# Cytochromes P450 from family 4 are the main omega hydroxylating enzymes in humans: CYP4F3B is the prominent player in PUFA metabolism[1]

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**Abstract Human CYP450 ω-hydroxylases of the CYP4 family are known to convert arachidonic acid (AA) to its metabolite 20-hydroxyeicosatetraenoic acid (20-HETE). This study deals with hydroxylations of four PUFAs, eicosatrienoic acid (ETA), AA, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) by either human recombinant CYP4s enzymes or human liver microsomal preparations. CYP4F3A and CYP4F3B were the most efficient ω-hydroxylases of these PUFAs. Moreover, the differences in the number of unsaturations of ETA, AA, and EPA allowed us to demonstrate a rise in the metabolic rate of hydroxylation when the double bond in 14-15 or 17-18 was missing. With the CYP4F enzymes, the main pathway was always the ω-hydroxylation of PUFAs, whereas it was the (ω-1)-hydroxylation with CYP1A1, CYP2C19, and CYP2E1. Finally, we demonstrated that the ω9 and ω3 PUFAs (ETA, EPA, and DHA) could all be used as alternative substrates in AA metabolism by human CYP4F2 and -4F3B. Thus, they decreased the ability of these enzymes to convert AA to 20-HETE. However, although ETA was the most hydroxylated substrate, EPA and DHA were the most potent inhibitors of the conversion of AA to 20-HETE. These findings suggest that some physiological effects of ω3 FAs could partly result from a shift in the generation of active hydroxylated metabolites of AA through a CYP-mediated catalysis.—** Fer, M., L. Corcos, Y. Dréano, E. Plée-Gautier, J-P. Salaün, F. Berthou, and Y. Amet. **Cytochromes P450 from family 4 are the main omega hydroxylating enzymes in humans: CYP4F3B is the prominent player in PUFA metabolism. *J. Lipid Res.* 2008.** 49: **2379–2389.**

**Supplementary key words** ω-hydroxylation • (ω-1)-hydroxylation • inhibition

Among the human cytochromes P450 (CYPs) (1), CYP families 1–3 are known to be largely involved in the oxidation of drugs and other xenobiotics essentially in the liver, whereas P450 families with higher numbers are involved in the metabolism of endobiotics, such as FAs, eicosanoids, and steroids. Families 1–3 are known to catalyze the epoxidation or the nonterminal hydroxylation of FAs (2, 3). The unusual terminal hydroxylation and, to a lesser extent, the subterminal hydroxylation of lauric acid (4, 5) and arachidonic acid (AA) (6, 7) are carried out by CYP4 enzymes. The oxidized compounds of the PUFAs, named oxylipids, play prominent physiological roles in vascular tone or inflammation. Indeed, 20-hydroxyeicosatetraenoic acid (20-HETE) is a potent vasoconstrictor (8, 9), in contrast to 19-HETE, known to have the opposite effect (10).

Among the CYP4 enzymes, the CYP4F subfamily is quite new (11–13) and endowed with catalytic activity as leukotriene B4 (LTB4) ω-hydroxylase (14, 15). To date, several isoforms have been described in the literature: CYP4F2 (15), CYP4F3A/3B (16), CYP4F8 (17, 18), CYP4F11 (19, 20), CYP4F12 (21), and CYP4F22 (22), corresponding to six genes that cluster to chromosome 19. CYP4F3A/3B enzymes result from an alternative splicing of the *CYP4F3* gene (16). All of these CYPs are involved in the hydroxylation of FAs, especially in prostaglandins and leukotriene oxidations (11, 12). The most characteristic reaction of the CYP4F subfamily is the ω-oxidation of LTB4. This leads to a dramatic loss of both its chemotactic and aggregation activities on leukocytes (14). However, despite the com-

Abbreviations: AA, arachidonic acid; APCI, atmospheric pressure chemical ionization; DBA, *n*-dibutylamine; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETA, eicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LTB4, leukotriene B4; VLCFA, very long-chain FA.

[1] CYPs, enzymes belonging to the cytochromes P450 families, are designated here from the nomenclature available on Dr. David Nelson's website for cytochrome P450s (http://drnelson.utmem.edu/human.P450.table.html).

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mon ability of CYP4F family members to successfully ω-hydroxylate AA, they display considerable differences in their substrate affinities (11, 12, 16). Furthermore, because of the differences in the *CYP4* gene between mouse, rat, and humans (12), no information about the catalytic function of the human CYP4 enzymes can be inferred from the knowledge of the rat CYP4F (23) or Cyp4a mouse (24) enzymes. And finally, the CYP4s are mostly expressed in the liver and the kidney, except the CYP4F3A isoform, which is expressed in myeloid cells (16, 21, 25). Among the AA metabolites formed by human liver microsomes and other tissues, 20-HETE is the main one and frequently the most abundant (6, 26–29).

Few studies have addressed the biological activities of eicosatrienoic acid (ETA), also named Mead acid, known to be a marker of deficiency in essential FAs. On the other hand, the ω3 FAs now widely used as dietary supplements, are assumed to be endowed, in humans, with a certain number of beneficial health-related actions, including an antihypertensive effect (30–33). Despite the widespread use of ω3 PUFAs, only limited data are available on their effect at the biochemical level. Recently, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were described to be ω-hydroxylated by CYP4F3B (34), suggesting that some functional effects of ω-3 FA supplementation may be due to inhibition of 20-HETE formation or the conversion of EPA to the corresponding omega-oxidized product. The goal of our study was to extend these findings to additional PUFAs, such as AA and ETA, and to additional recombinant enzymes. Importantly, knowledge of the consequences of PUFA metabolic activities in bioactive oxylipids and of the ability of PUFA to antagonize 20-HETE production upon competitive inhibition remains incomplete (6, 7, 34). We decided to analyze the metabolic profiles of PUFAs using recombinant CYP in order to determine the impact of the position of the last olefinic unsaturation in ω9 (ETA), ω6 (AA), and ω3 (EPA, DHA) compounds on the fate of the CYP-catalyzed reac-

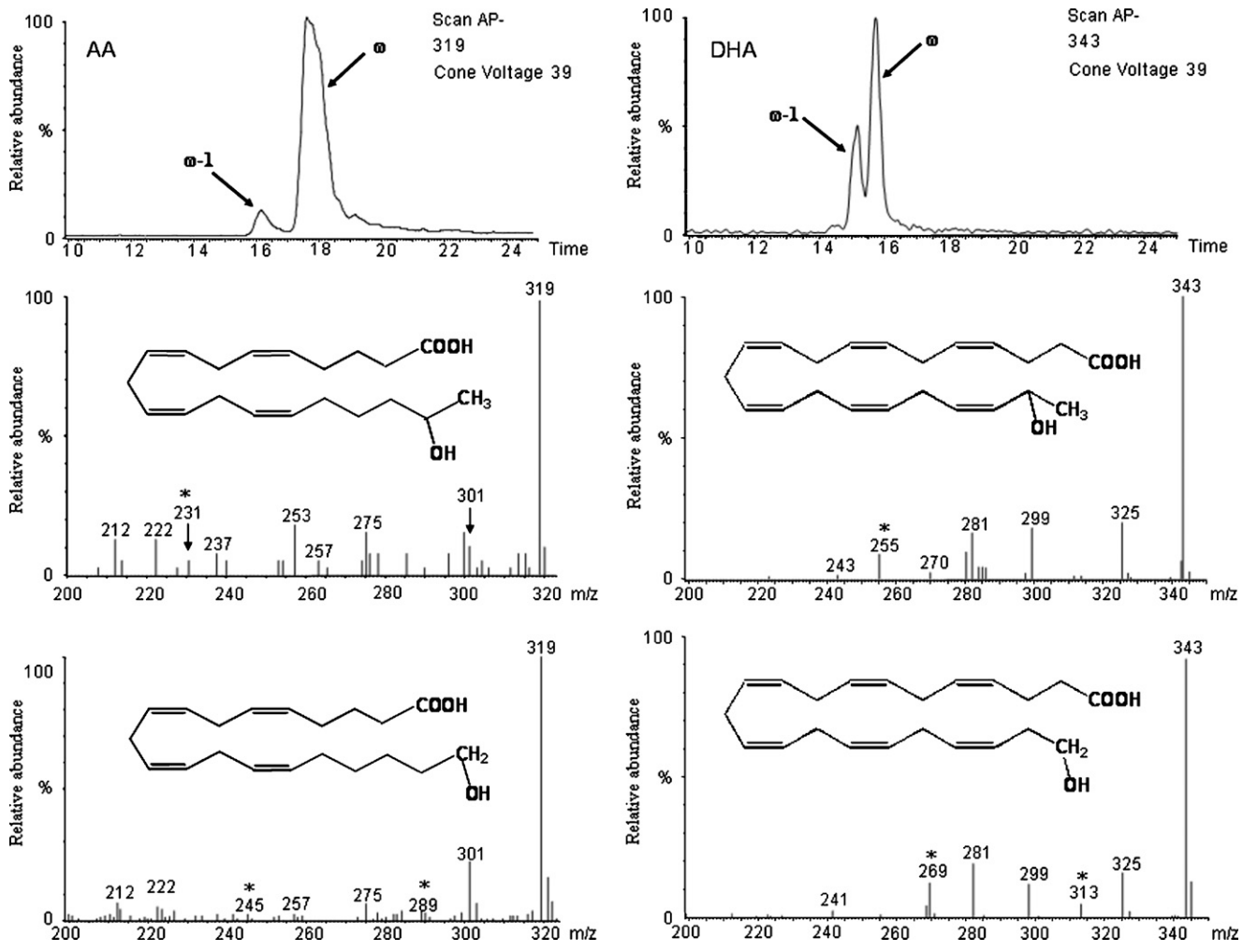


**Fig. 1.** LC-MS analysis of ω- and (ω-1)-hydroxylated metabolites of arachidonic acid (AA) and docosahexaenoic acid (DHA) incubated with recombinant CYP4F3B (10 pmol). The products were separated by ion pairing reverse-phase (RP)-HPLC and subjected to atmospheric pressure chemical ionization mass spectrometry (APCI-MS) analysis in a negative mode. The figure shows reconstructed ion chromatograms with molecular ions of hydroxylated derivatives of AA at *m/z* 319 or DHA at *m/z* 343 and the resulting mass spectra of ω- and (ω-1)-OH-AA or of ω- and (ω-1)-OH-DHA. The characteristic ions are noted by an asterisk.

tion. Then we focused on the inhibition of 20-HETE formation by ω3 FAs, and the inhibition of AA hydroxylation by EPA and DHA was compared with AA inhibition of DHA hydroxylation by CYP4F2 and -4F3B.

We examined the hydroxylation of 5,8,11-ETA, AA, EPA, and DHA through the use of 18 human recombinant CYPs from families 1–4, or with four preparations of human liver microsomes. Among the PUFAs studied, 5,8,11-ETA, AA, and EPA differ by their number of unsaturations, whereas DHA was used to probe the effect of the length of the alkyl chain of the ω3 FAs on the hydroxylation rate of methylene and methyl terminus. Only CYP4F proteins could efficiently produce the PUFA ω-hydroxylated derivatives. CYP4F3A and -4F3B were found to be the most efficient in this reaction, whereas CYP4A11 was the least efficient. Finally, we demonstrated that ETA, EPA, and DHA can be used as alternative substrates of AA by CYP4F2 and -4F3B, which decrease the ability of these enzymes to convert AA to 20-HETE. Although ETA was the most hydroxylated substrate, EPA and DHA were more potent inhibitors of the conversion of AA to 20-HETE.

## MATERIALS AND METHODS

### Chemicals

All chemicals of analytical grade were from Sigma-Aldrich (l'Isle d'Abeau, France). HPLC-purity-grade solvents were obtained from Carlo Erba (Val de Reuil, France). The HPLC counter-ion, *n*-dibutylamine (DBA), in its acetate concentrated form, was from Fluka (Buchs, Switzerland). The PUFAs (5,8,11-ETA, AA, EPA, and DHA) and the 20-HETE were from Cayman Chemical (Spi-Bio, Montigny le Bretonneux, France).

### Radioactive compounds

The specific activities of $^{14}$C-radiolabeled FAs were: 2.07 GBq/mmol for [1-$^{14}$C]AA, from Amersham Biosciences (UK); 2.03 GBq/mmol for [1-$^{14}$C]5,8,11-ETA and [1-$^{14}$C]EPA, both from MP Bio-

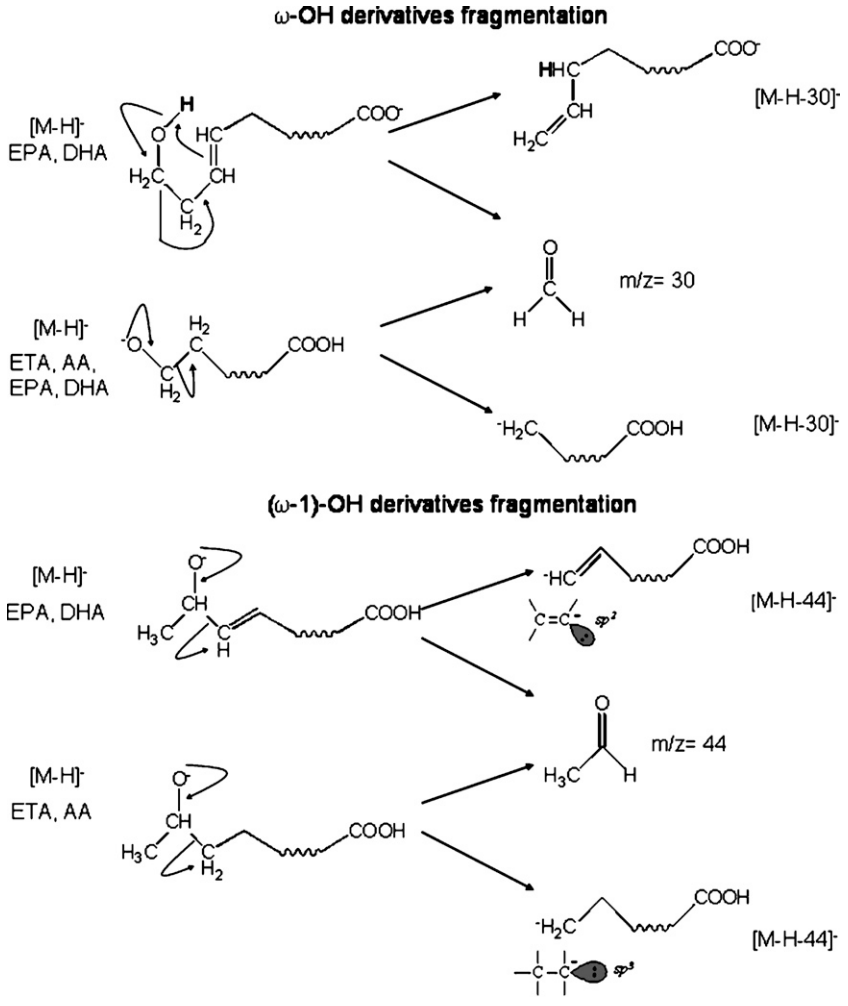


**Fig. 2.** Comparison from fragmentation mechanisms of ω- and (ω-1)-hydroxylated metabolites of PUFAs in APCI-MS in a negative mode. The resulting characteristic ions of ω-OH derivatives are: 291, 289, 287, and 313 for $[M-H-30]^-$ of eicosatrienoic acid (ETA), AA, eicosapentaenoic acid (EPA), and DHA, respectively; 247, 245, 243, and 269 for $[M-H-30-44]^-$ of ETA, AA, EPA, and DHA, respectively. The characteristic ions of (ω-1)-OH-derivatives are: 233, 231, 229, and 269 for $[M-H-44-44]^-$ of ETA, AA, EPA, and DHA, respectively.

medicals (Irvine, CA); and 1.92 GBq/mmol for [1-$^{14}$C]DHA obtained from Moravek Biomedicals (Brea, CA).

### Human recombinant P450s

The microsomes containing the human recombinant P450s (CYP1A1, -1A2, -1B1, -2A6, -2B6, -2C8, -2C9, -2C18, -2C19, -2D6, -2E1, -2J2, -3A4, -4A11, -4F2, -4F3A, -4F3B, and -4F12) were from BD-Biosciences Gentest (Woburn, MA, as the Supersome Enzymes® product line). BD P450 Supersomes® are recombinant cDNA-expressed cytochrome P450 enzymes prepared from the baculovirus-infected insect cell system, BTI-TH-5B1-4. The microsomes prepared from this system contained the necessary cytochrome P450 reductase and cytochrome b5. Control assays were systematically performed with insect cell microsomes (BTI-TN-5B1-4) infected with wild-type baculovirus (*Autographa californica*) with neither cDNA P450 nor reductase (BD-Biosciences as insect cell control Supersomes®). PUFAs were not oxidized in these control assays. Furthermore, preliminary experiments have shown that the addition of BSA to the incubation medium did not modify the metabolic profile (35).

### Human liver microsomes

Four human liver microsomes were assayed for PUFA metabolism. They were obtained from a liver microsome bank built up over many years in our laboratory (36). Ethical committee approval was obtained before studies in accordance with French law. The microsomal protein concentrations were determined by the Bradford method (BioRad, Munich, Germany), and P450 content was measured by differential spectrophotometry (37). The total P450 content was 553 ± 91 pmol/mg protein.

### FA metabolism assays

For the four PUFAs, all of the reaction mixtures contained 1 nmol of radiolabeled substrate corresponding to 2.07, 2.03, 2.03, and 1.92 kBq for [$^{14}$C]AA, [$^{14}$C]ETA, [$^{14}$C]EPA, and [$^{14}$C]DHA, respectively, in 0.12 M phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ and 0.15 mg microsomal liver proteins or 10 pmol of recombinant CYPs (in a final volume of 100 μl). After a 5 min preincubation at 37°C, the reaction was started by addition of 1 mM NADPH. The incubation times were 20, 30, and 15 min for recombinant CYP4 enzymes, recombinant CYPs from families 1–3, and human liver microsomes, respectively. The reaction was stopped by the addition of 0.1 ml acetonitrile containing 0.2% acetic acid (v/v). The FAs and their metabolites were twice extracted by 2 ml ethyl acetate. After drying under nitrogen, the samples were dissolved in 50 μl ethanol, and the metabolites were resolved by reverse-phase (RP)-HPLC. Radioactivity was measured with a β-radiometric Flo-One 500Tr apparatus (Perkin-Elmer-Packard Biosciences), with Ultima Flo scintillation cocktail added at a flow rate of 1 ml/min to the mobile phase. The formation rates of radiolabeled metabolites were calculated from peak areas integrated according to the Flo-One software. Enzymatic activity was expressed as $min^{-1}$ for recombinant P450s, or pmol/$mg^{-1}$ microsomal protein/$min^{-1}$ for human liver microsomes. All reactions were carried out at least in duplicate and the inter-assay variation was less than 10%. With a substrate concentration of 10 μM, the limit of detection was 0.004 pmol/min/pmol or $min^{-1}$ or 0.5 pmol/min/mg liver microsomal protein.

The effects of ω3 and ω9 FAs on the hydroxylation of AA were also determined by incubating 10 μM [$^{14}$C]AA with 10 pmol of CYP4F2 or -4F3B with or without either 10 or 50 μM 5,8,11-ETA, EPA, or DHA, under the conditions described above. And finally, incubations of 10 pmol CYP4F2 or -4F3B and 10 μM [$^{14}$C]DHA in the absence or presence of 10 μM AA were also carried out.

### HPLC conditions

The ion-pairing RP-HPLC method (38) used to separate the epoxidized metabolites was slightly modified. The analytical column used for the separation of the regioisomers was an RP-Capcell Pak C18 120 Å (250 mm × 4.6 mm, ID), particle diameter 5 μm (Shiseido Co. Ltd, Japan, distributed by C.I.l, Ste Foy La Grande, France). For AA metabolism, the mobile phase, A, was a mixture of water-methanol-acetonitrile (54:8:38; v/v/v) containing 15 mM DBA; the pH was adjusted to 7.5 with acetic acid. One should note that for each substrate, the composition of A was modified by slightly increasing the proportion of water. After elution of the metabolites, the unsaturated FA parent was eluted with a mobile phase, B, composed of a mixture of water-methanol-acetonitrile (17:8:75; v/v/v) containing 15 mM of DBA, pH adjusted to 7.5 for all the PUFAs.

TABLE 1. Hydroxylation of PUFAs by human CYP4 enzymes

| | 5,8,11-ETA | | | AA | | | EPA | | | DHA | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | ω | ω-1 | Total | ω | ω-1 | Total | ω | ω-1 | Total | ω | ω-1 |
| | | | | | | *10 μM* | | | | | | |
| CYP4A11 | 0.24* | 0.17 | 0.06 | 0.22* | 0.16 | 0.04 | 0.14 | 0.04 | 0.10 | 0.10 | 0.05 | 0.05 |
| CYP4F2 | 0.86* | 0.77 | 0.04 | 0.55 | 0.53 | 0.02 | 0.63* | 0.38 | 0.11 | 1.33* | 0.85 | 0.24 |
| CYP4F3A | 1.92* | 1.75 | 0.09 | 1.10* | 1.02 | 0.04 | 1.10* | 0.71 | 0.34 | 2.23* | 0.86 | 0.75 |
| CYP4F3B | 3.19* | 2.78 | 0.24 | 3.13* | 2.66 | 0.13 | 3.20* | 1.74 | 1.00 | 3.06* | 1.39 | 0.65 |
| | 5,8,11-ETA | | | AA | | | EPA | | | DHA | | |
| | Total | ω | ω-1 | Total | ω | ω-1 | Total | ω | ω-1 | Total | ω | ω-1 |
| | | | | | | *50 μM* | | | | | | |
| CYP4A11 | 1.1* | 0.6 | 0.3 | 0.9* | 0.6 | 0.2 | 0.4 | 0.1 | 0.3 | 0.4* | 0.1 | 0.2 |
| CYP4F2 | 2.4* | 2.1 | 0.1 | 1.6* | 1.4 | 0.1 | 1.0* | 0.7 | 0.2 | 3.0* | 2.0 | 0.6 |
| CYP4F3A | 7.8 | 7.4 | 0.4 | 5.2* | 4.8 | 0.2 | 2.3* | 1.0 | 0.7 | 6.4* | 3.1 | 2.8 |
| CYP4F3B | 8.9 | 8.1 | 0.8 | 8.6* | 7.6 | 0.4 | 4.1* | 2.6 | 1.4 | 5.3* | 3.4 | 1.1 |

ETA, eicosatrienoic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Specific metabolic rates of total, ω- and (ω-1)-hydroxylations of four PUFAs by human CYP4 enzymes. PUFAs were incubated at 10 μM and 50 μM concentration with human CYP4 enzymes (10 pmol) for 20 min. Formation of metabolites was quantitated by reverse-phase (RP)-HPLC using radiometric detection as described in Materials and Methods. All reactions were carried out in duplicate, and the inter-assay variation was found to be less than 10%.

*Indicates that other minor hydroxylated metabolites, probably in bis-allylic position, were detected; they were not formally identified.

### Identification of metabolites

The ω- and (ω-1)-hydroxylated metabolites of PUFAs formed by CYP were separated by HPLC as described above, and identified by mass spectrometry (MS) on a mass spectrometer (Navigator model; Thermo-Electron, Les Ulis, France) equipped with an ionization source at atmospheric pressure and operated in a negative-ion mode. The cone voltage was optimized and set between 30 V and 45 V to enhance molecular fragmentation. In atmospheric pressure chemical ionization (ACPI) mode, the temperatures of the vaporizer and source were 350° and 150°C, respectively. The drying nitrogen flow was 352 l/h. The energies of the corona pin needle, skimmer, and quadrupole were set, respectively, at 3 kV, 0.9 V, and 2.6 V. For the identification of ω- and (ω-1)-hydroxylated derivatives, the negative ions were monitored in full scan mode from *m/z* 60 to 500.

## RESULTS

### Identification of ω- and (ω-1)-hydroxylated derivatives of PUFAs by LC-MS

The HPLC profile obtained by incubation of both AA and DHA with human recombinant CYP4F3B and the mass spectra of their metabolites are presented in **Fig. 1**. The molecular ions $[M-1]^-$ of monooxygenated metabolites were 319 and 343 for AA and DHA, respectively. Furthermore, the mass spectra of ω- and (ω-1)-hydroxylated metabolites showed some noncharacteristic ion fragments corresponding to the loss of $H_2O$ $[M-1-18]^-$, $CO_2$ $[M-1-44]^-$ or the loss of both $H_2O$ and $CO_2$ $[M-1-62]^-$. On the other hand, the cleavage in the α position to the alcohol function led to characteristic ions that differed according to the class of alcohols: the ω-OH derivatives, i.e., primary alcohol, produced characteristic ions $[M-1-30]^-$ and $[M-1-30-44]^-$; the former resulted from the loss of $O = CH_2$ and the latter from the associated loss of $O = CH_2$ and $CO_2$. The (ω-1)-hydroxylated derivatives, i.e., secondary alcohol, exhibited a characteristic fragment $[M-1-88]^-$ issued from the loss of two *m/z* 44 fragments corresponding to $CH_3$-CHO and $CO_2$. The fragmentation mechanisms of ω- and (ω-1)-hydroxylated metabolites of the four PUFAs are shown in **Fig. 2**. The common and characteristic ion fragments issued from the fragmentation of the ω- and (ω-1)-hydroxylated metabolites of ETA, AA, EPA, and DHA proved to be similar. However, their intensities (in relative percentage) in particular, of specific ions, were substantially higher for EPA and DHA compared with the other FAs (data not shown). Such a behavior was probably due to an additional six-center rearrangement that was only encountered for the primary alcohol metabolites of ω3 FAs. This rearrangement is associated with a hydrogen migration and a six-membered ring transition, as in McLafferty rearrangement, requiring less energy than for the common cleavage in the α position to the alcohol function. For the secondary alcohol metabolites of ω3 FAs, the higher electronegativity of the carbon $sp^2$ of the proximal double bond versus that of the carbon $sp^3$ of other FAs facilitates the shift of one electron toward $Csp^2$, as illustrated in Fig. 2. Furthermore, the greater s-character of the charge-bearing-atom for ω3 (sp2) versus ω6 and ω9 (sp3) increases the stability of the formed anion. Regardless of which PUFAs were incubated with the CYP4 enzymes, no significant formation of (ω-2)-OH, except for CYP4F12, or (ω-3)-OH metabolites was detected, as shown by the absence of $[M-H-C_2H_5\text{-}CHO]^-$ and $[M-H-C_3H_7\text{-}CHO]^-$ ions.

### Hydroxylation of PUFA by CYP4 enzymes family

The total catalytic hydroxylation activity of a range of ω9 to ω3 PUFA substrates by five CYP4 enzymes was investigated (**Table 1**). Moreover, **Fig. 3** shows the metabolic rates of total hydroxylation of the PUFAs incubated at three different concentrations. Under our working conditions, ω- and (ω-1)-hydroxylated derivatives were the major

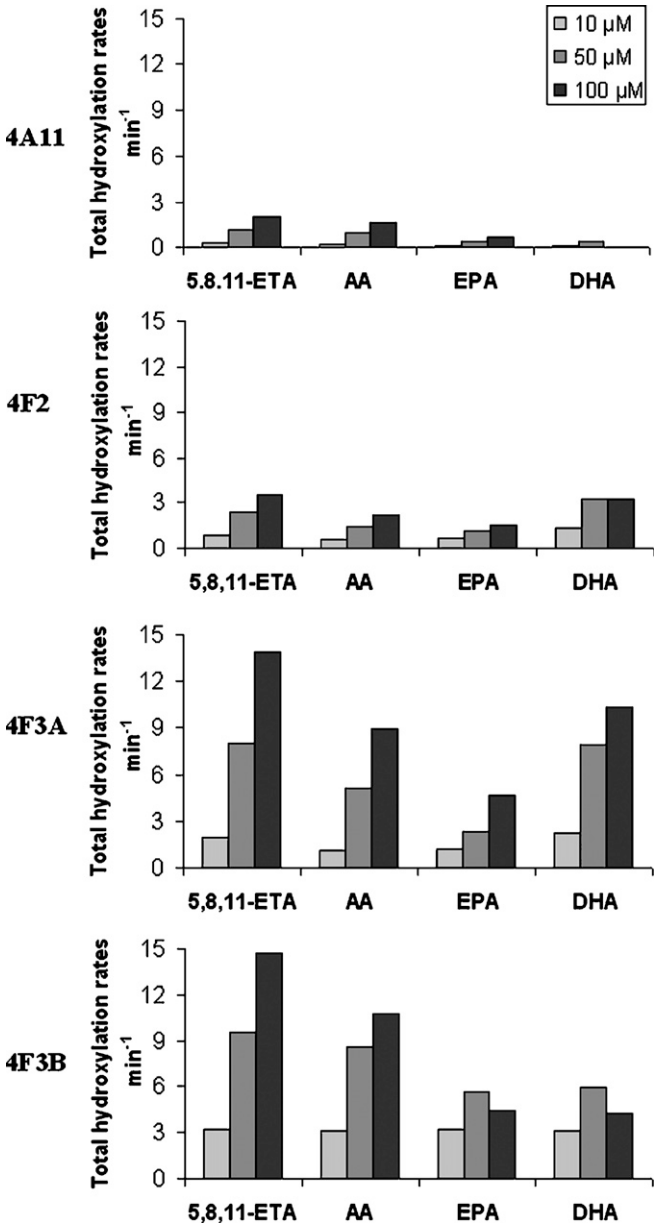


**Fig. 3.** Specific metabolic rates ($min^{-1}$) of total hydroxylation of PUFAs (5,8,11-ETA, AA, EPA, and DHA) incubated at three concentrations (10, 50, and 100 µM) with CYP4A11, CYP4F2, CYP4F3A, and CYP4F3B (10 pmol) for 20 min. Detection was made by radioactivity as described in Materials and Methods.

products (except for CYP4F12, which exhibited a dramatically lower activity than the other CYP4s, with hydroxylation rates in the range 0.04–0.06 $min^{-1}$). Furthermore, because the hydroxylation of PUFAs by CYP4F12 took place only at the (ω-1) and (ω-2) positions for ω9 and ω6 series FAs (data not shown), CYP4F12 was excluded from the data presented below. Among the other CYP4s, the total hydroxylation rates of PUFAs across the range of substrate concentrations used (10–100 μM) exhibited Michaelis-Menten kinetics. Nevertheless, the finding of saturating concentrations at 50 μM of DHA with CYP4F2 and 50 μM of EPA or DHA with CYP4F3B led us to carry out experiments regarding the rate and regioselectivity of hydroxylation of PUFAs at 50 μM.

All PUFAs were hydroxylated mainly at the ω and/or (ω-1) positions, but PUFA hydroxylation by CYP4A11 was low and only slightly regiospecific. Total CYP4A11 hydroxylase activities were within 0.4 and 1.1 $min^{-1}$ for every PUFA with a ω/ω-1 ratio in the range 0.3–3.0. In contrast, CYP4F enzymes showed a higher ω-hydroxylase activity. Among them, CYP4F3A and CYP4F3B displayed the highest catalytic hydroxylation activity toward PUFAs. Total hydroxylase metabolic rates were in the range 1–3.0 $min^{-1}$ for CYP4F2, 2.3–7.8 $min^{-1}$ for CYP4F3A, and 4.1–8.9 $min^{-1}$ for CYP4F3B. Nevertheless, it is worth noting that all CYP4 enzymes showed a substantial decrease in ω-hydroxylase activity when the number of double bonds was increased from three to five in the eicosa FA series. Therefore, EPA was less ω-hydroxylated than 5,8,11-ETA and AA when incubated with CYP4A11, -4F2, -4F3A, and -4F3B (Table 1). On the other hand, the (ω-1)-hydroxylation of EPA was higher than that of AA (its ω6 analog). Thus, the 19-hydroxyeicosapentaenoic acid or (ω-1)-OH-EPA accounted for 75, 22, 41, and 35% of the total ω- and (ω-1)-hydroxylated products of 50 μM EPA incubated with CYP4A11, 4F2, 4F3A and 4F3B, respectively. Likewise, the 21-hydroxydocosahexaene acid or (ω-1)-OH-DHA represented 66, 23, 47 and 24% of total ω- and (ω-1)-hydroxylated products of 50 μM DHA incubated with CYP4A11, -4F2, -4F3A, and -4F3B, respectively (Fig. 3). However, in contrast to EPA,

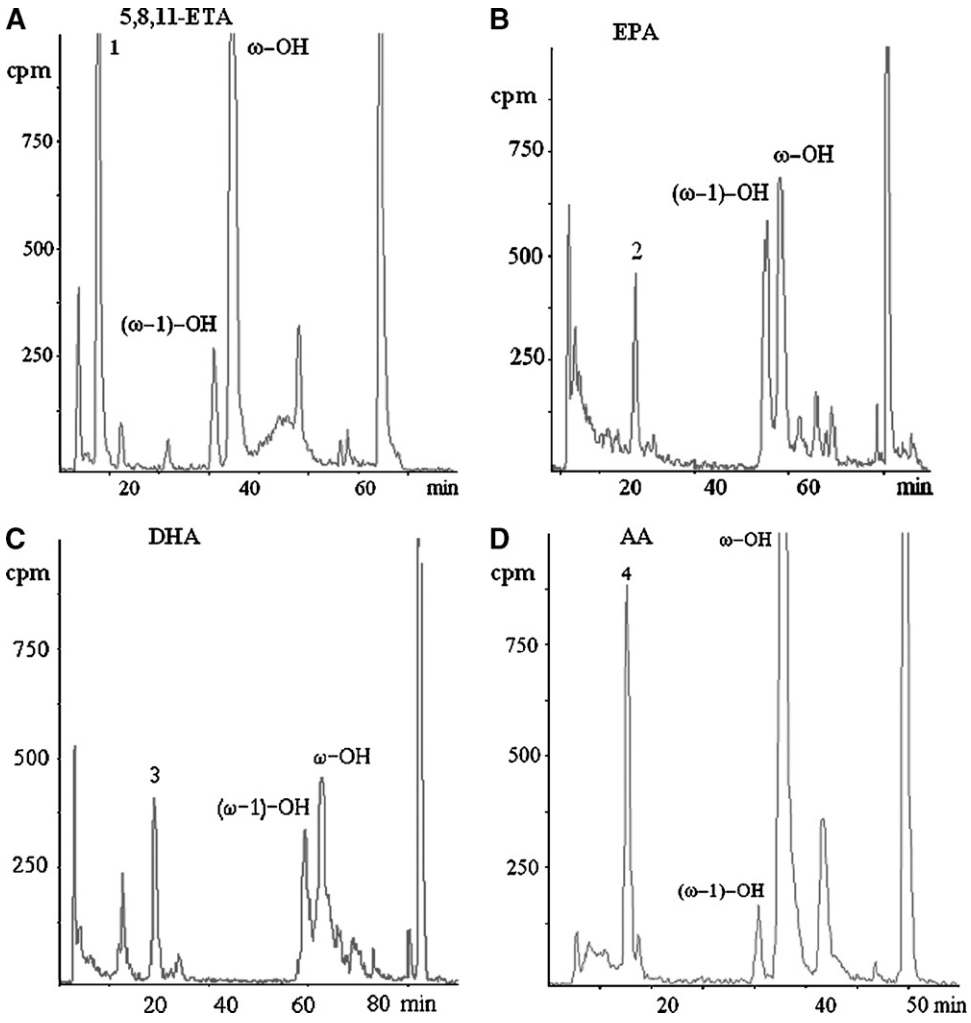


**Fig. 4.** RP-HPLC profiles of metabolism of PUFAs (A: 5,8,11-ETA; B: EPA; C: DHA; and D: AA) incubated at 10 μM in the presence of 10 pmol of CYP4F3B. Detection was made by radioactivity as described in Materials and Methods. Peaks 1, 2, 3, and 4 were identified as 20-carboxy-ETA, 20-carboxy-EPA, 20-carboxy-DHA, and 20-carboxy-AA, respectively.

DHA was not always the less hydroxylated FA, particularly with CYP4F2. Interestingly, the 8,11,14-ETA was weakly hydroxylated, in contrast to AA and 5,8,11-ETA, which suggests a favorable effect of the 5,6-double bond or an inhibitory role of the 14,15-double bond (data not shown).

Minor hydroxylated metabolites of EPA and DHA were detected and assumed to result from a bis-allylic hydroxylation (39), although they were not formally identified (**Fig. 4**). Moreover, incubation of PUFAs with CYP4F3A/3B led to the detection of strongly polar products probably issued from the oxidation of hydroxylated metabolites into 20- or 22-carboxy-PUFAs. The quasi-molecular ions of ETA, AA, EPA, and DHA polar metabolites were found by LC-MS at *m/z* 335, 333, 331, and 356, respectively. These metabolites probably result from the sequential oxidations of ω-OH-PUFAs into dicarboxylic FAs.

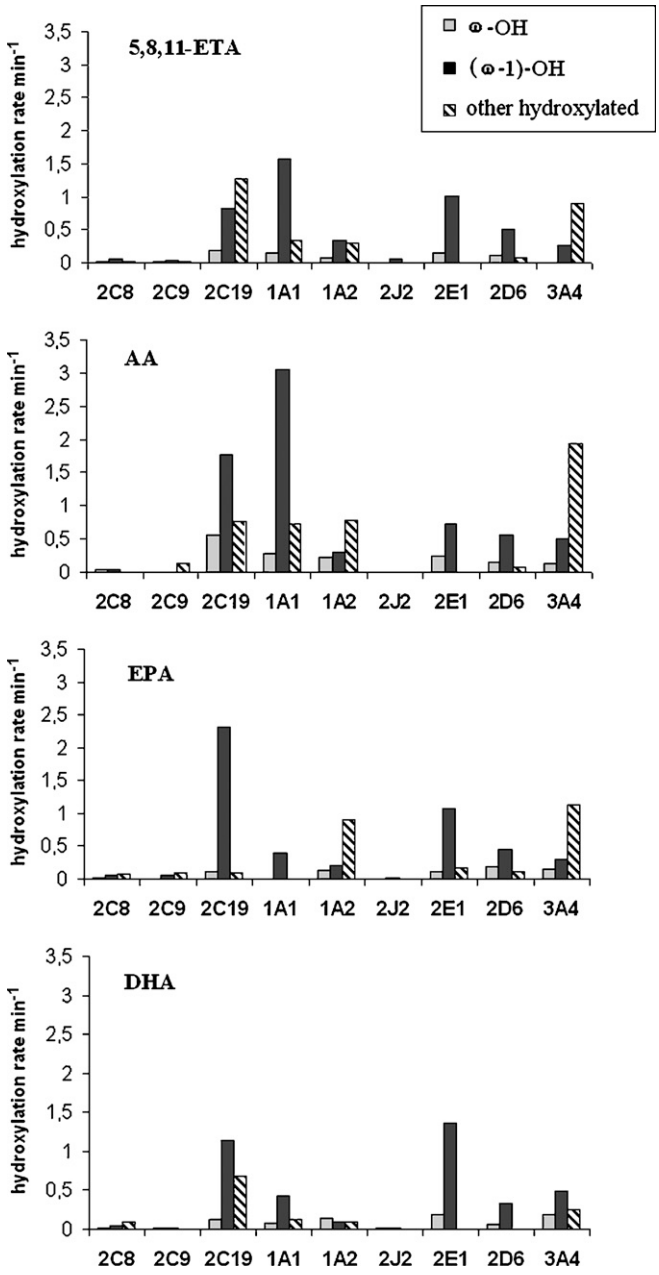


**Fig. 5.** Specific metabolic rates of hydroxylation ($min^{-1}$) of 50 μM FAs (5,8,11-ETA, AA, EPA, and DHA) incubated with various human CYPs. One should note that FAs were incubated at 10 μM with CYP2C8 and -2C9, because a decrease in hydroxylation was observed at 50 μM.

### Participation of other CYPs in the hydroxylation of PUFA

**Figure 5** shows hydroxylated metabolites obtained from PUFAs with a series of recombinant human P450s (families 1–3) expressed in insect cells (Supersomes®). With the exception of CYP1B1, -2A6, -2B6, and -2C18, the P450s were all involved in hydroxylations of PUFAs. Nevertheless, none of these exhibited substantial PUFA ω-hydroxylase activity. CYP1A1, -2E1, -2C19, and -2D6 were mainly (ω-1)-hydroxylases, whereas CYP1A2 and -3A4 were mid-chain hydroxylases. In comparison to the other FAs, particularly with CYP2C19, -1A1, and -3A4, AA was usually converted at higher rates. Total hydroxylation rates were 1.8 ± 0.5, 3.2 ± 0.7, 1.5 ± 1.0 $min^{-1}$, and 1.1 ± 0.7 $min^{-1}$ (mean of these three CYPs ± SD) for ETA, AA, EPA, and DHA, respectively.

### Metabolism by human liver microsomes

Four human liver microsomal samples were used to compare the patterns issued from the hydroxylation of 50 μM of PUFAs. **Table 2** shows that PUFAs were hydroxylated mainly in the ω position. However, in the eicosa FA series, the rate of ω-hydroxylation decreased with the increase in the unsaturation number. Thus, the ω/ω-1 ratios of hydroxylated products were 6.9, 3.1, 2.1, and 2.7 for ETA, AA, EPA, and DHA, respectively, and the ω-hydroxylated derivative of ETA was almost exclusively formed. Moreover, only AA and EPA formed significant amounts of medium-chain hydroxylated metabolites, and the epoxygenase pathway of PUFA metabolism by liver accounted for less than 50% of total oxidative metabolism.

### Inhibition studies

As shown in **Fig. 6A, B**, the addition of ω3 or ω9 FAs to the microsomal CYP4F2 or CYP4F3B enzymatic system dramatically reduced the production of total hydroxylated metabolites of AA. The percentages of inhibitions obtained were 23, 41, and 49% with CYP4F2 and 32, 56,

TABLE 2. Hydroxylation of PUFA by human liver microsomes

| | Hydroxylated Metabolites | | |
|---|---|---|---|
| | (ω-1)-OH Metabolites | ω-OH Metabolites | Other Hydroxylated Metabolites |
| | *pmol/min/mg* | | |
| 5,8,11-ETA | 26 ± 6 | 180 ± 31 | 4 ± 4 |
| AA | 50 ± 12 | 156 ± 28 | 41 ± 10 |
| EPA | 52 ± 27 | 108 ± 35 | 34 ± 12 |
| DHA | 51 ± 13 | 140 ± 60 | 11 ± 11 |

Regioisomeric distribution of hydroxylated metabolites from four 50 μM PUFAs incubated with four human liver microsomes (0.15 mg microsomal liver proteins) for 15 min. Formation of metabolites was quantitated by RP-HPLC using radiometric detection as described in Materials and Methods. Results are expressed as the mean of four individual determinations (± SD).

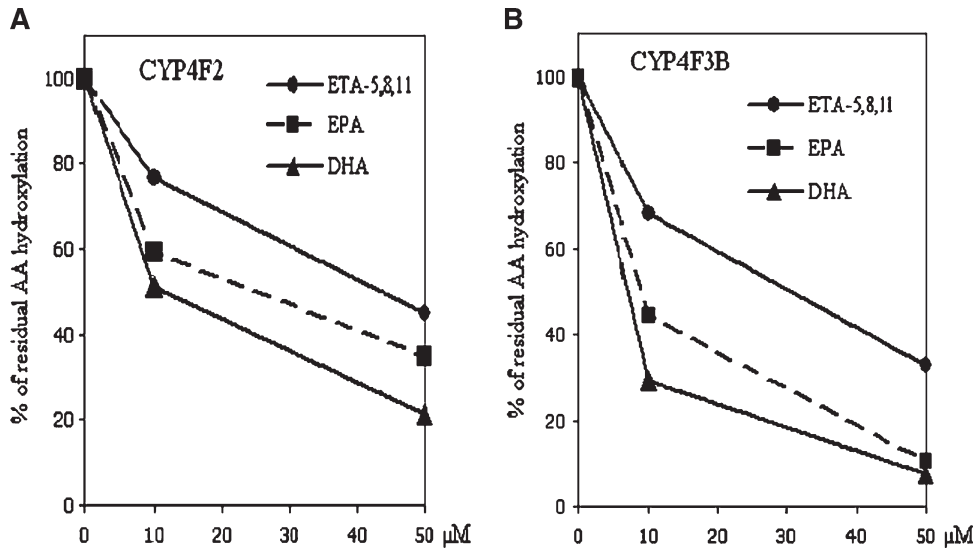


**Fig. 6.** Inhibition of hydroxylated derivatives formation from AA by ω3 and ω9 FAs. [$^{14}$C]AA (10 μM) was incubated with 10 pmol CYP4F2 (A) or CYP4F3B (B) in the absence or presence of cold 5,8,11-ETA, EPA, or DHA at two concentrations (10 and 50 μM).

and 70% with CYP4F3B when 10 μM ETA, EPA, or DHA was added to the reaction medium, respectively. When the DHA concentration was raised to 50 μM, the formation of AA-hydroxylated metabolites by CYP4F2 or -4F3B was inhibited by about 78% and 92%, respectively.

In another experiment, incubation of 10 μM [$^{14}$C]DHA with CYP4F2 or CYP4F3B enzymatic system in the presence of 10 μM cold AA revealed that DHA was a more potent inhibitor of AA hydroxylation than was AA for the hydroxylation of DHA. Effectively, AA reduced the production of DHA-hydroxylated derivatives only by about 27% and 45% with CYP4F2 and -4F3B, respectively, whereas DHA reduced the production of AA-hydroxylated derivatives by 49% and 70% with CYP4F2 and -4F3B, respectively.

## DISCUSSION

Several studies have highlighted that CYP4 enzymes are effective catalysts of ω-hydroxylation of medium- and long-chain FAs (6, 7, 16, 20, 23, 39, 40). However, the CYP-dependent metabolism of ω3 PUFAs and the metabolic interaction between AA and ω3 PUFAs are both poorly understood. This study is the first to address in an exhaustive manner the roles played individually by human CYP family members in the metabolism of PUFAs.

Here we decided to investigate the formation of ω-hydroxylated regioisomers of four PUFAs by enzymes of the CYP4 family through the use of an RP ion-pair HPLC method developed in our laboratory (38). It showed that the CYP4 enzymes ω-hydroxylated the four PUFAs under study with different efficiencies. CYP4F12 proved to be about ten- and fifty-fold less efficient than CYP4A11 and CYP4F2, respectively, which is in agreement with the literature data (25). One should note that the activity value was so low that it was nearly at the limit of detection. As reported (6, 40) we also observed a low AA hydroxylase activity for CYP4A11, as well as a poor regiospecificity for the ω- and (ω-1)-hydroxylations of PUFAs. The low activity and the lack of regiospecificity of CYP4A11 in the hydroxylation of polyunsaturated C20 and C22 FAs probably result from the features of the access channel to the P450 heme iron. According to the literature (4, 5, 40, 41), lauric acid has the chain length most suited to the constricted access channel of CYP4A enzymes; consequently, PUFAs with a long alkyl chain should not be the best substrates for CYP4A11. In contrast, of the PUFAs under investigation, CYP4F2, -4F3A, and -4F3B enzymes exhibited a high capability of hydroxylation, in agreement with previous reports for the hydroxylation of AA by CYP4F2 and -4F3B (6, 7, 16). The rates of hydroxylation of the same amount of ETA, AA, EPA, and DHA by CYP4F3A and -4F3B were much higher than those by -4F2. This may indicate that the FA carboxylic group positioned at the entrance of the access channel is more distant from the heme iron active site in CYP4F than in CYP4A11 (6, 40, 42). Indeed, the medium-chain FAs will be preferred by CYP4A11 (5, 40), whereas long-chain FAs, and even very long-chain FAs (VLCFAs) (43), will be targeted by CYP4Fs. Nevertheless, the decrease of ω-hydroxylation rates by every CYP4 observed in this study when the number of unsaturations in the eicosa FAs series was elevated is consistent with the finding that AA is a better substrate for CYP4F3B than either EPA or DHA (34). These results suggest that the access to the hydroxylation site of CYP4Fs is hindered by the flatness of the carbon skeleton induced by the double bond in 14,15 or 17,18 in AA and EPA, respectively. In contrast, it is worth noting that a dramatic fall in the hydroxylation rate was observed when the 5,6-double bond was missing in the regioisomer 8,11,14-ETA (data not shown). Thus, with the CYP4 family, the presence of a double bond in the vicinity of the carboxylic function would be impor-

tant to increase ω oxidation; this assumption is in agreement with the literature on hydroxylation of C18 series by CYP4A11 and rat brain microsomes (5, 44). On the other hand, the finding here of a more efficient hydroxylation of DHA compared with EPA by the three CYP4F enzymes under study suggests that the lengthening of the alkyl chain is not at the origin of steric hindrance. This assumption is corroborated by the report in (43) of ω hydroxylation of saturated VLCFA by CYP4F3B and -4F2 with turnover values close to those found here for DHA.

The study of the hydroxylating metabolism of PUFAs by 18 recombinant human P450s from families 1–4 showed no significant ω-hydroxylase activity by any CYP from families 1–3. Indeed, CYP1A1, -2E1, -2D6, and -2C19 proved to be mainly (ω-1)-hydroxylases, whereas CYP1A2 and -3A4 were more mid-chain hydroxylases. These results are in agreement with previous studies on AA (5, 26, 27, 45, 46) or EPA (46) as substrate. Indeed, two different criteria determine the preferred site of hydroxylation of FAs by CYP FA hydroxylases; one criterion is thermodynamic and the other is steric. Regarding the hydroxylations of PUFAs by CYP4F2, -4F3A, and -4F3B, our experiments demonstrated that the direct attack of the carbon of the terminal methyl group was privileged. This finding is in agreement with the assumption by several authors of a constricted access channel of CYP4A (5, 40, 41, 47) or CYP52A21 (48) enzymes through which, despite the high conformational flexibility of FA, the substrate is strictly positioned in such a way that the methyl group is close to the reactive ferryl oxygen (49). Furthermore, the heme prosthetic group in CYP4A (50) and CYP4F enzymes (51, 52) is covalently attached to the protein through the critical Glu321/328 residue of the highly conserved EGHDTT sequence (50). In addition, literature data on AA (11, 17) showed preferential (ω-2)- and (ω-3)-hydroxylation by CYP4F8/4F12, both known for the lack of the heme-binding Glu residue. Taken together, these data suggest the involvement of Glu328 in the ω-hydroxylation of PUFAs. However, some differences observed in the regiospecificity of hydroxylation by CYP4s depended on the PUFAs under study.

Although human liver microsomes converted PUFAs to numerous metabolites, including (ω-1) and mid-chain hydroxylated, epoxidized compounds or *vic*-diols, the ω-hydroxylated derivatives were always among the most abundant metabolites, as expected (6, 26–28, 40). Further, in immuno-inhibition studies (6), CYP4A11 and CYP4F2 are thought to be largely responsible for AA ω-hydroxylation in human liver. The higher expression of CYP4F3B relative to CYP4F2 in human liver (16) and the higher catalytic activity of CYP4F3B versus CYP4F2 observed in this study suggest that CYP4F3B is also one of the major enzymes involved in the production of ω-PUFA in the liver. Furthermore, because in the eicosa series of FAs, the ω-hydroxylase activity by both liver microsomal preparations and recombinant CYP4F3s was decreased when the number of unsaturations was elevated, their involvement in this reaction can be suggested in human liver. Nevertheless, any assessment of the contribution of each CYP4 enzyme isoform to the ω-hydroxylation of PUFAs in human liver is quite impossible because of the lack of a specific inhibitor for each of them.

Inasmuch as all PUFAs under investigation in this study were ω-hydroxylated by CYP4 enzymes, ETA, EPA, and DHA were all expected to inhibit the formation of 20-HETE. In fact, this study demonstrated that ETA, EPA, and DHA decreased total HETE formation with CYP4F2 and -4F3B. Although ETA was more ω-hydroxylated than EPA or DHA by human CYP4Fs, these ω3 FAs proved to be more-potent inhibitors of AA hydroxylation. Consistent with the previous result, 10 μM DHA inhibited AA-hydroxylated metabolite formation by CYP4F2 or -4F3B more efficiently than 10 μM AA inhibited the formation of DHA-hydroxylated metabolites. Such a variable inhibitory effect could result from differences in the position at which the substrate is bound to the active site. Indeed, DHA was more efficiently metabolized into (ω-1)-hydroxyl derivative than was AA.

The P450 enzymes of the CYP4A/4F family found mainly in the human liver and kidney are known as ω-hydroxylases of FAs. These reactions facilitate the degradation of long-chain FAs by initiating their conversion into dicarboxylic acids and promoting their elimination through peroxisomal β-oxidation. With AA, 20-HETE plays an important role in regulation of blood pressure and renal tubular transport (8, 9, 39, 53), whereas 19-HETE has the opposite effect (10, 54). This study confirmed the work of Harmon et al. (34) and further demonstrated that the ω3 FAs EPA and DHA can inhibit the formation of 20-HETE substantially better than 5,8,11-ETA. It leads us to assume that in the case of essential FA deficiency, 20-OH-ETA is preferentially formed, whereas in the case of dietary ω3 FA supplementation, their ω- and (ω-1)-OH metabolites were both formed. The biological effects of ω- and (ω-1)-hydroxylated metabolites of ω3 FAs generated by CYP4 enzymes are not yet known. However, dietary ω3 PUFA supplementation has a number of beneficial health-related effects in humans, especially an anti-hypertensive action (30–32). The molecular mechanism of action of ω3 FAs remains poorly defined. Because this oxylipid is known to be involved in the regulation of blood pressure, some functional effects of ω3 FA supplementation may be due to a biochemical inhibition mechanism. The conversion of EPA and DHA to the corresponding ω-OH metabolites may also be in itself another mechanism. Indeed, it has been recently reported that these compounds activated PPARα (55, 56), suggesting that like 20-HETE, they may function as lipid mediators. Yet, the mechanisms through which ω3 FAs affect cell function remain poorly known. Much work needs to be done to take into account the antagonistic activities they could exert through their alternative metabolism by the different CYP4 family enzymes.

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