidermis, we investigated whether HEETs, EETs, and 20:4*n*−6 were oxidized by recombinant CYP4F22 and CYP4F8. We also performed a systematic study of the MS/MS spectra and the fragmentation mechanism of HEETs derived from 15- and 12-HPETE.

Materials and methods

Materials

20:4*n*−6 (>99%) was from Sigma. 12(*S*)-Hydroperoxyeicosa-5(*Z*),8(*Z*),10(*E*),14(*Z*)-tetraenoic acid (12*S*-HPETE) was from Larodan Fine Chemicals (Malmö, Sweden). 14(*S*),15(*S*)-*trans*-epoxy-13(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoate (13*S*,14*S*,15*S*-HEET) methyl ester, 14(*S*),15(*S*)-*trans*-epoxy-11(*S*)-hydroxy-5(*Z*),8(*Z*),12-(*E*)-eicosatrienoate (11*S*,14*S*,15*S*-HEET) methyl ester, and 14(*S*),-15(*S*)-*trans*-epoxy-11(*R*)-hydroxy-5(*Z*),8(*Z*),12(*E*)-eicosatrienoate (11*R*,14S,15S-HEET) methyl ester were from Lipidox (Stockholm, Sweden). 15*S*-HPETE and [5,6,8,9,11,12,14,15-$^2H_8$]15*S*-HPETE were prepared using soybean LOX-1 from 20:4*n*−6 and [5,6,8,9,11,-12,14,15-$^2H_8$]20:4*n*−6 (99%), a gift of the late Dr. van Dorp. HPETE were obtained by vitamin E controlled autoxidation of 20:4*n*−6 [30]. Rac 16-HETE and 16*R*-HETE were obtained as described [31]. The Kromasil-100Si-column was from Dalco ChromTech (Sollentuna, Sweden). The ReproSil Chiral-NR-R, ReproSil Chiral-AM, and ReproSil 100Si-columns were from Dr. Maisch GmbH (Ammerbuch, Germany). Octadecasilane silica columns were purchased from Skandinaviska GeneTec AB (Västra Frölunda, Sweden). Recombinant CYP4F8 was expressed in a yeast system, and microsomes were prepared as described [5]. Platelets were from Uppsala Academic Hospital (Uppsala, Sweden). TA cloning kits and *Escherichia coli* strain (Top10) were from Invitrogen. The enhanced avian RT-PCR kit and NADPH were from Sigma. Restriction enzymes were from New England BioLabs and Fermentas. QIAquick gel extraction kits were from Qiagen. Yeast extract and peptone from soybean were from Merck. *Taq* DNA polymerase was from Promega. PCR primers were from Cybergene (Huddinge, Sweden) and from TIB Molbiol (Berlin, Germany). Sequencing was performed at Uppsala Genomic Center (Rudbeck Laboratories, Uppsala University). Human cornea was obtained from Uppsala Academic Hospital (Uppsala Human Ethics Committee; Ups 03-546). Other solvents were from Merck (Darmstadt, Germany), and were of *pro analysis* quality.

Expression of CYP4F8 and CYP4F22

Recombinant CYP4F8 has previously been expressed in *Saccharomyces cerevisiae* strain W(R) [5]. CYP4F22 was now expressed using the same method. The full length coding region was amplified by PCR, along with two linker sequences and three additional adenine residues before the ATG to increase the efficiency. The amplicon was sequenced, and then subcloned into the yeast expression vector pYeDP60, between the linker sequences, and under a galactose promoter. The vector was transformed into the *S. cerevisiae* strain W(R), with the lithium acetate method. Uracil and adenine were used for selection. To verify successful transformation, genomic DNA was isolated [32] and analyzed by PCR (conditions: 10 pmol of each primer, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase in 25 μl 10 mM Tris–HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100; 30 reaction cycles, annealing temperature 60 °C). A 325-bp fragment of the CYP4F22 gene was amplified using 5′-CACCACTACCTCGACTTC as forward primer, and 5′-GGC ATTTCTCCTGGTATTC as reverse primer. Yeast was initially grown on minimal media (casamino acids 1 g/L, yeast nitrogen base 7 g/L, glucose 20 g/L) for 24 h, before changing to complete media (yeast extract 10 g/L, bactopeptone 10 g/L, ethanol 16 g/L, glucose 5 g/L). For induction of CYP4F22 and P450 reductase, the culture was grown on galactose (2% w/v) for 15 h before harvesting the cells. Microsomes were obtained by breaking the cell walls by glass beads, differential centrifugation (20,000*g* for 10 min, +4 °C; 100,000*g* for 60 min, +4 °C), resuspension of the pellet in a buffer with 0.05 M Tris–HCl, 20% glycerol, 1 mM EDTA, and homogenization. Microsomes were stored at −80 °C in the same buffer. As negative controls in PCR and expression studies, we used yeast transfected with vector without insert and untransfected yeast (the selection step was excluded).

Synthesis of 12R-HPETE

Hydroperoxides from 20:4*n*−6 (100 mg) were produced by autoxidation [30] under oxygen (36 h, 37 °C) in the presence of 5–10% vitamin E. The reaction was followed by spectrophotometry at 235 nm. The sample was dissolved in 7% diethyl ether in hexane and applied on an open column with 6 g of silicic acid (Mallinckrodt; activated at 110 °C for >2 h). The column was washed with 100 mL of 7% diethyl ether in hexane and the products were eluted with 100 mL of 20% diethyl ether in hexane in 10 mL fractions. The fractions were analyzed by TLC (Kieselgel 60; hexane/ethyl acetate, 50/50), and those containing oxygenated products were combined, evaporated to dryness, and dissolved in isopropanol/hexane for purification of 12*R*-HPETE by preparative CP-HPLC (see below).

Synthesis of HEETs and EETs

HEETs were prepared by treatment of 15*S*-HPETE, 12*S*-HPETE and 12*R*-HPETE with 1–10 eq. hematin (hemin dissolved in 0.01 M NaOH) in 0.2 mL 0.1 M K_2HPO_4 [33,34]. After 10 min the mixture

was diluted in water, extracted on a SepPak C_{18} column, eluted in ethyl acetate, evaporated to dryness, and dissolved in ethanol. For preparation of 14(*S*),15(*S*)-epoxy-13(*R*)-hydroxy-5(*Z*),8(*Z*),11(*Z*)-eicosatrienoic acid (13*R*,14*S*,15*S*-HEET), hematin was added directly after the synthesis of 15*S*-HPETE with sLOX-1, the products were extracted as above, and 13*R*,14*S*,15*S*-HEET was isolated by NP-HPLC.

HEETs of 12*S*-HPETE and [$^2H_8$]12*S*-HPETE were prepared *in situ* using outdated human platelets. 20:4*n*−6 and [$^2H_8$]20:4*n*−6 were incubated with the cytosolic fraction of human platelets (1.5 mL) in the presence of hematin (8 eq., 10 min, 37 °C) [35]. After extractive isolation, the HEETs were separated and analyzed by NP-HPLC. HEETs were typically generated from 50–60 μg of 12*R*-HPETE and *threo* 13,14,15-HEET from 60 μg 15*S*-HPETE. Methyl ester derivatives of authentic HEETs were hydrolyzed by treatment with 200 μl rat blood plasma (20 min, 37 °C), followed by extractive isolation. The HEETs were quantified by MS/MS (*m/z* 335 → full scan) by comparison with known concentrations of authentic HEETs. Hydrolysis to trihydroxy compounds was not detected by MS/MS analysis (*m/z* 353 → full scan) under our experimental conditions.

EETs were prepared by oxidation of 20:4*n*−6 with 1.1 eq. *m*-chloroperoxybenzoic acid as described [36]. The EETs were separated on HPLC (see below) into 14,15-, 11,12-, and 8,9-EET.

Enzyme assay

The substrates (usually 10–50 μM) were incubated (37 °C) with microsomes in 0.1 M $KHPO_4$ buffer (pH 7.4) in a total volume of 100 μl (CYPF8), or 250 μl (CYP4F22), in the presence of NADPH (1 mM). As negative controls, reactions without NADPH were used. The reactions were terminated after 30 min with 1 mL ethanol, centrifuged (15,000*g*, 10 min, +4 °C), diluted to 10 mL with water, and the products were extracted on a SepPak/C_{18} column, evaporated to dryness, and dissolved in ethanol for RP-HPLC analysis, or in 3–5% isopropanol in hexane for NP-HPLC analysis (see below).

Analysis of HEETs in cornea

Human cornea was homogenized in $KHPO_4$ buffer (pH 7.4), and incubated with 20:4*n*−6 (100 μM, 30 min, 37 °C). The reaction was stopped with ethanol (4 vols.), centrifuged (5 min, 15,000*g*, +4 °C), and the supernatant was evaporated and extracted on a SepPak/C_{18} column. The extracts were dissolved in isopropanol/hexane for analysis by NP-HPLC (see below).

LC–MS/MS analysis

NP-HPLC of HEETs and their corresponding metabolites was performed on an analytical silica column (Kromasil, 250 × 2 mm, 5 μm, 100 Å) using the solvent system hexane/isopropanol/acetic acid, 97/3/0.01 or 95/5/0.01, and a flow rate of 0.3 mL/min. Post column the effluent was combined in a T-junction with isopropanol/water (3/2; 0.2–0.3 mL/min) from a Surveyor MS pump. Preparative purification of enantiomers of 13,14*S*,15*S*-HEET was performed in the same way on a ReproSil 100 Si-column (250 × 10 mm, 5 μm), and a flow rate of 2 mL/min; 1 min fractions were collected.

Separation of 12*R*-HPETE from other HPETEs was achieved on a ReproSil Chiral-NR-R column (250 × 4 mm, 8 μm) eluted at 0.6 mL/min with 1% isopropanol in hexane as mobile phase [37]. The effluent was collected in 1 min fractions, which were analyzed by MS/MS (direct injection). The fractions with 12*R*-HPETE were evaporated to dryness, dissolved in ethanol, and stored at −20 °C.

8,9-EET was purified by NP-HPLC on a ReproSil 100 Si-column (250 × 10 mm, 5 μm), whereas 11,12-EET and 14,15-EET were separated by preparative RP-HPLC (Phenomenex, 200 × 8 mm; eluted at 1.2 mL/min with methanol/water/acetic acid, 80/20/0.1). The column was connected to a splitter, allowing 5% of the effluent into the mass spectrometer and 95% to the fraction collector. Mass spectra of all EETs were in accordance with previously published data [38].

Metabolites of EETs and 20:4*n*−6 were analyzed by RP-HPLC as described [39]. The column (Phenomenex, 150 × 2 mm) was eluted at 0.3 mL/min with methanol/water/acetic acid (75/25/0.01). Steric analysis of 16-HETE was performed on amylose tris(3,5-dimethylphenylcarbamate) coated on silica gel (ReproSil Chiral-AM, 250 × 2 mm, 5 μm), eluted at 0.15 mL/min with hexane/ethanol/acetic acid, 95/5/0.1, as described [31]. The effluent from the chiral columns was combined with isopropanol/water, as above, for MS/MS analysis.

Effluent from the columns was subjected to electrospray ionization (spray voltage 4.3 kV) in an ion trap mass spectrometer (LTQ, ThermoFisher) with monitoring of negative ions. Temperature of the heated transfer capillary was set to 310 °C, and collision energy to 35% (arbitrary units). Activation time was 30 ms, and the *q*-value 0.25. Ion isolation width was 1.5 Da with the exception of MS/MS/MS analysis of HPETE (*m/z* 335 → 317 → full scan), where an isolation width of 5 Da was used in the first selection (*m/z* 335), and 1.5 Da in the second selection (*m/z* 317) [37].

Results

LC–MS/MS analysis of HEETs

HEETs from 15S-HPETE

HEETs were generated by hematin treatment of 15*S*-HPETE. Fig. 2 shows the separation of the HEETs on NP-HPLC (3% isopropanol in hexane). Four HEETs were generated as main products: the *erythro* isomer 13*S*,14*S*,15*S*-HEET (peak I) and the *threo* isomer 13*R*,14*S*,15*S*-HEET (peak II), 11*S*,14*S*,15*S*-HEET (peak III), and 11*R*,14*S*,15*S*-HEET (peak IV). The minor peaks likely consisted of the HEETs with *cis* epoxy conformation as judged from their MS/MS spectra.

11,14*S*,15*S*-HEET and 13,14*S*,15*S*-HEET fragmented adjacent to the hydroxyl group and in the epoxy ring (see Fig. 3A and C). The fragmentation patterns were confirmed by MS/MS analysis (*m/z* 335 → full scan) of the [^{2}H]labeled isotopomers (Fig. 3B and D). Shared fragments were *m/z* 317 (A^- – H_2O) (in the [^{2}H]labeled iso-

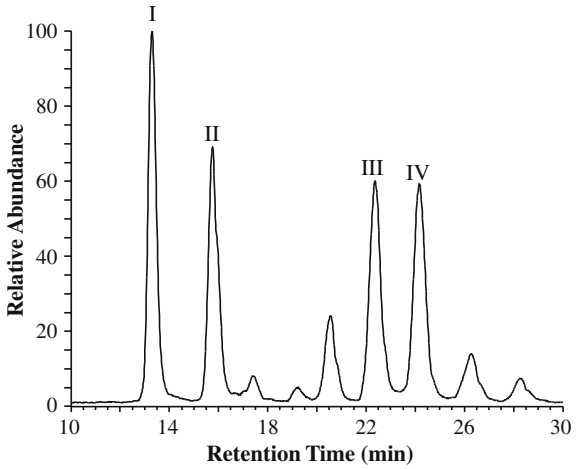

Fig. 2. NP-HPLC analysis of HEETs formed from 15*S*-HPETE by hematin. The four major products were (I) 13(*S*)-hydroxy-*trans*-14,15-epoxyeicosatrienoic acid, (II) 13(*R*)-hydroxy-*trans*-14,15-epoxyeicosatrienoic acid, (III) 11(*S*)-hydroxy-*trans*-14,15-epoxyeicosatrienoic acid, (IV) 11(*R*)-hydroxy-*trans*-14,15-epoxyeicosatrienoic acid. The products were eluted with hexane/isopropanol/acetic acid, 95/5/0.01, and the effluent was mixed with isopropanol/water, 60/40, and subject to electrospray ionization.

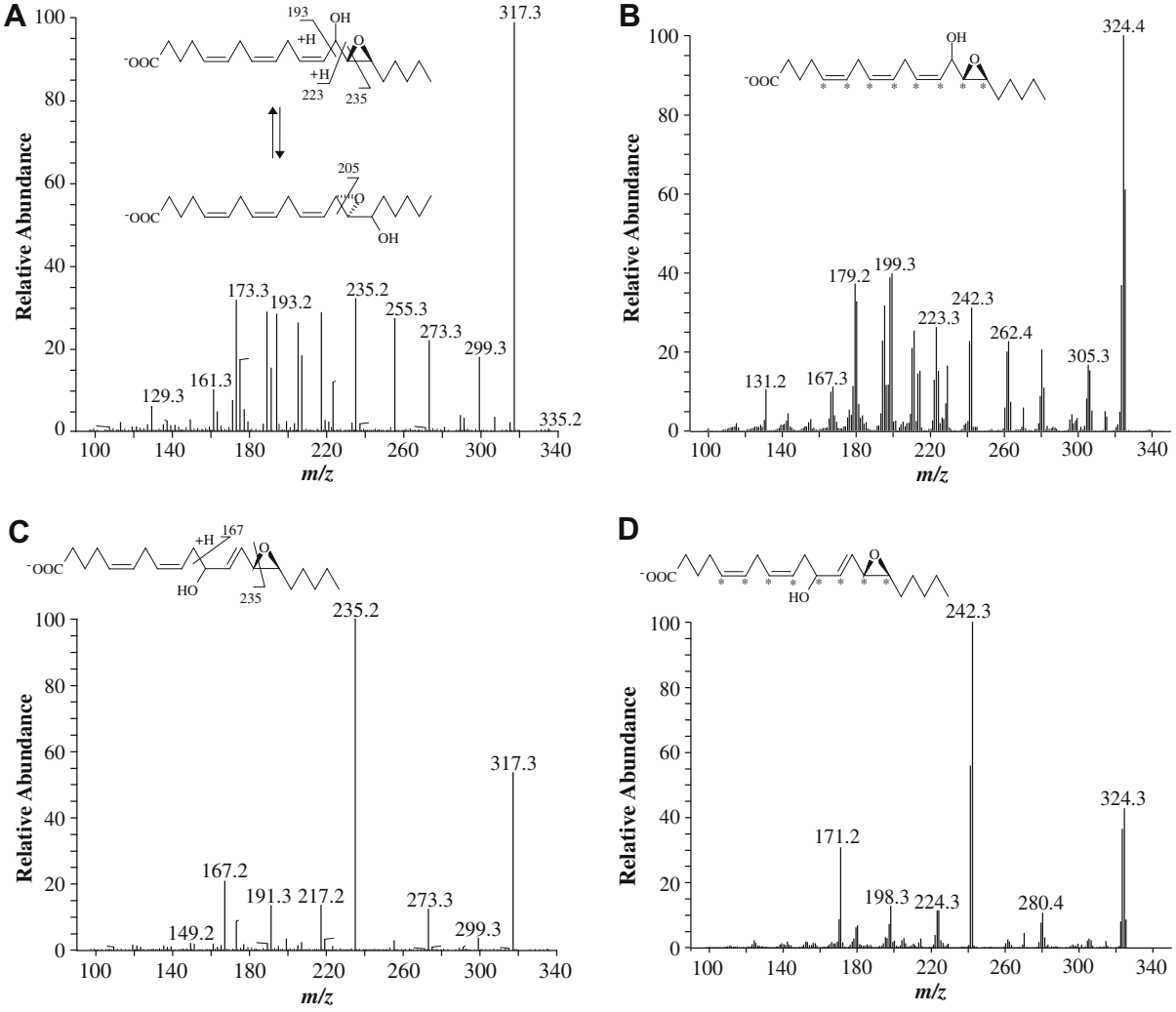

Fig. 3. MS/MS analysis of HEETs derived from 15*S*-HPETE. (A) 13-Hydroxy-*trans*-14,15-epoxyeicosatrienoic acid (m/z 335 → full scan) with evidence of keto-enol tautomerism (see insert). (B) [$^2H_8$]13*S*-Hydroxy-*trans*-14,15-epoxyeicosatrienoic acid (m/z 343 → full scan). (C) 11-Hydroxy-*trans*-14,15-epoxyeicosatrienoic acid. (D) [$^2H_8$]11-Hydroxy-*trans*-14,15-epoxyeicosatrienoic acid. Deuterium is marked with an asterisk. The inserts show the proposed fragmentations.

topomers m/z_{2H} 324), m/z 299 (317 – H_2O) (m/z_{2H} 305–306), m/z 273 (A^- – H_2O – CO_2) (m/z_{2H} 280), m/z 255 (273 – H_2O) (m/z_{2H} 262), m/z 235 (A^- – O=CH–$(CH_2)_4$–CH_3) (m/z_{2H} 242), m/z 217 (235 – H_2O) (m/z_{2H} 223–224), m/z 191 (235 – CO_2) (m/z_{2H} 198), m/z 173 (191 – H_2O) (m/z_{2H} 179–180). The discriminating ions were m/z 167 ($^-$OOC–$(CH_2)_3$–CH=CH–CH_2–CH=CH–CH_3) (m/z_{2H} 171) for 11,14*S*,15*S*-HEET, and m/z 193 ($^-$OC–$(CH_2)_3$–CH=CH–CH_2–CH=CH–CH_2–CH=CH_2) (m/z_{2H} 199) for 13,14*S*,15*S*-HEET. In the spectrum of 13-hydroxy derivative the signal at m/z 205 was identified as originating from a Payne rearrangement [40] of the epoxy group during the MS analysis (insert Fig. 3A).

HEETs from 12-HPETE

Fig. 4 shows the separation of the HEET products of 12*S*-HPETE on NP-HPLC. The four main products were identified as 11,12-*trans*-epoxy-10(*R*/*S*)-hydroxyeicosatrienoic acid (10,11,12-HEET; peak I and II), and 11,12-*trans*-epoxy-8(*R*/*S*)-hydroxyeicosatrienoic acid (8,11,12-HEET; peaks III and IV).

Both mass spectra (m/z 335 → full scan) (Fig. 5A and C) showed signals at m/z 317 (A^- – H_2O) (m/z_{2H} 324), m/z 299 (317 – H_2O) (m/z_{2H} 305–306), m/z 273 (A^- – H_2O – CO_2) (m/z_{2H} 280), m/z 255 (273 – H_2O) (m/z_{2H} 262), and m/z 195 ($^-$OOC–$(CH_2)_3$–CH=CH–CH_2–

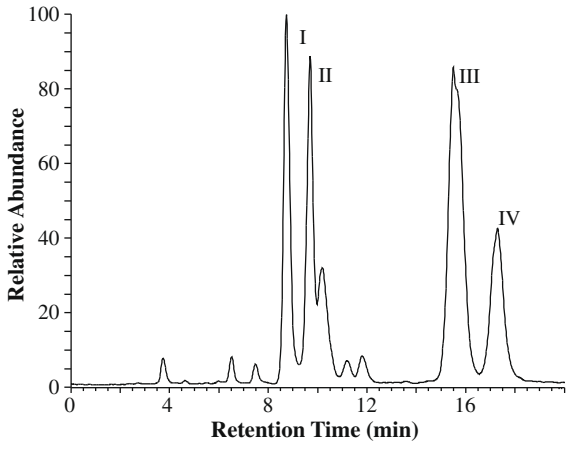

Fig. 4. NP-HPLC separation of HEETs formed from 12*S*-HPETE by hematin. The four major peaks contained (I) 10(*S*)-hydroxy-*trans*-11,12-epoxyeicosatrienoic acid, (II) 10(*R*)-hydroxy-*trans*-11,12-epoxyeicosatrienoic acid, (III/IV) 8(*S*/*R*)-hydroxy-*trans*-11,12-epoxyeicosatrienoic acid. The products were eluted with hexane/isopropanol/acetic acid, 95/3/0.01, and the effluent was mixed with isopropanol/water and subject to electrospray ionization.

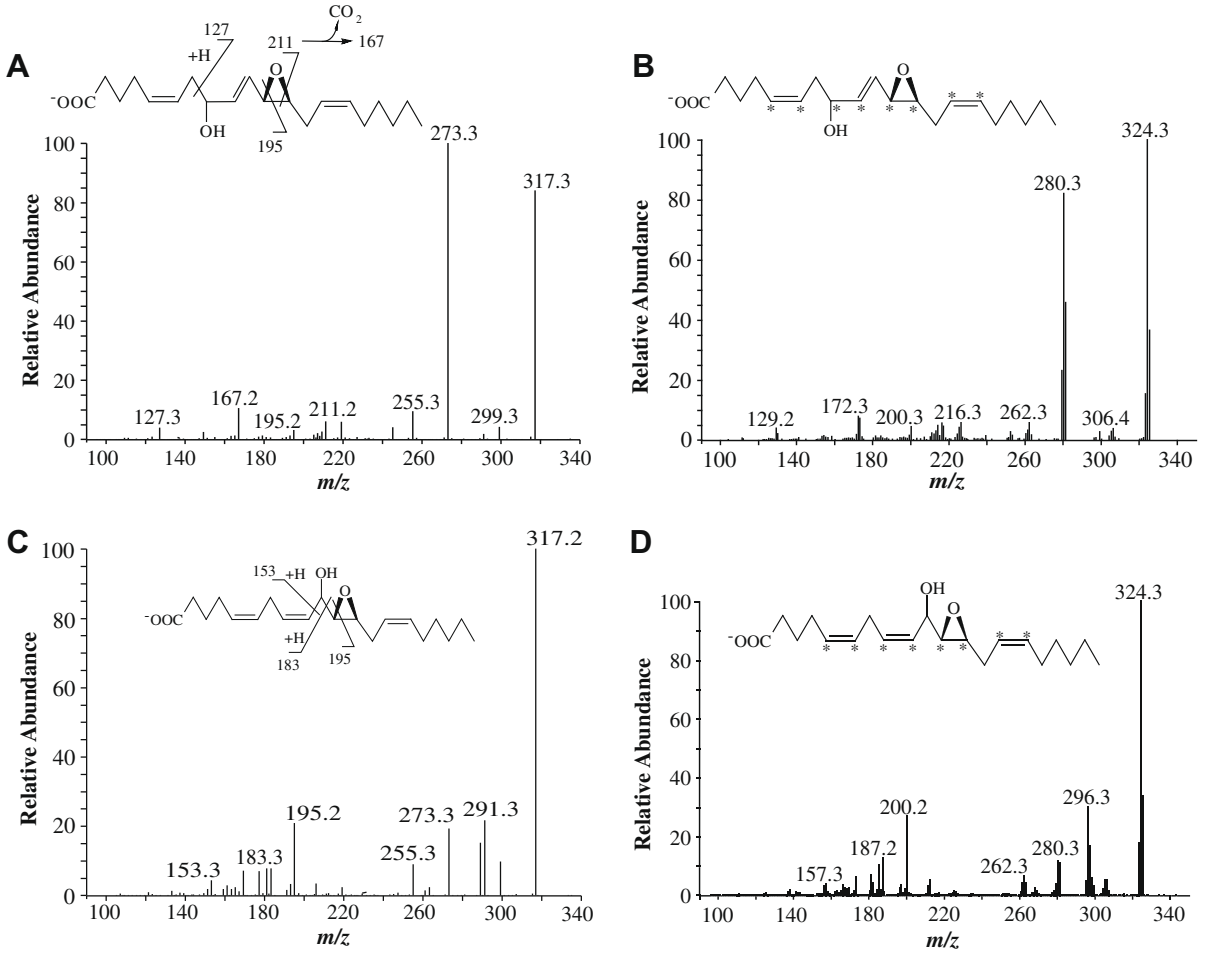

Fig. 5. MS/MS analysis of HEETs derived from 12*S*-HPETE. (A) 8-Hydroxy-*trans*-11,12-epoxyeicosatrienoic acid (*m*/*z* 335 → full scan). (B) [$^2H_8$]8-Hydroxy-*trans*-11,12-epoxyeicosatrienoic acid (*m*/*z* 343 → full scan). (C) 10-Hydroxy-*trans*-11,12-epoxyeicosatrienoic acid. (D) [$^2H_8$]10-Hydroxy-*trans*-11,12-epoxyeicosatrienoic acid. Deuterium is marked with an asterisk. The inserts show the proposed fragmentations.

C(OH)═CH–CH═CH_2 and $^-$OOC–$(CH_2)_3$–CH═CH–CH_2–CH═CH–CH(OH)═CH_2, respectively) (*m*/z_{2H} 200). 8-Hydroxy-11,12-epoxyeicosatrienoic acid also showed characteristic signals at *m*/*z* 127 ($^-$OOC–$(CH_2)_3$–CH═CH–CH_3) (*m*/z_{2H} 129), and *m*/*z* 211 ($^-$OOC–$(CH_2)_3$–CH═CH–CH_2–CH═CH–CH(OH)–CHO) (*m*/z_{2H} 216). 10-Hydroxy-11,12-epoxyeicosatrienoic acid shows signals at *m*/*z* 153 ($^-$OC–$(CH_2)_3$–CH═CH–CH_2–CH═CH_2) (*m*/z_{2H} 156–157), and *m*/*z* 183 ($^-$OOC–$(CH_2)_3$–CH═CH–CH_2–CH═CH–CH_2–OH) (*m*/z_{2H} 187). As expected, the MS/MS spectra of HEETs derived from 12*R*-HPETE and 12*S*-HPETE were virtually identical.

Formation of HEETs from 20:4n−6 by human cornea

Several products were detected by NP-HPLC–MS/MS (*m*/*z* 335 → full scan) from human cornea when incubated with 20:4*n*−6 (Fig. 6). Peak I was identified as 13*S*,14*S*,15*S*-HEET and peak II as 13*R*,14*S*,15*S*-HEET, as judged from the MS/MS spectra and retention times above. Peak III likely contained the two stereoisomers of 11,14*S*,15*S*-HEET, which were only partly resolved.

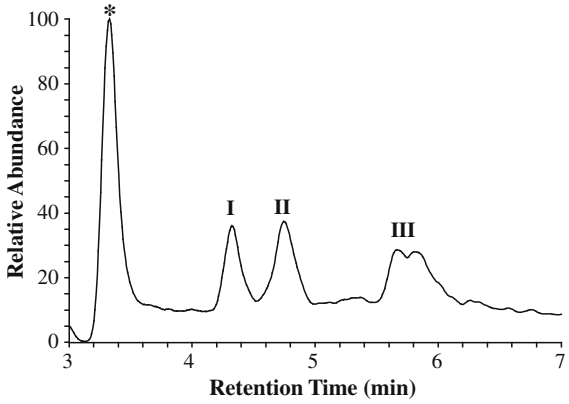

Fig. 6. NP-HPLC analysis of HEETs formed from 20:4*n*−6 by homogenates of human cornea. Materials in peaks I and II were identified as isomers of 13,14,15-HEET, and peak III as 11,14,15-HEET by MS/MS analysis (*m*/*z* 335 → full scan). Peak denoted with an asterisk labels unrelated compounds. The products were eluted with hexane/isopropanol/acetic acid, 95/5/0.01, and the effluent was mixed with isopropanol/water, 60/40, and subject to electrospray ionization.

Oxidation of EETs and HEETs by CYP4F8

Oxidation of EETs

Microsomes of CYP4F8 oxidized 8,9-EET and 11,12-EET to their corresponding 18-hydroxy metabolites (Fig. 7). This was confirmed by a characteristic signal at *m*/*z* 277 (A^- – 58; loss of CH_3–CH_2–CHO) in both mass spectra (*m*/*z* 335 → full scan). We could not detect any dihydroxy products, which validates that the epoxides were not subjected to epoxide hydrolase activity during enzyme assay. We could not detect any oxidation of 14,15-EET.

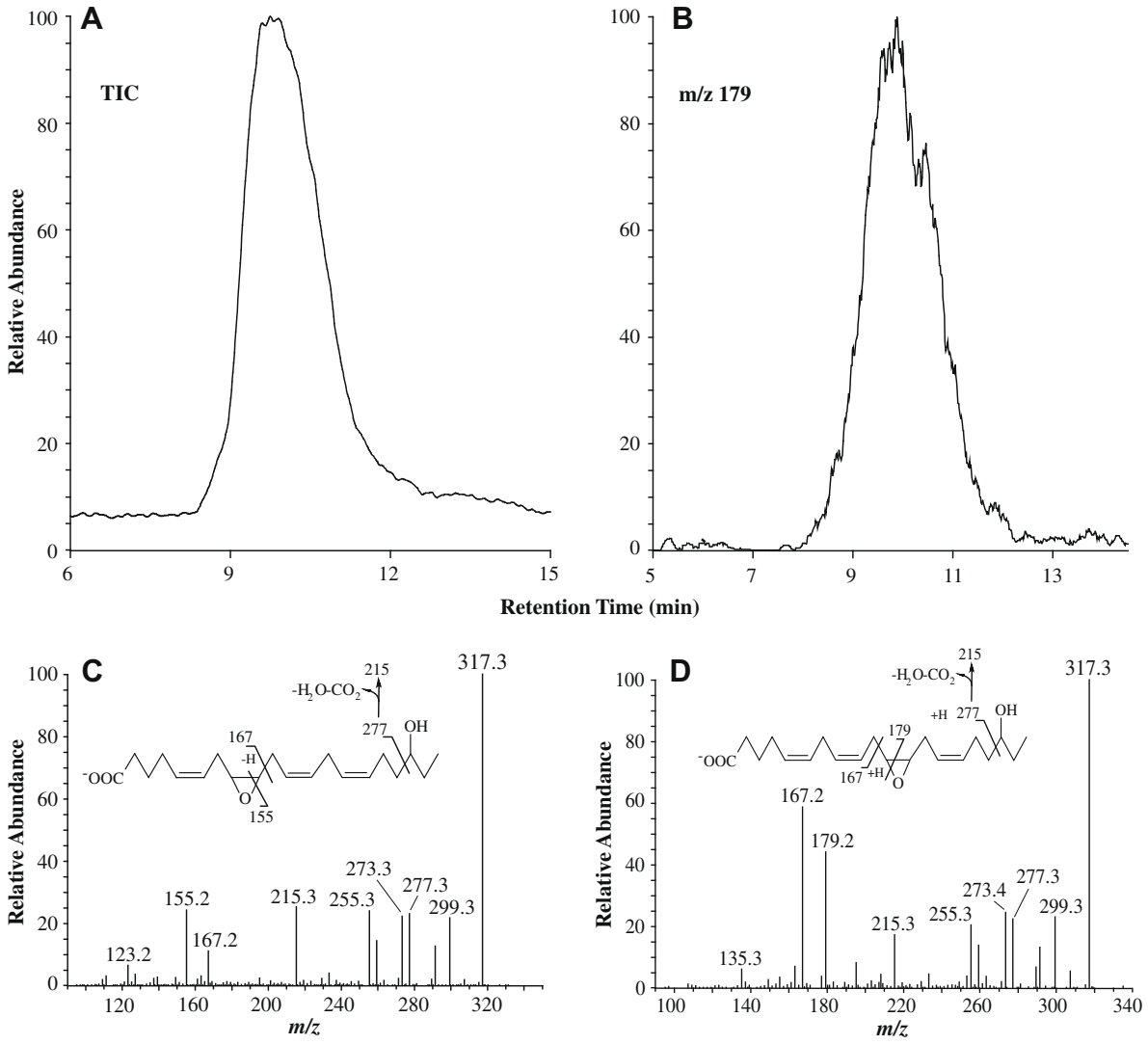

Fig. 7. RP-HPLC analysis of metabolites formed by recombinant CYP4F8. (A) Partial total ion current chromatogram (TIC) showing the elution of a metabolite formed by microsomes of CYP4F8 and 8,9-EET. The MS/MS spectrum is shown in C. (B) Partial ion chromatogram (*m/z* 179) showing the elution of a metabolite formed by microsomes of CYP4F8 and 11,12-EET. The MS/MS spectrum is shown in (D). (C and D) The MS/MS spectra (*m/z* 335 → full scan) of the main products formed from 8,9-EET and 11,12-EET showed a characteristic ion at *m/z* 277 (335 − 58), and were identified as the ω3 hydroxy metabolites (cf. Ref. [38]).

Oxidation of HEETs

Microsomes of CYP4F8 oxidized 10,11*R*,12*R*-HEET to the 18-hydroxy metabolite (Fig. 8A). The MS/MS spectrum (*m/z* 351 → full scan) showed a characteristic signal at *m/z* 293 (loss of O=CH–CH_2–CH_3), *m/z* 231 (293-44-18, loss of CO_{2+} H_2O), and *m/z* 213 (231 − 18) (Fig. 8B). Recombinant CYP4F8 did not oxidize 8(*R*/*S*),11*R*,12*R*-HEET, 11*S*,14*S*,15*S*-HEET, 11*R*,14*S*,15*S*-HEET, or 13*S*,14*S*,-15*S*-HEET significantly (data not shown).

Expression of recombinant CYP4F22 in yeast

The sequence of the amplified CYP4F22 open reading frame was in accordance with the previously published sequence (GenBank Accession No. NM_173483), except in position 582, where A was exchanged for G (a reported synonymous SNP; GenBank, rs11666601). The CYP4F22 sequence was ligated with the vector pYeDP60 and used to transform the yeast strain W(R). Transformation was confirmed by CYP4F22 specific PCR analysis of genomic DNA, which was isolated from transformed W(R) cells (Fig. 9). The expression of recombinant CYP4F22 (and CYP4F8) in microsomes was too low for quantification by the absorbance at 450 nm after $Na_2S_2O_4$ reduction and treatment with CO.

Oxidation by recombinant CYP4F22

20:4n−6

Microsomes of CYP4F22 were incubated with 200 μM 20:4*n*−6. LC–MS/MS analysis (*m/z* 319 → full scan) demonstrated the formation of two products, 16-hydroxyeicosa-5(*Z*),8(*Z*),11(*Z*),14(*Z*)-tetraenoic acid (16-HETE) (Fig. 10, top) and 18-hydroxyeicosa-5(*Z*),8(*Z*),11(*Z*),14(*Z*)-tetraenoic acid (18-HETE) (Fig. 10, bottom). Addition of cytochrome b_5 did not increase the amounts of products formed (data not shown). The MS/MS spectrum of 18-HETE was as reported [5], with a characteristic signal at *m/z* 261 (A^- – 58; loss of CH_3–CH_2–CHO). 16-HETE had a characteristic signal at *m/z* 233 (A^- – 86; loss of CH_3–$(CH_2)_3$–CHO). Control experiments with microsomes of yeast transformed with plasmid vector without the CYP4F22 sequence, and microsomes of untransformed yeast revealed the formation of rac 16-HETE (insert top Fig. 10), which was therefore not associated with expression of CYP4F22.

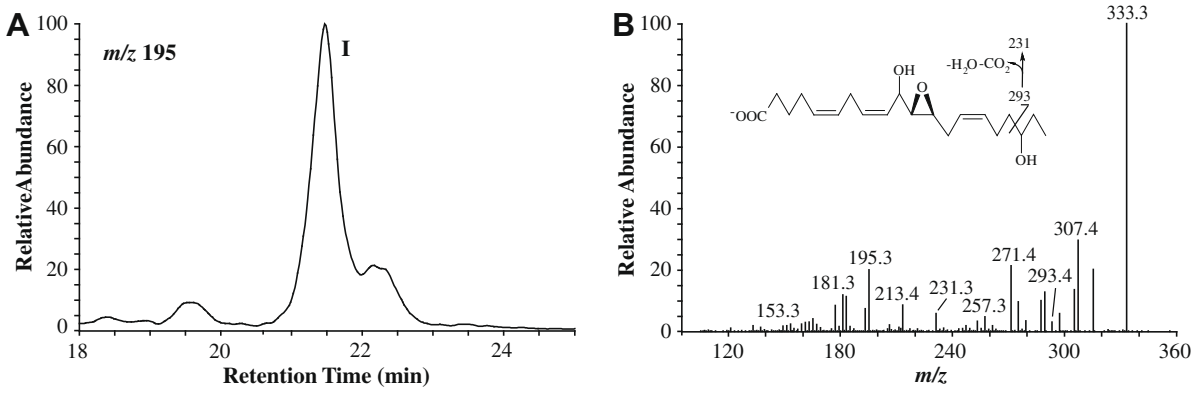

Fig. 8. LC–MS/MS analysis of products formed by recombinant CYP4F8 and 10,11,12-HEET. (A) NP-HPLC analysis shows separation of products (hexane/isopropanol/acetic acid, 95/5/0.01) with MS/MS analysis (m/z 351 → full scan). (B) The MS/MS spectrum (m/z 351 → full scan) of the material eluting at 21–22 min showed characteristic ions at m/z 231, and m/z 213, consistent with 18-hydroxy-10,11,12-HEET.

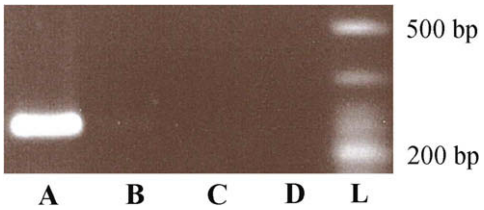

Fig. 9. PCR analysis of transformation of yeast W(R) with CYP4F22 analyzed by agarose gel electrophoresis. (A) PCR analysis of genomic DNA from W(R) transformed with vector with the CYP4F22 open reading frame. (B) PCR analysis of genomic DNA from W(R) transformed with vector without insert. (C) PCR analysis of genomic DNA of untransformed W(R). (D) PCR analysis without template DNA. L, low MW ladder (New England Biolabs).

Furthermore, the formation was NADPH dependent. We conclude that 16-HETE was likely formed by endogenous CYP of yeast as a minor product. Importantly, 18-HETE was not detected in any of these control experiments, and 18-HETE can not be formed non-enzymatically. We conclude that this metabolite was formed by CYP4F22. In comparison with recombinant CYP4F8, the catalytic activity of recombinant CYP4F22 was 1–2 orders of magnitude lower.

HEETs

We could not detect any metabolites formed by recombinant CYP4F22 in two experiments with 8,11*R*,12*R*-HEET or with 10,11*R*,12*R*-HEET. Under similar conditions, 10,11*R*,12*R*-HEET was oxidized by CYP4F8, as described above.

Discussion

We report expression of recombinant CYP4F22 with arachidonate ω3 hydroxylase activity. This confirms that the open reading frame of *CYP4F22* codes for a functional enzyme, but unfortunately the enzyme appeared to be poorly expressed. As we expected that CYP4F22 also could metabolize HEETs, we performed a systematic study of the MS/MS spectra of HEETs in order to determine metabolites formed by CYP4F22 and by CYP4F8.

18-HETE cannot be formed non-enzymatically. Biosynthesis of this metabolite by recombinant CYP4F22 is the first reported catalytic activity associated with this enzyme. 18-HETE is also formed by CYP4F8 [5], but the biological significance of this product is unknown. Seminal fluid contains ~0.1 mM concentration of 19-hydroxy-PGs, which are formed by ω2 hydroxylation of PGH by CYP4F8 [5], but 18-HETE has not been detected in this fluid.

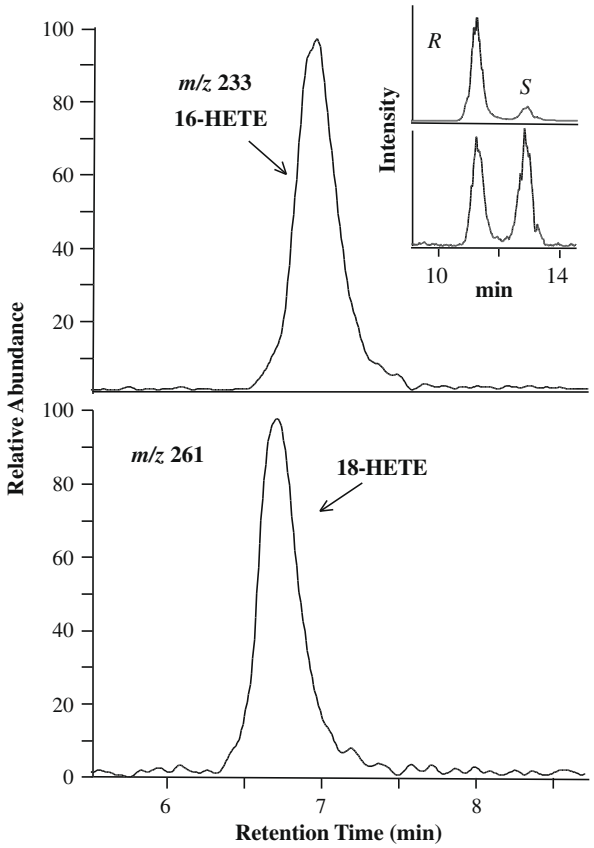

Fig. 10. LC–MS/MS analysis of products formed from recombinant CYP4F22 and 20:4*n*−6. Top, Selective ion monitoring of m/z 233 (319 − 86), a characteristic ion of 16-HETE. Bottom, selective ion monitoring of m/z 261 (319 − 58), a characteristic ion of 18-HETE. The insert in the top chromatogram shows CP-HPLC analysis of 16-HETE (16*R*-HETE above and the racemic biological sample below).

CYP4F8 may thus be functionally linked to COX-2 of seminal vesicles rather than to phospholipases and release of 20:4*n*−6. It is conceivable that CYP4F22 could be linked to the 12*R*-LOX and eLOX3 pathway in a similar way.

We report a systematic study of MS/MS spectra of HEETs derived from 12- and 15-HPETE, the two main LOX products in skin. The MS/MS spectra of the HEETs followed the fragmentation pattern described for the corresponding epoxyalcohols of linoleic acid [34]. The proposed fragmentation was supported by MS/MS spectra of [$^2H_8$]labeled isotopomers. We found that NP-HPLC–MS/MS

analysis was useful for analysis of HEETs in biological samples, as illustrated in human corneal tissue.

8*R*,11*R*,12*R*-HEET is produced in human epidermis by 12*R*-LOX and eLOX3 [19]. A linkage between mutations in the gene of CYP4F22 and lamellar ichtyosis was reported by Lefèvre et al., and the authors proposed that CYP4F22 could be involved in oxidation of 8*R*,11*R*,12*R*-HEET [9]. However, neither CYP4F22 nor CYP4F8 appeared to oxidize 8,11*R*,12*R*-HEET. Recombinant CYP4F8 oxidized 10,11*R*,12*R*-HEET to its 18-hydroxy metabolite (Fig. 8A), but 11*S*,14*S*,15*S*-HEET, 11*R*,14*S*,15*S*-HEET and 13*S*,14*S*,15*S*-HEET were not substrates.

The selective oxygenation of 10,11*R*,12*R*-HEET is interesting, as both 12*R*-LOX and CYP4F8 are up regulated in psoriatic lesions [8,11]. To investigate whether the position of the epoxide group at C11/C12 contributed to this selectivity, we tested the oxygenation of EETs. We found that 8,9-EET and 11,12-EET were oxidized at the ω3 position, but 14,15-EET was not a substrate. The position of the epoxide group was apparently important. No significant non-enzymatic hydrolysis of the HEETs or EETs was observed in our enzyme assays.

HEETs can be hydrolyzed to triols by epoxide hydrolases in tissues, and these products might be substrates of CY4F members. It is therefore possible that CYP4F22 might be involved in a subsequent step in the 12*R*-LOX/eLOX3 pathway. However, CYP4F22 is also expressed in testes, where 12*R*-LOX has not been described. It is conceivable that CYP4F22 could be involved in oxygenation of other lipids in analogy with CYP4F8 and ω2 hydroxylation of PGH in seminal vesicles [5]. Further studies of the function of CYP4F22 in reproductive organs seem merited.

In summary, we report that recombinant CYP4F22 catalyzed ω3 hydroxylation of 20:4*n*−6, whereas oxygenation of 8,11*R*,12*R*-HEET was not detected. CYP4F8 oxidized 8,9-EET, 11,12-EET, and 10,11*R*,12*R*-HEET. The latter is formed from 12*R*-HPETE, which suggests a functional link between CYP4F8 and 12*R*-LOX in epidermis.

Acknowledgments

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