

Anandamide Metabolism by Human Liver and Kidney Microsomal Cytochrome P450 Enzymes to Form Hydroxyeicosatetraenoic and Epoxyeicosatrienoic Acid Ethanolamides

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ABSTRACT

The endocannabinoid anandamide is an arachidonic acid derivative that is found in most tissues where it acts as an important signaling mediator in neurological, immune, cardiovascular, and other functions. Cytochromes P450 (P450s) are known to oxidize arachidonic acid to the physiologically active molecules hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs), which play important roles in blood pressure regulation and inflammation. To determine whether anandamide can also be oxidized by P450s, its metabolism by human liver and kidney microsomes was investigated. The kidney microsomes metabolized anandamide to a single mono-oxygenated product, which was identified as 20-HETE-ethanolamide (EA). Human liver microsomal incubations with anand-

amide also produced 20-HETE-EA in addition to 5,6-, 8,9-, 11-12, and 14,15-EET-EA. The EET-EAs produced by the liver microsomal P450s were converted to their corresponding dihydroxy derivatives by microsomal epoxide hydrolase. P450 4F2 was identified as the isoform that is most probably responsible for the formation of 20-HETE-EA in both human kidney and human liver, with an apparent K_m of 0.7 μ M. The apparent K_m values of the human liver microsomes for the formation of the EET-EAs were between 4 and 5 μ M, and P450 3A4 was identified as the primary P450 in the liver responsible for epoxidation of anandamide. The in vivo formation and biological relevance of the P450-derived HETE and EET ethanolamides remains to be determined.

The endocannabinoid system, which consists of cannabinoid receptors, endocannabinoids, and their associated signaling pathways, is known to play an important role in a number of physiological and pathophysiological processes, including drug addiction, obesity, inflammation, and cancer (Di Marzo et al., 2004). Progress in the development of novel therapies based on this system is in part dependent on our understanding of the metabolic pathways that regulate the endocannabinoid tone in vivo.

The discovery of anandamide, an ethanolamide of arachidonic acid, as the first endocannabinoid (Devane et al., 1992) was followed by a great number of studies designed to understand the factors that regulate its bioavailability in vivo. The enzyme fatty acid amide hydrolase (FAAH) catalyzes the

hydrolysis of anandamide to produce arachidonic acid and ethanolamine and is known to be the chief enzyme that terminates the activity of anandamide in vivo (Giang and Cravatt, 1997). In addition to this hydrolytic mode of metabolism, it is possible that anandamide can also be subjected to oxidative metabolism by a number of fatty acid oxygenases that are known to metabolize endogenous arachidonic acid. These include the cyclooxygenase (COX), lipoxygenases (LOX), and cytochrome P450 (P450) enzymes. Studies involving cell-free systems, and cellular assays have revealed that anandamide can be converted into several prostaglandin ethanolamides by COX-2 (Yu et al., 1997; Burstein et al., 2000; Kozak et al., 2002) and 12- and 15-hydroperoxyeicosatetraenoic acid ethanolamides by 12-LOX and 15-LOX, respectively (Hampson et al., 1995; Ueda et al., 1995; Edgemond et al., 1998). However, the biological significance of these metabolic pathways remains to be elucidated.

In contrast to the studies with COX-2 and LOX, the oxidative metabolism of anandamide by P450s has not been inves-

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ABBREVIATIONS: FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; LOX, lipoxygenase; P450, cytochrome P450; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; EA, ethanolamide; mEH, microsomal epoxide hydrolase; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; HET0016, *N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)-formamide; HKM, human kidney microsome; MS/MS, tandem mass spectrometry; HLM, human liver microsome; LTB₄, leukotriene B₄.

tigated in detail. P450s are microsomal heme-containing mono-oxygenases that are involved in the metabolism of a variety of endogenous substrates such as steroids, fatty acids, and neurotransmitters as well as many xenobiotics, including most clinically used drugs (Coon, 2005). Several P450 isoforms mainly belonging to subfamilies 2C, 2J, 4A, and 4F act on endogenous arachidonic acid to produce the epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (HETEs), which are potent signaling mediators and have a major role in blood pressure regulation as well as other important physiological processes (Capdevila and Falck, 2002; Sarkis and Roman, 2004). Because of the structural similarity of anandamide to arachidonic acid, it is likely that some P450s could be involved in the metabolism of anandamide. It has been shown previously that mouse brain and liver microsomes metabolize anandamide to give a number of oxygenated products (Bornheim et al., 1995); however, their structural identity was not elucidated. To our knowledge, the role of human P450 isoforms in the metabolism of anandamide has not been investigated thus far.

This article describes the metabolism of anandamide by human liver and kidney microsomes and the formation of EET- and HETE-ethanolamides (EAs) and demonstrates a role for P450 3A4 in the formation of 5,6-, 8,9-, 11,12-, and 14,15-EET-EAs in the liver and a potential role for P450 4F2 in the formation of 20-HETE-EA in both liver and kidney. Furthermore, the EET-EAs formed by the human liver microsomal P450s undergo secondary metabolism by microsomal epoxide hydrolase (mEH) to form the corresponding dihydroxyeicosatrienoic acid-EAs.

Materials and Methods

Human Kidney and Liver Microsomes. The human liver microsomes used for these studies were from six individual subjects and have been described previously (Teiber and Hollenberg, 2000). The pooled human liver microsomes that were used in the kinetic studies were purchased from BD Biosciences (San Jose, CA). Human kidney microsomes from normal donors were a gift from Dr. Lawrence Lash (Wayne State University, Detroit, MI).

P450 Enzymes. The P450 4F2, 4F3b, and 4A11 supersomes were purchased from BD Biosciences. P450 3A4 and P450 NADPH reductase were expressed in *Escherichia coli* and purified as described previously (Hanna et al., 1998; He et al., 1998).

Anandamide Metabolism Assays. For the initial studies, the conversion of anandamide to oxygenated metabolites was assessed in incubation mixtures (0.5 ml) containing 100 mM KPO₄ buffer, pH 7.4, anandamide (20 or 100 μ M, as specified in the legends to the figures), 1 mM NADPH, and one of the following enzyme sources: human kidney or human liver microsomes (100 μ g of protein) or P450 4F2, 4F3b, or 4A11 supersomes (25 pmol). Catalase (500 U) was also added to reaction mixtures using supersomes. The P450 3A4 reconstituted system consisted of 25 pmol of purified P450 3A4; 50 pmol of P450 reductase; 10- μ g mixture of L- α -dilauroyl-phosphocholine, L- α -dioleoyl-*sn*-glycero-3-phosphocholine, and L- α -phosphatidylserine (1:1:1); 50 U of catalase; and 50 mM HEPES in a final reaction volume of 0.5 ml. For the reaction mixtures that contained mEH, P450 3A4 (25 pmol) was reconstituted as above with the addition of mEH (25 pmol). All reactions were initiated by the addition of NADPH. For the initial studies, the samples were incubated at 37°C for 45 min. The reactions were terminated by the addition of 2 ml of nitrogen-purged ethyl acetate, and the samples were vortexed for 1 to 2 min. The samples were then centrifuged for 5 to 10 min at 1200 rpm to separate the organic layer, which was extracted and dried down under a constant stream of nitrogen gas. The dried samples

were resuspended in 200 μ l of methanol, and 10- μ l fractions were subjected to electrospray ionization (ESI)-liquid chromatography (LC)/mass spectrometry (MS) analysis as described below.

For the inhibition studies using chemical or antibody inhibitors, the microsomal protein mixtures were preincubated with either the inhibitory monoclonal antibody to the specified P450 or HET0016 (10 pM to 1 μ M), a highly selective inhibitor of arachidonic acid ω hydroxylation for 5 min before the addition of anandamide. The samples were then incubated in the presence of NADPH for 45 min.

For the determination of the K_m and V_{max} values, the incubation conditions were optimized for time and protein concentration and performed within the linear range of metabolite formation. The reaction mixture compositions were similar to those outlined above. For these studies, 75 μ g of human kidney microsomal protein was incubated for 8 min, and 25 μ g of liver microsomal protein was incubated for 15 min. In experiments using the P450 4F2 supersomes, the reaction mixtures were incubated for 8 min with 2 pmol of enzyme. After extraction and drying of the extracts, the samples were resuspended in 100 μ l of methanol, and 10- μ l aliquots were injected for analysis by ESI-LC/MS. Standard curves for 5,6-EET-EA, 14,15-EET-EA, and 20-HETE-EA were generated by extracting varying known amounts of the authentic standards from a 0.5-ml reaction mixture that did not contain anandamide and NADPH. Rates of formation for 8,9- and 11,12-EET-EA were determined based on the standard curves for 5,6- and 14,15-EET-EA, respectively.

ESI-LC/MS Analysis. Samples (10 μ l of each) were injected onto a Hypersil ODS column (5 μ m, 4.6 \times 100 mm; Thermo Electron Corporation, Waltham, MA) that had been equilibrated with 75% solvent B (0.1% acetic acid in methanol) and 25% solvent A (0.1% acetic acid in water). The metabolites were resolved using the following gradient: 0 to 5 min, 75% B; 5 to 20 min, 75 to 100% B; 20 to 25 min, 100% B; 25 to 26 min, 100 to 75% B; and 26 to 30 min, 75% B. The flow rate was 0.3 ml/min. The column effluent was directed into the LCQ mass analyzer (Thermo Electron Corporation, Waltham, MA). The ESI conditions were as follows: sheath gas, 90 arbitrary units; auxiliary gas, 30 arbitrary units; capillary temperature, 200°C; and spray voltage, 4.5 V. Data were acquired in positive ion mode using the Xcalibur software package (Thermo Electron Corporation) with one full scan from 300 to 500 mass to charge ratio (m/z) followed by one data dependent scan of the most intense ion.

Chemistry. 5,6-EET was synthesized by iodolactonization of arachidonic acid, as described previously (Corey et al., 1980). 14,15-EET was prepared using an arachidonic acid-selective epoxidation (Corey et al., 1979). 20-HETE was prepared from 14,15-EET with a known three-step procedure using the Wittig reaction as described previously (Manna et al., 1983). All three compounds were transformed into the corresponding ethanolamides using a standard procedure involving the intermediate *N*-hydroxysuccinimide/*N,N'*-dicyclohexylcarbodiimide activation of the carboxylic moiety followed by the reaction with ethanolamine (Sehgal and Vijay, 1994). All of the products were purified by high-performance liquid chromatography and characterized by nuclear magnetic resonance and mass spectrometry.

Data Analysis. Nonlinear regression analysis of the data were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA; <http://www.graphpad.com>).

Reagents. Anandamide, arachidonic acid, and HET0016 were purchased from Cayman Chemical (Ann Arbor, MI). mEH, catalase, NADPH, L- α -dilauroyl-phosphatidylcholine, L- α -dioleoyl-*sn*-glycero-3-phosphatidylcholine, and L- α -phosphatidylserine were purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal inhibitory antibodies to the various human P450s were kindly provided by Dr. Harry Gelboin (National Institutes of Health, Bethesda, MD). All other chemicals were of highest quality and available from commercial sources.

Results

Metabolism of Anandamide by Human Kidney Microsomes and P450s 4F2, 4F3b, and 4A11. Figure 1A shows the selected ion chromatogram from the metabolism of anandamide by human kidney microsomes. Anandamide formed a positive ion in the mass spectrometer with an m/z of 348 and eluted at 23 min under the conditions used here. A single mono-oxygenated metabolite with an m/z value of 364 and a retention time of approximately 15 min, labeled M1, was observed (Fig. 1A).

The identity of the P450 that is primarily responsible for the production of M1 in the human kidney then was investigated. Members of the family 2 P450s, such as 2C8, 2C19, and 2J2, are known to be the predominant arachidonic acid epoxygenases in humans, whereas hydroxylations of arachidonic acid are mainly carried out by P450s belonging to family 4, with 4A11, 4F2, and 4F3b playing the major roles in humans (Lasker et al., 2000; Capdevila and Falck, 2001; Christmas et al., 2001). To investigate the identity of the P450 responsible for the formation of M1, human kidney microsomes (HKMs) were preincubated with the antibodies that have been described previously (Gelboin and Krausz, 2006) and that possess inhibitory activity against specific P450s to test for their effect on the formation of M1 following the initiation of metabolism by the addition of NADPH. Preincubation with antibodies against P450s 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 had no significant

effect on the formation of M1 (data not shown), suggesting that these P450s did not play a role in the formation of M1. Because inhibitory antibodies specific for human P450s 4A and 4F were unavailable, the highly selective arachidonic acid ω -hydroxylase inhibitor, HET0016, was used to test inhibition of M1 formation. HET0016 has been shown to inhibit 20-HETE formation by 4A11 and 4F2, with a reported IC_{50} value of 8.9 nM in human kidney microsomes (Miyata et al., 2001). As shown in Fig. 1B, inset, preincubation of the HKMs with 10 nM HET0016 (solid line) for 5 min before the addition of anandamide and NADPH resulted in a greater than 90% reduction in the formation of M1 compared with control (dotted line). Furthermore, as shown in Fig. 1B, the effect of HET0016 was dose-dependent, exhibiting an IC_{50} value of 2.5 nM. This result was not surprising because human kidneys have been found to express P450s belonging to family 4 at much higher levels than most other P450s investigated.

To determine whether the profiles of metabolite formation by the human P450s 4F2, 4F3b, or 4A11 resembled those seen with HKMs, anandamide was incubated with supersomes containing these P450s. As shown in Fig. 1C, M1 formation differed significantly among the three enzymes tested when using equal protein amounts, substrate concentration, and incubation conditions, as described under *Materials and Methods*. The ratios of formation of M1, as determined by the corresponding peak heights, were approx-

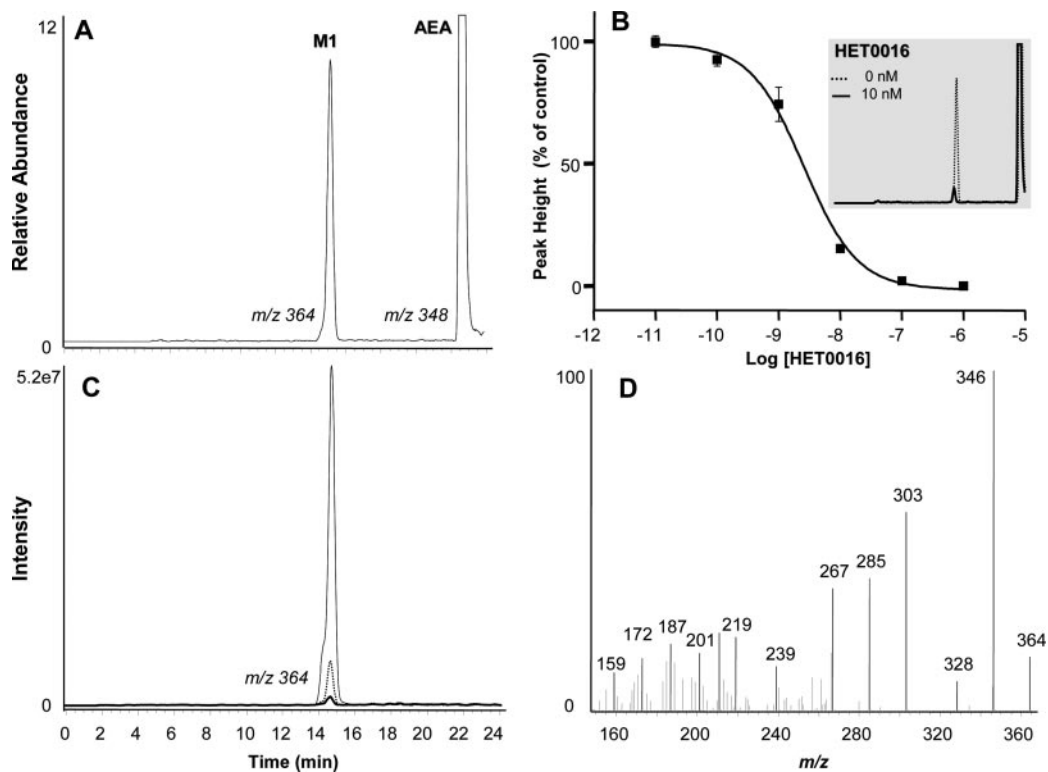


Fig. 1. Metabolism of anandamide by human kidney microsomes and supersomes containing P450s 4F2, 4F3b, or 4A11. Anandamide (20 μ M) was incubated with human kidney microsomes (100 μ g) in the presence of NADPH for 45 min, and the samples were analyzed as described under *Materials and Methods*. The selected ion chromatogram in A shows a single mono-oxygenated product (M1, m/z 364) and the anandamide parent ion (AEA; m/z 348). Human kidney microsomes were then preincubated with the P450 4A/4F arachidonic acid hydroxylase inhibitor HET0016 (10 nM) for 5 min before the addition of anandamide and NADPH. B, inset, peaks for M1 in the absence (---) and presence (—) of 10 nM HET0016. The dose-response curve (average of three experiments) for the inhibition of human kidney microsomal formation of M1 by HET0016 is shown in B. C, selected ion chromatograms (m/z 364) observed following the incubation of P450 4F2 (—), 4F3b (---), or 4A11 (---) supersomes (25 pmol) with 20 μ M anandamide for 45 min. D, MS/MS spectrum of M1 isolated from a P450 4F2 incubation mixture, which was identical to the MS/MS spectrum of the kidney metabolite (data not shown).

imately 58:7:1 (4F2:4F3b:4A11). To rule out significant differences in protein activity as a reason for the observed results, a parallel experiment was performed with arachidonic acid as a substrate (data not shown). The ratios of 20-HETE formation were 2.3:2:1 (4F2:4F3b:4A11), suggesting that the protein activity in the supersomes is representative of the established role of each of the enzymes in the ω hydroxylation of arachidonic acid and is not a factor in the results seen with anandamide.

Although metabolism by the individual P450s in supersomes may not exactly reflect metabolism by those P450s in the kidney microsomes, these data suggest that the main P450 involved in the formation of M1 by the HKMs is P450 4F2. In addition to having the same retention time as M1, the metabolite of anandamide formed by P450 4F2 also exhibited MS/MS fragmentation identical to that seen with human microsomes (Fig. 1D). In combination, these results suggest that the human kidney microsomes metabolize anandamide to give a single mono-oxygenated product, probably a hydroxylated metabolite, and that this reaction is most probably catalyzed by P450 4F2.

Metabolism of Anandamide by Human Liver Microsomes. In addition to forming product M1, human liver microsome (HLM) incubations led to the formation of several minor mono-oxygenated (m/z 364) metabolites eluting between 16 and 21 min (M2–M6), as shown in Fig. 2A. Incubation of the HLMs in the presence of anandamide also resulted in the formation of four additional metabolites (M7–M10), with retention times between 10 and 18 min and m/z values

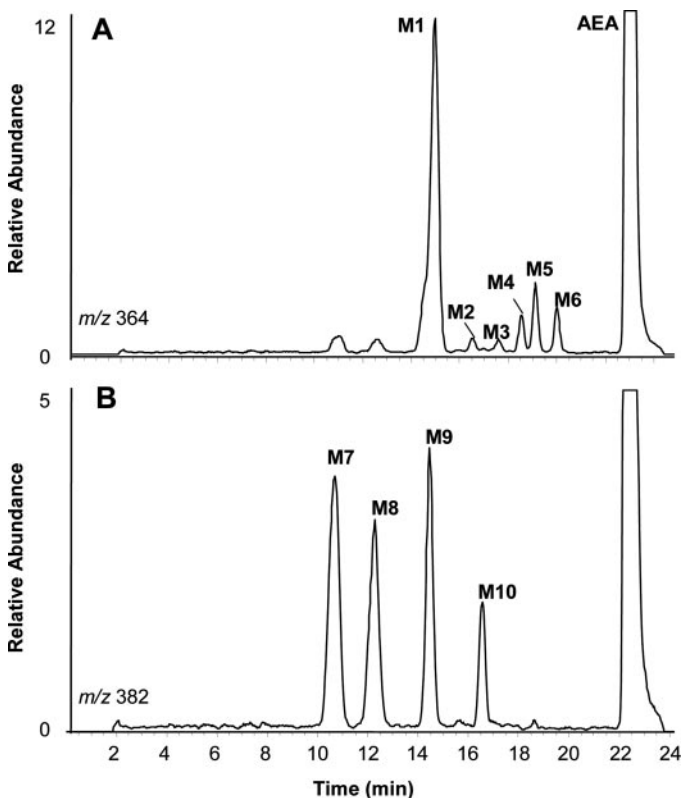


Fig. 2. Metabolism of anandamide by human liver microsomes. Human liver microsomes (100 μ g) were incubated in the presence of 100 μ M anandamide and NADPH for 45 min. The selected ion chromatogram (A) shows several mono-oxygenated products (M1–M6, m/z 364) and the anandamide ion (AEA; m/z 348). Four additional products (M7–M10) were detected from the same incubation having m/z values of 382 (B).

of 382 (Fig. 2B). The mass to charge ratio of these products corresponded to that of one mono-oxygenated metabolite (m/z 364) plus a water molecule ($M + 18$). Therefore, the possibility that metabolites M7–M10 may represent secondary products of the mono-oxygenated metabolites was considered because, in addition to P450s, the HLMs also contain other phase I and phase II metabolizing enzymes including mEH, which catalyzes the addition of water to epoxides (Morisseau and Hammock, 2005). Because exactly four secondary products were seen and because there are four possible sites for epoxidation on the anandamide structure, these data suggest the possibility that some of the minor mono-oxygenated metabolites that were observed could be anandamide epoxides that are subsequently converted to their corresponding dihydroxy derivatives.

To determine the involvement of specific P450 isoforms in the formation of the HLM anandamide metabolites, a panel of monoclonal inhibitory antibodies and the chemical inhibitor HET0016 were used again. Once again, the formation of M1 was inhibited by HET0016 in a dose-dependent manner with an IC_{50} of approximately 93 nM (data not shown). As shown in Fig. 3A, preincubation of the HLMs with the P450 3A4 antibody significantly inhibited the formation of metabolites M7–M10. The level of inhibition shown in Fig. 3A was the maximum inhibition observed with respect to the amount of antibody added. Inhibition of metabolism was not observed for incubations in the presence of inhibitory antibodies against P450s 1A1, 1A2, 2A6, 2B6, 2D6, 2C8, 2C9, and 2E1, whereas antibody against P450 2C19 produced modest inhibition of M7–M10 ranging between 5 and 16% for the six HLMs tested (data not shown).

Anandamide was also incubated with recombinant P450 3A4 to determine how the metabolite profile for the single enzyme compared with those seen with the products obtained from the liver microsomes. Figure 3B (solid line) shows that P450 3A4 converted anandamide to four mono-oxygenated products (m/z 364). These products have identical retention times as the metabolites M3–M6 that were produced by the liver microsomes. To determine whether these metabolites were indeed epoxides, P450 3A4 was reconstituted together with human recombinant mEH before incubation with anandamide and NADPH. Figure 3, B and C (dotted lines), depicts the metabolic profile of the anandamide metabolites generated by P450 3A4 in the presence of mEH. This profile was similar to the one observed with the liver microsomes (Fig. 2, A and B). A significant decrease in the formation of M3–M6 (m/z 364) was seen, whereas at the same time, the appearance of four new products that correspond to M7 to M10 (m/z 382) was observed. This experiment confirms that products M3 to M6 are epoxygenated metabolites of anandamide that undergo secondary metabolism by microsomal epoxide hydrolase in the liver microsomes.

The incomplete inhibition of M7–M10 formation by the antibody against P450 3A4 (Fig. 3A) could be indicative of the participation of another hepatic P450 in the formation of these products or the inability of the antibody to completely inhibit the activity of P450 3A4. To determine whether the latter holds true, the effectiveness of the same antibody to inhibit the activity of reconstituted P450 3A4 was measured in the context of anandamide metabolism as well as the *O*-debenzylation of 7-benzyloxy-4-(trifluoromethyl)- coumarin (a probe substrate for P450 3A4). In both cases, the level

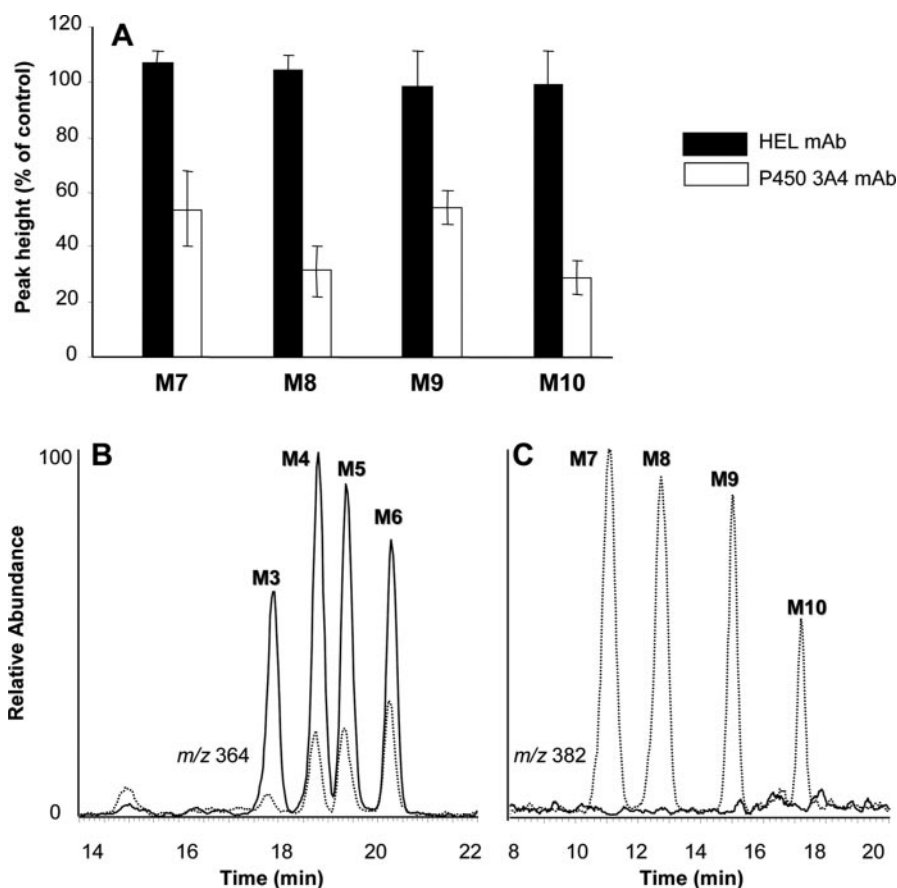


Fig. 3. Involvement of P450 3A4 in the metabolism of anandamide by human liver microsomes and the effect of microsomal epoxide hydrolase. Human liver microsomes were preincubated in the presence of control antibody [hen egg lysozyme (HEL)] or P450 3A4 inhibitory antibody (A). The peak heights for metabolites M7 to M10, expressed as a percent of control (no antibody), are plotted on the y-axis. The data show the mean and S.D. of six experiments. Human recombinant P450 3A4 (25 pmol) was reconstituted in the presence (·) or absence (—) of microsomal epoxide hydrolase and was incubated with anandamide (100 μ M) for 45 min (B and C). The selected ion chromatograms for the ions at m/z 364 (B) and m/z 382 (C) are shown.

of inhibition was similar (approximately 50%) to that observed with the HLM incubations (data not shown), suggesting that P450 3A4 is the primary enzyme involved in the human liver epoxidation of anandamide, and the incomplete inhibition reflects the fact that the antibody is not fully inhibitory in these experiments.

Structural Confirmation of Anandamide Metabolites. Chemical standards of 20-HETE-EA, 14,15-EET-EA, and 5,6-EET-EA were synthesized for structural confirmation, as described under the *Materials and Methods* section. Data from the ESI-LC/MS analysis of the chemical standards are shown in Fig. 4, A, C, and E, and reveal that 20-HETE-EA, 14,15-EET-EA, and 5,6-EET-EA have the same retention times as M1 (15 min), M3 (17 min), and M6 (20 min), respectively. The MS/MS spectra of each of the standards, which were identical to the MS/MS spectra of the corresponding products obtained through the metabolic reaction, are also shown in Fig. 4, B, D, and F.

The MS/MS spectra looked similar to each other with respect to the major fragments seen (m/z 346, 328, 303, 285, 267). The m/z ions 346 and 328 can be formed as a result of two sequential water losses (-18) from m/z 364, whereas 303 is the result of a loss of the ethanolamine group (-61), and 285 and 267 are the fragments of two sequential water losses (-18) from m/z 303. In addition to these common m/z fragments, 20-HETE-EA consistently displayed the unique fragment 219, whereas 14,15-EET-EA formed the unique fragments m/z 187 and 248, and 5,6-EET-EA exhibited the fragments m/z 154 and 172. Standards for 19-HETE-EA, 11,12-, and 8,9-EET-EA are currently unavailable; however, they most probably corre-

spond to M2, M4, and M5, respectively, because the location of the functional group on the acyl chain would lead to that order of elution observed from the C18 column used in the study (Nithipatikom et al., 2001).

Kinetic Analysis of Anandamide Hydroxylation by Human Kidney and Liver Microsomes and P450 4F2 Supersomes. The human kidney and liver microsomes converted anandamide to 20-HETE-EA in a time- and protein-dependent manner that was linear for at least 12 min of reaction time at 37°C and 75 μ g of kidney microsomal protein and 15 min and 25 μ g of liver microsomal protein (data not shown). Pooled kidney microsomes from three subjects were used in the incubation reactions for the kinetic analysis of renal 20-HETE-EA formation. Over the range of substrate concentrations used (0.25–10 μ M), anandamide metabolism to 20-HETE-EA exhibited simple Michaelis-Menten kinetics (Fig. 5A, open circles) that were consistent with reaction catalysis by a single enzyme. Nonlinear regression analysis was used to analyze the data and derive an apparent K_m of 2.6 ± 0.4 μ M and a V_{max} of 122 ± 7.4 pmol of 20-HETE-EA formed/min/mg kidney microsomal protein.

Similar kinetics were observed with the HLM incubations (Fig. 5A, filled circles), for which an apparent K_m of 2.4 ± 0.6 μ M and a V_{max} of 266 ± 26 pmol of 20-HETE-EA formed/min/mg protein were derived. The increase in turnover number in the HLM incubations relative to the HKM incubations is consistent with the higher level of expression of P450 4F2 in liver tissue compared with kidney (Nishimura et al., 2003).

The kinetics of 20-HETE-EA formation by P450 4F2 supersomes are shown in Fig. 5B. The V_{max} for ω hydroxylation of anandamide by P450 4F2 was 11.5 ± 0.4 pmol of 20-HETE-

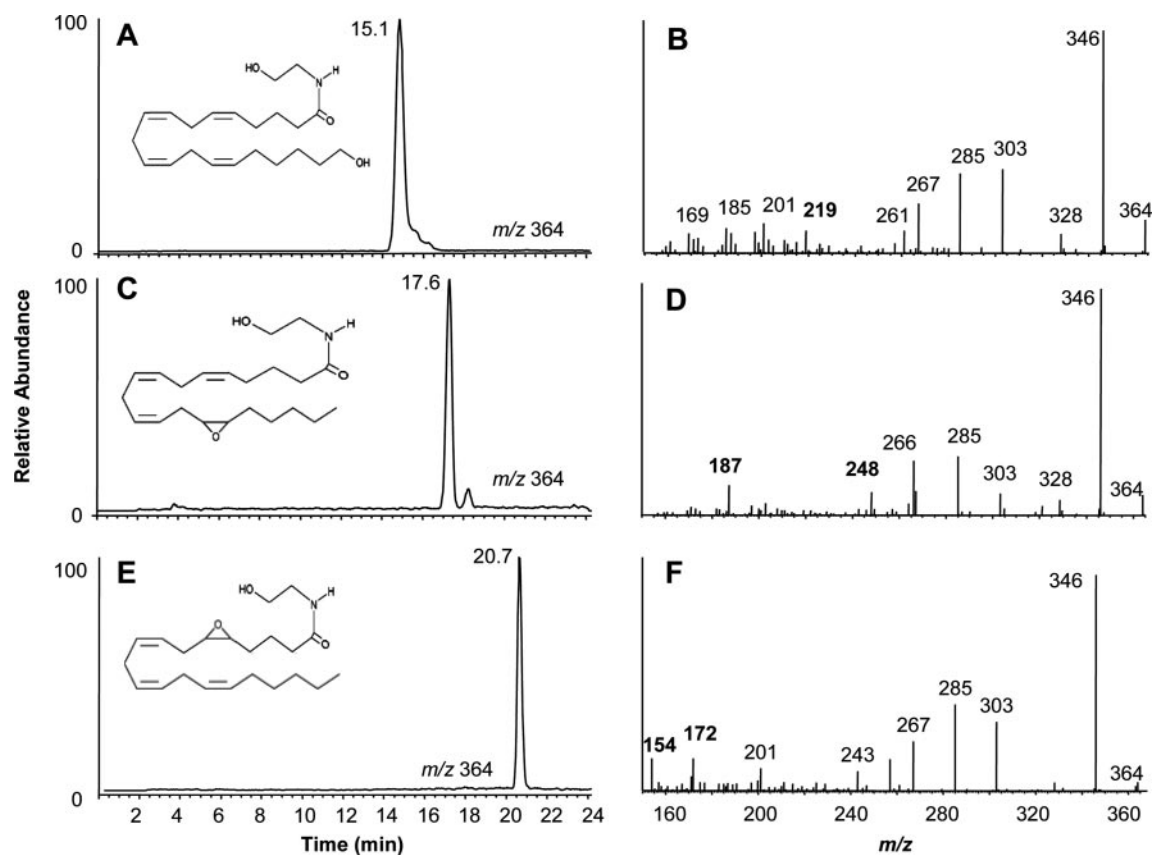


Fig. 4. Structural confirmation of 20-HETE-EA, 14, 15-EET-EA, and 5,6-EET-EA. ESI-LC/MS/MS analyses of 50 pmol of the authentic metabolite standards are shown in A to F. Selected ion chromatograms (m/z 364) of the three standards (A, C, and E) and their corresponding MS/MS spectra (B, D, and F) are shown.

EA/min/pmol P450 4F2 protein, and the apparent K_m was $0.7 \pm 0.07 \mu\text{M}$. In comparison, P450 4F3b supersomes exhibited an apparent K_m of $3.4 \pm 0.79 \mu\text{M}$ and a V_{max} of 0.1 ± 0.01 pmol of 20-HETE-EA/min/pmol P450 4F3b protein (data not shown). Combined, these data suggest that anandamide is a high-affinity substrate for P450 4F2 and that this P450 may be the one that leads primarily to the formation of 20-HETE-EA in human kidney and liver microsomes.

Kinetic Analysis of Anandamide Epoxidation by Human Liver Microsomes. The human liver microsomes converted anandamide to 5,6-, 8,9-, 11,12-, and 14,15-EET-EA in a time- and protein-dependent manner that was linear for all products for 15 min of reaction time at 37°C and $25 \mu\text{g}$ of microsomal protein (data not shown). Pooled liver microsomes were used for the kinetic analyses of hepatic EET-EA formation from anandamide at concentrations ranging from 1 to $25 \mu\text{M}$. Nonlinear regression analysis of the data were used to derive apparent K_m values ranging from 4 to $5 \mu\text{M}$ (Table 1).

The rates of formation of the EET-EAs by the liver microsomes ranged from around 40 pmol/min/mg for 14,15- and 11,12-EET-EA to 180 pmol/min/mg for 5,6-EET-EA and to 480 pmol/min/mg for 8,9-EET-EA. Because standards for 11,12- and 8,9-EET-EA were unavailable, their rates of formation were estimated from standard curves of 14,15- and 5,6-EET-EA, respectively, which could have been a factor in obtaining more accurate values. In combination, these data implicate anandamide as a relatively high-affinity substrate

for human liver microsomal P450s that undergoes significant turnover *in vitro* to produce EET ethanolamides.

Discussion

Identification of enzymes that may participate in the metabolism of the endogenous cannabinoid anandamide is necessary for a better understanding of the regulation and action of this potent signaling mediator. Because inhibition of the anandamide-inactivating enzyme FAAH represents a novel intervention strategy in potentially treating a wide range of nervous system and peripheral disorders (Cravatt and Lichtman, 2003), it is crucial to identify alternative routes of anandamide metabolism. Knowledge of the involvement of human cytochrome P450 enzymes in the oxidative metabolism of anandamide is lacking, and the studies reported here are the first ones to begin examining these potential metabolic pathways.

A number of novel oxygenated products of anandamide produced by human liver and kidney microsomal P450s were identified here. The data presented show that anandamide can undergo hydroxylation by human kidney microsomes and epoxygenation and hydroxylation by human liver microsomes. Lack of detection of epoxygenated metabolites in the reactions carried out with human kidney microsomes is in agreement with previously published studies on the lack of detection of EET production from arachidonic acid in this tissue (Lasker et al., 2000). This also points to the possibility

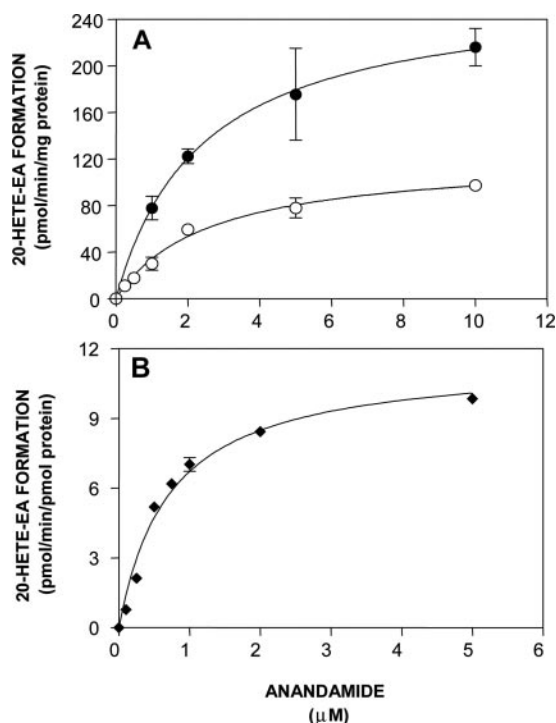


Fig. 5. Kinetics of 20-HETE-EA formation by human kidney and liver microsomes and P450 4F2 supersomes. Seventy-five micrograms of human kidney microsomes (○) or 25 μg of human liver microsomes (●) were incubated with various concentrations of anandamide (0.1–10 μM) for 8 and 15 min, respectively (A). P450 4F2 supersomes (2 pmol) were incubated in the presence of anandamide (0.1–5 μM) for 8 min (B). The amount of 20-HETE-EA formed was determined from a standard curve, and the rate data (average of four experiments) were fitted to a one-enzyme Michaelis-Menten model using Prism software.

of tissue-dependent production of anandamide metabolites in accordance with the presence of specific P450 isoforms in various tissues.

The finding that P450 4A11 exhibited negligible anandamide ω -hydroxylase activity compared with P450 4F2, although somewhat surprising, has precedent from at least one other example of a similar phenomenon in the literature. A study by Jin et al. (1998) found that CYP4F2 converted leukotriene B4 (LTB4) to 20-OH LTB4 at a turnover rate of 392 pmol/min/nmol P450, whereas CYP4A11 exhibited negligible LTB4 ω -hydroxylase activity.

The studies presented here indicate a specific role for P450 4F2 in the formation of 20-HETE-EA in both the kidney and the liver and a role for P450 3A4 in the formation of EET-EAs in the liver. Although P450 2C and 2J families are known to be the major arachidonic acid epoxygenases in humans, it has been reported previously that monkey endothelial P450 3A4 can also act as an arachidonic acid epoxygenase to produce the vasorelaxant EETs (Ayajiki et al., 2003). EETs are known to play an important function in the vascular and renal systems and are also produced by the endothelium and

can function as the endothelium-derived hyperpolarizing factors in the coronary circulation (Spector and Norris, 2006). The vasorelaxant properties of anandamide have been well documented, and one mechanism involves the transient receptor potential channel TRPV4, which is also known to be activated by EETs (Watanabe et al., 2003). 20-HETE is an inhibitor of renal tubular Na^+/K^+ -ATPase activity and a potent constrictor of kidney microvessels (Miyata and Roman, 2005). Anandamide has also been reported to have vasoconstrictor properties in renal vascular beds through a mechanism that is independent of its action at the cannabinoid receptor (Gardiner et al., 2002).

Conversion of anandamide to arachidonic acid via hydrolysis by FAAH and subsequent metabolism of the free fatty acid by P450s to vasoactive eicosanoids is one mechanism to explain the vascular properties of anandamide, and this mechanism has been proposed previously (Watanabe et al., 2003). However, the possibility that P450s can directly act on anandamide to produce HETE and EET ethanolamides that may have pharmacological activity should also be considered and further examined. Human kidney microsomes and purified P450 4F2 metabolize arachidonic acid to 20-HETE with apparent K_m values of 43 and 23.5 μM , respectively (Lasker et al., 2000). According to the data presented here, anandamide appears to be a better substrate than arachidonic acid for human kidney microsomes and P450 4F2, exhibiting apparent K_m values of 2.3 and 0.7 μM , respectively. This raises the possibility that 20-HETE-EA could be formed in vivo, and it may be responsible for mediating some of the renal vasoconstrictor properties of anandamide that have been reported previously and for which a mechanism has not yet been identified. EET-EAs could also be potentially formed in vivo, considering the relatively low apparent K_m values of 4 to 5 μM in the human liver microsomes. These are similar to the K_m values published for arachidonic acid-derived EETs of 9 to 11 μM with human liver microsomes (Rifkind et al., 1995). Although under normal conditions the tissue concentration of nonesterified arachidonic acid in vivo is believed to be in the low micromolar range (Brash, 2001), whereas anandamide is found in tissues in the low nanomolar range, local intracellular anandamide concentrations could be favorable to this route of metabolism. In addition, the K_m values reported here are similar to those reported for the human FAAH enzyme, which is 2 μM (Maccarrone et al., 1998), again pointing to the possibility of the P450 pathway occurring in vivo. The biological significance of this pathway remains to be determined, and the studies presented here represent an aid to that effort by making available an ESI-LC/MS method for the separation and detection of the HETE-EA, EET-EA, and dihydroxyeicosatrienoic acid-EA anandamide metabolites that could be useful in determining their presence and levels in biological samples. Because of the wide-ranging effects of anandamide on many organ systems, its metabolism by

TABLE 1

Anandamide epoxidation by human liver microsomes

K_m and V_{max} values were assessed from 15-min incubations at 37°C of 0.5-ml reaction mixtures containing 25 μg of microsomal protein, 100 mM KPO_4 buffer, pH 7.4, anandamide (1–25 μM), and NADPH (1 mM) and represent the average of five experiments. Rates of formation were determined as described under *Materials and Methods*.

	14,15-EET-EA	11,12-EET-EA	8,9-EET-EA	5,6-EET-EA
K_m (μM)	5.6 \pm 2.3	4.2 \pm 1.6	4.4 \pm 1.4	5.1 \pm 1.9
V_{max} (pmol/min/mg)	48 \pm 6	44 \pm 4	480 \pm 56	184 \pm 24

P450s from various tissues such as the brain and the spleen needs to be examined in detail, and this is the focus of current and future investigations.

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References

- Ayajiki K, Fujioka H, Toda N, Okada S, Minamiyama Y, Imaoka S, Funae Y, Watanabe S, Nakamura A, and Okamura T (2003) Mediation of arachidonic acid metabolite. (s) produced by endothelial cytochrome P-450 3A4 in monkey arterial relaxation. *Hypertens Res* **26**:237–243.
- Bornheim LM, Kim KY, Chen B, and Correia MA (1995) Microsomal cytochrome P450-mediated liver and brain anandamide metabolism. *Biochem Pharmacol* **50**: 677–686.
- Brash AR (2001) Arachidonic acid as a bioactive molecule. *J Clin Invest* **107**:1339–1345.
- Burstein SH, Rossetti RG, Yagen B, and Zurier RB (2000) Oxidative metabolism of anandamide. *Prostaglandins Other Lipid Mediat* **61**:29–41.
- Capdevila JH and Falck JR (2001) The CYP P450 arachidonic acid monooxygenases: from cell signaling to blood pressure regulation. *Biochem Biophys Res Commun* **285**:571–576.
- Capdevila JH and Falck JR (2002) Biochemical and molecular properties of the cytochrome P450 arachidonic acid monooxygenases. *Prostaglandins Other Lipid Mediat* **68–69**:325–344.
- Christmas P, Jones JP, Patten CJ, Rock DA, Zheng Y, Cheng SM, Weber BM, Carlsson N, Scadden DT, Rettie AE, et al. (2001) Alternative splicing determines the function of CYP4F3 by switching substrate specificity. *J Biol Chem* **276**: 38166–38172.
- Coon MJ (2005) Cytochrome P450: nature's most versatile biological catalyst. *Annu Rev Pharmacol Toxicol* **45**:1–25.
- Corey EJ, Niwa H, and Falck JR (1979) Selective epoxidation of eicosa-cis-5,8,11,14-tetraenoic (arachidonic) acid and eicosa-cis-8,11,14-trienoic acid. *J Am Chem Soc* **101**:1586–1587.
- Corey EJ, Niwa H, Falck JR, Mioskowski C, Arai Y, and Marfat A (1980) Recent studies on the chemical synthesis of eicosanoids. *Adv Prostaglandin Thromboxane Res* **6**:19–25.
- Cravatt BF and Lichtman AH (2003) Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. *Curr Opin Chem Biol* **7**:469–475.
- Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffing G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258**:1946–1949.
- Di Marzo V, Bifulco M, and De Petrocellis L (2004) The endocannabinoid system and its therapeutic exploitation. *Nat Rev Drug Discov* **3**:771–784.
- Edgemond WS, Hillard CJ, Falck JR, Kearn CS, and Campbell WB (1998) Human platelets and polymorphonuclear leukocytes synthesize oxygenated derivatives of arachidonylethanolamide (anandamide): their affinities for cannabinoid receptors and pathways of inactivation. *Mol Pharmacol* **54**:180–188.
- Gardiner SM, March JE, Kemp PA, and Bennett T (2002) Complex regional haemodynamic effects of anandamide in conscious rats. *Br J Pharmacol* **135**:1889–1896.
- Gelboin HV and Krausz K (2006) Monoclonal antibodies and multifunctional cytochrome P450: drug metabolism as paradigm. *J Clin Pharmacol* **46**:353–372.
- Giang DK and Cravatt BF (1997) Molecular characterization of human and mouse fatty acid amide hydrolases. *Proc Natl Acad Sci USA* **94**:2238–2242.
- Hampson AJ, Hill WA, Zan-Phillips M, Makriyannis A, Leung E, Eglen RM, and Bornheim LM (1995) Anandamide hydroxylation by brain lipoxigenase:metabolite

- structures and potencies at the cannabinoid receptor. *Biochim Biophys Acta* **1259**: 173–179.
- Hanna IH, Teiber JF, Kokones KL, and Hollenberg PF (1998) Role of the alanine at position 363 of cytochrome P450 2B2 in influencing the NADPH- and hydroperoxide-supported activities. *Arch Biochem Biophys* **350**:324–332.
- He K, Iyer KR, Hayes RN, Sinz MW, Woolf TF, and Hollenberg PF (1998) Inactivation of cytochrome P450 3A4 by bergamottin, a component of grapefruit juice. *Chem Res Toxicol* **11**:252–259.
- Jin R, Koop DR, Raucy JL, and Lasker JM (1998) Role of human CYP4F2 in hepatic catabolism of the proinflammatory agent leukotriene B₄. *Arch Biochem Biophys* **359**:89–98.
- Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R, Jakobsson PJ, and Marnett LJ (2002) Metabolism of the endocannabinoids, 2-arachidonylethanolamide and anandamide, into prostaglandin, thromboxane, and prostacyclin glycerol esters and ethanolamides. *J Biol Chem* **277**:44877–44885.
- Lasker JM, Chen WB, Wolf I, Blawick BP, Wilson PD, and Powell PK (2000) Formation of 20-hydroxyeicosatetraenoic acid, a vasoactive and natriuretic eicosanoid, in human kidney: role of Cyp4F2 and Cyp4A11. *J Biol Chem* **275**:4118–4126.
- Maccarrone M, van der Stelt M, Rossi A, Veldink GA, Vliegthart JF, and Agro AF (1998) Anandamide hydrolysis by human cells in culture and brain. *J Biol Chem* **273**:32332–32339.
- Manna S, Falck JR, Chacos N, and Capdevila J (1983) Synthesis of arachidonic acid metabolites produced by purified kidney cortex microsomal cytochrome P-450. *Tetrahedron Lett* **24**:33–36.
- Miyata N and Roman RJ (2005) Role of 20-hydroxyeicosatetraenoic acid (20-HETE) in vascular system. *J Smooth Muscle Res* **41**:175–193.
- Miyata N, Taniguchi K, Seki T, Ishimoto T, Sato-Watanabe M, Yasuda Y, Doi M, Kametani S, Tomishima Y, Ueki T, et al. (2001) HET0016, a potent and selective inhibitor of 20-HETE synthesizing enzyme. *Br J Pharmacol* **133**:325–329.
- Morrisseau C and Hammock BD (2005) Epoxide hydrolases: mechanisms, inhibitor designs, and biological roles. *Annu Rev Pharmacol Toxicol* **45**:311–333.
- Nishimura M, Yaguti H, Yoshitsugu H, Naito S, and Satoh T (2003) Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high-sensitivity real-time reverse transcription PCR. *Yakugaku Zasshi* **123**:369–375.
- Nithipatikom K, Grall AJ, Holmes BB, Harder DR, Falck JR, and Campbell WB (2001) Liquid chromatographic-electrospray ionization-mass spectrometric analysis of cytochrome P450 metabolites of arachidonic acid. *Anal Biochem* **298**:327–336.
- Rifkind AB, Lee C, Chang TK, and Waxman DJ (1995) Arachidonic acid metabolism by human cytochrome P450s 2C8, 2C9, 2E1, and 1A2: regioselective oxygenation and evidence for a role for CYP2C enzymes in arachidonic acid epoxyoxygenation in human liver microsomes. *Arch Biochem Biophys* **320**:380–389.
- Sarkis A and Roman RJ (2004) Role of cytochrome P450 metabolites of arachidonic acid in hypertension. *Curr Drug Metab* **5**:245–256.
- Sehgal D and Vijay IK (1994) A method for the high efficiency of water-soluble carbodiimide-mediated amidation. *Anal Biochem* **218**:87–91.
- Spector AA and Norris AW (2006) Action of epoxyeicosatrienoic acids (EETs) on cellular function. *Am J Physiol*, in press.
- Teiber JF and Hollenberg PF (2000) Identification of the human liver microsomal cytochrome P450s involved in the metabolism of *N*-nitrosodi-*n*-propylamine. *Carcinogenesis* **21**:1559–1566.
- Ueda N, Yamamoto K, Kurahashi Y, Yamamoto S, Ogawa M, Matsuki N, Kudo I, Shinkai H, Shirakawa E, and Tokunaga T (1995) Oxygenation of arachidonylethanolamide (anandamide) by lipoxigenases. *Adv Prostaglandin Thromboxane Leukotriene Res* **23**:163–165.
- Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, and Nilius B (2003) Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature (Lond)* **424**:434–438.
- Yu M, Ives D, and Ramesha CS (1997) Synthesis of prostaglandin E₂ ethanolamide from anandamide by cyclooxygenase-2. *J Biol Chem* **272**:21181–21186.

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