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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 (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 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 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-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.

Keywords

Metabolism; Transport; Pharmacogenomics

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

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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 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 neuropsychiatric 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 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, 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 di-oxygenated products.

Methods

Reagents

Arachidonic acid, anandamide, 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). Immunoblotting antibody against cytochrome c was purchased from Abcam (Cambridge, MA). All other chemicals were of highest quality and available from commercial sources.

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Protein purification

NADPH-cytochrome P450 reductase and 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 the microsomes and mitochondria.

Preparation of human brain microsomes and mitochondria and immunoblotting for cytochrome c and CYP2D6

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-PAGE gel, and blotted onto a PVDF membrane. The membranes were blocked overnight in blocking buffer (20mM Tris, 140mM NaCl, 0.1% Tween 20, 5% milk) followed by incubation with primary antibody against cytochrome c and subsequent incubation with secondary HRP-conjugated antibodies. All antibody dilutions were according to manufacturer 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. 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 figure legends. 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

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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 (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 × 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 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 (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. 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 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. 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 b5 and superoxide dismutase were also included in the reactions. As shown in Figure 1C, the formation of 20-HETE-ethanolamide and the EET-ethanolamides was not significantly affected under

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these conditions. These data demonstrate that 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 Figure 2A show the metabolic profiles obtained after 2 minutes (solid line) and 20 minutes (dotted line) of reaction time. The amounts of products M1-M5 (*m/z* 380) as well as 20-HETE-EA formed are significantly increased after 20 minutes, whereas the amounts of the EET-EAs are increased to a much lesser extent, raising the possibility that M1-M5 are the secondary products of the EET-EAs. To investigate that further, the peak intensities for 14,15-EETEA 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 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. Results obtained from reactions where CYP2D6 was incubated in the presence of each individual EET-EA are shown in Figure 3 (top 4 panels). Multiple oxygenated products resulted from the incubation of an individual EET-EA with CYP2D6 and these products matched the retention times of the di-oxygenated metabolites formed from CYP2D6 incubation with anandamide. 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 M1 and M2 which were observed in the presence of CYP2D6 (Figure 3, bottom panel).

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 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 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

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Figure 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 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 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 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 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 (Figure 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 Figure 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 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. 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., 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 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

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(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.

The potential for CYP2D6-mediated metabolism could be increased in situations where the activity of FAAH is inhibited or where CYP2D6 levels are elevated. Since 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 regards to CYP2D6 expression among the population, Miksys et al. 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. The regional origin of the brain tissue used in these studies (frontal and temporal cortex) is therefore relevant to both the endocannabinoid system and CYP2D6 expression.

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 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 CYP-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 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, CYP3A4 protein in the human brain has been detected in the striatum, cerebellum and the 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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Abbreviations

CYP, cytochrome P450; AA, arachidonic acid; AEA, anandamide; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; EA, ethanolamide; FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; LOX, lipoxygenase; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry.

References

Bhagwat SV, Boyd MR, Ravindranath V. Multiple forms of cytochrome P450 and associated monooxygenase activities in human brain mitochondria. Biochem Pharmacol 2000;59:573–582. [PubMed: 10660123]

Burstein SH, Rossetti RG, Yagen B, Zurier RB. Oxidative metabolism of anandamide. Prostaglandins Other Lipid Mediat 2000;61:29–41. [PubMed: 10785540]

Capdevila JH, Falck JR. The CYP P450 arachidonic acid monooxygenases: from cell signaling to blood pressure regulation. Biochem Biophys Res Commun 2001;285:571–576. [PubMed: 11453630]

Craib SJ, Ellington HC, Pertwee RG, Ross RA. A possible role of lipoxygenase in the activation of vanilloid receptors by anandamide in the guinea-pig bronchus. Br J Pharmacol 2001;134:30–37. [PubMed: 11522594]

Cravatt BF, Lichtman AH. Fatty acid amide hydrolase: an emerging therapeutic target in the endocannabinoid system. Curr Opin Chem Biol 2003;7:469–475. [PubMed: 12941421]

Di Marzo V, Bifulco M, De Petrocellis L. The endocannabinoid system and its therapeutic exploitation. Nat Rev Drug Discov 2004;3:771–784. [PubMed: 15340387]

Dorado P, Penas-Lledo EM, Llerena A. CYP2D6 polymorphism: implications for antipsychotic drug response, schizophrenia and personality traits. Pharmacogenomics 2007;8:1597–1608. [PubMed: 18034624]

Gelboin HV, Krausz K. Monoclonal antibodies and multifunctional cytochrome P450: drug metabolism as paradigm. J Clin Pharmacol 2006;46:353–372. [PubMed: 16490812]

Gervasini G, Carrillo JA, Benitez J. Potential role of cerebral cytochrome P450 in clinical pharmacokinetics: modulation by endogenous compounds. Clin Pharmacokinet 2004;43:693–706. [PubMed: 15301574]

Hampson AJ, Hill WA, Zan-Phillips M, Makriyannis A, Leung E, Eglen RM, Bornheim LM. Anandamide hydroxylation by brain lipoxygenase:metabolite structures and potencies at the cannabinoid receptor. Biochim Biophys Acta 1995;1259:173–179. [PubMed: 7488638]

Hanna IH, Kim MS, Guengerich FP. Heterologous expression of cytochrome P450 2D6 mutants, electron transfer, and catalysis of bufuralol hydroxylation: the role of aspartate 301 in structural integrity. Arch Biochem Biophys 2001;393:255–261. [PubMed: 11556812]

Hanna IH, Teiber JF, Kokones KL, Hollenberg PF. Role of the alanine at position 363 of cytochrome P450 2B2 in influencing the NADPH- and hydroperoxide-supported activities. Arch Biochem Biophys 1998;350:324–332. [PubMed: 9473308]

Ingelman-Sundberg M. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. Pharmacogenomics J 2005;5:6–13. [PubMed: 15492763]

Kozak KR, Crews BC, Morrow JD, Wang LH, Ma YH, Weinander R, Jakobsson PJ, Marnett LJ. Metabolism of the endocannabinoids, 2-arachidonylglycerol and anandamide, into prostaglandin,

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thromboxane, and prostacyclin glycerol esters and ethanolamides. J Biol Chem 2002;277:44877–44885. [PubMed: 12244105]

Kozak KR, Marnett LJ. Oxidative metabolism of endocannabinoids. Prostaglandins Leukot Essent Fatty Acids 2002;66:211–220. [PubMed: 12052037]

Krausz KW, Yang TJ, Gonzalez FJ, Shou M, Gelboin HV. Inhibitory monoclonal antibodies to human cytochrome P450 2D6. Biochem Pharmacol 1997;54:15–17. [PubMed: 9296346]

Llerena A, Edman G, Cobaleda J, Benitez J, Schalling D, Bertilsson L. Relationship between personality and debrisoquine hydroxylation capacity. Suggestion of an endogenous neuroactive substrate or product of the cytochrome P4502D6. Acta Psychiatr Scand 1993;87:23–28. [PubMed: 8424321]

Maccarrone M, van der Stelt M, Rossi A, Veldink GA, Vliegenthart JF, Agro AF. Anandamide hydrolysis by human cells in culture and brain. J Biol Chem 1998;273:32332–32339. [PubMed: 9822713]

Miksys S, Rao Y, Hoffmann E, Mash DC, Tyndale RF. Regional and cellular expression of CYP2D6 in human brain: higher levels in alcoholics. J Neurochem 2002;82:1376–1387. [PubMed: 12354285]

Pacher P, Batkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. Pharmacol Rev 2006;58:389–462. [PubMed: 16968947]

Petersdorf EW, Deeg HJ. Diagnostic use of molecular probes before and after marrow transplantation. Clin Lab Med 1992;12:113–128. [PubMed: 1563237]

Roberts RL, Luty SE, Mulder RT, Joyce PR, Kennedy MA. Association between cytochrome P450 2D6 genotype and harm avoidance. Am J Med Genet B Neuropsychiatr Genet 2004;127:90–93. [PubMed: 15108188]

Rowland P, Blaney FE, Smyth MG, Jones JJ, Leydon VR, Oxbrow AK, Lewis CJ, Tennant MG, Modi S, Eggleston DS, Chenery RJ, Bridges AM. Crystal structure of human cytochrome P450 2D6. J Biol Chem 2006;281:7614–7622. [PubMed: 16352597]

Siegle I, Fritz P, Eckhardt K, Zanger UM, Eichelbaum M. Cellular localization and regional distribution of CYP2D6 mRNA and protein expression in human brain. Pharmacogenetics 2001;11:237–245. [PubMed: 11337939]

Snider NT, Kornilov AM, Kent UM, Hollenberg PF. Anandamide metabolism by human liver and kidney microsomal cytochrome p450 enzymes to form hydroxyeicosatetraenoic and epoxyeicosatrienoic acid ethanolamides. J Pharmacol Exp Ther 2007;321:590–597. [PubMed: 17272674]

Steffens M, Schulze-Bonhage A, Surges R, Feuerstein TJ. Fatty acid amidohydrolase in human neocortex-activity in epileptic and non-epileptic brain tissue and inhibition by putative endocannabinoids. Neurosci Lett 2005;385:13–17. [PubMed: 15923084]

Voirol P, Jonzier-Perey M, Porchet F, Reymond MJ, Janzer RC, Bouras C, Strobel HW, Kosel M, Eap CB, Baumann P. Cytochrome P-450 activities in human and rat brain microsomes. Brain Res 2000;855:235–243. [PubMed: 10677595]

Woodland C, Huang TT, Gryz E, Bendayan R, Fawcett JP. Expression, activity and regulation of CYP3A in human and rodent brain. Drug Metab Rev 2008;40:149–168. [PubMed: 18259987]

Woodward DF, Liang Y, Krauss AH. Prostamides (prostaglandinethanolamides) and their pharmacology. Br J Pharmacol 2008;153:410–419. [PubMed: 17721551]

Yu AM, Idle JR, Byrd LG, Krausz KW, Kupfer A, Gonzalez FJ. Regeneration of serotonin from 5-methoxytryptamine by polymorphic human CYP2D6. Pharmacogenetics 2003a;13:173–181. [PubMed: 12618595]

Yu AM, Idle JR, Gonzalez FJ. Polymorphic cytochrome P450 2D6: humanized mouse model and endogenous substrates. Drug Metab Rev 2004;36:243–277. [PubMed: 15237854]

Yu AM, Idle JR, Herraiz T, Kupfer A, Gonzalez FJ. Screening for endogenous substrates reveals that CYP2D6 is a 5-methoxyindolethylamine O-demethylase. Pharmacogenetics 2003b;13:307–319. [PubMed: 12777961]

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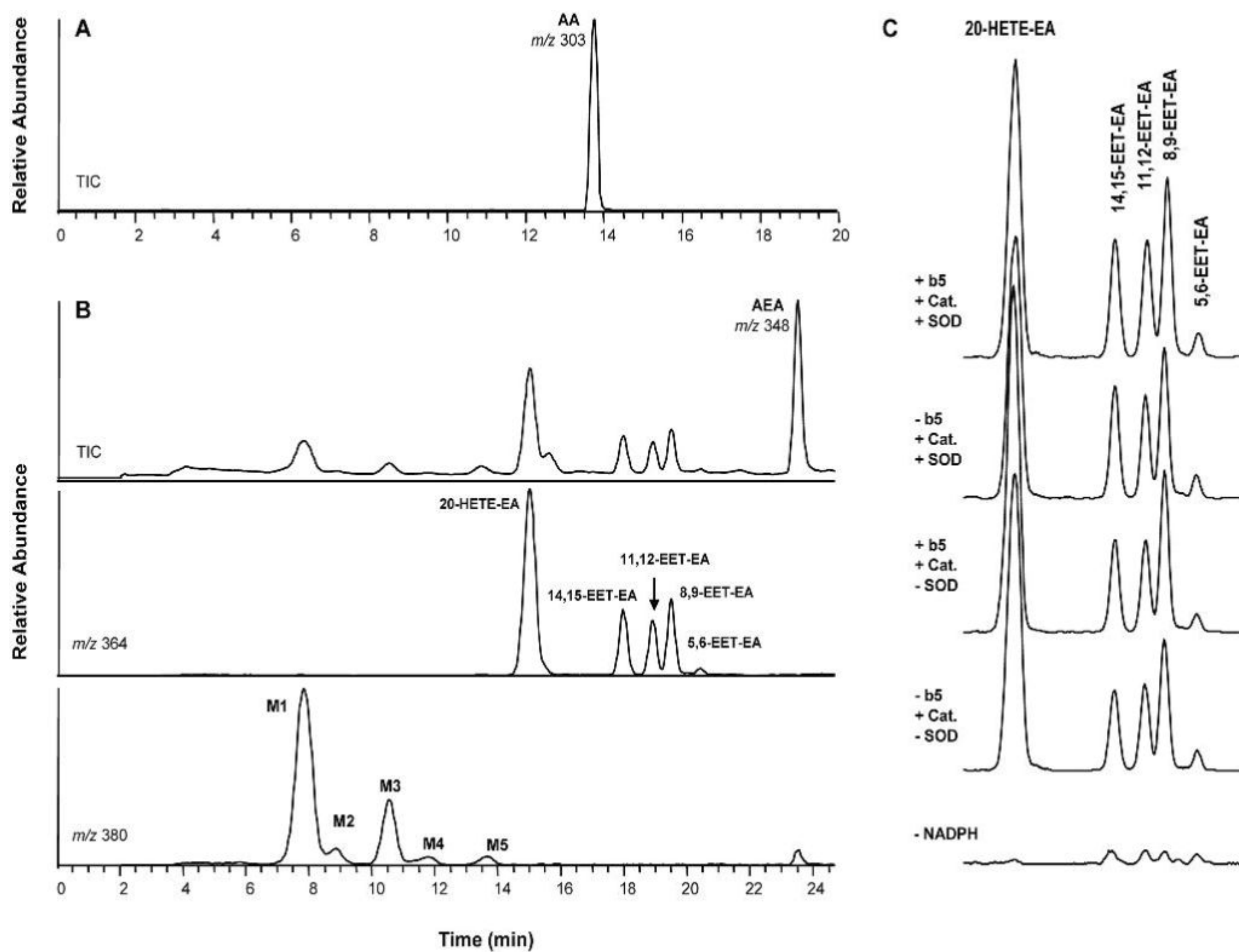

Figure 1. Metabolism of arachidonic acid and anandamide by human recombinant CYP2D6

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 Methods. The total ion chromatogram (TIC) in A shows the arachidonic acid (AA) peak (*m/z* 303) and the absence of any metabolic products. The TIC in panel B shows the anandamide (AEA) peak (*m/z* 348) and the presence of several products. The selected ion chromatograms of the monooxygenated (*m/z* 364) and dioxygenated (*m/z* 380) M1-M5 anandamide products are shown in the bottom two panels. Shown in panel C are 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 b5 (1:1 molar ratio with CYP2D6) or superoxide dismutase (SOD).

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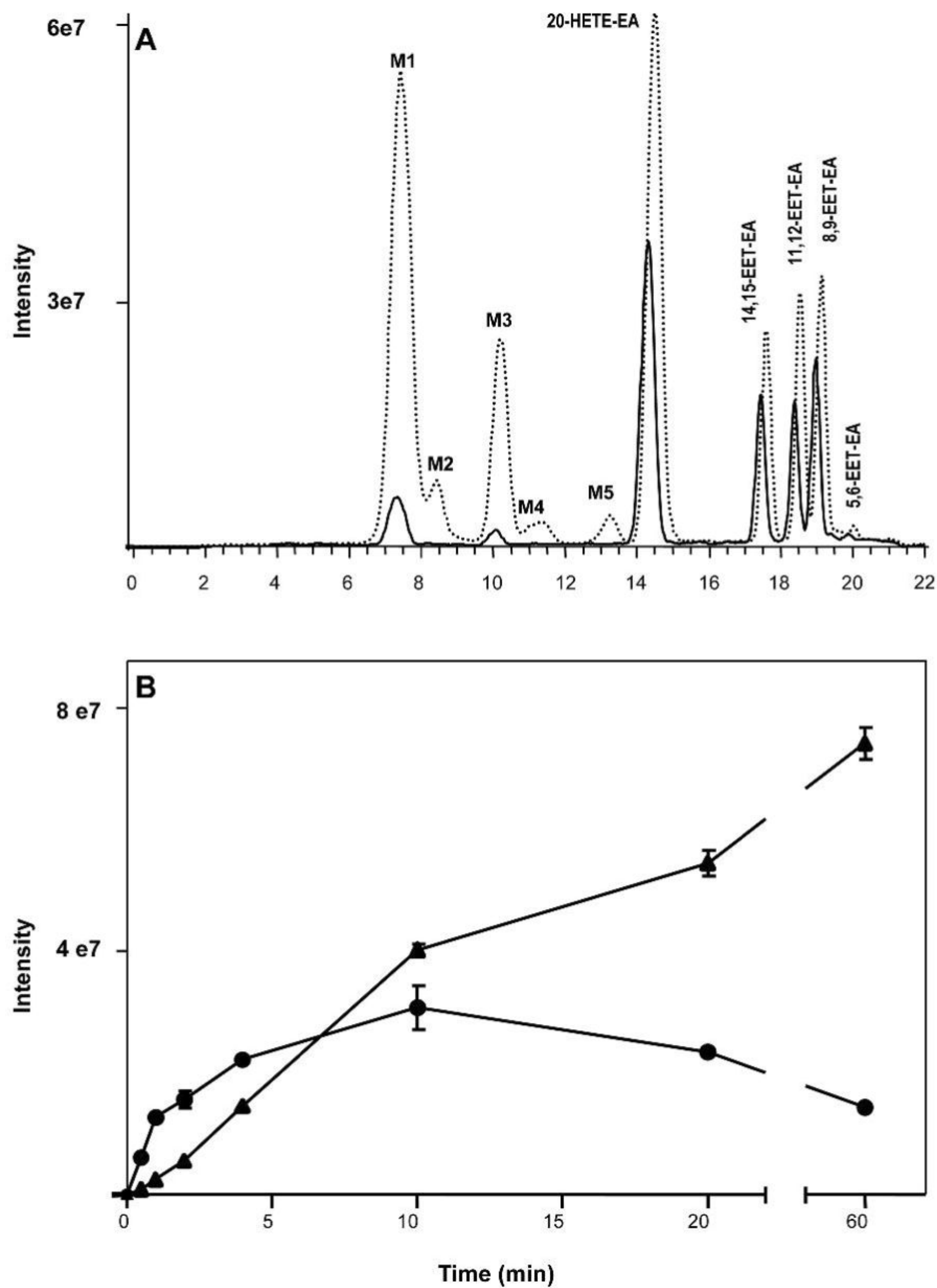

Figure 2. Time course for the formation of metabolites of anandamide by CYP2D6

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 in Methods. The selected ion chromatograms (*m/z* 364, 380) in A depict the metabolic profiles after 2 min (solid line) and 20 min (dotted line) of reaction time. Shown in panel B are the peak intensities for 14,15-EET-EA (●) and product M1 (▲) observed for incubations carried out for the time periods indicated.

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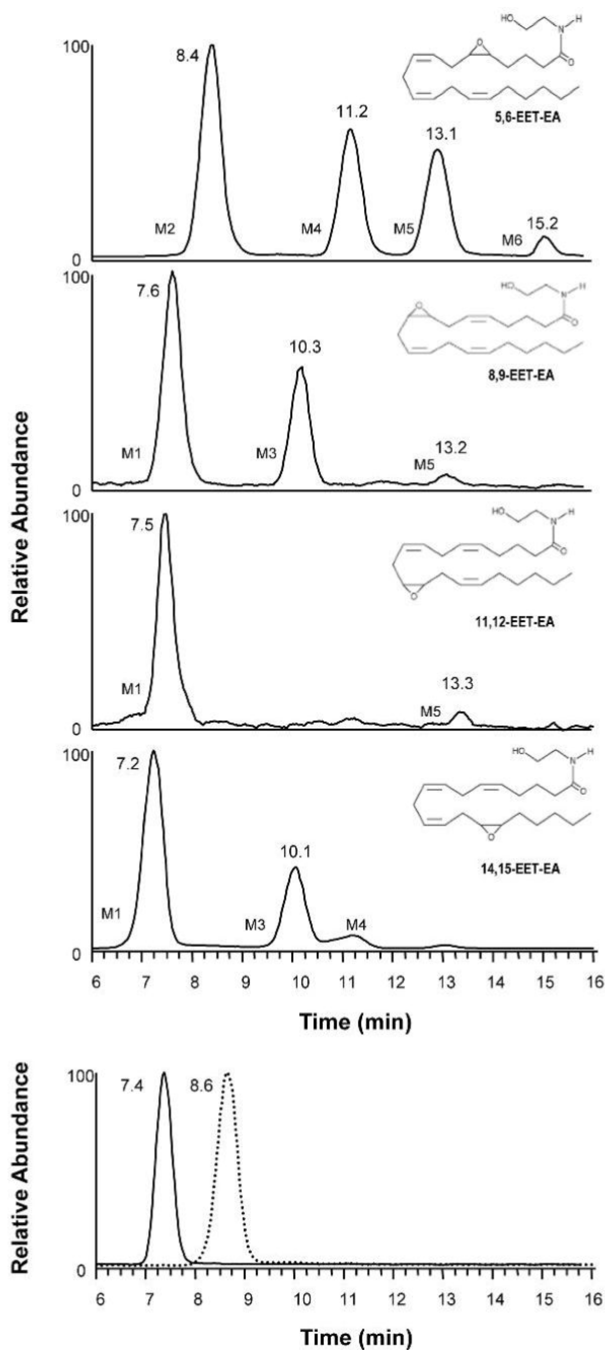

Figure 3. Metabolism of 5,6-, 8,9-, 11,12- and 14,15-EET-EA by CYP2D6

The four EET-EAs (10 μM each) were incubated with 25 pmol CYP2D6 in the reconstituted system (top 4 chromatograms). Bottom panel shows 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 minutes and samples were analyzed as described under Methods. Shown are selected ion chromatograms (*m/z* 380) of the metabolites formed under these conditions. The labels (M1-M5) correspond to the M1-M5 peaks from Figure 1 according to retention times.

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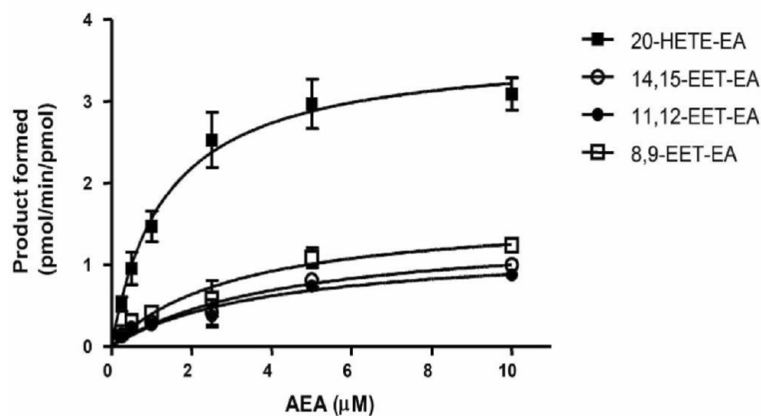

Figure 4. Kinetics of anandamide metabolite formation by human recombinant CYP2D6

Reaction mixtures containing 5 pmol of 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.

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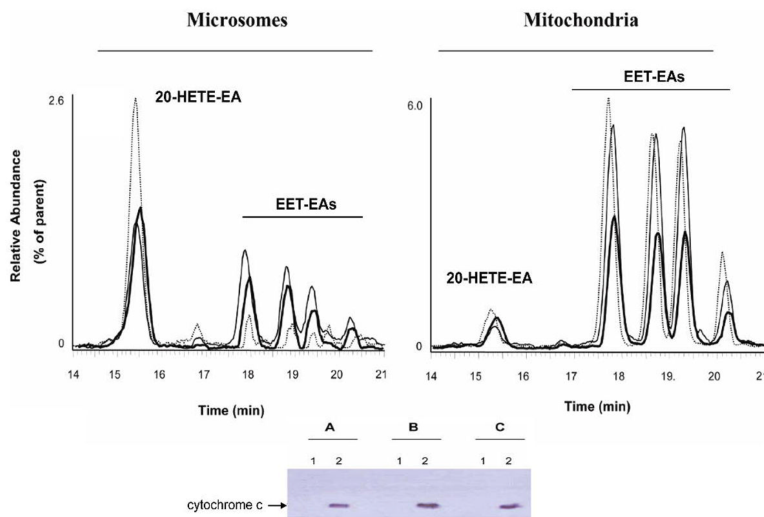

Figure 5. Anandamide metabolism by human neocortical brain microsomes and mitochondria
Anandamide (20 μM) was incubated in the presence of 100 μg of microsomal or mitochondrial protein from subjects A (dotted line), B (solid bold line), or C (solid line) for 10 min and the samples were analyzed as described under Methods. Shown are the selected ion chromatograms at *m/z* 364. For immunoblot, the SDS-PAGE 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 in Methods.

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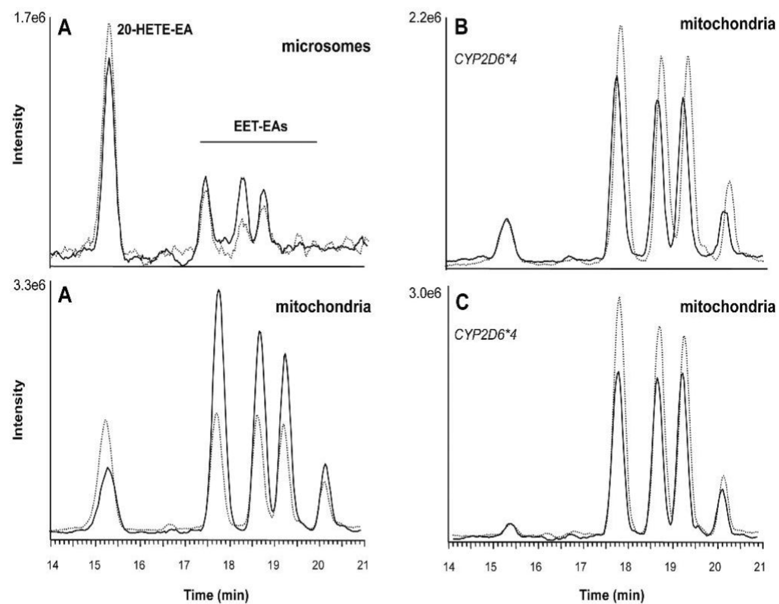

Figure 6. Effect of a monoclonal inhibitory antibody against CYP2D6 on anandamide metabolism by human brain microsomes and mitochondria

Microsomal protein sample from subject A and mitochondrial protein samples from subjects A, B and C were pre-incubated in the presence of hen egg lysozyme (control) antibody (solid line) or antibody specific against CYP2D6 (dotted line) for 5 minutes. Anandamide (q0 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 Methods. The CYP2D6*4 genotype was assigned to subjects B and C based on a genotype analysis which was performed as described in Methods.