OXIDATION OF THE ENDOGENOUS CANNABINOID ARACHIDONOYL ETHANOLAMIDE (ANANDAMIDE) BY CYTOCHROME P450 ENZYMES

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in The University of Michigan
2009

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Acknowledgements

I am extremely grateful to have had the opportunity of working under the guidance of my thesis mentor, Dr. Paul Hollenberg, who invested countless hours on my training and development and supported this work financially and with incredible enthusiasm. Thank you to all of the current and former members of the Hollenberg lab who have made my graduate school experience enjoyable and rewarding, in particular Dr. Ute Kent, Dr. Namandjé Bumpus, Chitra Sridar, and Hsia-Lien Lin. I thank my committee members, Dr. William Pratt, Dr. William Smith, Dr. Yoichi Osawa, Dr. John Traynor, and Dr. Roger Sunahara for their invaluable input and guidance over the past four years. I would also like to acknowledge the help and support of all faculty, staff and students in the Department of Pharmacology as well as the Program in Biomedical Sciences (PIBS). A special thanks to Dr. Anne Garcia, one of the best educators I have encountered, who allowed me to present lectures in her psychology course at Washtenaw Community College. I am thankful for the help and support I have received from numerous collaborators over the years, especially Dr. Jeff Johnson and Dr. Andrei Kornilov from Cayman Chemical. Finally, I am thankful to all of my friends and family members for their help and support. Above all, I thank my husband Matt for his love and encouragement and for always being there for me.

This work was supported in part by National Institutes of Health Grants CA-16954 and T32 GM007767 and a Merck & Co. Pre-Doctoral Fellowship

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AA, arachidonic acid
AEA, anandamide
cAMP, cyclic adenosine monophosphate
CB1, cannabinoid receptor 1
CB2, cannabinoid receptor 2
CHO, Chinese hamster ovary
CNS, central nervous system
COX, cyclooxygenase
DHET, dihydroxyeicosatrienoic acid
EA, ethanolamide
EET, epoxyeicosatrienoic acid
EH, epoxide hydrolase
ESI, electrospray ionization
FAAH, fatty acid amide hydrolase
HET0016, *N*-hydroxy-*N*'-(4-butyl-2-methylphenyl)-formamidine
HETE, hydroxyeicosatetraenoic acid
HKM, human kidney microsome
HLM, human liver microsome
IFNγ, interferon gamma
IBMX, isobutylmethylxanthine
LC, liquid chromatography
LOX, lipoxygenase
LPS, lipopolysaccharide
LTB4, leukotriene B4
MS, mass spectrometry
MS/MS, tandem mass spectrometry
NADPH, nicotinamide adenine dinucleotide phosphate-oxidase
NAPE, N-arachidonoyl phosphatidyl ethanolamine
P450, cytochrome P450
PPAR, peroxisome proliferator-activated receptor
Δ^9-THC, delta-9-tetrahydrocannabinol

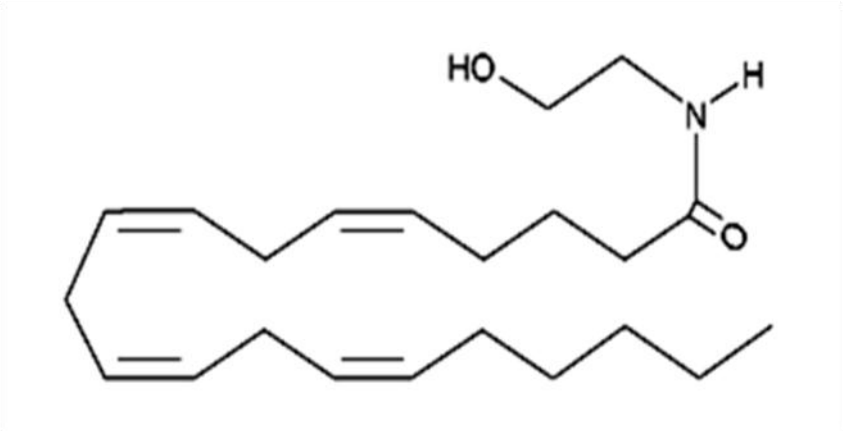
Chapter I

Introduction

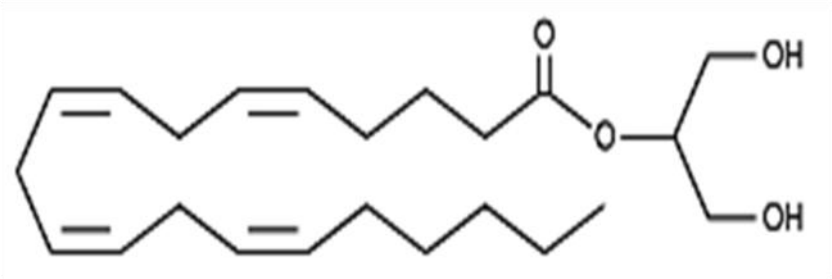
Marijuana and the endocannabinoid system. Marijuana (*Cannabis sativa*) is one of the most common drugs of abuse in the world, but it also has a long history of medicinal application, dating back as early as 2600 B.C (Mechoulam and Hanus, 2000; Robson, 2005). Several pharmaceuticals based on marijuana's main psychoactive cannabinoid, delta-9-tetrahydrocannabinol (Δ^9-THC), were developed even before the molecular mode of Δ^9-THC action was known. These include dronabinol and nabilone, prescribed as anti-emetics and appetite stimulants to patients afflicted with AIDS wasting syndrome or undergoing cancer chemotherapy (Mechoulam and Hanus, 2000). Nabilone is also used as an adjunct therapy for the management of chronic pain associated with fibromyalgia and multiple sclerosis (Wissel et al., 2006; Skrabek et al., 2008). Molecular investigations into cannabinoid action intensified after the cloning of the cellular targets of Δ^9-THC in the early 1990s (Matsuda et al., 1990; Munro et al., 1993). The two cannabinoid receptors identified to date are CB1 and CB2, which are found on many different cell types but are most abundantly expressed on neurons and immune cells, respectively. Both receptors are G-protein coupled and their activation by agonists leads to inhibition of the accumulation of cyclic adenosine monophosphate (cAMP) in cells *via* their associated proteins $G\alpha_{i/o}$ (Howlett, 2005; Pertwee, 2005).

In addition to the marijuana-derived and the synthetic cannabinoid analogs, there are also are endogenous ligands for the cannabinoid receptors (Devane et al., 1992; Mechoulam et al., 1995). The endogenous cannabinoids (endocannabinoids) that have been most studied and characterized are arachidonoylethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG), the amide and the ester, respectively, of arachidonic acid (Figure 1.1).

Therapeutic relevance of the endocannabinoid system. The CB1 receptor is expressed heterogeneously within the central nervous system (CNS), accounting for many of the characteristic actions of CB1 receptor agonists including marijuana-derived Δ^9-THC. For example, the presence of high levels of this receptor in the cerebral cortex, hippocampus, substantia nigra and cerebellum as well as in areas of the brain and spinal cord that modulate nociceptive information account for the ability of CB1 receptor agonists to impair cognition and memory, to alter the control of motor function, and to produce antinociception (Mackie, 2005). CB1 receptor activation also leads to increased food intake, which is the reason why the use of cannabis in various forms increases appetite in humans (Di Marzo and Matias, 2005). Activation of the CB1 receptor in the CNS also leads to neuroprotection, which is the rationale for attempts to exploit CB1 receptor activation therapeutically in the management of Parkinson's disease and multiple sclerosis (Kreitzer and Malenka, 2007; Maresz et al., 2007). However, barriers to the development of CB1 receptor agonists are their socially unacceptable psychoactive properties as well as the regulatory restrictions. On the other hand, successful management of obesity via blockade of the CB1 receptor has been clinically achieved with the drug rimonabant (Maresz et al., 2007). However, due to an unacceptable risk of



N-arachidonoylethanolamide
(AEA, Anandamide)



2-arachidonoyl glycerol
(2-AG)

Figure 1.1. Chemical structures of the endocannabinoids anandamide and 2-AG

psychological disturbances (e.g. depression and suicide ideation), rimonabant did not receive approval by the Food and Drug Administration to be marketed in the United States and marketing of rimonabant was recently suspended by the European Medicines Agency.CB2 receptors are expressed mainly on immune cells such as lymphocytes, macrophages, mast cells, natural killer cells, and microglia (Mackie, 2005), and activation of the CB2 receptors on immune cells alters cell migration and leads to immunosuppression (Miller and Stella, 2008). Since CB2 receptor-selective agonists are immunosuppressive, anti-inflammatory and lack psychoactive properties, they are being considered as potential therapeutics for the management of chronic pain and inflammation, including inflammation associated with neurodegenerative disease (Cabral et al., 2008). However, a much better understanding of their complex role in the regulation of immune responses is needed before further progress can be made.

Anandamide itself possesses neuroprotective and immunosuppressive properties that are mediated by cannabinoid receptor-dependent as well as cannabinoid receptor - independent pathways, such as the modulation of ion channel activity, including the transient receptor potential vanilloid type-1 receptor (TRPV1) and activation of the nuclear peroxisome proliferator-activated receptors (PPARs) (Rockwell and Kaminski, 2004; Eljaschewitsch et al., 2006; Hegde et al., 2008). Therefore, enhancing and/or prolonging the effects of anandamide by increasing its endogenous concentration also holds therapeutic potential. Anandamide is produced on-demand, acts locally due to its lipophilic character and is inactivated by the enzyme fatty acid amide hydrolase (FAAH), which metabolizes it into arachidonic acid and ethanolamine (Liu et al., 2006; Liu et al.,

2008). Therefore, inhibitors of FAAH are being developed as potential anti-inflammatory therapeutics (Cravatt and Lichtman, 2003).

Oxidation of anandamide. Anandamide appears to be synthesized "on demand" following a cell stimulus, such as neuronal depolarization, via a two step enzymatic process involving a calcium-dependent N-acyl transferase to form the anandamide precursor N-arachidonoyl phosphatidyl ethanolamine (NAPE) and a NAPE-specific phospholipase D, which releases anandamide from its precursor (Piomelli, 2003). Most of the intracellular pool of anandamide undergoes FAAH-mediated hydrolysis (Figure 1.2), but diminishing or eliminating the hydrolysis of anandamide by FAAH would increase the likelihood that anandamide might undergo alternative routes of metabolism, such as oxidation by the fatty acid oxygenases that are known to act on endogenous arachidonic acid; namely, the members of the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (P450) families of enzymes (Kozak et al., 2004). COX-2, which is expressed in an inducible manner during inflammation, converts anandamide to several prostaglandin ethanolamides (Yu et al., 1997; Kozak et al., 2002), whereas 12-LOX and 15-LOX form hydroxylated derivatives of anandamide (Hampson et al., 1995). Work regarding the synthesis and action of these metabolites has been previously reviewed (Starowicz et al., 2007; Woodward et al., 2008). In contrast, significantly less is known about the roles of the P450 enzymes in anandamide oxidation, as well as the fate of the metabolic products generated *via* these enzymatic pathways.

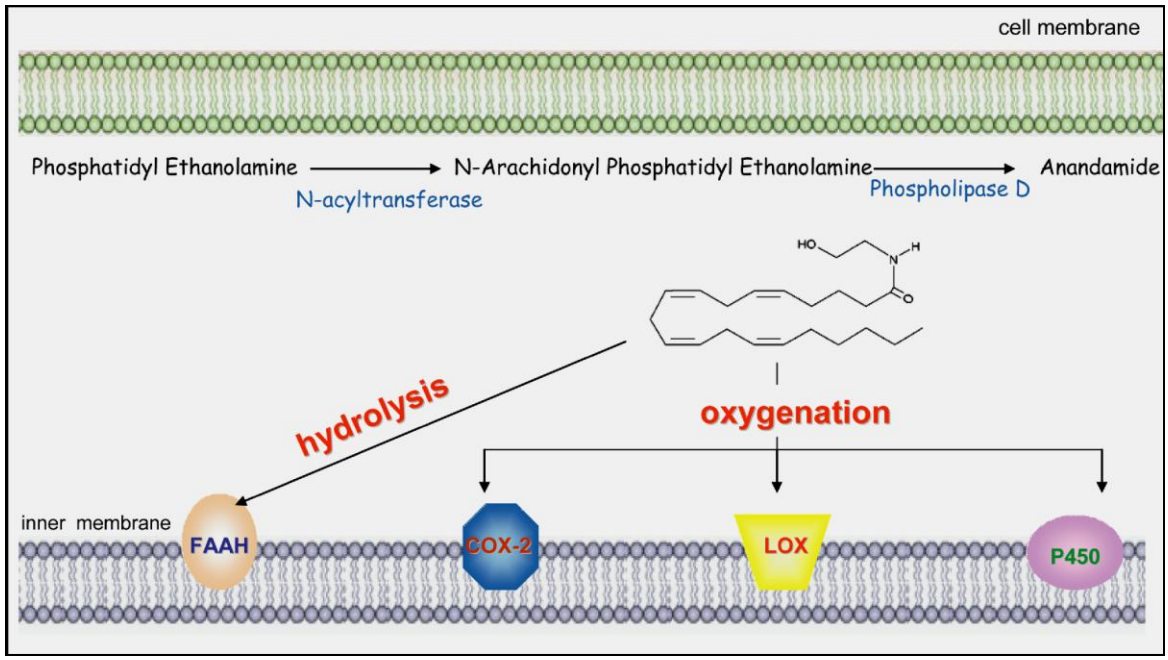


Figure 1.2. Intracellular synthesis and metabolism of anandamide.

The cytochrome P450 (P450) monooxygenases. The P450s are heme-containing monooxygenases that, as a group, are one of the most extensively studied enzyme systems because they play important roles in the biotransformation of most clinically used drugs, environmental chemicals and endogenous substrates (Coon, 2005). Many of the P450 genes are expressed constitutively in a tissue, gender and age-dependent fashion and may also be regulated by external factors such as drugs, environmental pollutants, food, hormones, and disease states, such as hypertension and diabetes (Ingelman-Sundberg et al., 2007). The nomenclature for the P450 enzymes is based on their amino acid sequence identity, determining their grouping into families (> 40% sequence identity) and subfamilies (>55 % sequence identity). The name of each P450 begins with a number indicating the family, followed by a letter indicating the subfamily, followed by another number indicating the specific isoform and signifying the sequence in which it was first identified (e.g. P450 1A1). The mammalian P450s are membrane-bound and are localized in the endoplasmic reticulum (ER) or the mitochondrial membrane of cells in the liver and most other tissues, including kidney, brain, intestine, lung, skin and heart. The tissue with the highest level of expression for most of the drug-metabolizing P450s is the liver (Omura, 2006; Meyer et al., 2007). The mono-oxygenation reactions carried out by P450s require a stepwise supply of electrons which are derived from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) and supplied by a redox partner (Coon, 2005). The redox partner in the case of the microsomal P450s is a single membrane bound enzyme (P450 reductase) whereas the mitochondrial P450s use a two-component electron shuttle system consisting of an iron-sulfur protein (adrenodoxin) and a flavoprotein (adrenodoxin reductase). During turnover, the P450s generally catalyze the

delivery of an active form of atomic oxygen delivered from molecular oxygen to the substrate molecule, and the additional oxygen is incorporated into water (Capdevila and Falck, 2002).

Fifty seven functional P450 genes have been identified in the human genome. However, P450s are also found across all living organisms and are involved in the biotransformation of a diverse range of xenobiotics as well as endogenous substrates (Nebert and Russell, 2002). Although the catalytic function for the majority of the human P450 enzymes is known, there are a significant number of "orphan" P450s belonging to families 2, 3, 4, 20 and 27 for which the catalytic activity is still unknown (Stark and Guengerich, 2007). Of those with known function, around 15 of the human isoforms that are primarily in families 1, 2 and 3 are involved in the metabolism of xenobiotics (Wienkers and Heath, 2005). Vitamins A and D in the kidney and the brain are metabolized by P450s from families 24, 26 and 27 (Sakaki et al., 2005; McCaffery and Simons, 2007), and certain steroid synthesis pathways are carried out by a large number of P450s classified into families 1, 7, 8, 11, 17, 19, 21, 27, 39, 46 and 51 (Miller, 2008). Most of the steroid-oxidizing enzymes are critical for normal physiological function, and their levels are relatively invariable among individuals. Deficiencies in these enzymes can lead to serious diseases, such as congenital adrenal hyperplasia, which is caused by a deficiency of P450 21A2 (Harada et al., 1987; Tajima et al., 1993). Many P450 members of families 2 and 4 are promiscuous in the sense that they can metabolize both drugs, such as the antibiotic erythromycin and the antihistamine ebastine, as well as endogenous substrates, such as fatty acids, including lauric and arachidonic acid, and eicosanoids, including leukotrienes and prostaglandins (Kalsotra and Strobel, 2006).

Arachidonic acid metabolism by the cytochrome P450 enzymes. Arachidonic acid, an ω-6 essential fatty acid found inside cells, is mostly esterified to membrane phospholipids. It is released from the membrane via the action of phospholipases and can subsequently undergo oxidation to form a number of physiologically active eicosanoids (Brash, 2001; Harizi et al., 2008). P450s metabolize arachidonic acid to hydroxylated and epoxygenated products which are known to have important physiological roles, primarily in blood pressure regulation and inflammation (Capdevila et al., 2007; Levick et al., 2007). In humans, the main arachidonic acid epoxygenases are P450s in subfamilies 2C and 2J, which produce the 5,6-, 8,9-, 11,12-, and 14,15- epoxyeicosatrienoic acids (EETs) (Daikh et al., 1994; Wu et al., 1996). The EETs are vasodilatory and exert anti-inflammatory actions upon the vascular endothelium (Liu et al., 2005; Spector, 2008). Decreases in the EET levels in the kidney and the vasculature have been associated with an increase in blood pressure and endothelial dysfunction (Imig, 2005). The EETs are inactivated by being converted to their dihydroxy derivatives by the enzyme soluble epoxide hydrolase (Yu et al., 2000). Epoxide hydrolase is a phase one enzyme that catalyzes the addition of water to epoxides, producing the corresponding dihydro diol product (Morisseau and Hammock, 2005). Two forms of this enzyme, the soluble and the microsomal epoxide hydrolase, catalyze hydrolysis of epoxides in humans (Morisseau and Hammock, 2005). Inhibition of soluble epoxide hydrolase leading to elevation of endogenous EET levels, and inhibitors are a potential novel treatment for renal and cardiovascular disorders (Chiamvimonvat et al., 2007).

Arachidonic acid can also be hydroxylated at the terminal carbons by P450s 4A11, 4F2 and 4F3. The omega alcohol, 20-hydroxyeicosatetraenoic acid (20-HETE) is

the major hydroxylated product formed in humans (Powell et al., 1998; Lasker et al., 2000; Fer et al., 2008). This eicosanoid is produced in a tissue-specific manner, and changes in its levels have been observed in ischemic cerebrovascular pathologies, cardiac ischemia-reperfusion injury, kidney dysfunction, hypertension, diabetes and pregnancy (Miyata and Roman, 2005; Mayer et al., 2006; Minuz et al., 2008). By acting in an opposing manner to that of the EETs, 20-HETE is a potent vasoconstrictor in the cerebral and renal microcirculation (Mayer et al., 2006). Inhibitors of 20-HETE synthesis have been developed and are being used to better understand the role of this eicosanoid, as well as to evaluate their potential clinical application in neuro- and cardioprotection (Sato et al., 2001; Chen et al., 2005; Kroetz and Xu, 2005; Nithipatikom et al., 2006).

Anandamide metabolism by rodent cytochrome P450 enzymes. Because of its structural similarity to arachidonic acid, anandamide has also been thought to be a candidate to undergo oxygenation by P450s. Bornheim and colleagues were the first to report on anandamide metabolism by mouse liver and brain microsomal P450s (Bornheim et al., 1995). In the presence of NADPH, mouse liver microsomes converted anandamide to approximately 20 products, whereas brain microsomes produced two metabolites. Classical inducers of several P450s were utilized in an effort to narrow down and identify the specific isoforms involved in the formation of the liver microsomal metabolites. Mice were pretreated with 3-methylcholanterene, phenobarbital, dexamethasone or clofibrate, chemical inducers thought to be somewhat specific for P450 1A, 2B, 3A and 4A isoforms, respectively. The most profound induction of anandamide metabolism was seen following incubations with liver microsomes from dexamethasone-treated mice. In particular, the formation of four of the metabolites increased

approximately 5 to 15 fold relative to untreated animals. Furthermore, pre-incubation of the microsomes with an antibody against P450 3A significantly diminished production of all four metabolites, suggesting they were specifically formed by P450 3A. Small increases in the formation of several oxygenated products of anandamide were seen with microsomal incubations from phenobarbital (1.5 to 2 fold) and 3-methylcholanterene (3 to 4 fold) treated mice, but the authors reported no changes in the metabolite profiles with microsomes from clofibrate-treated mice. Taken together, the data provided evidence that microsomal P450 3A and to a lesser extent 2B and 1A, but not 4A, contribute to anandamide metabolism in the mouse liver. Using liquid chromatography/mass spectrometry (LC/MS) analysis of the metabolites, it was determined that the brain microsomal metabolites were mono-oxygenated products and that P450 3A was involved in the formation of one of the two products, based on antibody inhibition experiments. The structures of all of the reported anandamide metabolites formed by the mouse microsomal P450s were not determined. The likelihood that all of the metabolites would be produced in vivo is not clear since the concentration of anandamide used in the microsomal incubation studies (720 μM) appears to be several orders of magnitude higher than its physiological levels. Although there are discrepancies over the reported physiological levels of anandamide, its tissue and plasma levels appear to be in the low nanomolar range (Monteleone et al., 2005; Piomelli et al., 2006), but the levels are likely higher inside the cell where it is being produced on demand. Nevertheless, the study by Bornheim et al. (1995) clearly demonstrated the possibility that P450s could play a role in the metabolism of anandamide.

For several years there were no further investigations into this topic until a study published by Costa et al. (2002) examining the effect of acute and chronic administration of anandamide to rats on the expression of P450 enzymes. They found that treatment of the rats with 20 mg/kg of anandamide, both acutely (one dose) and chronically (15 doses on consecutive days), caused a statistically significant induction in the expression of P450 3A and 2B isoforms in the rat liver microsomes. The total microsomal brain P450 content in acutely, but not chronically, treated rats also increased. It is possible for a P450 substrate to induce the expression of the particular isoform that is involved in its metabolism, especially in the case of the P450 3A enzymes (Zhou, 2008). Assuming that is the case with anandamide, the results of this study suggest that, similar to the mouse, P450 3A and 2B isoforms are likely to be involved in anandamide metabolism in the rat.

In light of the important roles of the P450 enzymes in the metabolism of arachidonic acid, as well as the abovementioned findings that demonstrated involvement of the rodent P450s in anandamide metabolism, **the central hypothesis of this thesis was that anandamide is metabolized by human P450s to form novel oxygenated metabolites which may be of physiological and/or pharmacological importance.** The hypothesis was investigated by the following aims:

Aim 1: Determine the metabolism of anandamide by P450 enzymes expressed in various human tissues and identify the specific P450 isoforms involved.

Aim 2: Elucidate the chemical structures of the metabolites, synthesize authentic standards, and investigate the kinetics of anandamide metabolism by the various P450 isoforms.

Aim 3: Investigate the potential physiological significance of the P450-derived anandamide metabolites, such as cannabimimetic activity and metabolic stability.

Anandamide metabolism by human cytochrome P450 enzymes. With regards to addressing aims 1 and 2 of the hypothesis, our laboratory has recently reported studies investigating the participation of human P450s in anandamide metabolism (Snider et al., 2007; Snider et al., 2008). By using synthetically prepared authentic standards, the anandamide metabolites were structurally characterized (Figure 1.3) and a LC/MS method for their separation and detection was developed (Snider et al., 2007). In addition, the specific P450 isoforms that participate in the formation of the various metabolites by human liver and kidney microsomal and brain microsomal and mitochondrial preparations were identified (Snider et al., 2007; Snider et al., 2008). While the metabolic reactions are essentially analogous to arachidonate metabolism, there are significant differences with respect to the identities of the specific P450 isoforms involved.

Kidney microsomal anandamide metabolism: involvement of P450 4F2. Human kidney microsomes convert anandamide to a single mono-oxygenated product, 20-HETE-ethanolamide (20-HETE-EA) (Snider et al., 2007). The formation of 20-HETE-EA is inhibited by pre-incubating the microsomes with N-hydroxy-N'-(4-n-butyl-2-methylphenyl) formamidine (HET0016), an inhibitor of arachidonic acid omega hydroxylase.

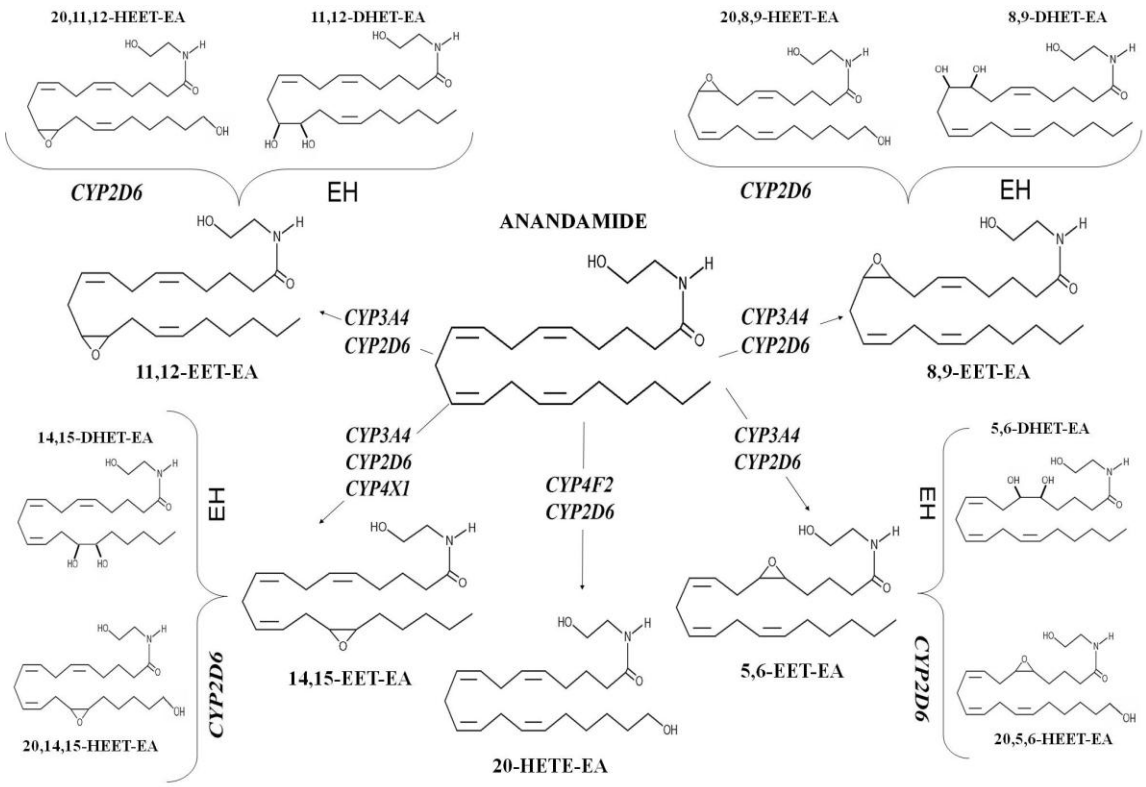


Figure 1.3. Metabolism of anandamide by human cytochrome P450 (CYP) enzymes. Anandamide can undergo epoxidation by CYPs 3A4, 2D6 and 4X1 to form the four EET-EAs. Hydroxylation of anandamide can be carried out by CYP4F2 or CYP2D6 to form 20-HETE-EA. The EET-EAs can be further metabolized by epoxide hydrolase to form the corresponding DHET-EAs or by CYP2D6 to form the corresponding HEET-EAs.

Microsomes from insect cells specifically expressing 4A11, 4F2 or 4F3b also formed 20-HETE-EA, although the significant difference in the ratios of the rates of formation among the three isoforms, which was 58:7:1 (4F2:4F3b:4A11), suggests that human kidney microsomal P450 4F2 is most likely the predominant isoform forming 20-HETE-EA (Snider et al., 2007). These data suggest that P450 4A11 most likely plays a relatively minor role in anandamide metabolism, which is in contrast to arachidonate metabolism where both P450 4A11 and 4F2 are major hydroxylases. However, an important factor to consider when determining the relative roles for the P450 4A11 and 4F2 enzymes in the metabolism of anandamide is the regulation of their expression levels in the various tissues and cells, which could dictate the extent of their involvement in the production of 20-HETE-EA. For example, it has been reported that retinoic acid is a potent suppressor of P450 4A11 and an inducer or P450 4F2 expression (Zhang et al., 2000; Antoun et al., 2006). Similar reciprocal regulation by PPAR agonists has also been reported for these two isoforms (Antoun et al., 2006; Siest et al., 2008). Comparison of the Km values between P450 4F2 (0.7 μM) and the other enzymes involved in anandamide metabolism, FAAH (2.4 μM), COX-2 (24 μM) and 12-LOX (6 μM), indicates that anandamide is likely to be a natural substrate for P450 4F2. The results also suggest that anandamide is a higher affinity substrate for P450 4F2 relative to arachidonic acid, which is metabolized by 4F2 to 20-HETE with a Km of 24 μM (Powell et al., 1998). It should be noted that Bornheim et al. (1995) found no change in anandamide metabolism upon pretreatment of the mice with clofibrate, an inducer of P450 4A, suggesting a species similarity between mouse and human with regards to the lack of 4A-mediated metabolism of anandamide. Whether there is a species similarity with regards

to the involvement of the P450 4F enzymes is difficult to assess, because there are a larger number of mouse 4F isoforms and they are differentially-regulated by clofibrate treatment (Cui et al., 2001).

Liver microsomal anandamide metabolism: involvement of P450s 4F2 and 3A4. Human liver microsomal metabolism of anandamide leads to the P450 4F2-mediated formation of 20-HETE-EA in addition to the formation of 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EET-EAs), which are products of metabolism by P450 3A4 (Snider et al., 2007). The major role of P450 3A4 in anandamide epoxidation is different from that observed for arachidonate metabolism where the main epoxygenases are P450s 2C8, 2C9 and 2J2. The levels of liver microsomal EET-EAs formed relative to 20-HETE-EA are significantly lower because they undergo secondary metabolism by epoxide hydrolase, which leads to the formation of the four dihydroxyeicosatrienoic acid ethanolamides (DHET-EAs). It is likely that the four anandamide metabolites that were produced at significantly higher levels in the dexamethasone-pretreated mice (Bornheim et al., 1995) were the four EET-EAs (or their corresponding DHET products). In that regard, we could speculate that the role of P450 3A in anandamide oxidation is similar between mouse and human. However, unlike mouse, the human P450 2B (2B6) does not appear to be involved in anandamide metabolism as evidenced by a lack of metabolic inhibition in the presence of a P450 2B6 antibody as well as by the absence of anandamide metabolism by recombinantly expressed 2B6 (unpublished data).

Brain microsomal and mitochondrial anandamide metabolism: involvement of P450s 2D6, 3A4, and 4F2. In recent years, the importance of P450 expression in the

brain has come to the forefront of research in this area because of evidence that metabolites produced by brain P450s (from both endo- and xenobiotics) may cause physiologically relevant responses (Liu et al., 2004; Meyer et al., 2007). However, performing in vitro metabolism reactions using human brain tissue preparations is difficult for several reasons, such as availability of tissue, the large quantities of protein needed for the reactions (due to the low levels of P450 expression), and the labile nature of brain microsomal proteins (Miksys and Tyndale, 2004).

Our lab recently reported on the metabolism of anandamide by human brain tissue microsomal and mitochondrial preparations (from non-diseased sections surrounding a brain tumor) which were obtained from 3 subjects (Snider et al., 2008). In all three samples, anandamide was converted primarily to 20-HETE-EA or to the four EET-EAs by the microsomal and the mitochondrial preparations, respectively. Based on the results of antibody and chemical inhibition studies, the P450 isoforms that participate in kidney and liver microsomal metabolism of anandamide (4F2 and 3A4) also appear to be the ones responsible for brain metabolism. Additionally, based on antibody inhibition experiments, mitochondrial P450 2D6 also appears to be involved in the epoxidation of anandamide in human brain. P450 2D6 is one of the major drug-metabolizing P450 isoforms expressed in neurons, and polymorphisms in 2D6 have been implicated in neurodegeneration, psychosis, schizophrenia, and personality traits (Dorado et al., 2007b), which have been proposed to be due, at least in part, to the involvement of 2D6 in the metabolism of endogenous substrates, such as several psychoactive tryptamines (Yu et al., 2004). However, since P450 2D6 is highly polymorphic, more studies utilizing a larger pool of brain samples are needed in order to confidently establish the extent of its

involvement in anandamide oxidation in the human brain and a possible physiological significance.

Anandamide metabolism by recombinant P450 2D6. The finding that P450 2D6 participates in anandamide metabolism is unexpected, since 2D6 does not metabolize arachidonic acid and its prototypical substrates (many cardiovascular and CNS - acting drugs) are structurally very different from anandamide. Recombinant P450 2D6 metabolizes anandamide to give 20-HETE-EA, the four EET-EAs, and several novel, di-oxygenated derivatives that are likely to be the hydroxylated metabolites of the EET-EAs (at the ω, ω-1, ω-2 and ω-3 positions). This result is similar to a previous report characterizing P450 4A-derived ω and ω-1 hydroxylated metabolites of arachidonate-derived EETs that were found to be high-affinity ligands for the peroxisome proliferator-activated receptor (Cowart et al., 2002). The proposed ω-hydroxylated product of each of the EET-EAs, the 20-hydroxy-epoxyeicosatrienoic acid ethanolamide (20-HEET-EA), appeared to be the predominant metabolite produced by 2D6 in each case (Figure 1.3). The identification of anandamide and the EET-EAs as substrates for 2D6 raises the possibility that this polymorphic enzyme may be involved in the metabolism of several other bioactive fatty acid amides (Farrell and Merkler, 2008) and eicosanoid-like molecules with the potential to yield novel, physiologically relevant metabolites.

Anandamide metabolism by recombinant "orphan" P450 4X1. Of the 57 human P450s, the functional significance of 25% of them is still unknown and they are refered to collectively as "orphan" P450s (Stark and Guengerich, 2007). Detection of mRNA for the orphan human P450 4X1 in several tissues was recently reported by Stark

et al. (2008). The highest levels of expression were found in the prostate, which were approximately 15 fold higher than the liver, and also the skin and the amygdala of the brain, both of which had approximately 30 fold higher levels of P450 4X1 mRNA than the liver (Stark et al., 2008). The catalytic activity of recombinantly expressed P450 4X1 towards anandamide was tested, revealing the formation of a single product, 14,15-EET-EA, with a Km of 65 μM and a catalytic rate of 65 pmol of product formed/min/nmol P450. The high levels of mRNA for 4X1 were detected in the amygdala, skin and prostate, suggesting that anandamide may be epoxygenated to 14,15-EET-EA by 4X1 in those tissues (Stark et al., 2008).

Physiological relevance of the P450-mediated oxidation of anandamide. There are at least three different possibilities regarding the biological significance of the oxidative pathways for the metabolism of anandamide (Kozak et al., 2004). The oxygenation of anandamide may represent an inactivation pathway leading to the production of metabolites with decreased cannabimimetic activity. An alternative to the inactivation pathway is that the oxygenated metabolites could possess enhanced activity at the cannabinoid receptor or enhanced metabolic stability compared to anandamide, and, in that regard, oxygenation could represent an activation pathway. Lastly, since the oxidative metabolism of anandamide by COX-2, LOX and P450 leads to the formation of a structurally diverse set of molecules, it is possible that some of these novel lipids may possess potent biological activities that are distinct from their precursor molecules.

In order to address aim 3 of the hypothesis, our laboratory has recently begun to investigate the relevance of the P450-mediated oxidation of anandamide, and we have determined that the anandamide epoxide 5,6-EET-EA is a potent, CB2 receptor -

selective agonist. Radioligand binding analysis revealed that 5,6-EET-EA binds to the human CB1 and CB2 receptors with Ki values of 3.2 μM and 8.9 nM, respectively. Ki values obtained from parallel studies using anandamide were 155 nM and 11.4 μM for CB1 and CB2, respectively. The 5,6-EET-EA metabolite is also able to functionally activate the CB2 receptor as evidenced by its ability to inhibit the forskolin-stimulated accumulation of cAMP in Chinese hamster ovary (CHO) cells expressing the CB2 receptor. The level of CB2-mediated cAMP inhibition by 5,6-EET-EA (IC_{50} = 9.8 nM) was found to be similar to that of the synthetic CB2-selective agonist (R)-3-(2-Iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1H-indole (AM1241) (Marriott and Huffman, 2008). Additionally, when incubated in mouse brain homogenate to estimate its biological stability, 5,6-EET-EA underwent epoxide-hydrolase mediated degradation with a half life of 32 min, whereas anandamide disappeared more rapidly with a half life of 14 min.

Taken together, the ability of 5,6-EET-EA to activate the CB2 receptor at low nanomolar concentrations and its increased biological stability suggest that P450-mediated epoxidation of anandamide to form 5,6-EET-EA represents a bioactivation pathway which may be most relevant in the context of immune cell function. Much more work remains to be done in order to fully understand the physiological and pharmacological significance of anandamide oxidation by the P450 enzymes. Additional *in vitro* and *in vivo* functional studies with 20-HETE-EA and the EET-EAs are needed to fully understand their relevance. Synthesis of authentic standards of the DHET-EAs and the HEET-EAs will be essential in determining their pharmacological activities and will also aid in determining if they are produced physiologically.

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Chapter II

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

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 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 if 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 (20-HETE-EA). Human liver microsomal incubations with anandamide also produced 20-HETE-EA in addition to 5,6-, 8,9-, 11-12, and 14,15-EET ethanolamide (EET-EA). The EET-EAs produced by the liver microsomal P450s were converted to their corresponding dihydroxy derivatives by microsomal epoxide hydrolase (mEH). P450 4F2 was identified as the isoform that is most likely responsible for the formation of 20-HETE-EA in both human kidney and

human liver, with an apparent K_m of 0.7 μM. The apparent K_m values of the human liver microsomes for the formation of the EET-EAs were between 2 and 5 μM, 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.

Introduction

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 cyclooxygenases (COX), lipoxygenases

(LOX) and cytochromes P450 (P450). 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 investigated in detail. P450s are microsomal heme-containing monooxygenases 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) that are potent mediators of signaling 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 previously been shown 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 paper describes the metabolism of anandamide by human liver and kidney microsomes and the formation of EET- and HETE- ethanolamides (EET-EAs and HETE-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 ethanolamides (DHET-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).

P450 enzymes. The P450 4F2, 4F3b and 4A11 supersomes were purchased from BD Biosciences (San Jose, CA). 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 KPO_4 buffer (pH 7.4), anandamide (20 or 100 μM), 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 μg 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 μg mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine (1:1:1), 50 U catalase, and 50 mM HEPES in a final reaction volume of 0.5 mL. For the reaction mixtures that contained microsomal epoxide hydrolase (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 ºC for 45 min. The reactions were terminated by the addition of 2 mL of nitrogen-purged ethyl acetate and the samples were vortexed for 1-2 min. The samples were then centrifuged for 5-10 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 re-suspended in 200 μL methanol and 10 μL fractions were subjected to electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS) analysis as described below.

For the inhibition studies using chemical or antibody inhibitors, the microsomal protein mixtures were pre-incubated with either the inhibitory monoclonal antibody to the specified P450 or HET0016 (10 pM-1 μM), a highly selective inhibitor of arachidonic acid omega hydroxylation for 5 min prior to 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 the 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 μg of human kidney microsomal protein was incubated for 8 min and 25 μg 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. Following extraction and drying of the extracts, the samples were re-suspended in 100 μL of methanol and 10 μL 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.

Electrospray ionization - liquid chromatography-mass spectrometry (ESI-LC-MS) Analysis. Samples (10 μL of each) were injected onto a Hypersil ODS column (5 micron, 4.6 X 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: 0-5 min, 75% B; 5-20 min, 75-100% B; 20-25 min, 100 % B; 25-26 min, 100-75% B; and 26-30 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 *m/z* followed by one data dependent scan of the most intense ion.

Chemistry. 5,6-Epoxyeicosatrienoic acid (5,6-EET) was synthesized by iodolactonization of arachidonic acid, as described previously (Corey et al., 1980). 14,15-Epoxyeicosatrienoic acic (14,15-EET) was prepared using an arachidonic acid selective epoxidation (Corey et al., 1979). 20-Hydroxyeicosatetraenoic acid (20-HETE) was prepared from 14,15-EET with a known 3-step procedure using the Wittig reaction as previously described (Manna et al., 1983). All three compounds were transformed into the corresponding ethanolamides using a standard procedure involving the intermediate NHS/DCC activation of the carboxylic moiety followed by the reaction with ethanolamine (Sehgal and Vijay, 1994). All of the products were purified by HPLC and characterized by NMR and mass spectrometry.

Data analysis. Non-linear regression analysis of the data was performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com)

Reagents – Anandamide, arachidonic acid and HET0016 were purchased from Cayman Chemical (Ann Arbor, MI). Microsomal epoxide hydrolase (mEH), catalase, NADPH, L-α-dilauroyl-phosphatidylcholine, L-α-dioleyl-*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 (NIH). All other chemicals were of highest quality and available from commercial sources.

Results

Metabolism of anandamide by human kidney microsomes (HKMs) and P450s 4F2, 4F3b and 4A11. Figure 2.1A shows the selected ion chromatogram from the metabolism of anandamide by human kidney microsomes. Anandamide formed a positive ion in the mass spectrometer with a mass to charge ratio (*m/z*) of 348 and eluted at 23 minutes under the conditions used here. A single mono-oxygenated metabolite with a *m/z* value of 364 and a retention time of approximately 15 min, labeled M1, was observed (Figure 2.1A).

Next, the identity of the P450 that is primarily responsible for the production of M1 in the human kidney 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). In order to investigate the identity of the P450 responsible for the formation of M1, HKMs were pre-incubated with antibodies previously described (Gelboin and Krausz, 2006) 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. Pre-incubation 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.

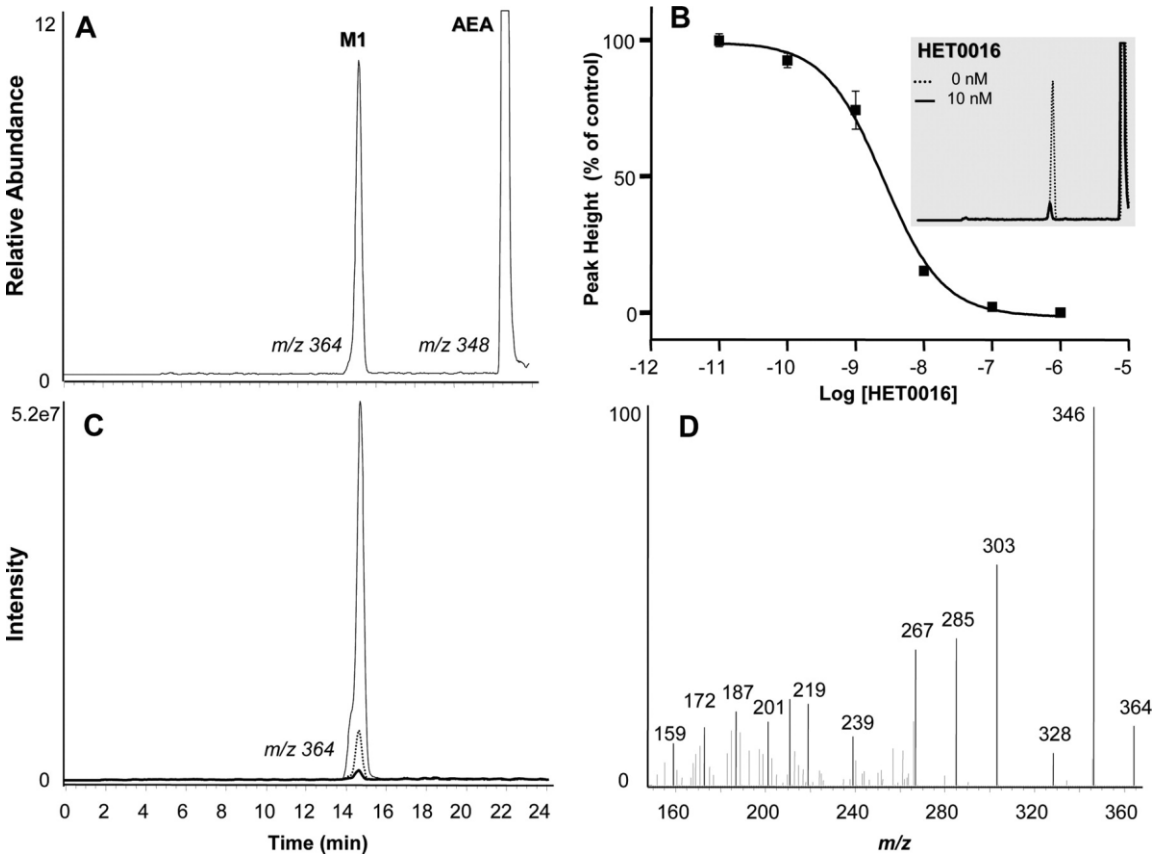

Figure 2.1. Metabolism of anandamide by human kidney microsomes and supersomes containing P450s 4F2, 4F3b, or 4A11. Anandamide (20 µM) was incubated with human kidney microsomes (100 µg) in the presence of NADPH for 45 min, and the samples were analyzed as described under *Materials and Methods*. The 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 µM 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).

Because inhibitory antibodies specific for human P450s 4A and 4F were unavailable, the highly selective arachidonic acid omega hydroxylase inhibitor, HET0016 (N-hydroxy-N'-(4-butyl-2-methylphenyl)-formamidine) 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 Figure 2.1B (inset), pre-incubation of the HKMs with 10 nM HET0016 (solid line) for 5 min prior to the addition of anandamide and NADPH resulted in a greater than 90% reduction in the formation of M1 compared to control (dotted line). Furthermore, as shown in Figure 2.1B, 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. In fact, the kidney levels of P450 4F2 and 4A11 are similar to the levels found in human liver (Nishimura et al., 2003).

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 Figure 2.1C, M1 formation differed significantly among the three enzymes tested when using equal protein amounts, substrate concentration and incubation conditions, as described in the Methods section. The ratios of formation of M1, as determined by the corresponding peak heights, were approximately 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 omega 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 (Figure 2.1D).

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 likely catalyzed by P450 4F2.

Metabolism of anandamide by human liver microsomes (HLMs). In addition to forming product M1, HLM incubations led to the formation of several minor monooxygenated (*m/z* 364) metabolites eluting between 16 and 21 minutes (M2-M6), as shown in Figure 2.2A. 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 minutes and having *m/z* values of 382 (Figure 2.2B). The mass to charge ratio of these products corresponded to that of one monooxygenated metabolite (*m/z* 364) plus a water molecule (M+18). Therefore, the possibility that metabolites M7-M10 may represent secondary products of the monooxygenated metabolites was considered because, in addition to P450s, the HLMs also contain other phase I and II metabolizing enzymes, including microsomal epoxide hydrolase (mEH), which catalyzes

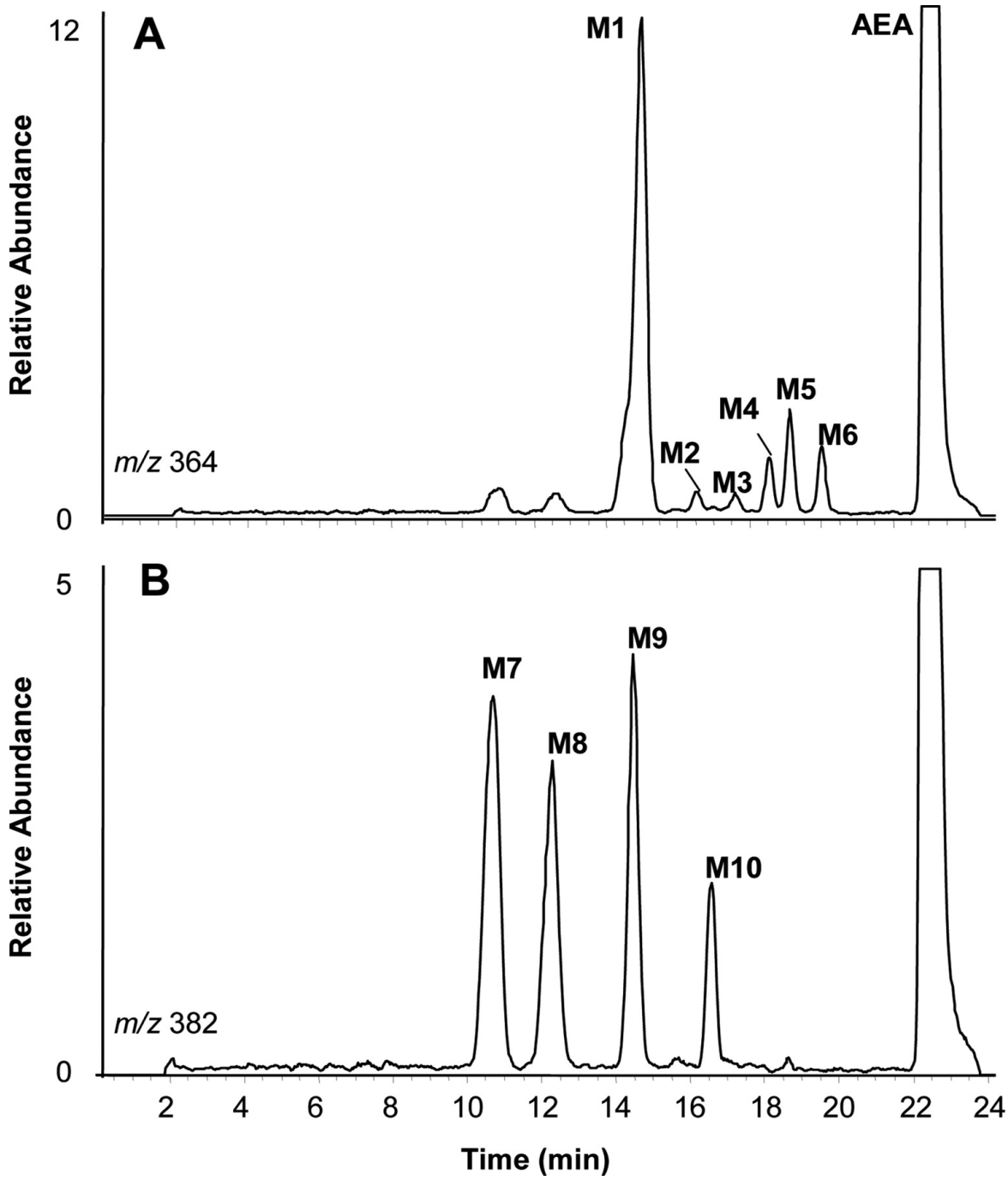

Figure 2.2. Metabolism of anandamide by human liver microsomes. Human liver microsomes (100 µg) were incubated in the presence of 100 µM anandamide and NADPH for 45 min. The selected ion chromatogram (A) shows several mono-oxygenated products (M1-M6, *m*/*z* 364) and the anandamide ion (AEA; *m*/*z* 348). Four additional products (M7-M10) were detected from the same incubation having *m*/*z* values of 382 (B).

the addition of water to epoxides (Morisseau and Hammock, 2005). Because exactly four possible secondary products were seen and since there are four possible sites for epoxidation on the anandamide structure, these data suggest the possibility that some of the minor monooxygenated 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 Figure 2.3A, pre-incubation of the HLMs with the P450 3A4 antibody significantly inhibited the formation of metabolites M7-M10.

The level of inhibition shown in Figure 2.3A 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 5-16% for the 6 HLMs tested (data not shown).

Anandamide was also incubated with recombinant P450 3A4 to determine how the metabolite profile for the single enzyme compared to those seen with the products obtained from the liver microsomes. Figure 2.3B (solid line) shows that P450 3A4 converted anandamide to four monooxygenated products (*m/z* 364). These products have identical retention times as the metabolites M3-M6 that were produced by the liver microsomes.

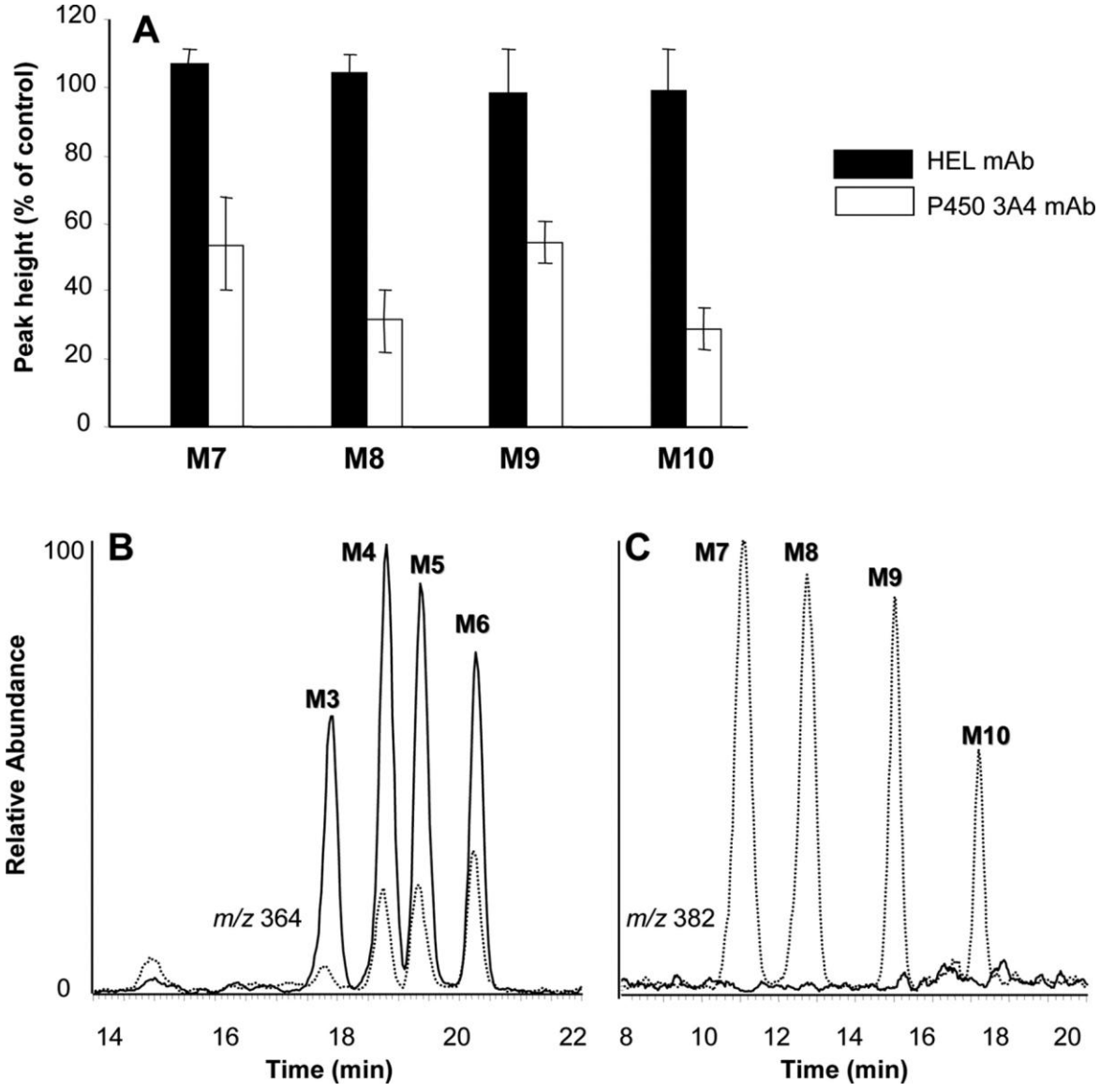

Figure 2.3. Involvement of P450 3A4 in the metabolism of anandamide by human liver microsomes and the effect of microsomal epoxide hydrolase. Human liver microsomes were preincubated in the presence of control antibody [hen egg lysozyme (HEL)] or P450 3A4 inhibitory antibody (A). The peak heights for 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 μM) 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.

To determine whether these metabolites were indeed epoxides, P450 3A4 was reconstituted together with human recombinant mEH prior to incubation with anandamide and NADPH. Figures 2.3 B and C (dotted lines) depict 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 (Figure 2.2 A and B). A significant decrease in the formation of M3-M6 (*m/z* 364) was seen while at the same time the appearance of four new products that correspond to M7-M10 (*m/z* 382) was observed. This experiment confirms that products M3-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 (Figure 2.3A) 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 if 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 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-hydroxyeicosatetraenoic acid ethanolamide (20-HETE-EA), 14,15-epoxyeicosatrienoic acid ethanolamide (14,15-EET-EA) and 5,6- epoxyeicosatrienoic acid ethanolamide (5,6-EET-EA) were synthesized for structural confirmation, as described in the Materials and Methods section.

Data from the ESI-LC-MS analysis of the chemical standards are shown in Figure 2.4 (A, C, 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 Figure 2.4 (B, D, 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 likely correspond 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).

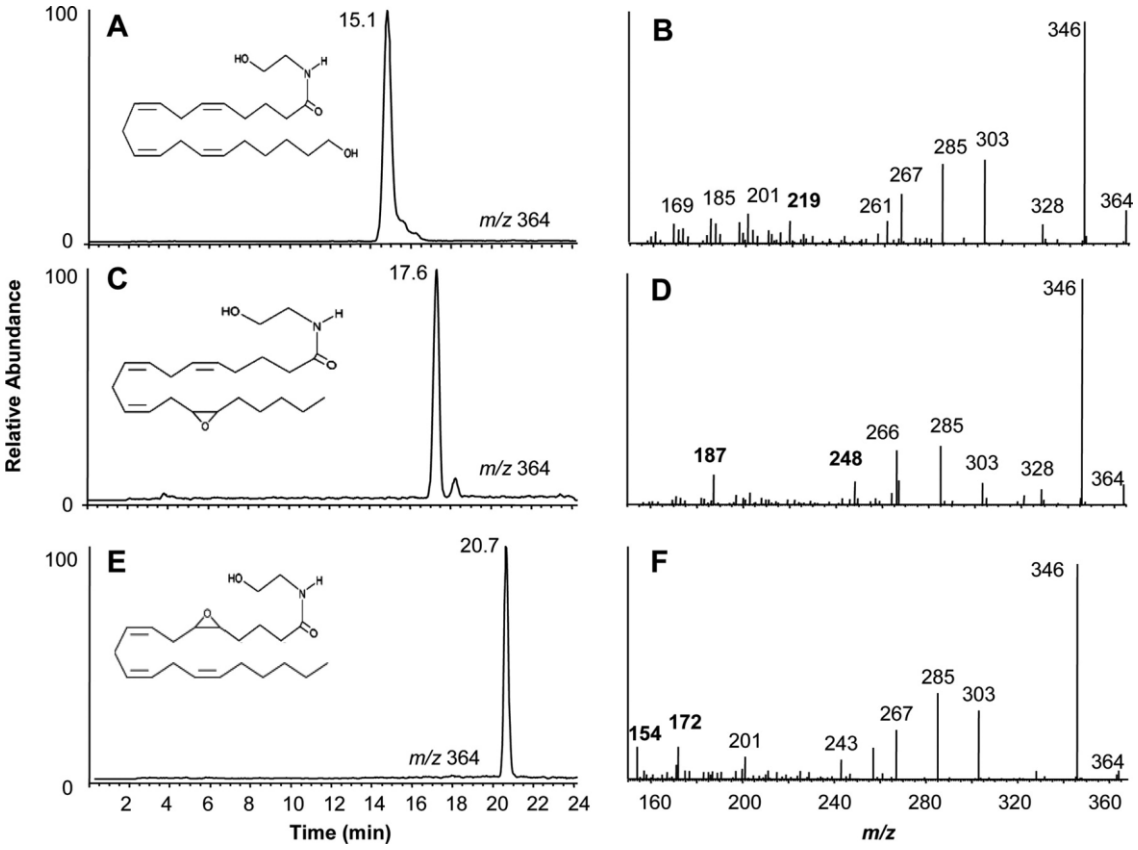

Figure 2.4. Structural confirmation of 20-HETE-EA, 14, 15-EET-EA, and 5,6-EET-EA. ESI-LC/MS/MS analyses of 50 pmol of the authentic metabolite standards are shown in A to F. Selected ion chromatograms (*m*/*z* 364) of the three standards (A, C, and E) and their corresponding MS/MS spectra (B, D, and F) are shown.

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 which was linear for at least 12 min of reaction time at 37 °C and 75 μg of kidney microsomal protein, and 15 min and 25 μg 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 μM), anandamide metabolism to 20-HETE-EA exhibited simple Michaelis-Menten kinetics (Fig. 2.5A, open circles) which 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 μM and a V_{max} of 122 ± 7.4 pmol of 20-HETE-EA formed/min/mg of kidney microsomal protein. Similar kinetics were observed with the HLM incubations (Fig. 2.5A, filled circles), for which an apparent K_m of 2.4 ± 0.6 μM and a V_{max} of 266 ± 26 pmol 20-HETE-EA formed/min/mg of 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 to kidney (Nishimura et al. 2003). The kinetics of 20-HETE-EA formation by P450 4F2 supersomes are shown in Figure 2.5B. The V_{max} for omega hydroxylation of anandamide by P450 4F2 was 11.5 ± 0.4 pmol 20-HETE-EA/min/pmol P450 4F2 protein and the apparent K_m was 0.7 ± 0.07 μM. In comparison, P450 4F3b supersomes exhibited an apparent K_m of 3.4 ± 0.79 μM and a V_{max} of 0.1 ± 0.01 pmol 20-HETE-EA/min/pmol P450 4F3b protein (data not shown).

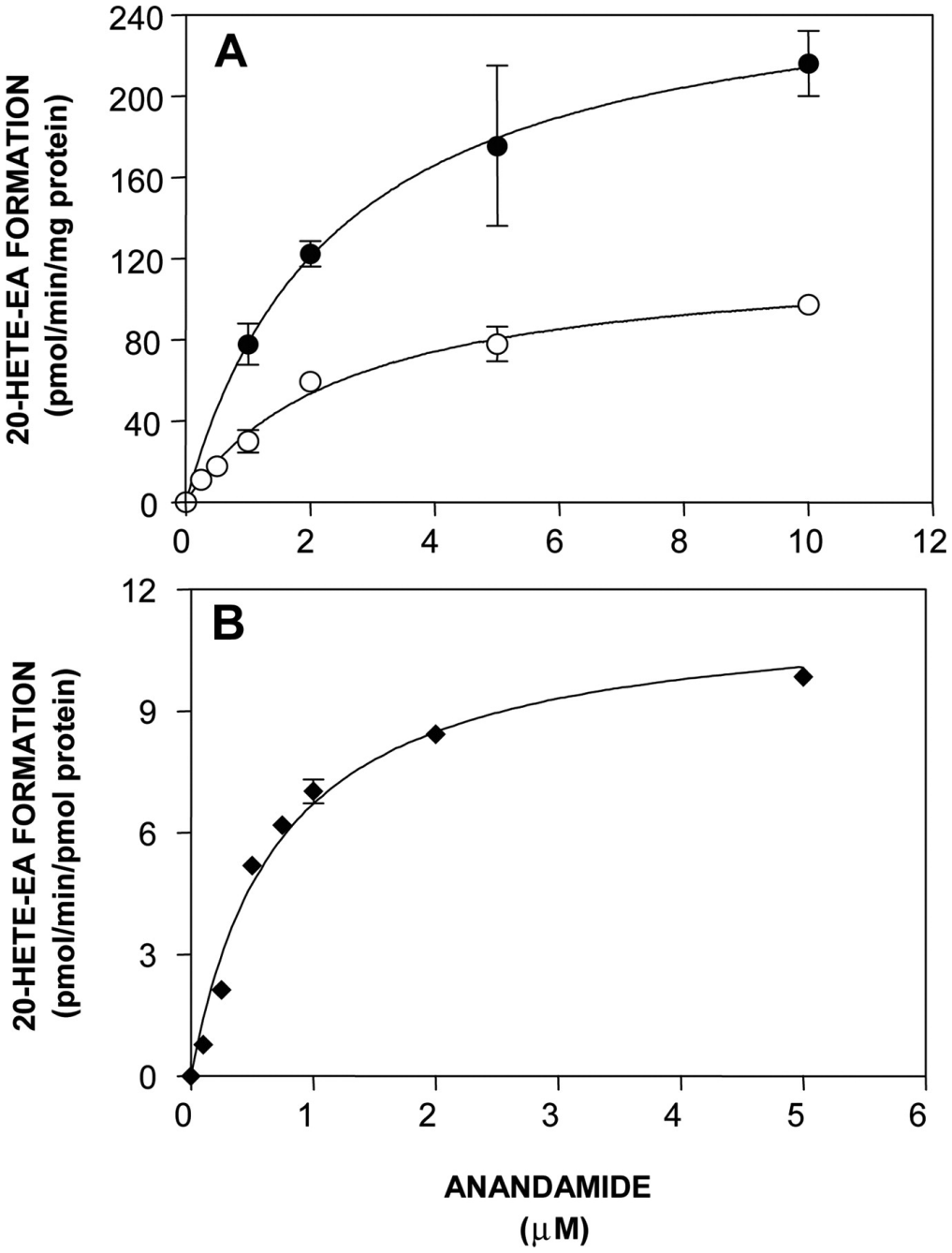

Figure 2.5. Kinetics of 20-HETE-EA formation by human kidney and liver microsomes and P450 4F2 supersomes. Seventy-five micrograms of human kidney microsomes (○) or 25 μg of human liver microsomes (●) were incubated with various concentrations of anandamide (0.1–10 μM) for 8 and 15 min, respectively (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.

These data suggest that anandamide is a high affinity substrate for P450 4F2 and that this P450 may be the one which 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 which was linear for all products for 15 min of reaction time at 37 °C and 25 μg of microsomal protein (data not shown). Pooled liver microsomes were used for the kinetic analysis of hepatic EET-EA formation from anandamide at concentrations ranging from 1-25 μM. Non-linear regression analysis of the data was used to derive apparent K_m values ranging from 4 to 5 μM (Table 2.1).

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.

These data suggest that anandamide is a relatively high-affinity substrate for and undergoes significant turnover by human liver microsomal P450s *in vitro* to produce EET ethanolamides.

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

TABLE 2.1. 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*.

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 the potential treatment of 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 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 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 omega hydroxylase activity when compared to 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.

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 omega-hydroxylase activity (Jin et al., 1998).

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 previously been reported 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 (EDHFs) 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 which 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 *Km* 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 *Km* values of 2.3 μM 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 *Km* values of 4-5 μM in the human liver microsomes. These are similar to the *Km* values published for arachidonic acid-derived EETs of 9-11 μM with human liver microsomes (Rifkind et al., 1995). Although under normal conditions the tissue concentration of non-esterified 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. Also, the *Km* 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 DHET-EA anandamide metabolites which 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 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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Chapter III

The Endocannabinoid Anandamide is a Substrate for the Human Polymorphic Cytochrome P450 2D6

Abstract

Members of the cytochrome P450 (CYP) family of drug metabolizing enzymes are present in the human brain and they may have important roles in the oxidation of endogenous substrates. The polymorphic CYP2D6 is one of the major brain CYP isoforms and has been implicated in neurodegeneration, psychosis, schizophrenia, and personality traits. The objective of this study was to determine if the endocannabinoid arachidonoylethanolamide (anandamide) is a substrate for CYP2D6. Anandamide is the endogenous ligand to the cannabinoid receptor CB1, which is also activated by the main psychoactive component in marijuana. Signaling via the CB1 receptor alters sensory and motor function, cognition and emotion. Recombinant CYP2D6 converted anandamide to 20-hydroxyeicosatetraenoic acid ethanolamide (20-HETE-EA) and 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EET-EAs) with low K_m values. CYP2D6 further metabolized the epoxides of anandamide to form novel di-oxygenated derivatives. Human brain microsomal and mitochondrial preparations metabolized anandamide to form hydroxylated and epoxygenated products, respectively. An inhibitory antibody against CYP2D6 significantly decreased the mitochondrial formation of the EET-EAs. To our knowledge, anandamide and its epoxides are the first eicosanoid molecules to be

identified as CYP2D6 substrates. Our study suggests that anandamide may be a physiological substrate for brain mitochondrial CYP2D6, implicating this polymorphic enzyme as a potential component of the endocannabinoid system in the brain. This study also offers support to the hypothesis that neuropsychatric phenotype differences among individuals with genetic variations in CYP2D6 could be ascribable to interactions of this enzyme with endogenous substrates.

Introduction

The endogenous cannabinoids (endocannabinoids) anandamide and 2-arachidonoylglycerol (2-AG) activate the two known G_i-protein coupled cannabinoid receptors CB1 and CB2, which are predominantly expressed on neurons and immune cells, respectively (Pacher et al., 2006). The cannabinoid receptors, the endocannabinoids and enzymes involved in their synthesis and degradation, as well as the associated signaling pathways are collectively known as the endocannabinoid system. The various components of this system represent novel and important pharmacological targets in the treatment of many disorders, including neurodegeneration, chronic pain, inflammation, cancer and others (Di Marzo et al., 2004). Inhibition of the enzyme fatty acid amide hydrolase (FAAH), which terminates the cannabimimetic activity of anandamide, represents one potential intervention strategy in the management of pain and neuropsychatric disorders (Cravatt and Lichtman, 2003). Because of the importance of the endocannabinoid system, it is critical to identify other enzymes that may be able to affect the anandamide tone in vivo.

We previously reported the metabolism of anandamide by human hepatic and renal CYPs, in particular the involvement of CYPs 3A4 and 4F2, which exhibit anandamide epoxygenase and hydroxylase activity, respectively (Snider et al., 2007). However, because anandamide is synthesized by neurons in the brain in an activity-dependent mechanism, it is necessary to also determine if it is a substrate for the CYP enzymes known to be present in the brain. Of particular interest is CYP2D6, which has been implicated in a number of neurological and neuropsychatric conditions due to a proposed interaction with endogenous substrates (Llerena et al., 1993; Yu et al., 2003a; Yu et al., 2003b; Gervasini et al., 2004; Yu et al., 2004).

CYP2D6 is a phase I xenobiotic metabolizing enzyme that is known to be involved in the oxidation of 20-30% of the most commonly prescribed drugs, many of which modulate cardiovascular and central nervous system (CNS) function (Ingelman-Sundberg, 2005). This enzyme is highly polymorphic with more than 80 variant alleles having been identified to date, including the non-functional CYP2D6*4 null allele, which is present in 12-21% of Caucasians (Ingelman-Sundberg, 2005). In addition to hepatic expression, 2D6 is one of the major CYP isoforms in human brain, having been demonstrated to be present in neurons using a variety of techniques, such as immunoblotting, in situ hybridization, reverse transcription-polymerase chain reaction, and metabolism of CYP2D6-specific probe substrates (Bhagwat et al., 2000; Siegle et al., 2001; Miksys et al., 2002). Aside from being involved in the metabolic disposition of xenobiotics, a role for CYP2D6 in the metabolism of endogenous substrates has also been demonstrated including its involvement in a critical step of the serotonin-melatonin cycle where it catalyzes the O-demethylation of 5-methoxytryptamine to form 5-

hydroxytryptamine (Yu et al., 2003a). However, more work remains to be done in identifying other endogenous substrates of this important enzyme to fully understand the physiological significance of these metabolic pathways. The work presented here demonstrates that the endocannabinoid anandamide is metabolized by CYP2D6, resulting in the formation of multiple mono- and di-oxygenated products.

Materials and Methods

Reagents. Arachidonic acid, anandamide and anandamide metabolite standards and the CYP 4A/4F chemical inhibitor HET0016 were purchased from Cayman Chemical (Ann Arbor, MI). Catalase, superoxide dismutase, NADPH, L-α-dilauroyl-phosphatidylcholine, L-α-dioleyl-*sn*-glycero-3-phosphatidylcholine, and L-α-phosphatidylserine were purchased from Sigma-Aldrich (St. Louis, MO). CYP4F2 supersomes were purchased from BD Biosciences (San Jose, CA). Monoclonal inhibitory antibodies to several CYPs, including CYP2D6 [MAb 50-1-3 (Gelboin and Krausz, 2006)] 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.

Protein purification. NADPH-cytochrome P450 reductase and cytochrome b5 were purified as described previously (Hanna et al., 1998). CYP2D6 protein was expressed and purified according to a previously published procedure (Hanna et al., 2001).

Human Brain Tissue. Fresh human neocortical tissue was obtained from three patients during surgical treatment of brain tumors. Before the operation each patient signed a declaration of consent as requested by the local Ethics Committee (Steffens et al.,2005). After removal, the tissue specimens were immediately placed in ice-cold saline and further processed within 10-15 min. The white matter was separated (and discarded) from the grey matter, which contained all six neocortical layers after preparation. Tissue macroscopically infiltrated with tumor was excluded. The regions of the human neocortical tissue included either frontal or temporal areas. Following separation, the human tissues were frozen at -80°C until used to prepare microsomes and mitochondria.

Preparation of human brain microsomes and mitochondria and immunoblotting for cytochrome c. Subcellular fractions from frozen human neocortical tissue (0.8-1.2 g) sections of three subjects were prepared by homogenization followed by differential centrifugation according to a published procedure by Voirol et al. (Voirol et al., 2000). For the immunoblot experiment, 40 μg of protein from each sample was resolved on a 15% SDS-PAGE gel, and blotted onto a PVDF membrane. The membrane was blocked overnight in blocking buffer (20mM Tris, 140mM NaCl, 0.1% Tween 20, 5% milk). Cytochrome c was detected using a monoclonal antibody commercially available from Abcam (Cambridge, MA).

CYP2D6 genotype analysis. CYP2D6 genotype was determined as described previously (Petersdorf and Deeg, 1992). Crude cell lysates were genotyped for CYP2D6*3, *4, and *6 alleles using the Applied Biosystems' Taqman Allelic Discrimination Assay (Foster City, CA) according to the manufacturer's instructions, with minor modifications. Briefly, 1μL of cell lysate was added to a 25μL reaction

mixture containing PCR master mix (Applied Biosystems, Foster City, CA), forward and reverse primers and allele specific probes. Samples were analyzed by using a Bio-Rad Thermo cycler (Hercules, CA). To control for the effects of the lysis buffer on PCR efficiency and probe fluorescence, samples were added to control DNA with known genotypes, and were found not to interfere with the genotyping assays.

Anandamide Metabolism Assays. CYP2D6 protein was reconstituted with reductase (1:2 ratio), a 10-µg mixture of L-α–dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine (1:1:1) and 500 U of catalase for 45 minutes on ice. Cytochrome b5 and superoxide dismutase were also included in the reaction mixture in some experiments (as described in the legend to Figure 3.1C). The metabolism of anandamide or the EET-EAs was assessed in incubation mixtures (0.5 mL) containing 100 mM KPO_4 buffer, pH 7.4, anandamide (0.25-10 µM, as specified in the legends to the figures) and one of the following enzyme sources: reconstituted CYP2D6 (5 or 25 pmol, specified in the legends to the figures), CYP4F2 supersomes (25 pmol), or human brain microsomal or mitochondrial protein (100 µg). All reactions were initiated by the addition of 1 mM NADPH and allowed to proceed for 10 minutes at 37°C, unless specified otherwise in the legends to the figures. Control reactions in the absence of either NADPH or protein were routinely performed. 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 under a constant stream of nitrogen gas. The dried samples were resuspended in 100 µl of methanol and 10-µl fractions were subjected to electrospray ionization (ESI)-liquid chromatography (LC)/mass spectrometry (MS)

analysis as described below. For the antibody inhibition studies using the microsomal or mitochondrial proteins, the reaction mixtures were pre-incubated with either the inhibitory monoclonal antibody to CYP2D6 or a non-immunogenic control antibody (hen egg lysozyme) for 5 min prior to the addition of anandamide and NADPH. Standard curves for the various metabolites used for the determination of the K_m and V_{max} values were generated by extracting various known amounts of the authentic standards from a 0.5-ml reaction mixture that did not contain anandamide and NADPH, followed by analysis by ESI-LC/MS.

ESI-LC/MS Analysis. Samples (10 µL of each) were injected onto a Hypersil ODS column (5 µm, 4.6 x 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 for anandamide and negative ion mode for arachidonic acid using the Xcalibur software package (Thermo Electron Corporation, Waltham, MA) 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.

Data Analysis. Nonlinear regression analysis of the data was performed using GraphPad Prism version 5.00 for Windows (Graph-Pad Software, San Diego, CA; http://www.graphpad.com).

Results

Metabolism of arachidonic acid and anandamide by human recombinant CYP2D6. Arachidonic acid is oxidized predominantly by the human CYP4A/4F and CYP2C enzymes to yield the physiologically active HETE and EET products, respectively (Capdevila and Falck, 2001). Not surprisingly, this fatty acid has not been shown to be a substrate for CYP2D6, which generally metabolizes molecules that contain a protonated nitrogen and a planar aromatic ring (Rowland et al., 2006). As shown in Figure 3.1A, arachidonic acid (AA) was not metabolized by human recombinant CYP2D6, as evidenced by the lack of product peaks in the total ion chromatogram (TIC). Similar results were obtained when using concentrations of AA between 1μM-250μM. To determine whether the presence of the ethanolamine group at the C1 position affects the substrate specificity of CYP2D6, the enzyme was also incubated with anandamide. Interestingly, and in contrast to arachidonic acid, anandamide is a substrate for the enzyme and it is extensively metabolized to give a number of products (Figure 3.1B). In addition to forming the hydroxylated and epoxygenated derivatives, the 20-HETE and 14,15-, 11,12-, 8,9 and 5,6-EET ethanolamides with mass to charge ratios (*m/z*) of 364, CYP2D6 also formed several di-oxygenated products with mass to charge ratios (*m/z*) of 380. These data demonstrate that anandamide is a substrate for CYP2D6 and that the CYP2D6 active site can accommodate an eicosanoid-like structure.

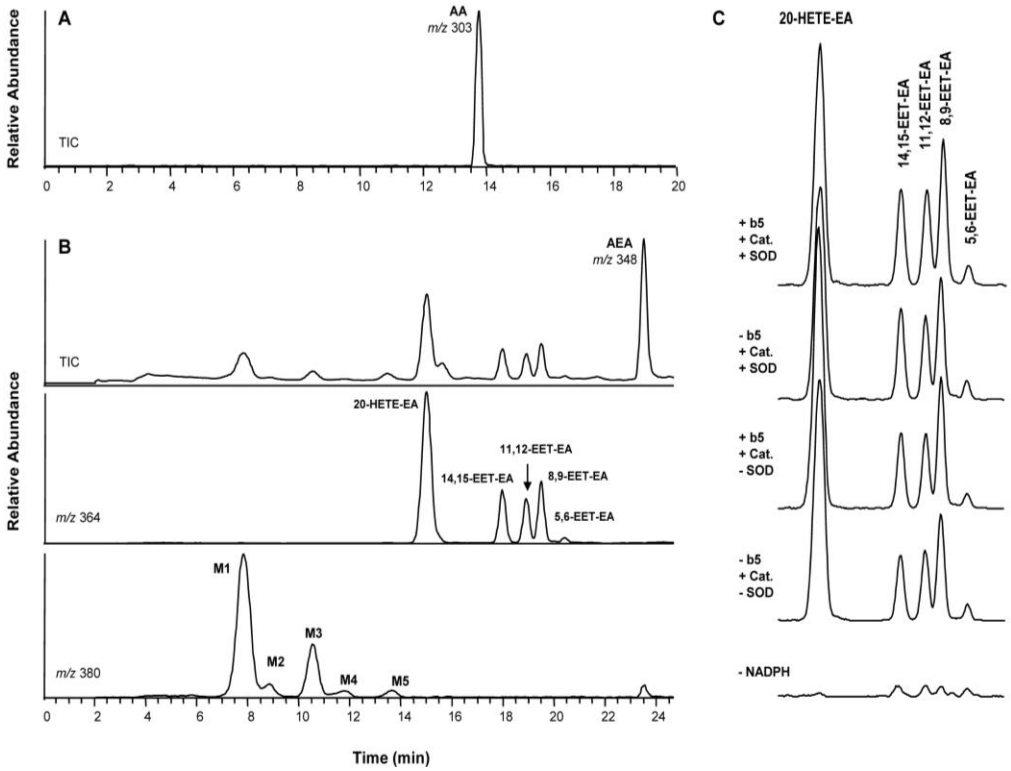

Figure 3.1. Metabolism of arachidonic acid and anandamide by human recombinant CYP2D6. Recombinant CYP2D6 protein (25 pmol) was incubated in the reconstituted system in the presence of arachidonic acid (10 µM) or anandamide (10 µM) for 10 min, and the samples were analyzed as described under *Materials and Methods*. The TIC in A shows the AA peak (*m*/*z*, 303) and the absence of any metabolic products. The TIC in B shows the anandamide (AEA) peak (*m*/*z*, 348) and the presence of several products. The selected ion chromatograms of the mono-oxygenated (*m*/*z*, 364) and dioxygenated (*m*/*z*, 380) M1 to M5 anandamide products are shown in the bottom two panels. C, representative chromatograms for the formation of 20-HETE-EA and the EET-EAs by CYP2D6 in the absence of NADPH or in the absence or presence of cytochrome-b_5 (1:1 M ratio with CYP2D6) or superoxide dismutase (SOD).

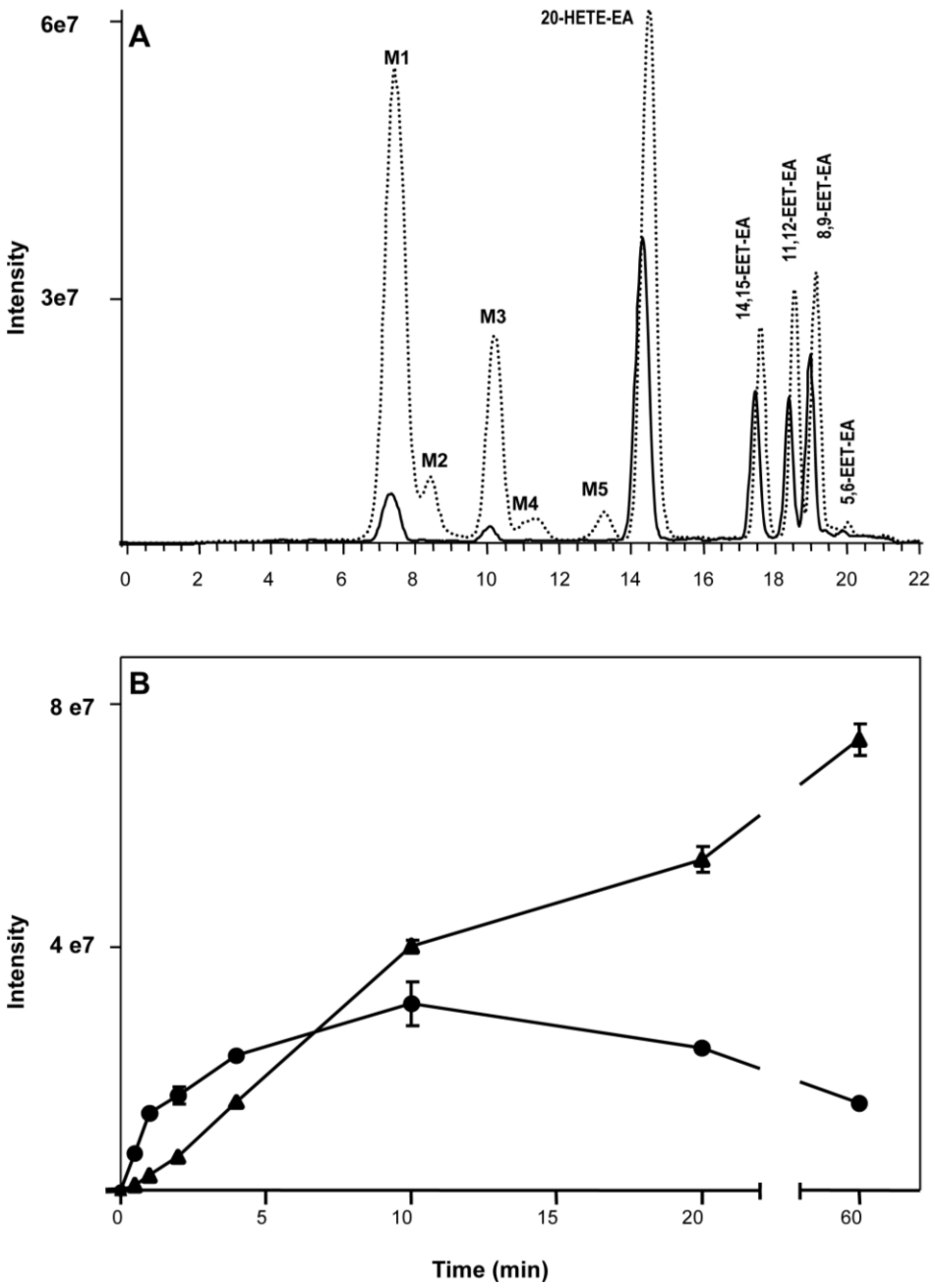

Figure 3.2. Time course for the formation of metabolites of anandamide by CYP2D6. Anandamide (20 μM) was incubated with 10 pmol CYP2D6 in the reconstituted system, and the reactions were terminated at the times indicated and analyzed as described under *Materials and Methods*. The selected ion chromatograms (*m*/*z*, 364, 380) in A depict the metabolic profiles after 2 (solid line) and 20 (dotted line) min of reaction time. B, peak intensities for 14,15-EET-EA (●) and product M1 (▲) observed for incubations carried out for the time periods indicated.

To investigate that further, the peak intensities for 14,15-EET-EA obtained from incubations which were terminated after various times of up to 60 minutes were compared to the peak intensities of M1 from those same incubations. This pair was chosen for the analysis as a potential precursor-product pair due to their order of elution from the column. As can be seen in Figure 3.2B, the peak intensity of product M1 continued to increase over the 60 minutes whereas the peak intensity of 14,15-EET-EA reached a maximum at approximately 10 minutes and continuously decreased thereafter. Similar results were obtained when the formation of the other EET-EAs was compared to the formation of the di-oxygenated metabolites over time (data not shown). These data show that CYP2D6 not only metabolizes anandamide, but is also able to metabolize the EET-EAs to give novel, di-oxygenated products. The precursor-product relationship was also confirmed in experiments where CYP2D6 was incubated with each individual EET-EA (Figure 3.3). As can be seen from in the four top panels, multiple oxygenated products resulted from the incubation of an individual EET-EA with CYP2D6. These products most likely result from the hydroxylation of the EET-EAs at positions C16-C20 since incubation of 5,6-EET-EA or 14,15-EET-EA with the anandamide ω-hydroxylase CYP4F2 resulted in the formation of metabolites with identical retention times to some of those observed in the presence of CYP2D6 (Figure 3.3, bottom panel).

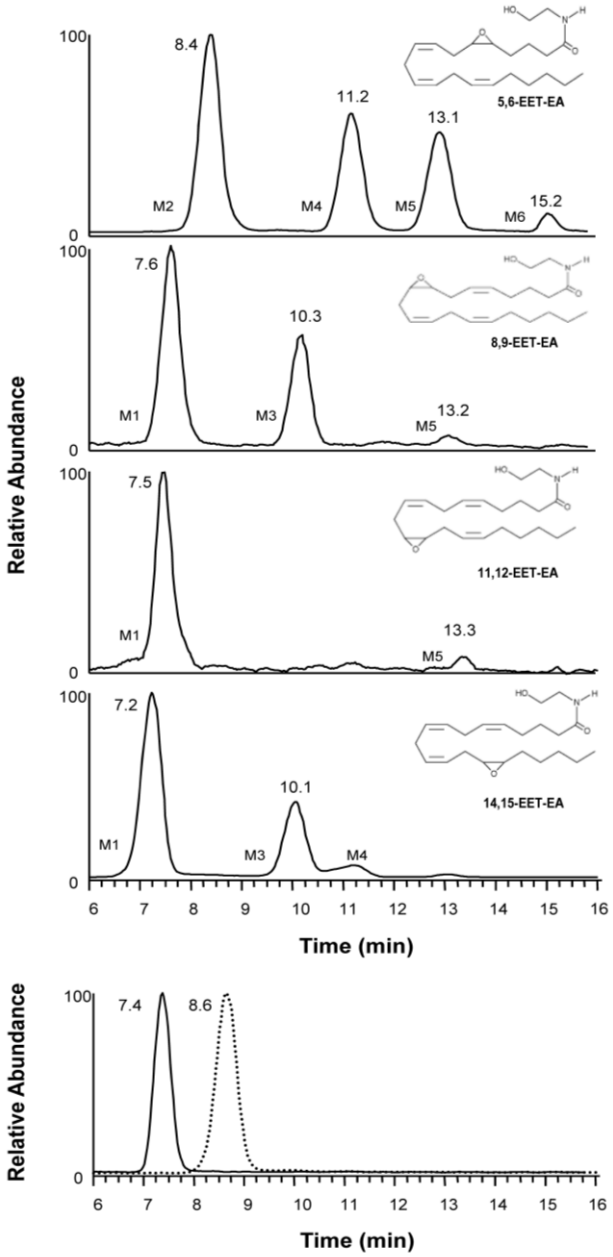

Figure 3.3. Metabolism of 5,6-, 8,9-, 11,12-, and 14,15-EET-EA by CYP2D6. The four EET-EAs (10 µM each) were incubated with 25 pmol CYP2D6 in the reconstituted system (top four chromatograms). Bottom panel, results obtained from incubations containing 25 pmol CYP4F2 supersomes and either 5,6-EET-EA (dotted line) or 14,15-EET-EA (solid line). The reactions were terminated after 20 min, and samples were analyzed as described under *Materials and Methods*. Shown are selected ion chromatograms (*m*/*z*, 380) of the metabolites formed under these conditions. The labels (M1–M5) correspond to the M1 to M5 peaks from Figure 3.1 according to retention times.

Kinetic analysis of anandamide hydroxylation and epoxidation by human recombinant CYP2D6. The reaction conditions used to determine the kinetic parameters for anandamide metabolite formation by CYP2D6 were optimized such that the formation of products was linear with respect to protein concentration and time of incubation. As shown in Figure 3.4, anandamide metabolism to 20-HETE- and 8,9-, 11,12- and 14,15-EET-EAs exhibited simple Michaelis-Menten kinetics with apparent K_m values of 1.3, 2.1, 2.6 and 2.8 micromolar and V_{max} values of 3.7, 1.6, 1.1, and 1.3 pmol product/min/pmol protein, respectively. The levels of 5,6-EET-EA formed under these conditions were too low to obtain accurate measures for K_m and V_{max}. These data demonstrate that anandamide is a high-affinity substrate for CYP2D6 and raise the possibility that this lipid mediator could be a physiological substrate of CYP2D6.

Metabolism of anandamide by human brain microsomes and mitochondria. Microsomal and mitochondrial fractions were prepared from human neocortical tissue from three human subjects (A,B and C) using a previously published procedure (Voirol et al., 2000). The protein obtained was subjected to immunoblot analysis to determine the presence of cytochrome c (a mitochondrial marker). As shown in Figure 3.5, the presence of cytochrome c was confirmed in the mitochondrial samples but it was absent from the microsomal preparations. Anandamide was incubated with 100 μg of either mitochondrial or microsomal protein from each of the subjects and the results from these experiments are also shown in Figure 3.5. Interestingly, the metabolic profiles obtained with the two fractions were very different. The major product formed by the microsomal preparations was 20-HETE-EA in each sample whereas the EET-EAs were the predominant metabolites formed by each of the liver microsomal metabolism of

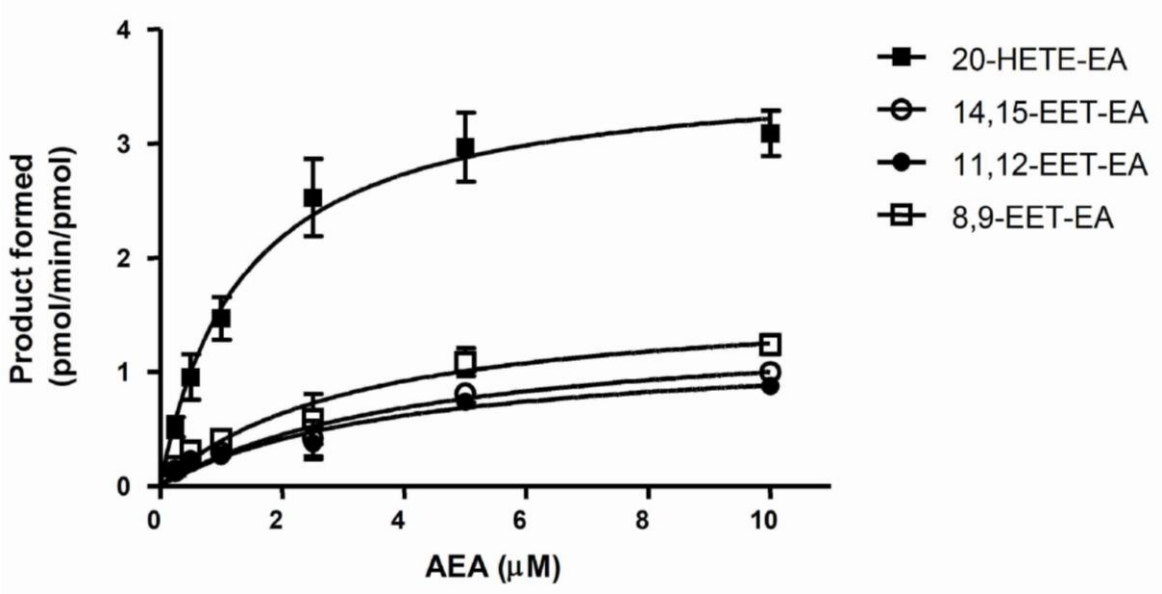

Figure 3.4. Kinetics of anandamide metabolite formation by human recombinant CYP2D6. Reaction mixtures containing 5 pmol CYP2D6 protein and the concentrations of anandamide indicated (0.25–10 μM) were incubated for 10 min at 37°C. The amount of products formed was determined based on a standard curve generated for each metabolite, and the rate data (average of three experiments) were fitted to a one-enzyme Michaelis-Menten model using Prism software.

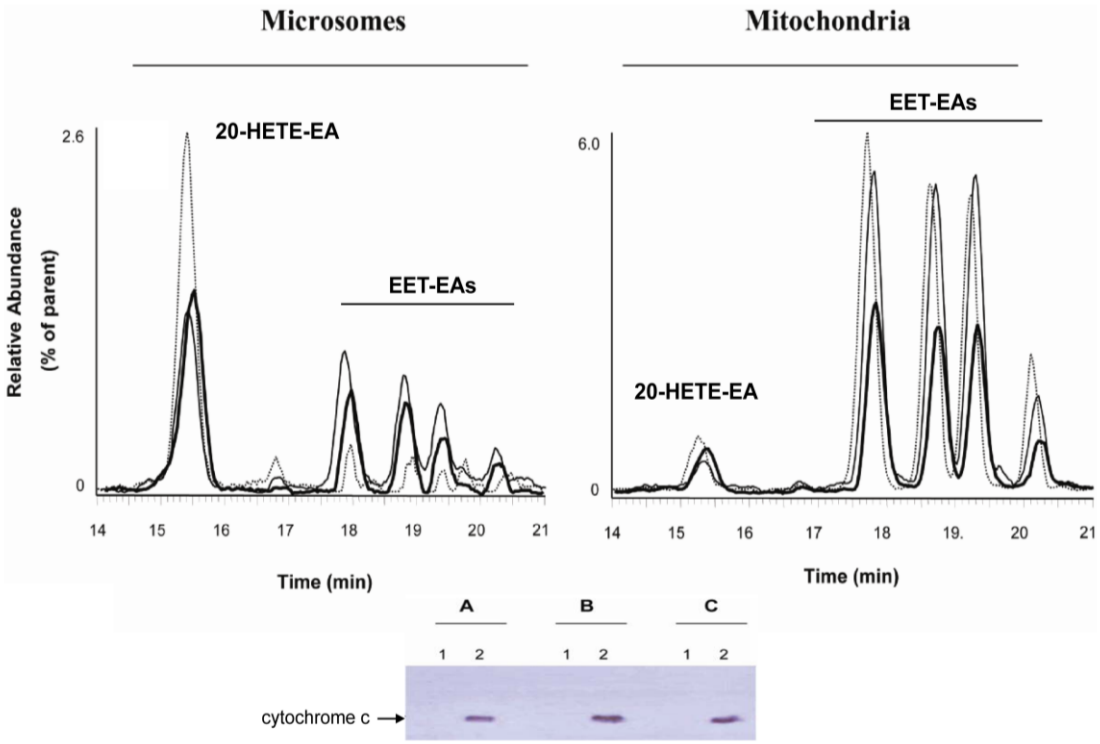

Figure 3.5. Anandamide metabolism by human neocortical brain microsomes and mitochondria. Anandamide (20 µM) was incubated in the presence of 100 µg of microsomal or mitochondrial protein from subjects A (dotted line), B (solid bold line), or C (solid line) for 10 min, and the samples were analyzed as described under *Materials and Methods*. Shown are the selected ion chromatograms at *m*/*z* 364. For immunoblot, the SDS-polyacrylamide gel electrophoresis gel lanes were loaded with 40 µg of either microsomal (1) or mitochondrial (2) protein, and the membrane was probed using a monoclonal antibody recognizing cytochrome *c* as described under *Materials and Methods*.

anandamide (Snider et al., 2007). We were unable to detect any dihydroxy derivatives of the EET-EAs from the brain incubations, despite the fact that microsomal epoxide hydrolase is also present in the brain.

Involvement of CYP2D6 in the metabolism of anandamide by human brain mitochondria. Although 2D6 is one of the major drug-metabolizing CYP enzymes in human brain, the presence of several other isoforms in the brain has also been demonstrated (Bhagwat et al., 2000). A monoclonal inhibitory antibody against CYP2D6 which has been previously described (Krausz et al., 1997) was utilized to determine if this enzyme was specifically involved in forming any of the products seen with the human brain microsomal and mitochondrial incubations. The inhibitory ability of the antibody was confirmed by pre-incubation of recombinant CYP2D6 in the reconstituted system with the antibody (5 μL antibody/0.5 mL reaction volume) for 5 min. Under these conditions, the amount of anandamide metabolism by CYP2D6 in the reconstituted system was reduced by 88 ± 2.2% in comparison to control antibody, therefore this concentration of antibody was used in subsequent experiments.

Pre-incubation of the microsomal preparations with the CYP2D6 inhibitory antibody had no effect on product formation (data not shown). However, a significant decrease in the formation of the EET-EAs by mitochondrial protein from subject A was observed in the presence of the CYP2D6 antibody, as can be seen in Figure 3.6, whereas the formation of 20-HETE-EA by both mitochondria and microsomes from subject A was

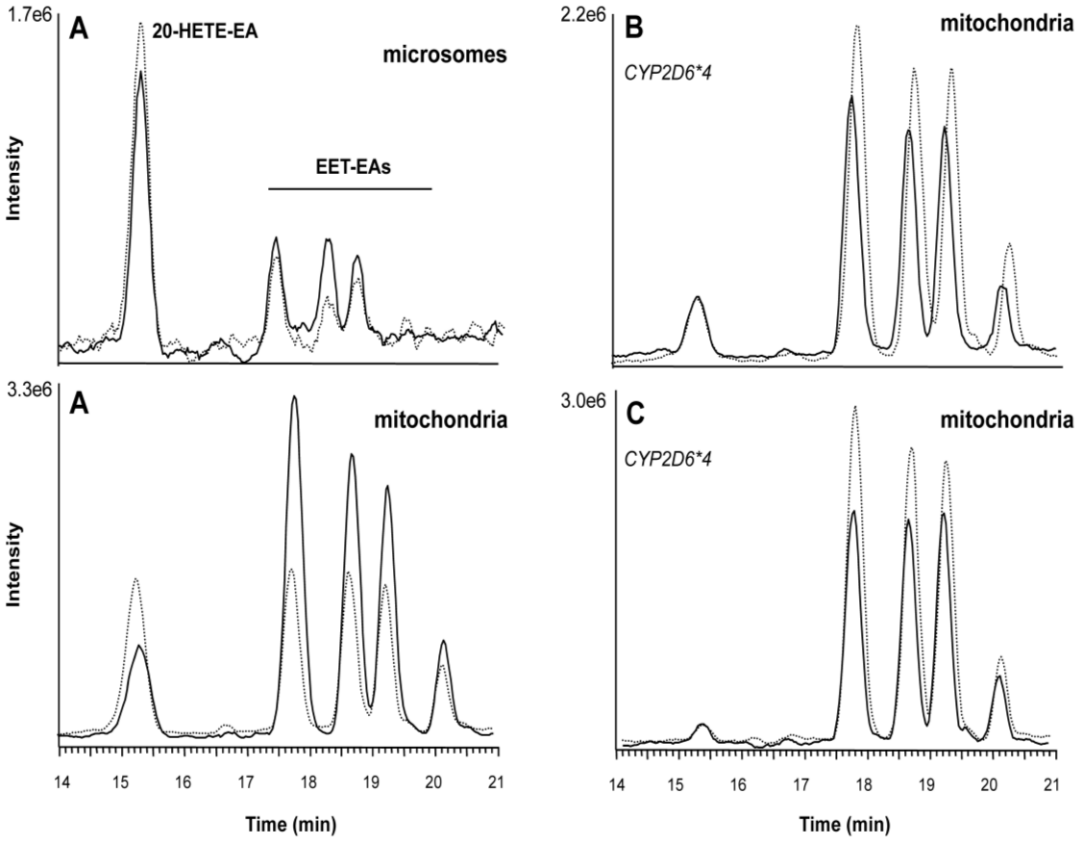

Figure 3.6. Effect of a monoclonal inhibitory antibody against CYP2D6 on anandamide metabolism by human brain microsomes and mitochondria. Microsomal protein sample from subject A and mitochondrial protein samples from subjects A, B, and C were preincubated in the presence of hen egg lysozyme (control) antibody (solid line) or antibody specific against CYP2D6 (dotted line) for 5 min. Anandamide (10 μM) and NADPH were then added, and the reaction mixture was incubated for an additional 10 min at 37°C with shaking. Samples were analyzed for anandamide metabolism activity as described under *Materials and Methods*. The CYP2D6*4 genotype was assigned to subjects B and C based on a genotype analysis that was performed as described under *Materials and Methods*.

slightly increased in the presence of CYP2D6 antibody. In contrast to subject A, there was a slight increase in the formation of EET-EAs from incubations using brain mitochondria from subjects B and C in the presence of the CYP2D6 antibody. To determine whether CYP2D6 polymorphisms could explain the observed difference between the three brain samples, the patient samples were genotyped for the common non-functional CYP2D6 alleles *4, *6, and *3 as described in Methods. Genotyping revealed that while subject A did not carry any one of the three mutations, subjects B and C were homozygous for CYP2D6*4, indicating that neither subject B nor subject C expresses any functional CYP2D6. This is consistent with our observation that CYP2D6 antibody has no effect on anandamide metabolism in subjects B and C. Formation of the anandamide epoxides by brain mitochondria from subjects B and C was inhibited in the presence of an antibody against CYP3A4 (EET-EA formation in the presence of CYP3A4 antibody was 62 ± 11% and 46 ± 21% relative to control antibody for subjects B and C, respectively). Therefore, anandamide can be metabolized by other brain CYP enzymes in the absence of functional CYP2D6.

Discussion

The importance of the human polymorphic CYP2D6 in the metabolism of drugs and other xenobiotics is well documented and appreciated, but its potential involvement in the metabolism of endogenous substrates is not well-characterized. Studies reporting a personality difference between individuals with non-functional CYP2D6 protein and those expressing the functional form of the protein suggest that CYP2D6 may play an

important role in the metabolism of psychoactive endogenous substrates (Llerena et al., 1993; Roberts et al., 2004; Dorado et al., 2007a). In strong support of this hypothesis are demonstrations of the neuronal expression of CYP2D6 and its proposed role in the O-demethylation of several psychotropic methoxyindolethylamines (Bhagwat et al., 2000; Siegle et al., 2001; Miksys et al., 2002; Yu et al., 2003a; Yu et al., 2003b).

Anandamide, an endogenous ligand for the CB1 receptor, is an important neuromodulator and, along with the other components of the endocannabinoid system, represents a novel drug target (Pacher et al., 2006). Therefore, a detailed examination of the metabolic pathways regulating the anandamide tone in the various tissues is needed in order to gain a better understanding about the involvement of this critical signaling mediator in physiological and pathophysiological situations. Anandamide is extensively metabolized by FAAH, leading to its inactivation and the termination of neuromodulatory activity (Maccarrone et al., 1998). Nevertheless, the possibility that a certain fraction of the pool of anandamide produced could also undergo oxidative metabolism can not be excluded.

With regards to the potential biological significance of the oxidative pathways of anandamide metabolism by CYPs as well as other fatty acid oxygenases, such as cyclooxygenase (COX) and lipoxygenase (LOX), several possibilities exist (Kozak and Marnett, 2002). Oxidation of anandamide may represent either an activation or an inactivation pathway, leading to the formation of products with either enhanced or decreased biostability and/or affinity for the cannabinoid receptors. Alternatively, oxidation of anandamide may result in the formation of novel signaling mediators which interact with their own specific targets. To address these questions, work has been done

in several laboratories focusing on the COX and LOX products of anandamide. Results obtained from these studies demonstrate various roles for this diverse set of molecules, including the ability of some COX-2- derived prostaglandin ethanolamides to regulate intraocular pressure by binding to novel targets, and vanniloid receptor activation by the LOX-derived anandamide metabolites (Hampson et al., 1995; Burstein et al., 2000; Craib et al., 2001; Kozak et al., 2002; Woodward et al., 2008). Ongoing work in our laboratory is aimed at addressing the physiological and pharmacological importance of the CYP-derived anandamide products.

In conclusion, there are several key findings from this study. First, the eicosanoid-like molecules anandamide and its epoxygenated derivatives are high-affinity CYP2D6 substrates, raising the possibility that this polymorphic enzyme could be involved in the metabolism of other endogenous signaling mediators which possess similar structural properties. Second, it demonstrates that anandamide can be metabolized to the same products by multiple microsomal and mitochondrial CYPs in the brain, such as 2D6 and 3A4, both of which form the EET-EAs. Although this may make the role of CYP2D6 in anandamide metabolism seem redundant, the regional distribution of these two proteins in the human brain would suggest otherwise. For example, CYP 3A4 protein in the human brain has been detected in the striatum, cerebellum and the hippocampus, whereas CYP2D6 protein is has been detected at highest levels in the substantia nigra and pyramidal neurons of the cortex (Miksys et al., 2002; Woodland et al., 2008). Finally, this study offers support to the hypothesis that neuropsychatric phenotype differences among individuals with genetic variations in CYP2D6 may, at least in part, be ascribable to interactions of this enzyme with endogenous substrates. Ongoing studies aimed at

elucidating the potential biological role of the oxygenated anandamide metabolites will further address the relevance of this hypothesis.

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Chapter IV

A Cytochrome P450-derived Epoxygenated Metabolite of Anandamide is a Potent, Cannabinoid Receptor 2-selective Agonist

Abstract

Oxidation of the endocannabinoid anandamide by cytochrome P450 (P450) enzymes has the potential to affect signaling pathways within the endocannabinoid system and pharmacological responses to novel drug candidates targeting this system. We previously reported that the human cytochrome P450s 2D6, 3A4 and 4F2 are high-affinity, high-turnover anandamide oxygenases *in vitro*, forming the novel metabolites hydroxyeicosatetraenoic acid ethanolamides (HETE-EAs) and epoxyeicosatrienoic acid ethanolamides (EET-EAs). The objective of this study was to investigate the possible biological significance of these metabolic pathways. We report that the 5,6-epoxide of anandamide (5,6-EET-EA) is a potent and selective cannabinoid receptor 2 (CB2) agonist. The Ki values for the binding of 5,6-EET-EA to membranes from Chinese hamster ovary (CHO) cells expressing either recombinant human CB1 or CB2 receptor were 11.4 μM and 8.9 nM, respectively. Additionally, 5,6-EET-EA inhibited the forskolin-stimulated accumulation of cyclic AMP in CHO cells stably expressing the CB2 receptor (IC_{50} = 9.8 ± 1.3 nM).

Within the central nervous system, the CB2 receptor is expressed on activated microglia and is a potential therapeutic target for neuroinflammation. BV-2 microglial cells stimulated with low doses of interferon-gamma exhibited an increased capacity for converting anandamide to 5,6-EET-EA which correlated with increased protein expression of microglial P450s 4F13 and 3A1. Finally, we demonstrate that 5,6-EET-EA is more stable than anandamide in mouse brain homogenates and is primarily metabolized by epoxide hydrolase. Combined, our results suggest that epoxidation of anandamide by P450s to form 5,6-EET-EA represents an endocannabinoid bioactivation pathway in the context of immune cell function.

Introduction

The endocannabinoids anandamide and 2-arachidonoylglycerol, the enzymes involved in their synthesis and degradation, and the cannabinoid CB1 and CB2 receptors are collectively known as the endocannabinoid system. The components of this system are novel pharmacological targets in the treatment of many disorders including neurodegeneration, chronic pain, inflammation, cancer and others (Di Marzo, 2008). The CB2 receptor, the main immune cell receptor, which in the brain appears to be primarily expressed on activated microglia, is considered to be a possible therapeutic target for the treatment of central nervous system (CNS) inflammation, including the type of inflammation implicated in the etiology of neurodegenerative disease (Cabral and Marciano-Cabral, 2005; Block et al., 2007; Cabral et al., 2008). CB2-selective agonists have the potential to alleviate inflammation by reducing the secretion of several pro-inflammatory cytokines while at the same time being devoid of the psychotropic activity

exhibited by CB1 receptor agonists (Maresz et al., 2005; Sheng et al., 2005; Eljaschewitsch et al., 2006; Shoemaker et al., 2007). A thorough understanding of the metabolic pathways that regulate the endocannabinoid tone *in vivo* is crucial to the development of novel therapeutic agents that can target the various components of the endocannabinoid system.

We have previously reported that anandamide, a structural relative of arachidonic acid, is oxygenated *in vitro* by the human cytochrome P450 (P450) enzymes 2D6, 3A4 and 4F2 to yield several metabolites, including 20-hydroxyeicosatetraenoic acid ethanolamide (20-HETE-EA) and the 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EET-EAs) in human kidney, liver and brain tissue (Snider et al., 2007; Snider et al., 2008b). The purpose of this study was to address the potential pharmacological and physiological significance of these P450-mediated oxidative pathways of anandamide metabolism.

Here we report on the ability of the 5,6- epoxide of anandamide (5,6-EET-EA) to bind and to functionally activate the recombinant human CB2 receptor expressed in Chinese hamster ovary (CHO) cells. In our studies the 5,6-EET-EA exhibited greater than a 300-fold selectivity for binding to CB2 over CB1 and its affinity for CB2 was more than 1000-fold greater than that of the parent molecule, anandamide. We also demonstrate that murine microglial BV-2 cells metabolize anandamide and that they produce significantly more 5,6-EET-EA after stimulation with the cytokine interferon gamma (IFNγ). This increase in 5,6-EET-EA formation corresponded with an increase in the protein levels of P450 4F13 and 3A1 in the IFNγ -stimulated microglia. In addition, incubations of anandamide and 5,6-EET-EA with mouse brain homogenates revealed that

the 5,6-EET-EA has a significantly increased biological stability compared to anandamide and that it is primarily metabolized by epoxide hydrolase to form 5,6-dihydroxyeicosatrienoic acid ethanolamide (5,6-DHET-EA).

Our results demonstrate that the P450-mediated epoxidation of anandamide to form 5,6-EET-EA represents a bioactivation pathway for endocannabinoid signaling, which may affect microglial activity during inflammatory states. At least two different classes of novel drug candidates have the potential to increase the levels of 5,6-EET-EA, including inhibitors of the anandamide inactivating enzyme fatty acid amide hydrolase (FAAH), which are being developed for the treatment of inflammatory pain and anxiety disorders (Cravatt and Lichtman, 2003), and inhibitors of the enzyme epoxide hydrolase which are being developed for the treatment of hypertension (Chiamvimonvat et al., 2007). Therefore, the current findings may provide additional insights into the mechanisms of action of these novel drug candidates.

Materials and Methods

Materials. Anandamide, 5,6-EET-EA, AM1241 and WIN-55212-2 were purchased from Cayman Chemical (Ann Arbor, MI). Radiolabeled CP-55940 was purchased from Perkin Elmer (Waltham, MA). The full length cDNA clones encoding human CB1 and CB2 receptors were purchased from the Missouri S&T cDNA Resource Center (Rolla, MO). Lipofectamine 2000 transfection reagent and geneticin were purchased from Invitrogen (Carlsbad, CA). Forskolin and IBMX were purchased from Sigma-Aldrich (Saint Louis, MO). Radioactivity-based cAMP assay kits were purchased from GE Healthcare (Piscataway, NJ). Recombinant mouse interferon gamma was

purchased from R&D Systems (Minneapolis, MN). Monoclonal antibodies against CYPs 4F13 and 3A1 and secondary anti-mouse and anti-rabbit horseradish peroxidase-conjugated antibodies were purchased from Abcam (Cambridge, MA). Polyclonal antibody against beta-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of highest quality and available from commercial sources.

Cell culture and transfection. Chinese hamster ovary (CHO-K1) cells were maintained in 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM) Nutrient Mixture F-12 supplemented with L-glutamine, 2.438 g/L Sodium Bicarbonate, 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL). Murine microglial BV-2 cells were a gift from Dr. Dennis Selkoe (Harvard Medical School) and were maintained in DMEM supplemented with L-glutamine, 4.5 g/L D-Glucose, 25 mM HEPES, 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). CHO cells were stably transfected with the pcDNA3.1 vector encoding N-terminal 3xHA-tagged human wild type CB1 or CB2 receptor using lipofectamine 2000 reagent following the manufacturer's protocol for using a 24-well plate format. Stable transformants were selected in growth medium containing geneticin (0.4 mg/ml). Colonies were picked approximately 2 weeks post-transfection and allowed to expand, then tested for expression of CB1 or CB2 protein by western blot using anti-HA antibody. Cell lines containing moderate to high levels of receptor were propagated in geneticin-containing medium and used for membrane preparations and for cAMP measurements.

Membrane preparation and saturation binding experiments. Membranes from CHO cells stably expressing either the CB1 (CHO-CB1) or the CB2 (CHO-CB2) receptor

were prepared by homogenization in TME buffer (50 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4) followed by centrifugation at 1100xg for 10 min. The supernatant was collected and centrifuged at 45,000xg for 30 min. The pellet was resuspended in TME buffer containing protease inhibitors and the protein concentration measured by the BCA method. Membrane preparations were frozen at -80°C until used for experiments.

For the saturation binding experiments, the 200 μL reaction mixtures contained TMEB buffer (TME buffer with 0.5% bovine serum albumin), various concentrations of radiolabeled CP-55940 (0.02 nM - 10 nM) and 5 μg or 20 μg membrane protein from CHO-CB1 or CHO-CB2 cells, respectively, in the presence or absence of WIN5212-2 (10 μM). The binding reactions were carried out in silanized amber vials in a 30°C shaking water bath for 1 hour. Bound radioactivity was separated from unbound ligand on 96-well microplates with hydrophilic GF/C filter mesh well bottoms (Whatman, Florham Park, NJ) which were washed once with 200 μL TMEB buffer prior to the application of sample mixtures and washed 3-4 times with 200 μL TMEB buffer after sample application. Upon vacuum drying of the filter plate, 50 μL of Microscint 0 reagent was added to each well and the radioactivity was counted on a TopCount instrument (Perkin Elmer, Waltham, MA).

Competition binding assays. Competition binding assays were carried out similarly to saturation binding experiments except the reaction mixtures contained TMEB buffer with 50 μM phenylmethanesulphonylfluoride, various concentrations of competitor (anandamide or 5,6-EET-EA) as described in the legend to Figure 4.1, radiolabeled CP-55940 at its Kd concentration (which was determined to be 5 nM for

CHO-CB1 and 1.4 nM for CHO-CB2 membranes) and membrane protein (5 μg from CHO-CB1 and 20 μg from CHO-CB2 cells). Radiolabeled-CP-55940 binding in the presence of 10 μM WIN-55212-2 and the absence of any competitor was considered to be non-specific.

cAMP inhibition assays. CHO-K1 or CHO-CB2 cells were plated onto 24-well plates in regular growth medium which was replaced with serum-free medium on the day of the experiment for 1 hour prior to the addition of drugs. At this point the cells were at 80-90% confluence. Cells were treated with either medium alone (control) or medium containing 10 μM forskolin and 100 μM IBMX and varying doses (10pM-1nM) of AM1241 (dissolved in DMSO) or 5,6-EET-EA (dissolved in ethanol). The final vehicle concentration was less than 0.04%. Upon the addition of the treatments, the cells were placed back in the 37°C incubator for a total of 10 min after which the medium was aspirated and replaced with a 3% solution of perchloric acid (1mL/well). After allowing time for solubilization (1 hour at 4°C), the samples were neutralized with a 2.5 M solution of potassium bicarbonate. Cyclic AMP levels were measured using a kit from GE Healthcare per manufacturer's instructions.

Whole cell metabolism assays. BV-2 microglial cells were plated onto 100 mm dishes in regular growth medium, which was replaced with serum-free medium when the cells reached 60-70% confluence. Two hours later, either medium alone or medium containing interferon gamma (10 ng/mL final concentration) was added and the cells were incubated for 24 hours. After the 24-hour cell activation, the medium was aspirated and replaced with serum- free medium containing 20 μM anandamide and the cells were

placed back in the incubator for 45 min to allow for metabolism to occur. The anandamide-containing medium, into which the cells were scraped, was collected and the cells were subjected to 2-3 freeze thaw cycles alternating between dry ice and a 37ºC water bath to ensure cell lysis. The samples were spiked with 100 pmol of deuterated anandamide (internal standard) and extracted with 4 volumes of ethyl acetate which was subsequently dried down. The samples were then resuspended in 50 μL of methanol and subjected to electrospray ionization liquid chromatography mass spectrometry (ESI-LC/MS) analysis as described previously (Snider et al., 2007). Standard curve for 5,6-EET-EA was generated by injecting various known amounts of an authentic standard. Linear regression analysis was performed after the peak area was expressed as a function of the amount injected.

Immunoblot experiments. BV-2 microglial cells were plated onto 60 mm dishes and activated with IFNγ or lipopolysaccharide (LPS) for 24 hours as described above. The medium was aspirated and the cells were lysed by incubating in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors at 4°C with constant agitation. The lysates were centrifuged at 12,000 RPM for 15 minutes. The supernatants were removed and the protein concentration was measured using the BCA method. The protein mixture from the cell lysates (60 μg protein/well) was separated on a 4-20 % SDS-PAGE gel and subsequently transferred onto a PVDF membrane. The membranes were probed with antibodies against CYP4F13, CYP3A1, or β-actin followed by HRP-conjugated secondary antibodies and the signals were detected using the ECL system.

Degradation of anandamide and 5,6-EET-EA in mouse brain homogenates. Brains from BALB/c mice were collected, quick frozen on dry ice and stored at -80°C until use. The brain tissue was homogenized in potassium phosphate buffer, pH 7.4 using a Polytron homogenizer. Protein concentrations were measured using the BCA method. Anandamide or 5,6-EET-EA (5 μM) was incubated in the presence of the mouse brain homogenate (0.5 mg protein/reaction) in phosphate buffer at 37°C for 0-180 min. At the designated time points and after addition of internal standard, reaction mixtures were extracted with 3 volumes of ethyl acetate, dried down and resuspended in 100 μL of methanol followed by ESI-LC/MS analysis. Control experiments contained the same components with the exception that the brain homogenate was first heat-inactivated by boiling for 10 min.

Data Analysis. Nonlinear regression and statistical analyses of the data were performed using GraphPad Prism version 5.01 for Windows (Graph-Pad Software, San Diego, CA; http://www.graphpad.com).

Results

5,6-EET-EA selectively binds the human CB2 receptor with high affinity. In order to understand the potential physiological and pharmacological relevance of the P450-mediated oxidation of anandamide, we compared the binding affinities of anandamide and its epoxygenated metabolite 5,6-EET-EA for the human CB1 and CB2 receptors. The ligand binding assay utilized the ability of the two molecules to compete

with radiolabeled (-) – cis–3 - [2 – Hydroxy – 4 - (1,1 - dimethylheptyl) phenyl] – trans - 4-(3 hydroxypropyl) cyclohexanol (CP-55940), a synthetic non-selective cannabinoid, for binding to the receptors. The sources for the CB1 and CB2 proteins were membrane preparations from CHO cells stably expressing the receptors (CHO-CB1 and CHO-CB2). The presence of the CB1 or CB2 receptors in the membrane preparations was confirmed by western blot and saturation binding experiments (not shown) as described in Methods. From the saturation binding experiments, the receptor densities (Bmax) were found to be 4900 ± 222 fmol/mg and 961 ± 54 fmol/mg for CHO-CB1 and CHO-CB2 membrane preparations, respectively. The Kd value for CP-55940 binding to the receptors was 5.1 ± 1.1 nM for CB1 and 1.4 ± 0.1 nM for CB2.

As shown in Figure 4.1A, the anandamide metabolite 5,6-EET-EA bound more weakly to the CB1 receptor relative to anandamide, as evidenced by the Ki values obtained from the competition curves, which were 155 nM and 3.2 μM for anandamide and 5,6-EET-EA, respectively. In contrast, the metabolite displayed a significantly higher affinity for the CB2 receptor relative to anandamide as can be seen from the competition curves in Figure 4.1B. In this case, the Ki values obtained were 8.9 nM and 11.4 μM for 5,6-EET-EA and anandamide, respectively.

5,6-EET-EA is an agonist at the human CB2 receptor. In order to determine if binding of 5,6-EET-EA to CB2 leads to functional activation of the receptor, we again utilized the CHO-CB2 cells and monitored intracellular cyclic adenosine monophosphate (cAMP) levels.

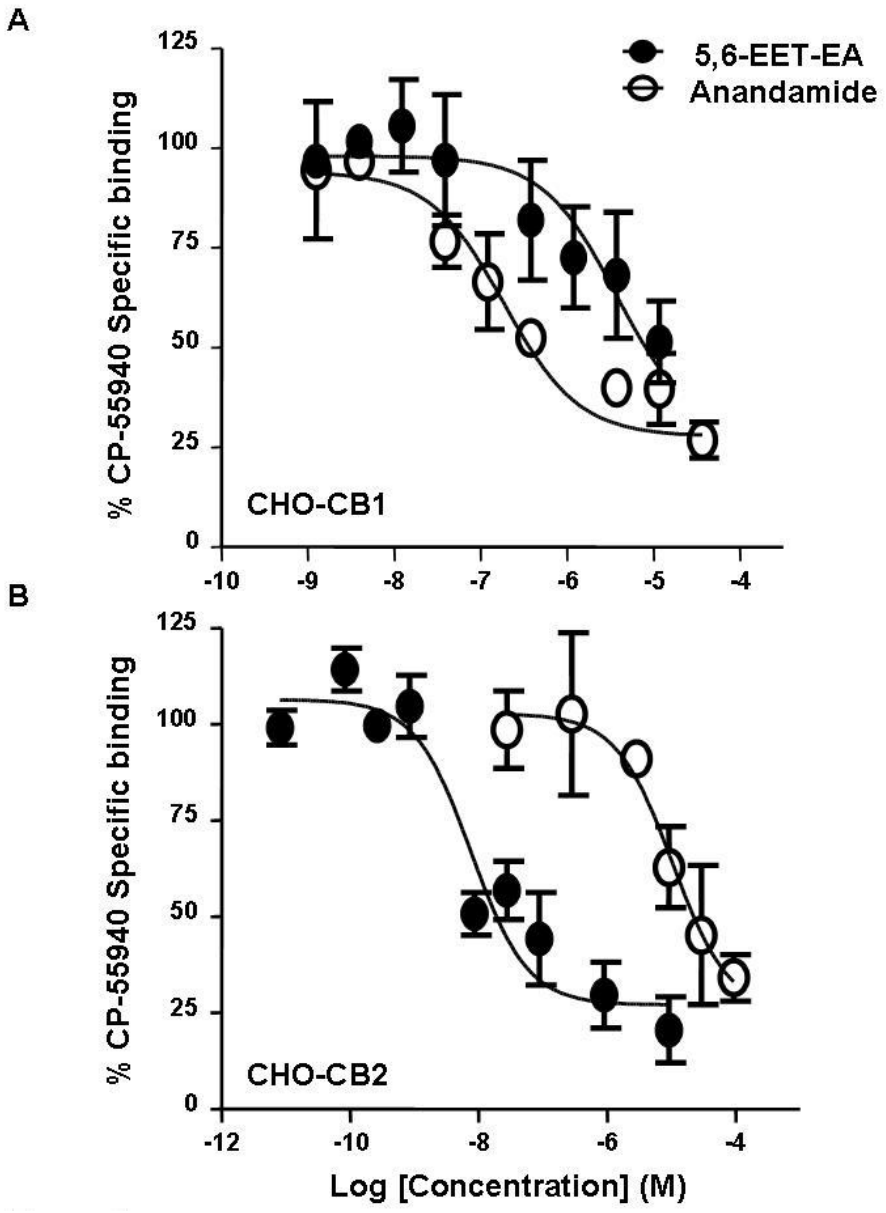

Figure 4.1. Binding of anandamide and 5,6-EET-EA to human CB1 and CB2 receptors. Membrane protein from CHO cells stably expressing either (A) the human CB1 or (B) the human CB2 receptor were incubated with radiolabeled CP-55940 at its Kd value (5.1 nM for CB1 and 1.4 nM for CB2) in the presence of vehicle (ethanol) or various concentrations of anandamide or 5,6-EET-EA (0.3 nM – 100 μM) and the reactions were allowed to reach equilibrium. The binding of CP55940 in the presence of a saturating concentration (10 μM) of the cannabinoid agonist WIN55212-2 was considered to be due to non-specific binding. The specific binding in the presence of the various concentrations of competitor was expressed as a percentage of the specific binding in the presence of vehicle.

The CB2 receptor is G-protein coupled and its activation by agonists leads to inhibition of the accumulation of cAMP within cells *via* its associated G-proteins Gαi/o (Howlett, 2005). As a positive control, we used the synthetic CB2-selective agonist (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]-methanone (AM1241). As shown in Figure 4.2A, acute (10 min) treatment of the CHO-CB2 cells with the adenylyl cyclase activator forskolin and the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) led to a significant increase in intracellular cAMP levels. Co-treatment of the forskolin/IBMX-stimulated cells with low doses of AM1241 caused a dose-dependent decrease in the cAMP levels. Similarly, co-treatment of the CHO-CB2 cells with the same low doses of 5,6-EET-EA also decreased the intracellular cAMP levels (Figure 4.2B).

The inhibition of cAMP was solely due to CB2 receptor activation, since co-treatment of forskolin/IBMX-stimulated untransfected CHO-K1 cells with varying doses of 5,6-EET-EA had no effect upon cAMP levels (Figure 4.3A), which was similar to results that were obtained using the CHO-CB1 cells (not shown). The estimated IC_{50} value for the 5,6-EET-EA-mediated inhibition of cAMP in the CHO-CB2 cells was estimated to be 9.8 ± 1.3 nM (Figure 4.3B), which is comparable to the Ki value of 8.9 nM for the binding of 5,6-EET-EA to the CB2 receptor.

IFNγ - stimulated BV-2 microglial cells have increased capacity for metabolizing anandamide to 5,6-EET-EA. The CB2 receptor is primarily expressed on immune cells, including microglia, which are resident macrophage-like cells in the brain. Microglia become activated in almost all brain pathologies and have been implicated in CNS inflammation related to neurodegeneration (Gao and Hong, 2008).

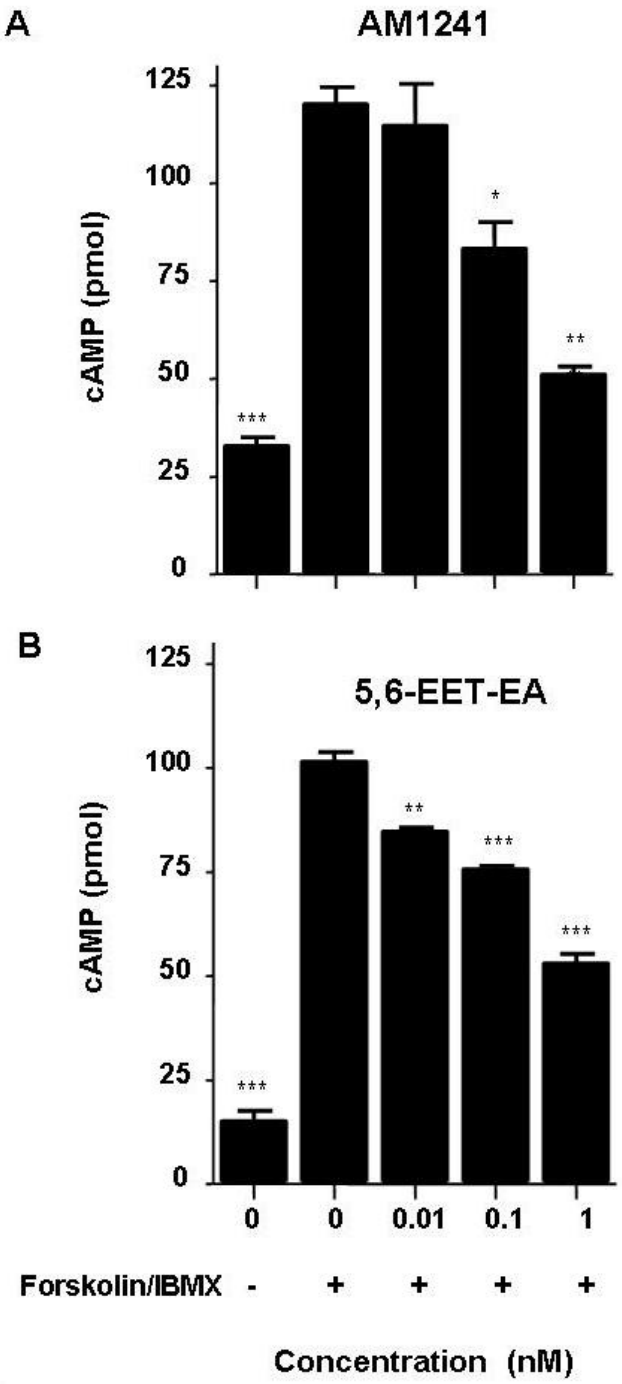

Figure 4.2. Inhibition of cyclic AMP accumulation in CHO-CB2 cells by AM1241 and 5,6-EET-EA. Cyclic AMP levels were measured in CHO-CB2 cells that were either untreated or treated with 10 μM forskolin and 100 μM IBMX in the presence or absence of (A), AM1241 or (B), 5,6-EET-EA for 10 min. Control cells (0,-) received medium alone. The results are the mean ± S.E. of triplicate cultures. *, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with vehicle (0,+) group.

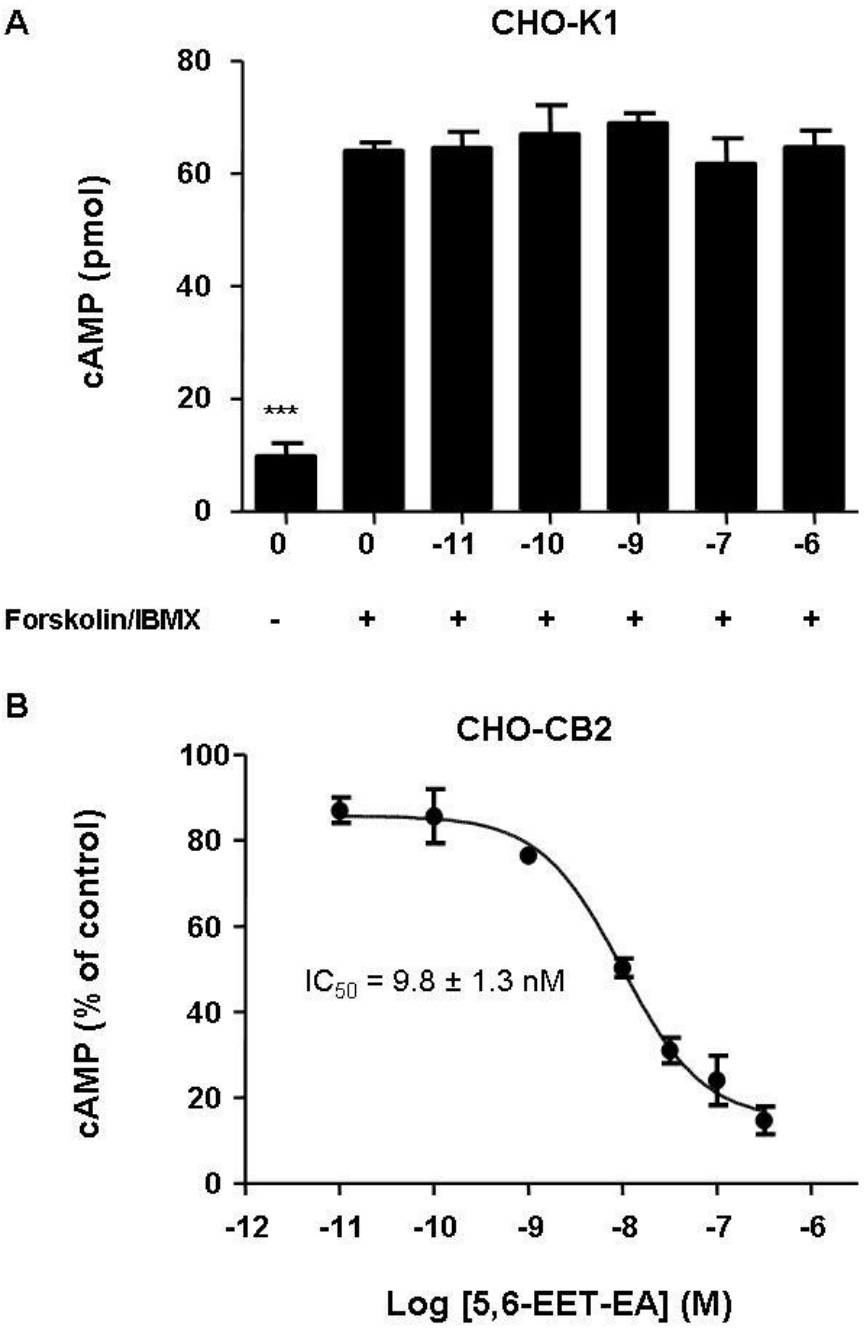

Figure 4.3. Effect of 5,6-EET-EA on intracellular cAMP levels in CHO-K1 and CHO-CB2 cells. Cyclic AMP levels were measured in untreated, or CHO-K1 (A) or CHO-CB2 (B) cells treated with 10 μM forskolin and 100 μM IBMX in the presence or absence of various doses of 5,6-EET-EA for 10 min. Control cells (0,-) received medium alone. The results are the mean ± S.E. of triplicate cultures. *** $p < 0.001$, compared with vehicle (0,+) group.

Upon stimulation with interferon gamma (IFNγ), a pleiotropic cytokine released by dendritic, natural killer and T cells, microglia acquire antigen presentation capacity ("primed" phenotype) and while in this state, expression of the microglial CB2 receptor has been found to be significantly upregulated (Carlisle et al., 2002; Maresz et al., 2005). It is possible that in addition to CB2 receptor upregulation, primed microglia also synthesize signaling mediators that can act upon CB2.

To determine if microglia produce more of the CB2 agonist, 5,6-EET-EA, following exposure to IFNγ we conducted *in vitro* whole cell metabolism experiments. For these studies we utilized the BV-2 murine microglial cells which have been extensively studied because they possess many characteristics of primary microglia.

Either unstimulated or BV-2 cells stimulated with 10 ng/mL IFNγ for 24 hours were exposed to 20 μM anandamide for 45 minutes, after which the production of anandamide metabolites by the cells was determined as described under Methods. A representative chromatogram for anandamide metabolism from this experiment is shown in Figure 4.4A. Characteristic peaks for the P450-derived anandamide metabolites having mass to charge (*m/z*) ratios of 364, which we have previously described (Snider et al., 2007), were observed in samples from unstimulated as well as the IFNγ–stimulated cells. No such peaks were seen when the anandamide-containing medium was incubated in the absence of cells (not shown). Changes in the levels of formation of each metabolite were observed between the two treatments with the two hydroxylated products decreasing (20-HETE-EA and 19-HETE-EA) and the four epoxygenated products primarily increasing in intensity (11,12-, 8,9- and 5,6-EET-EA).

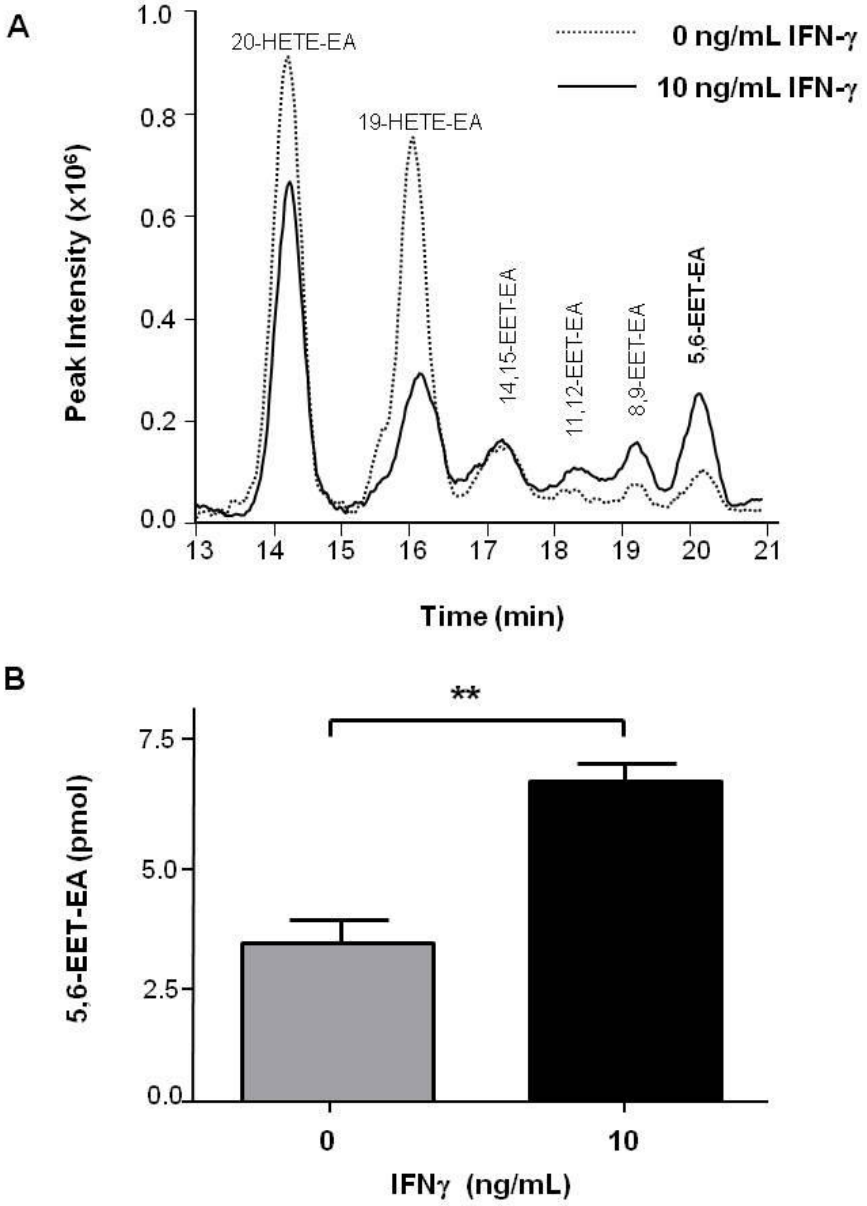

Figure 4.4. Metabolism of anandamide by microglial cells in culture. Unstimulated or BV-2 microglial cells stimulated with IFNγ (10 ng/mL) for 24 hours were incubated in serum-free medium containing anandamide (20 μM) for 45 min. Metabolites were extracted and analyzed by ESI-LC/MS as described under Methods. A, selected ion chromatogram showing monooxygenated anandamide metabolites with mass to charge (*m/z*) ratios of 364. B, quantitation of 5,6-EET-EA based on a standard curve generated using an authentic standard. The results are the mean ± S.E. from triplicate cultures. ** $p < 0.01$.

Of particular importance is the observation that the increase in formation of the CB2 agonist 5,6-EET-EA by the stimulated cells was highly reproducible and statistically significant, as shown in Figure 4.4B, suggesting that the expression of certain P450 isoforms which metabolize anandamide may be upregulated in the IFNγ-stimulated microglia.

Expression of anandamide-metabolizing P450 isoforms in microglia is increased upon exposure to IFNγ. We have previously reported on the participation of human P450s 2D6, 3A4 and 4F2 in the metabolism of anandamide (Snider et al., 2007; Snider et al., 2008b). Therefore, we wanted to determine if P450s belonging to these same subfamilies were upregulated in the mouse microglial cells upon IFNγ treatment. BV-2 microglial cells were stimulated as described under Methods. Since bacterial lipopolysaccharide (LPS) has previously been shown to induce several mouse P450 4F isoforms (Cui et al., 2001), we also included LPS treatment in the analysis of P450 4F13 expression as a positive control.

As shown in Figure 4.5A, the presence of P450 4F13 was not detected in unstimulated microglia. However, a band migrating at approximately 50 kDa corresponding to this protein was detected in the LPS-, and more strongly, the IFNγ-stimulated cells. Furthermore, the level of P450 4F13 expression was dependent on the IFNγ dose used to activate the cells (Figure 4.5A).

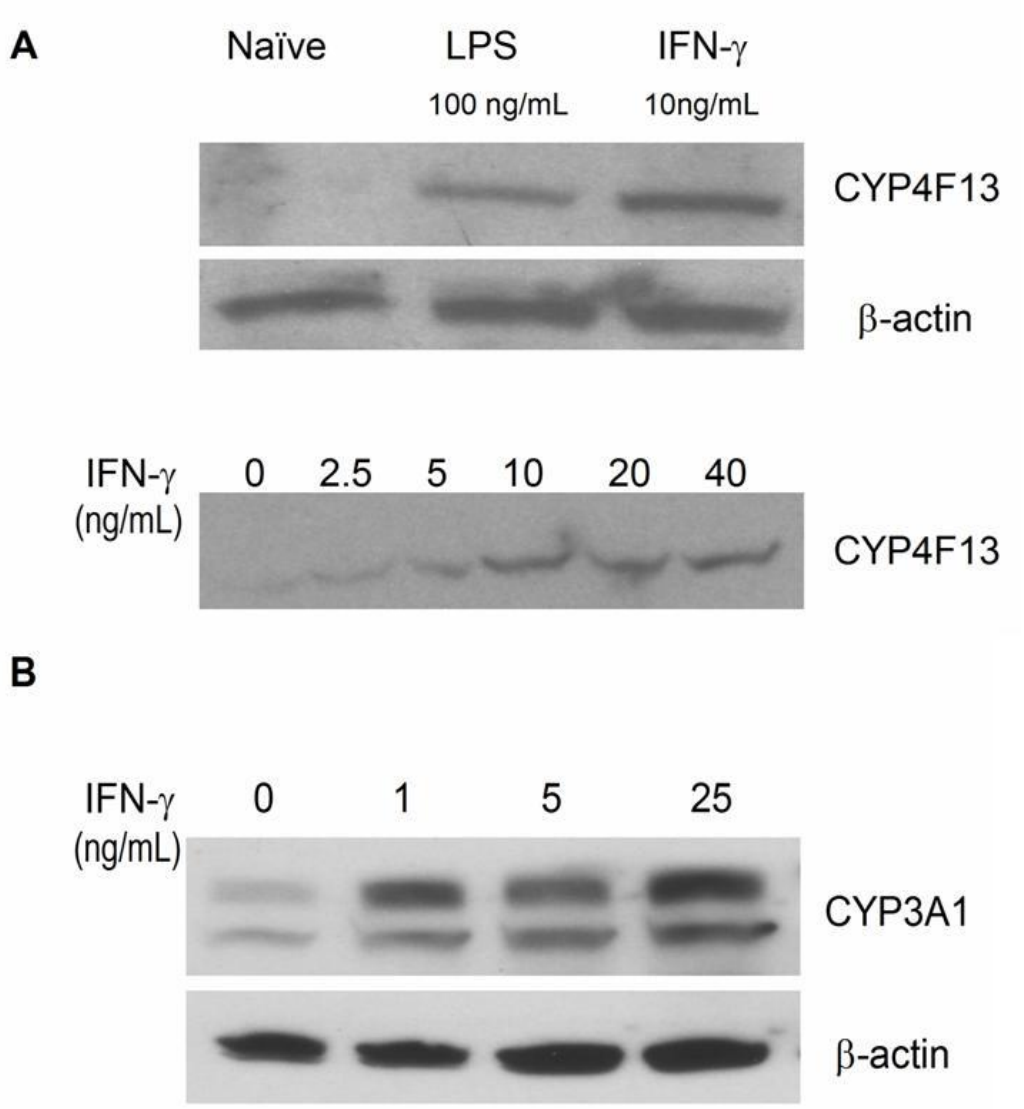

Figure 4.5. Induction of anandamide-metabolizing cytochrome P450 enzymes in activated microglial cells. Mouse microglial BV-2 cells were stimulated for 24 hours with the treatments indicated. Whole cell lysates (60 μg protein/lane) from the BV-2 cells were separated on a 4-20 % SDS-PAGE gel and transferred onto PVDF membranes. The membranes were probed with antibodies against either P450 4F13 (A), P450 3A1 (B) or β-actin (A,B) followed by HRP-conjugated secondary antibodies. The signals were detected using the ECL system.

Since the expression of P450 4F13 increased in the IFNγ-stimulated cells while the formation of the HETE-EA metabolites of anandamide decreased under these conditions (Figure 4.4A), we could speculate that the murine 4F13 enzyme, unlike the human 4F2, is not an anandamide hydroxylase. However, this hypothesis needs to be further investigated.

The expression of P450 3A protein in the BV-2 cells was also induced in a dose-dependent manner by IFNγ treatment (Figure 4.5B). Consistent with previous reports by others (Matheny et al., 2004), we also detected a double band of 3A protein around 50 kDa using an antibody against P450 3A1. This is most likely due to the presence of multiple 3A isoforms in mouse cells. As a loading control, immunoblots for β-actin were performed which demonstrated that the observed differences in the expression levels of P450 were not simply due to differences in the amounts of protein loaded onto the gel. The expression of 2D6 appeared to be very low and we were unable to detect any significant changes among the different treatment groups (data not shown).

5,6-EET-EA has increased stability in mouse brain homogenates relative to anandamide. Anandamide is known to be extensively degraded by the enzyme fatty acid amide hydrolase (FAAH) which is abundantly expressed in the brain (Giang and Cravatt, 1997). In order to compare the relative stabilities of anandamide and its CB2 agonist metabolite 5,6-EET-EA, we incubated each molecule in mouse brain homogenates for 0-180 min and monitored the amount remaining as described under Methods. As a control, we used heat-inactivated protein in the incubations.

As can be seen from Figure 4.6A, neither molecule was significantly degraded in the control samples. This was in contrast to samples which contained active mouse brain proteins where both anandamide and 5,6-EET-EA disappeared over time. Notably, however, decrease in the level of 5,6-EET-EA was significantly slower than that of anandamide at each time point. The estimated half lives were 32.2 min and 14.3 min for 5,6-EET-EA and anandamide, respectively. Since the plateau of the decay curve for anandamide was approximately 7% compared to 22% for 5,6-EET-EA, the stability difference may be even greater than what is simply estimated by the respective half lives.

We previously reported that the human liver microsomal P450-derived epoxides of anandamide are further metabolized by epoxide hydrolase to their corresponding dihydroxy derivatives which have *m/z* ratios of 382, corresponding to the mass of the EET-EA metabolite plus a water molecule (Snider et al., 2007). Since epoxide hydrolase is also expressed in brain (Shin et al., 2005; Sura et al., 2008), we monitored the formation of 5,6-dihydroxyeicosatrienoic acid ethanolamide (5,6-DHET-EA) in the mouse brain incubations to which we added 5,6-EET-EA. We found that the amount of 5,6-DHET-EA increased over time as the amount of 5,6-EET-EA decreased. This can be seen in Figure 4.6B which shows the peaks for the two molecules from representative incubations at times 0 and 120 min. The time-dependent formation of the 5,6-DHET-EA peak normalized to internal standard is shown in the inset to Figure 4.6B.

We did not observe significant formation of 5,6-EET under these conditions (not shown), which would be the product of hydrolysis by FAAH, suggesting that in the brain 5,6-EET-EA is degraded almost exclusively by epoxide hydrolase and not by FAAH.

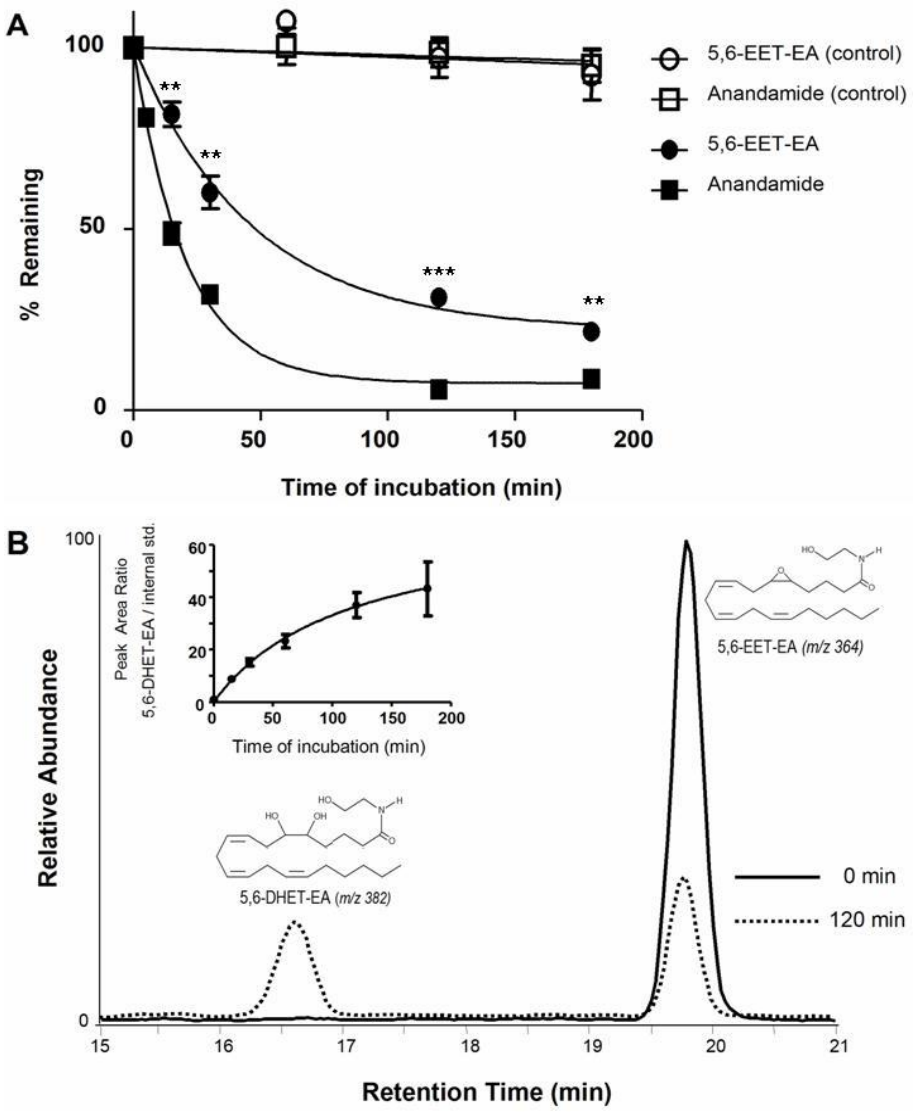

Figure 4.6. Degradation of anandamide and 5,6-EET-EA by mouse brain proteins. Anandamide or 5,6-EET-EA (5 μM) were incubated in the presence of mouse brain homogenate (0.5 mg protein/reaction) in phosphate buffer (pH 7.4) at 37°C for 0-180 min. At the designated time points and after the addition of internal standards, reaction mixtures were extracted with 3 volumes of ethyl acetate and analyzed by ESI-LC/MS as described under Methods. A, the amount of 5,6-EET-EA or anandamide remaining at each time point plotted as a percent of the starting amount (at time 0). Control samples contained 0.5 mg of heat-inactivated mouse brain homogenate. ** $p < 0.01$, *** $p < 0.001$ compared with the anandamide group at the same time point. B, selected ion chromatograms of 5,6-EET-EA and its epoxide hydrolase-derived metabolite 5,6-DHET-EA at time 0 and 120 min. B (inset), time course for the formation 5,6-DHET-EA.

Discussion

Since the initial discovery of the receptors for the main psychoactive constituent in marijuana, Δ^9-tetrahydrocannabinol, and the subsequent identification of anandamide and 2-arachidonoylglycerol as the endogenous ligands to these receptors (Devane et al., 1988; Devane et al., 1992; Kaminski et al., 1992; Mechoulam et al., 1995), much work has been done to understand the role of the endocannabinoid signaling system because it appears to be involved in controlling physiological homeostasis and it is dysregulated in numerous pathological conditions (Di Marzo, 2008). Progress in the development of novel therapeutics designed to manipulate the various components of this system is largely dependent on a comprehensive understanding of the various metabolic pathways that exert control over the action of endocannabinoids.

The evidence thus far suggests that anandamide is produced on demand and acts locally, most probably due to its lipophilic character. Its duration of action is relatively short as it is rapidly metabolized by FAAH to arachidonic acid and ethanolamide. A protective role for anandamide in several pathologies, including pain, inflammation and anxiety has been clearly demonstrated and therefore inhibitors of FAAH are being developed for the treatment of these disorders (Cravatt and Lichtman, 2003). Increases in the endogenous levels of anandamide via inhibition of the primary enzymes responsible its metabolic inactivation would prolong its action but would also increase the likelihood for anandamide to undergo oxidation by a number of fatty acid oxygenases, including cyclooxygenase-2 (COX-2) lipoxygenase (LOX) and the cytochrome P450 enzymes. Understanding the biological significance of these oxidative pathways would lead to a

better understanding of the actions of these novel drug candidates. Indeed, recent work with the COX-2 and LOX enzymes has already started to shed light on this topic (Craib et al., 2001; Woodward et al., 2008). Our current report that the P450-derived epoxide of anandamide, 5,6-EET-EA is a potent, CB-2 selective agonist with increased biological stability significantly increases our understanding of both the physiological and pharmacological consequences of the P450 branch of anandamide oxidation.

The combination of our previous findings showing that several P450s are high-affinity, high-turnover anandamide oxygenases (Snider et al., 2007; Snider et al., 2008b) combined with the current data showing that the P450-derived anandamide metabolite 5,6-EET-EA activates the CB2 receptor at sub- and low nanomolar concentrations and has higher biological stability than anandamide lead us to conclude that oxidation of anandamide by P450s is not only likely to occur under physiological conditions but also that it may have important functional consequences. In addition, the data demonstrating that the anandamide-metabolizing P450s are induced in IFNγ-stimulated microglial cells points to the possible involvement of the P450 monooxygenases in mediating neuro-immune interactions.

The data supporting a role for epoxide hydrolase in the metabolism of 5,6-EET-EA may lead to a better understanding of the physiological mechanisms of action of epoxide hydrolase inhibitors. Soluble epoxide hydrolase metabolizes several fatty acid epoxides that are known to have vasodilatory properties (Imig, 2005). Therefore, inhibition of epoxide hydrolase is a promising avenue for the treatment of hypertension (Chiamvimonvat et al., 2007). Since inhibition of either FAAH or epoxide hydrolase could lead to the endogenous production of increased levels of 5,6-EET-EA, the findings

presented here may aid in understanding the pharmacological activity and the mechanism of action of some of these novel drug molecules.

In conclusion, the data presented here provide evidence for a functional connection between the endocannabinoid system and the cytochrome P450 monooxygenase family of enzymes in the context of immune cell function.

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Chapter V

Discussion and Future Directions

The endocannabinoid anandamide is a lipid mediator that has been shown to be important for CNS and immune system function and inhibitors of FAAH, the enzyme primarily responsible for inactivating it, are being developed as novel therapeutics for the treatment of neuropathic pain, inflammation and anxiety, among other indications (Giang and Cravatt, 1997; Cravatt and Lichtman, 2003; Di Marzo, 2008). The structural similarity of anandamide to arachidonic acid suggests that it would be amenable to oxidation by several enzymes, including the cytochrome P450 heme oxygenases (Bornheim et al., 1995; Snider et al., 2007; Snider et al., 2008; Stark et al., 2008). Some of the potential outcomes of anandamide oxidation by the P450s would include degradation, bioactivation, or formation of novel biologically active molecules. Therefore, we thought it would be important to explore these possibilities for the purpose of gaining a better understanding of endocannabinoid signaling and insights into the mechanisms of action of novel drug molecules like the FAAH inhibitors (Burstein et al., 2000; Kozak and Marnett, 2002; Kozak et al., 2004; Farrell and Merkler, 2008). The formation of 5 major mono-oxygenated products is described and these are the 20-HETE-EA and the 5,6-, 8,9-, 11,12- and 14,15-EET-EA (Snider et al., 2007). Also reported here

is the formation of at least 8 additional di-oxygenated products that are secondary metabolites from the EET-EAs resulting from epoxide hydrolase-mediated hydrolysis to form the DHET-EAs and 2D6-mediated hydroxylation to form the HEET-EAs (Snider et al., 2008). With respect to the biological significance, the P450 product of anandamide generated by epoxidation of the 5,6- double bond (5,6-EET-EA) was found to be a potent and selective agonist at the CB2 cannabinoid receptor, which is primarily expressed on immune cells (Munro et al., 1993; Cabral et al., 2008; Miller and Stella, 2008). In addition, 5,6-EET-EA exhibits increased biological stability in brain homogenates relative to anandamide and is metabolized primarily by epoxide hydrolase, rather than fatty acid amide hydrolase. Therefore, it is likely that 5,6-EET-EA may be an important endogenous immune mediator and that its formation represents a bioactivation pathway. These findings establish a functional connection between the endocannabinoid system and the cytochrome P450 monooxygenases.

The studies reported in Chapter II characterize the metabolism of anandamide by human kidney and liver microsomal P450s (Snider et al., 2007). The novel metabolites were structurally identified by using ESI-LC/MS/MS analysis and synthetic chemical standards. Due to the limited formation of unique fragment ions in the MS/MS analysis, the enzyme epoxide hydrolase was used to differentiate between the epoxides and the alcohols of anandamide. This type of use for epoxide hydrolase could be applied more widely towards distinguishing between the *in vitro* formation of epoxygenated and hydroxylated P450 products of other substrate molecules as well. Chemical and antibody inhibitors were used to identify the specific isoforms involved in forming the various

metabolites, revealing a role for P450s 3A4 and 4F2 in anandamide epoxidation and hydroxylation, respectively. This is one of the main differences between arachidonic acid and anandamide metabolism with regards to the particular isoforms involved. The ESI-LC/MS/MS method which we have developed for the separation and detection of the anandamide metabolites along with the availability of several authentic standards for the various products will aid in characterizing their *in vivo* formation and in determining the importance of anandamide oxidation by the P450s in normal physiology as well as in pathological conditions. Additionally, more valuable insights into their roles and importance will be provided by coupling the results from our work with knowledge that is being generated from the newly established LIPID MAPS project (Fahy et al., 2007; Sud et al., 2007) which is aimed at developing an integrated metabolomic system to characterize global changes in lipid metabolites.

The studies in Chapter III have identified P450 2D6 as a high-affinity anandamide hydroxylase and epoxygenase, as well as a high-affinity EET-EA hydroxylase (Snider et al., 2008b). The data presented in this chapter also support a role for mitochondrial P450 2D6 in the formation of the EET-EAs in human brain neocortical tissue. These findings are significant for several reasons. First, anandamide and the EET-EAs are the first eicosanoid-like molecules to be identified as P450 2D6 substrates, raising the possibility that P450 2D6 could be involved in the metabolism of other bioactive eicosanoids or other fatty acid amides such as the sleep-inducing oleamide and the anti-inflammatory and anti-nociceptive palmitoylethanolamide (Farrell and Merkler, 2008). Second, the highly polymorphic nature of P450 2D6 and the previously reported neurological and psychiatric differences among individuals with the various 2D6 phenotypes (Funae et al.,

2003; Miksys and Tyndale, 2004; Yu et al., 2004; Dorado et al., 2007b; Ingelman-Sundberg et al., 2007) coupled with our findings suggest that these differences might possibly be due to the involvement of this enzyme in the metabolism of anandamide as well as similar psychoactive substances. However, since the reported experiments utilized brain tissue from only 3 subjects, additional studies involving a larger pool of human brain tissue as well as various other anatomical regions of the brain are required in order to determine the extent of P450 2D6 involvement in endocannabinoid signaling in the human brain.

The data presented in Chapter IV demonstrate the ability of the anandamide epoxide, 5,6-EET-EA to activate the CB2 receptor and to exhibit increased refractoriness for undergoing enzymatic hydrolysis in brain tissue (Figure 5.1). The CB2 receptor is highly expressed on most immune cells and its activation generally leads to immunusuppression in part via transcriptional regulation of pro-inflammatory cytokines, such as tumor necrosis factor alpha and interleukin-6 (Klein et al., 2003). Within the CNS, the CB2 receptor appears to be predominantly expressed on activated microglial cells (Maresz et al., 2005; Cabral et al., 2008). Microglia, which are the resident macrophages in the brain have a "surveillance" phenotype under normal conditions, but upon perturbation of their microenvironment they undergo morphological and functional changes resembling a "reactive" phenotype which allows them to respond to the altered homeostasis via migration and the release of various immune modulators (Hanisch and Kettenmann, 2007; Gao and Hong, 2008). Both beneficial and detrimental effects of microglial activation upon neurons have been documented, depending on the stimulus as well as the spatial and temporal dynamics of the activation process (Nathan et al., 2005;

El Khoury et al., 2007; Fan et al., 2007; Majumdar et al., 2007; Takata et al., 2007). Utilization of anti-inflammatory therapy for reducing the harmful effects of microglia has been proposed based on numerous studies (Craft et al., 2005; Skaper, 2007). Selective cannabinoid receptor 2 agonists, which lack psychotropic properties, are one class of anti-inflammatory agents that have therapeutic potential in reducing inflammation associated with chronic conditions, such as Alzheimer's and multiple sclerosis as well as acute CNS injury such as stoke and trauma (Carrier et al., 2004; Maresz et al., 2005; Ortega-Gutierrez et al., 2005; Ramirez et al., 2005; Ashton and Glass, 2007; Ashton et al., 2007; Fernandez-Ruiz et al., 2007; Sagredo et al., 2007). Based on the data reported here, 5,6-EET-EA could be an endogenously produced mediator which can alter microglial activity. Therefore, its formation by microglial P450s was investigated in more detail and the data from those experiments are also reported in Chapter IV.

The difficulty in obtaining human microglia prompted us to use the murine microglial BV-2 cell line because it has been established as an appropriate model for studying the function of this cell type (Bocchini et al., 1992). It was determined that unstimulated BV-2 cells in culture metabolize anandamide to form the P450-derived hydroxylated and epoxygenated products. However, upon activation of the cells with the cytokine interferon gamma (IFNγ), microglia produce significantly more 5,6-EET-EA relative to the unstimulated cells, suggesting that the expression of P450 isoforms which are involved in the formation of this metabolite is induced upon IFNγ stimulation.

Immunoblot analysis revealed a significant, dose-dependent upregulation in the expression of mouse P450 proteins recognized by monoclonal antibodies against 4F13 and 3A1, whereas protein expression was not detected using an antibody against 2D6.

The lack of 2D6 detection is in agreement with the primarily neuronal localization of 2D6 within the CNS which has been previously reported (Siegle et al., 2001; Chinta et al., 2002; Funae et al., 2003). There are five mouse P450 4F isoforms (4F13, 4F14, 4F15, 4F16 and 4F18) and the physiological function of 4F13 is not known thus far (Kalsotra and Strobel, 2006). However, in contrast to the human P450 4F2, the data suggest that this particular isoform is not involved in anandamide hydroxylation based on the metabolic profile of the activated microglia where a decrease in 20-HETE-EA formation was detected. However, it would be a future interest to explore the catalytic activity of this enzyme in light of its apparent regulation by cytokine signaling.

On the other hand, P450 3A induction concomitant with an increase in 5,6-EET-EA formation by the activated microglia strongly suggests a role for P450 3A in the formation of the metabolite and this conclusion is further supported by the previously published data on the involvement of P450 3A in anandamide metabolism in the mouse brain and liver (Bornheim et al., 1995). In contrast to the IFNγ-stimulated induction of P450 3A protein expression which we observed in the microglia, previous reports by others have demonstrated an IFNγ-stimulated downregulation of 3A in human and rat hepatocytes (Tapner et al., 1996; Aitken and Morgan, 2007). This points to the possibility of cell-specific regulation of the expression of P450 3A by this cytokine and is in agreement with data demonstrating differential regulation of 3A protein expression in liver and brain (Robertson et al., 2003). Interestingly, the P450 3A4 *4 polymorphism (Ile118Val mutation) which produces an enzyme with decreased catalytic activity, was recently reported to be strongly associated (P=0.0006) with an increased risk for for hemorrhagic stroke in the Japanese population (Yamada et al., 2008). Whether a decrease

in the endogenous production of 5,6-EET-EA from anandamide is somehow behind this effect remains to be investigated, and measuring in vitro catalytic activity of P450 3A4*4 for the conversion of anandamide to 5,6-EET-EA is a possible future experiment which would provide some insight into this mechanism.

Another finding reported in Chapter IV which may have both physiological and pharmacological importance is the involvement of epoxide hydrolase as the primary enzyme responsible for the metabolism of 5,6-EET-EA in mouse brain homogenate to form the diol product, 5,6-DHET-EA (Figure 5.1). An important question is whether 5,6-DHET-EA, and not 5,6-EET-EA is the product which activates the CB2 receptor. Although that issue has not been investigated directly due to lack of a synthetic standard for 5,6-DHET-EA, we can speculate that 5,6-EET-EA is the active product based on its ability to inhibit cAMP accumulation in the CHO-CB2 cells during short, 10 min incubations and the previously reported low endogenous epoxide hydrolase activity of CHO cells (Sandberg et al., 2000).

During ischemic stroke in laboratory animals as well as in human patients, there is an increased release of several fatty acid ethanolamides, including anandamide (Schabitz et al., 2002; Muthian et al., 2004). A recent study reported that administration of the soluble epoxide hydrolase inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDA-BE) either before or after experimental ischemic stroke reduces infarct size by 40-50% (Zhang et al., 2007). This protective effect is almost completely reversed by co-administration of the P450 epoxygenase inhibitor 6-(2-propargyloxyphenyl) hexanoic acid (PPOH). However, it was found that the mechanism of protection by AUDA-BE was not due to vascular effects that would be characteristic of arachidonate-derived EETs

or altered ischemic severity, as determined by blood flow rates. This suggests an alternate mechanism for the protection by AUDA-BE. Because of the involvement of epoxide hydrolase in the metabolism of 5,6-EET-EA, it is possible that the observed protective mechanism of AUDA-BE in ischemic stroke may in part be due to prolongation of the action of 5,6-EET-EA at the CB2 receptor on microglia and/or infiltrating lymphocytes (Figure 5.1). Co-administration of a CB2-selective antagonist such as 6-iodo-2-methyl-1-[2-(4-morpholinyl) ethyl]-1H-indol-3-y l](4-methoxyphenyl) methanone (AM630) with AUDA-BE in the experimental cerebral ischemia model could provide more insight into this mechanism. Additionally, it would be beneficial to determine the effect of the epoxide hydrolase inhibitors upon the expression of the P450 anandamide epoxygenases since their upregulation could lead to the formation of higher levels of 5,6-EET-EA and this may be another possible mechanism for the observed protection from ischemic stroke.

The interaction of the P450 enzymes with the endocannabinoid system is likely to be important in other pathophysiological situations, in particular liver pathologies. A number of recent studies have demonstrated a protective role for the CB2 receptor (Figure 5.1) in hepatic ischemia/reperfusion injury as well as fibrogenesis associated to chronic liver diseases (Julien et al., 2005; Batkai et al., 2007; Mendez-Sanchez et al., 2007). It would be of interest to apply the knowledge on the identified anandamide metabolites, in particular 5,6-EET-EA, towards understanding the protective mechanism of CB2 receptor activation in liver disease, especially since many P540 isoforms, including 3A4 have been found to be downregulated during chronic liver disease (Yang et al., 2003; Horiike et al., 2005). Chemical synthesis of more stable and/or potent

analogs of 5,6-EET-EA will allow for functional studies in animal models of disease and a better understanding of its physiological role. Synthesis of the authentic standards for all of the reported metabolites will be of great value in elucidating their potential biological relevance.

In conclusion, the work presented in this thesis provides a better understanding of the endogenous roles of some of the cytochrome P450 monooxygenases by identifying various oxygenated metabolites of the endocannabinoid anandamide, the specific isoforms involved the formation of the metabolites in human liver, kidney and brain tissue and the pharmacological characterization of one of the products as a potent agonist at the cannabinoid CB2 receptor (Figure 5.1). It is our hope that this work will lead to a better understanding of the metabolic regulation of the anandamide tone in vivo as well as the involvement of the P450 enzymes in CNS and immune system function. Additionally, we hope that the findings will provide valuable insights into the potential physiological effects of inhibitors of fatty acid amide hydrolase and epoxide hydrolase and their pharmacological optimization for use in the treatment of various pathologies related to inflammation, vascular abnormalities and other disease processes (Figure 5.1).

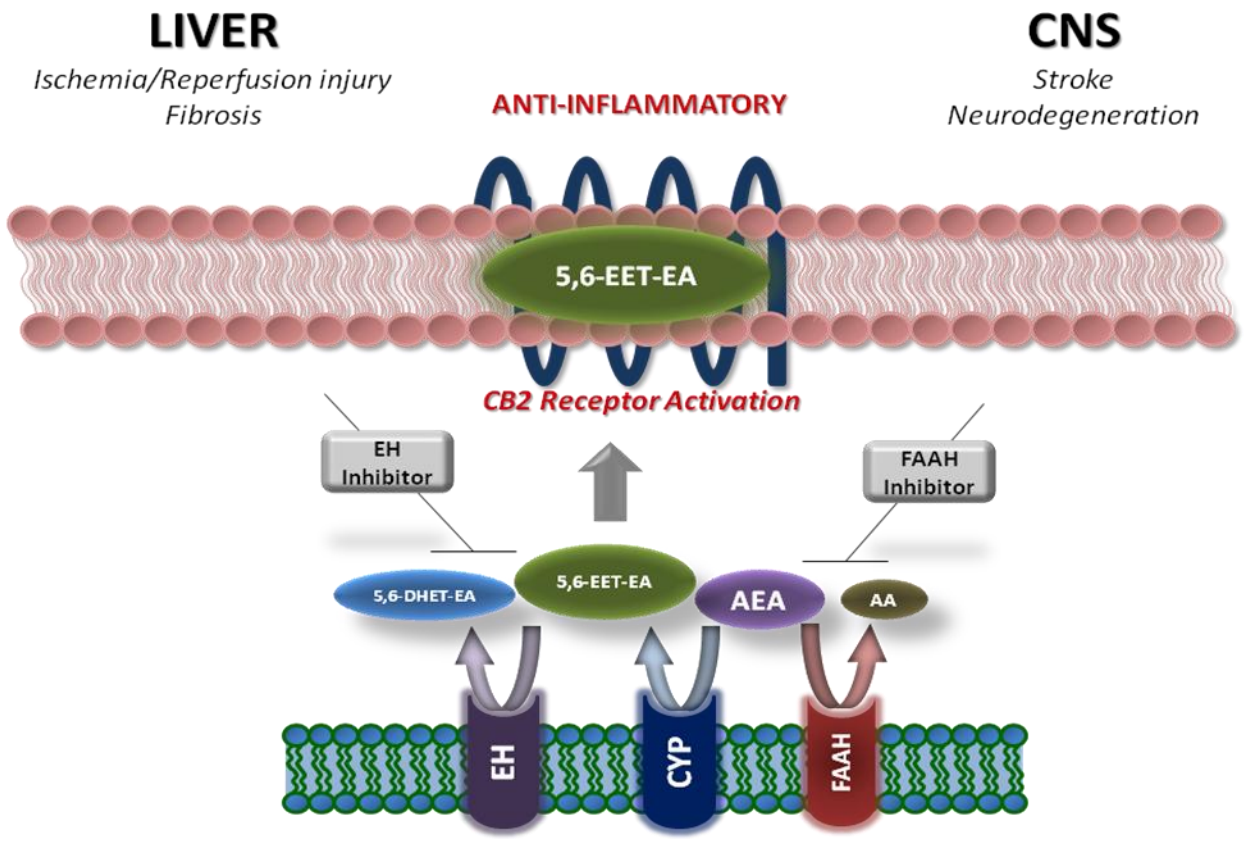

Figure 5.1. Potential anti-inflammatory actions of 5,6-EET-EA in hepatic and central nervous system (CNS) pathologies. Anandamide (AEA) is metabolized by fatty acid amide hydrolase (FAAH) to arachidonic acid (AA) and by cytochrome P450 (CYP) to 5,6-EET-EA. 5,6-EET-EA is in turn metabolized by epoxide hydrolase (EH) to 5,6-DHET-EA. Novel drug candidates such as the FAAH and the EH inhibitors could lead to an increase in the formation of endogenously produced 5,6-EET-EA by affecting its synthesis or its degradation. The cannabinoid receptor 2 (CB2), which is primarily expressed on immune cells, is activated by 5,6-EET-EA and this activation may lead to important anti-inflammatory events which could alter the pathological outcome in both acute and chronic conditions affecting the liver and the CNS. EET-EA, epoxyeicosatrienoic acid ethanolamide; DHET-EA, dihydroxyeicosatrienoic acid ethanolamide.

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