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

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ABSTRACT

Members of the cytochrome P450 (P450) 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 P450 isoforms and has been implicated in neurodegeneration, psychosis, schizophrenia, and personality traits. The objective of this study was to determine whether 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 and 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EET-EAs) with low micromolar K_m values. CYP2D6 further metabolized the epoxides of anandamide to form novel dioxygenated 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-like 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 neuropsychiatric phenotype differences among individuals with genetic variations in CYP2D6 could be ascribable to interactions of this enzyme with endogenous substrates.

The endogenous cannabinoids (endocannabinoids) anandamide and 2-arachidonoylglycerol 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, and 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 neuropsychiatric 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 cytochromes P450 (P450s), in particular the involvement of P450s 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 whether it is a substrate for the P450 enzymes known to be present in the brain. Of particular interest is CYP2D6, which has been implicated in a number of neurological and neuropsychiatric conditions due to a proposed interaction with endogenous substrates (Llerena et al., 1993; Yu et al., 2003a,b, 2004; Gervasini et al., 2004).

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ABBREVIATIONS: FAAH, fatty acid amide hydrolase; P450, cytochrome P450; EET-EA, epoxyeicosatrienoic acid ethanolamide; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry; HETE, hydroxyeicosatetraenoic acid; AA, arachidonic acid; TIC, total ion chromatogram; COX, cyclooxygenase; LOX, lipoxygenase; HET0016, *N*-hydroxy-*N*′-(4-butyl-2-methylphenyl)formamidine.

CYP2D6 is a phase I xenobiotic metabolizing enzyme that is known to be involved in the oxidation of 20 to 30% of the most commonly prescribed drugs, many of which modulate cardiovascular and central nervous system function (Ingelman-Sundberg, 2005). This enzyme is highly polymorphic with more than 80 variant alleles identified to date, including the nonfunctional CYP2D6*4 null allele, which is present in 12 to 21% of Caucasians (Ingelman-Sundberg, 2005). In addition to hepatic expression, 2D6 is one of the major P450 isoforms in the human brain and has 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 and 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 dioxygenated products.

Materials and Methods

Reagents. Arachidonic acid, anandamide, anandamide metabolite standards, and the CYP4A/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 P450s, including CYP2D6 (MAb 50-1-3) (Gelboin and Krausz, 2006) were kindly provided by Dr. Harry Gelboin (National Institutes of Health, Bethesda, MD). Immunoblotting antibody against cytochrome *c* was purchased from Abcam Inc. (Cambridge, MA). All other chemicals were of highest quality and available from commercial sources.

Protein Purification. NADPH-cytochrome P450 reductase and b_5 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 to 15 min. The white matter was separated (and discarded) from the gray 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. After separation, the human tissues were frozen at −80°C until used to prepare the 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 (Voirol et al., 2000). For the cytochrome *c* immunoblot experiments, 40 μg of protein from each sample was resolved on a 15% SDS-polyacrylamide gel electrophoresis gel, and blotted onto a polyvinylidene difluoride membrane. The membranes were blocked overnight in blocking buffer (20 mM Tris, 140 mM NaCl, 0.1% Tween 20, 5% milk) followed by incubation with primary antibody against cytochrome *c* and subsequent incubation with secondary horseradish peroxidase-conjugated antibody. All antibody dilutions were according to the manufacturer's recommendations.

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. In brief, 1 μl of cell lysate was added to a 25-μl reaction mixture containing polymerase chain reaction master mix (Applied Biosystems), 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 polymerase chain reaction 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 min on ice. Cytochrome-b_5 and superoxide dismutase were also included in the reaction mixture in some experiments, as described in the figure legends. The metabolism of anandamide or the epoxyeicosatrienoic acid ethanolamides (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 min 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 down 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-liquid chromatography/mass spectrometry (ESI-LC/MS) analysis as described below. For the antibody inhibition studies using the microsomal or mitochondrial proteins, the reaction mixtures were preincubated with either the inhibitory monoclonal antibody to CYP2D6 or a nonimmunogenic control antibody (hen egg lysozyme) for 5 min before 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 × 100 mm; Thermo Fisher Scientific, 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 Fisher Scientific). 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 Fisher Scientific) with one full scan from 300 to 500 mass/charge ratio (*m/z*) followed by one data-dependent scan of the most intense ion.

Data Analysis. Nonlinear regression analyses of the data were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., 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). It is not surprising that 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 Fig. 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 1 and 250 μM (data not shown). 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. It is interesting that in contrast to arachidonic acid, anandamide is a substrate for the enzyme, and it is extensively metabolized to give a number of products (Fig. 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 *m/z* of 364, CYP2D6 also formed several dioxygenated products with *m/z* of 380. To improve coupling of the P450 catalytic cycle and to rule out the possibility that superoxide or hydroxyl radicals may contribute to the formation of the anandamide epoxides, cytochrome-b_5 and superoxide dismutase were also included in the reactions. As shown in Fig. 1C, the formation of 20-HETE-ethanolamide and the EET-ethanolamides was not significantly affected under these conditions. These data demonstrate that

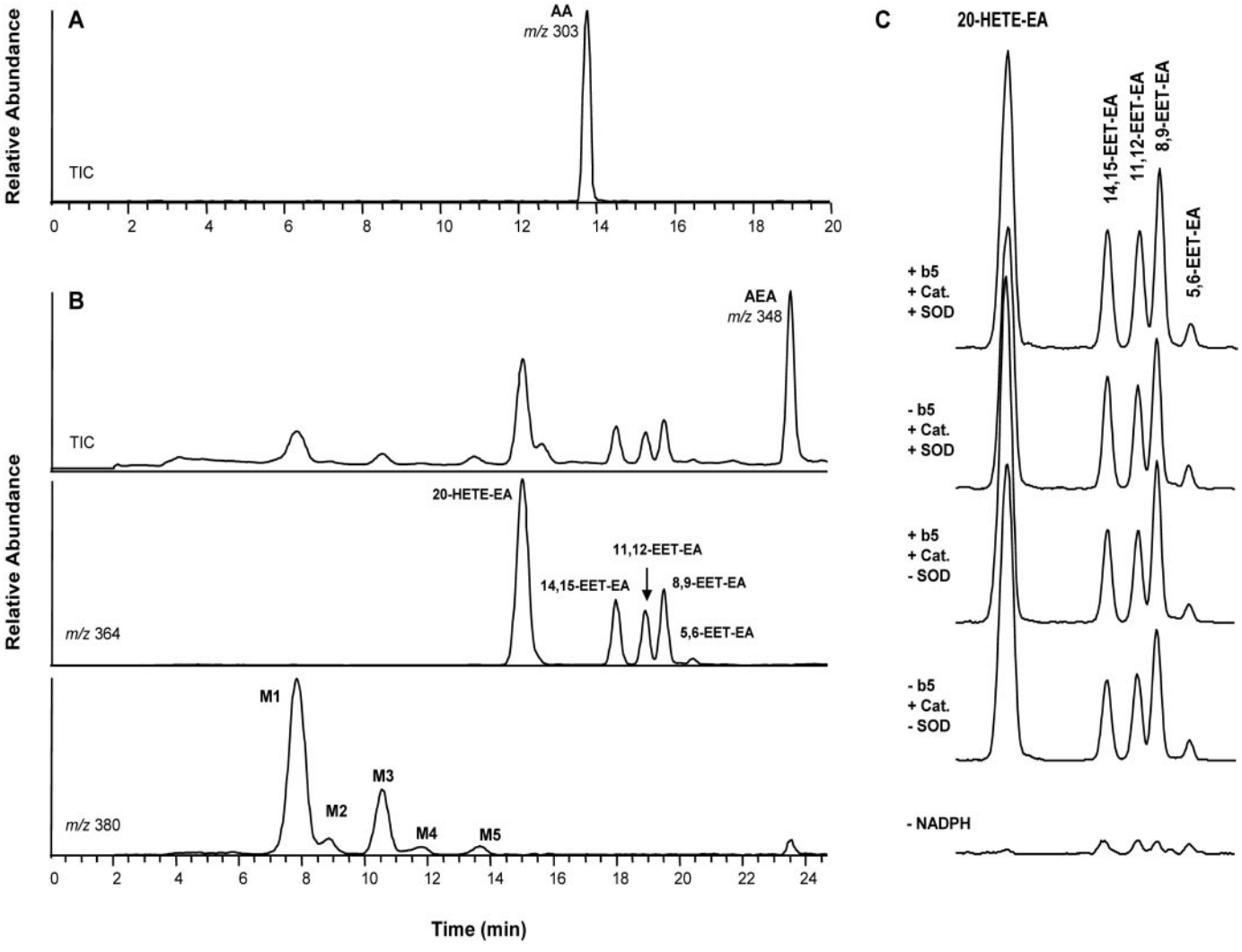

Fig. 1. Metabolism of arachidonic acid and anandamide by human recombinant CYP2D6. Recombinant CYP2D6 protein (25 pmol) was incubated in the reconstituted system in the presence of arachidonic acid (10 μM) or anandamide (10 μM) for 10 min, and the samples were analyzed as described under *Materials and 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).

anandamide is a substrate for CYP2D6 and that the CYP2D6 active site can accommodate an eicosanoid-like structure.

Time Dependence of Anandamide Metabolite Formation by Human Recombinant CYP2D6 and Secondary Metabolism of the EET-EAs. The possibility that the dioxygenated metabolites were secondary products formed from the metabolism of 20-HETE and/or EET ethanolamides was investigated by monitoring their formation over time. The selected ion chromatograms (*m/z*, 364, 380) in Fig. 2A show the metabolic profiles obtained after 2 (solid line) and 20 (dotted line) min of reaction time. The amounts of products M1 to M5 (*m/z*, 380) and 20-HETE-EA formed are significantly increased after 20 min, whereas the amounts of the EET-EAs are increased to a much lesser extent, raising the possibility that M1 to M5 are the secondary products of the EET-EAs. To investigate that further, the peak intensities for 14,15-EET-EA obtained from incubations that were terminated after various times of up to 60 min were compared with 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 Fig. 2B, the peak intensity of product M1 continued to increase over the 60 min, whereas the peak intensity of 14,15-EET-EA reached a maximum at approximately 10 min and continuously decreased thereafter. Similar results were obtained when the formation of the other EET-EAs was compared with the formation of the dioxygenated 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, dioxygenated products. Results obtained from reactions where CYP2D6 was incubated in the presence of each individual EET-EA are shown in Fig. 3 (top four panels). Multiple oxygenated products resulted from the incubation of an individual EET-EA with CYP2D6, and these products matched the retention times of the dioxygenated metabolites formed from CYP2D6 incubation with anandamide. These products most likely result from the hydroxylation of the EET-EAs at positions C16 to C20 because incubation of 5,6-EET-EA or 14,15-EET-EA with the anandamide ω-hydroxylase CYP4F2 re-

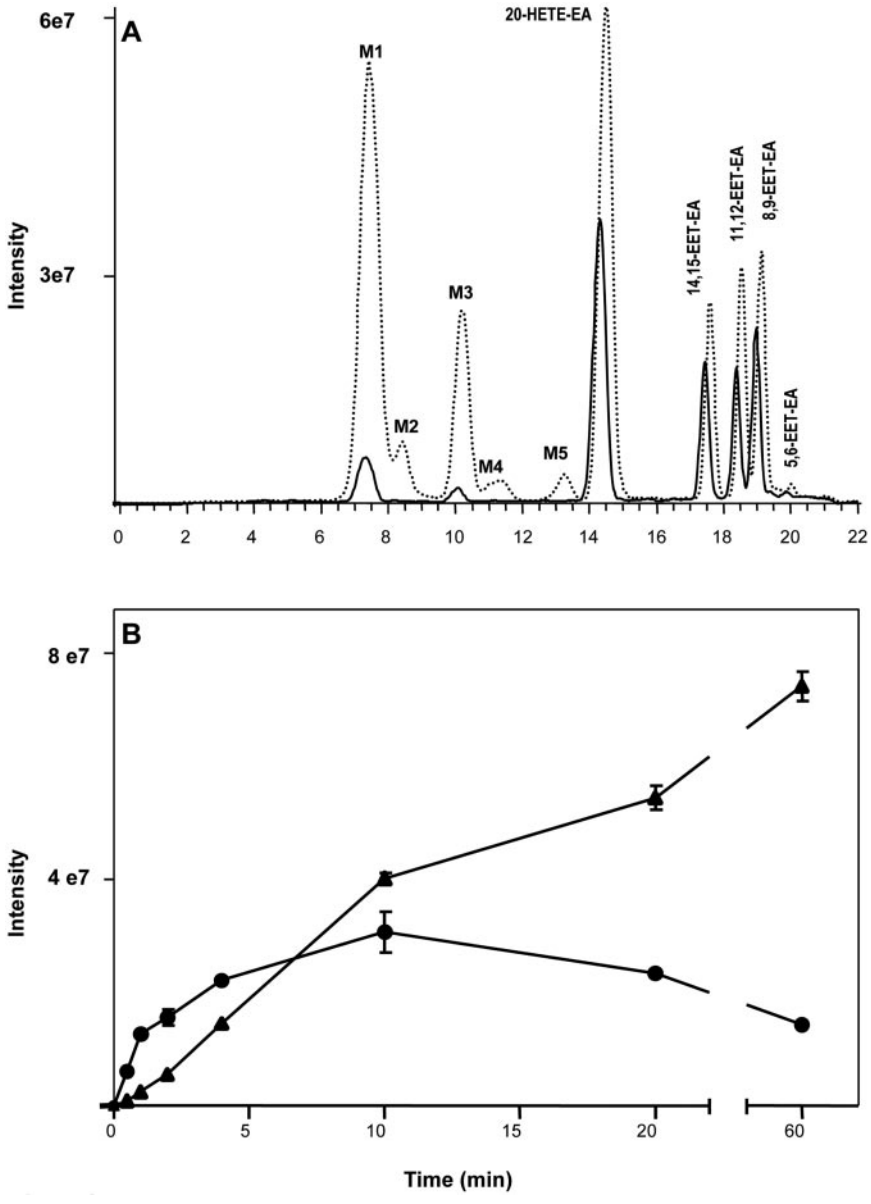

Fig. 2. Time course for the formation of metabolites of anandamide by CYP2D6. Anandamide (20 μM) was incubated with 10 pmol CYP2D6 in the reconstituted system, and the reactions were terminated at the times indicated and analyzed as described under *Materials and Methods*. The selected ion chromatograms (*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.

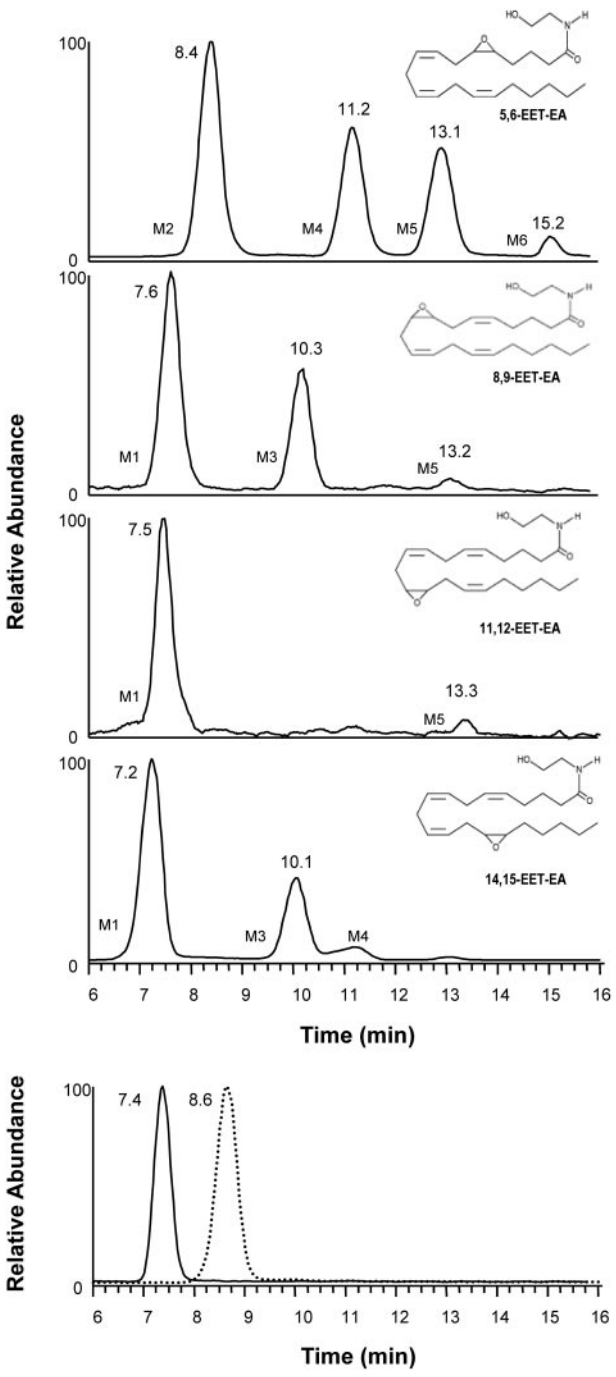

Fig. 3. Metabolism of 5,6-, 8,9-, 11,12-, and 14,15-EET-EA by CYP2D6. The four EET-EAs (10 μM each) were incubated with 25 pmol CYP2D6 in the reconstituted system (top four chromatograms). Bottom panel, results obtained from incubations containing 25 pmol CYP4F2 supersomes and either 5,6-EET-EA (dotted 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 Fig. 1 according to retention times.

sulted in the formation of metabolites with identical retention times to M1 and M2, which were observed in the presence of CYP2D6 (Fig. 3, bottom).

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 Fig. 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 μM 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–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 Fig. 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 Fig. 5. It is interesting to note that 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 mitochondrial preparations. However, unlike our previous finding involving the liver microsomal metabolism of 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 known to be 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 P450 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 that has been previ-

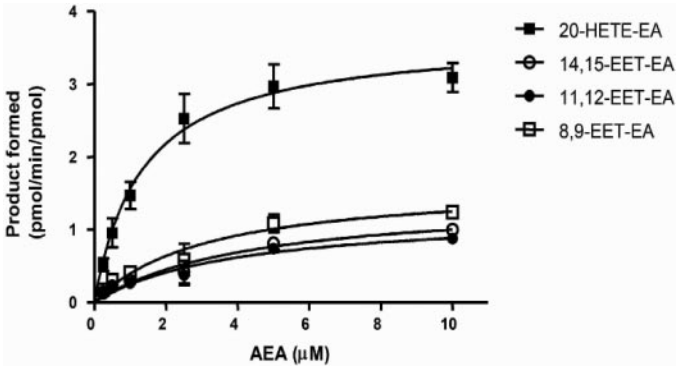

Fig. 4. Kinetics of anandamide metabolite formation by human recombinant CYP2D6. Reaction mixtures containing 5 pmol CYP2D6 protein and the concentrations of anandamide indicated (0.25–10 μM) were incubated for 10 min at 37°C. The amount of products formed was determined based on a standard curve generated for each metabolite, and the rate data (average of three experiments) were fitted to a one-enzyme Michaelis-Menten model using Prism software.

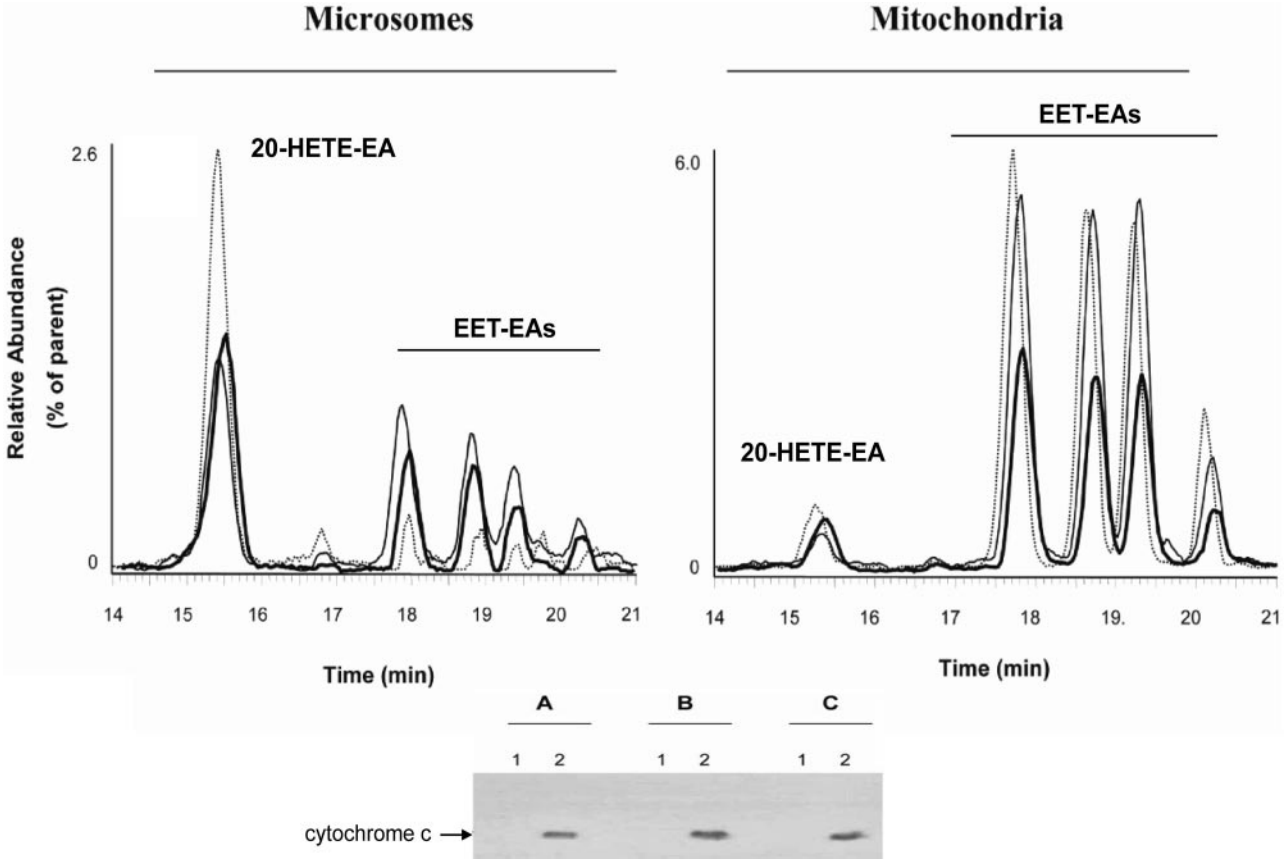

Fig. 5. Anandamide metabolism by human neocortical brain microsomes and mitochondria. Anandamide (20 μM) was incubated in the presence of 100 μg of microsomal or mitochondrial protein from subjects A (dotted line), B (solid bold line), or C (solid line) for 10 min, and the samples were analyzed as described 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*.

ously described (Krausz et al., 1997) was used to determine whether 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 preincubation of recombinant CYP2D6 in the reconstituted system with the antibody (5 μl of antibody/0.5-ml reaction volume) for 5 min. Under these conditions, the amount of anandamide metabolism by CYP2D6 was reduced by 88 ± 2.2% in comparison with the control antibody; therefore, this concentration of antibody was used in subsequent experiments. Preincubation of the microsomal preparations from all three samples with the CYP2D6 inhibitory antibody essentially had no effect on 20-HETE-EA formation, as shown in a representative chromatogram obtained using microsomes from subject A (Fig. 6A, top). 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 Fig. 6A, bottom. 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 among the three brain samples, the patient samples were genotyped for the common nonfunctional CYP2D6 alleles *4, *6, and *3 as described under *Materials and Methods*. Genotyping revealed that although 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 C expresses any functional CYP2D6. 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 P450 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 nonfunctional 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., 2007). 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

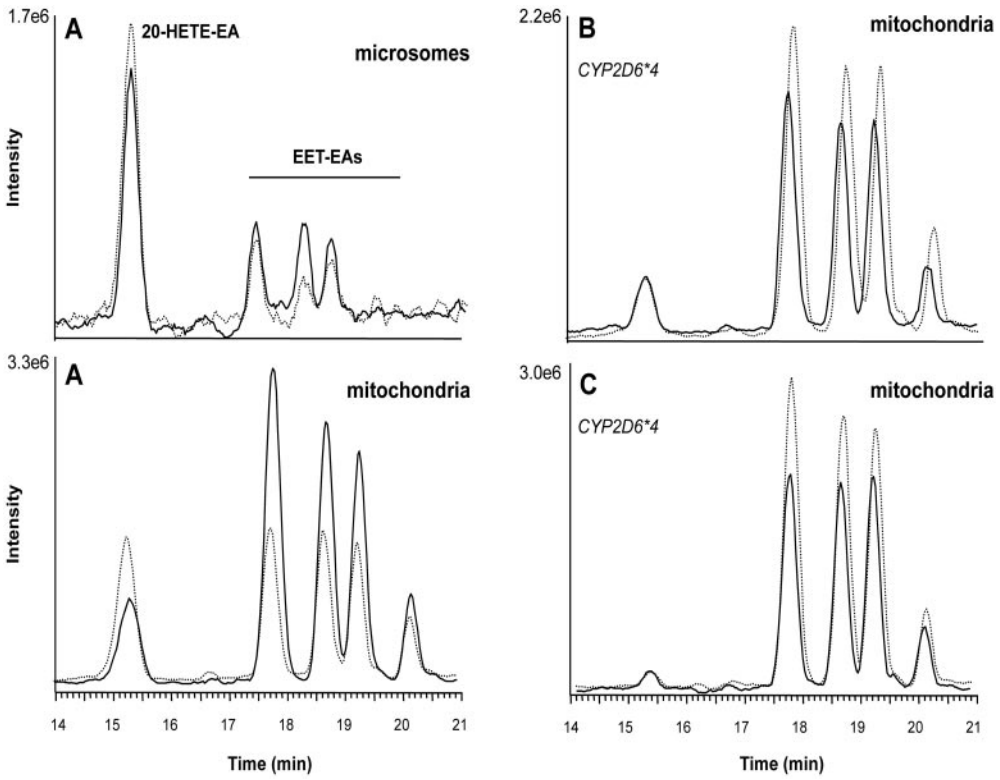

Fig. 6. Effect of a monoclonal inhibitory antibody against CYP2D6 on anandamide metabolism by human brain microsomes and mitochondria. Microsomal protein sample from subject A and mitochondrial protein samples from subjects A, B, and C were preincubated in the presence of hen egg lysozyme (control) antibody (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*.

et al., 2000; Siegle et al., 2001; Miksys et al., 2002; Yu et al., 2003a,b).

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 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 cannot be excluded.

The potential for CYP2D6-mediated metabolism could be increased in situations where the activity of FAAH is inhibited or where CYP2D6 levels are elevated. Because FAAH inhibition is considered a promising therapeutic intervention in the management of inflammatory pain and anxiety disorders (Cravatt and Lichtman, 2003), the oxidative route of anandamide metabolism catalyzed by CYP2D6 may become of greater importance under those conditions. With regard to CYP2D6 expression among the population, Miksys et al. (2002) found a significant increase in CYP2D6 protein level in 13 different brain regions of alcoholics, including the frontal cortex, temporal cortex, hippocampus, and substantia nigra, all of which are areas of high CB1 expression and where anandamide is known to be produced. Therefore, the regional origin of the brain tissue used in these studies (frontal and temporal cortex) is relevant to both the endocannabinoid system and CYP2D6 expression.

With regard to the potential biological significance of the oxidative pathways of anandamide metabolism by P450s and 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 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 that 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 vanilloid 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 P450-derived anandamide products.

In conclusion, there are several key finding 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 that possess similar structural properties. Second, it demonstrates that anandamide can be metabolized to the same products by multiple microsomal and mitochondrial P450s 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, CYP3A4 protein in the human brain has been detected in the striatum,

cerebellum, and hippocampus, whereas CYP2D6 protein 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 neuropsychiatric 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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