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Oxidation of the Endogenous Cannabinoid Arachidonoyl Ethanolamide by the Cytochrome P450 Monooxygenases: Physiological and Pharmacological Implications

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Abstract——**Arachidonoyl ethanolamide (anandamide) is an endogenous amide of arachidonic acid and an important signaling mediator of the endocannabinoid system. Given its numerous roles in maintaining normal physiological function and modulating pathophysiological responses throughout the body, the endocannabinoid system is an important pharmacological target amenable to manipulation directly by cannabinoid receptor ligands or indirectly by drugs that alter endocannabinoid synthesis and inactivation. The latter approach has the possible advantage of more selectivity, thus there is the potential for fewer untoward effects like those that are traditionally associated with cannabinoid receptor ligands. In that regard, inhibitors of the principal inactivating enzyme for anandamide, fatty acid amide hydrolase (FAAH), are currently in development for the treatment of pain and inflammation. However, several pathways involved in anandamide synthesis, metabolism, and inactivation all need to be taken into account when evaluating the effects of FAAH inhibitors and similar agents in preclinical models and assessing their clinical potential. Anandamide undergoes oxidation by several human cytochrome P450 (P450) enzymes, including CYP3A4, CYP4F2, CYP4X1, and the highly polymorphic CYP2D6, forming numerous structurally diverse lipids, which are likely to have important physiological roles, as evidenced by the demonstration that a P450-derived epoxide of anandamide is a potent agonist for the cannabinoid receptor 2. The focus of this review is to emphasize the need for a better understanding of the P450-mediated pathways of the metabolism of anandamide, because these are likely to be important in mediating endocannabinoid signaling as well as the pharmacological responses to endocannabinoid-targeting drugs.**

I. Introduction

The molecular and biochemical components of the endocannabinoid system have emerged as important new pharmacological targets because of their ability to control normal physiological responses and modulate disease-related processes (Pacher et al., 2006). As an endogenous ligand for the cannabinoid receptors CB1[1] and CB2, the endocannabinoid anandamide participates in the regulation of a variety of cellular responses within the immune, cardiovascular, gastrointestinal, and central nervous systems (Howlett, 2005). Numerous studies have demonstrated that anandamide possesses antinociceptive, anti-inflammatory, and neuroprotective properties, providing a solid rationale for the development of pharmacologic agents that can selectively elevate endog-

[1] Abbreviations: Δ^9-THC, Δ^9-tetrahydrocannabinol; 20-HEET-EA, 20-hydroxyepoxyeicosatrienoic acid ethanolamide; 20-HETE, 20-hydroxyeicosatetraenoic acid; 20-HETE-EA, 20-hydroxyeicosatetraenoic acid-ethanolamide; 2-AG, 2-arachidonoylglycerol; ABHD4, α/β-hydrolase 4; AM1241, (*R*)-3-(2-iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1*H*-indole; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl(4-methoxyphenyl) methanone; AUDA-BE, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CNS, central nervous system; COX, cyclooxygenase; CP-55940, (1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol; DHET, dihydroxyeicosatrienoic acid; EA, ethanolamide; EG, epoxyeicosatrienoyl glycerol; EET, epoxyeicosatrienoic acid; FAAH, fatty acid amide hydrolase; FABP, fatty acid binding protein; GDE1, glycerophosphodiesterase 1; GP, glycerophospho; HET0016, *N*-hydroxy-*N*′-(4-*n*-butyl-2-methylphenyl) formamidine; HSC, hepatic stellate cell; IFNγ, interferon γ; LC/MS, liquid chromatography/mass spectrometry; LOX, lipoxygenase; LPS, lipopolysaccharide; LY2318912, 5-((4-azido-3-iodobenzoylamino)methyl)tetrazole-1-carboxylic acid dimethylamide; NAE, *N*-acyl ethanolamine; NAPE, *N*-arachydonoyl-phosphatidylethanolamine; NAT, *N*-acyltransferase; OL-135, 1-oxo-1-[5-(2-pyridyl)-2-yl]-7-phenylheptane; P450, cytochrome P450; PD, Parkinson's disease; PF-3845, 4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)-N-(pyridin-3-yl)piperidine-1-carboxamide; PLD, phospholipase D; PPAR, peroxisome proliferator-activated receptor; PTPN22, protein tyrosine phosphatase 22; SNL, spinal nerve-ligated; sPLA2, soluble phospholipase A2; TRPV1, transient receptor potential vanilloid type-1 receptor; TRPV4, transient receptor potential channel 4; URB597, cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester.

enous anandamide levels (Di Marzo, 2008). Inhibitors of fatty acid amide hydrolase (FAAH), the enzyme that primarily inactivates anandamide, are being developed as one such class of drugs, and they hold major potential for providing a new approach to the clinical management of disorders affecting a significant percentage of the population (Schlosburg et al., 2009). However, a thorough understanding of all the potential pathways that can exert control over the endogenous anandamide levels is crucial in order for this pharmacologic approach to be clinically successful. In addition to hydrolysis by FAAH, anandamide undergoes oxidation via the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (P450) enzyme systems, resulting in the generation of a large number of structurally diverse molecules, the significance of which is poorly understood at this point, particularly with regard to the P450-mediated pathways (Hampson et al., 1995; Yu et al., 1997; Snider et al., 2007). We will provide an overview of the current understanding of anandamide metabolism by P450s and integrate findings from various recent studies in an attempt to provide a foundation in directing further research into this area.

II. Cannabis, the Endocannabinoid System, and Therapeutic Relevance

A. Marijuana and Cannabinoids

The medicinal use of cannabis (marijuana), currently one of the most frequently used recreational drugs, dates back to 2600 BCE (Mechoulam and Hanus, 2000; Robson, 2005). Clinical agents based on marijuana's principal psychoactive cannabinoid, Δ^9-tetrahydrocannabinol (Δ^9-THC), were developed before our current understanding of the molecular mechanism of Δ^9-THC action. Such pharmaceuticals include dronabinol and nabilone, which are prescribed as antiemetics and appetite stimulants to patients afflicted with the AIDS wasting syndrome or receiving 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). The true potential of the cannabinoid-based agents as potential therapeutics became more apparent after the cloning of the receptors for Δ^9-THC (Matsuda et al., 1990; Munro et al., 1993). So far, two cannabinoid receptors have been identified, CB1 and CB2; they are expressed on many different cell types but most abundantly on neurons and immune cells, respectively (Mackie, 2005). Both cannabinoid receptors are coupled to G-proteins, and their activation by agonists leads to inhibition of the accumulation of cAMP in cells via $G\alpha_{i/o}$ (Howlett, 2005; Pertwee, 2005). In addition to the marijuana-derived and synthetic cannabinoid analogs, endogenous ligands for the cannabinoid receptors have been identified (Devane et al., 1992; Mechoulam et al., 1995). The endogenous cannabinoids (endocannabinoids) that have been most thoroughly studied and characterized are arachidonoyl ethanolamide (anandamide) and 2-arachidonoylglycerol (2-AG), the amide and the ester, respectively, of arachidonic acid.

B. Therapeutic Relevance of the Cannabinoid Receptors

The CB1 receptor is expressed heterogeneously within the central nervous system (CNS), where its activation leads to many of the characteristic actions of CB1 receptor agonists, including the marijuana-derived Δ^9-THC. For example, the elevated levels of the CB1 receptor in the cerebral cortex, hippocampus, substantia nigra, cerebellum, and areas of the brain and spinal cord that modulate nociceptive information account for the actions of cannabinoids on the impairment of cognition and memory, alterations in the control of motor function, and antinociception (Mackie, 2005). CB1 receptor activation also leads to increased appetite and body weight by a number of central and peripheral mechanisms that are mediated by both neurotransmitters and hormones (Di Marzo and Matias, 2005). Activation of the CB1 receptor in the CNS also leads to neuroprotection, raising the potential that CB1 receptor activation can lead to favorable therapeutic outcomes in the management of Parkinson's disease (PD) and multiple sclerosis (Kreitzer and Malenka, 2007; Maresz et al., 2007). In support of that, the oromucosal spray Sativex, which is an extract from marijuana plants specifically grown to contain approximately 1:1 ratio of Δ^9-THC and cannabidiol, a nonpsychoactive cannabinoid that modulates the activity of THC in vivo, is effective in alleviating neuropathic pain and spasticity due to multiple sclerosis and is approved for use in Canada and several European countries (Russo et al., 2007). Significant obstacles in the development of CB1 receptor agonists as clinical drugs are their socially unacceptable psychoactive properties and the regulatory restrictions on their usage. On the other hand, successful management of obesity via blockade of the CB1 receptor has been achieved clinically with the drug rimonabant (Maresz et al., 2007). However, because it was determined that rimonabant carried an unacceptable risk for psychological disturbances (e.g., depression and suicide ideation), it did not receive approval by the Food and Drug Administration to be marketed in the United States, and its European marketing was recently suspended by the European Medicines Agency (Le Foll et al., 2009).

CB2 receptors are expressed mainly on immune cells such as lymphocytes, macrophages, mast cells, natural killer cells, and microglia (Mackie, 2005), and activation of these receptors alters cell migration and leads to immunosuppression (Miller and Stella, 2008). Because CB2 receptor-selective agonists are immunosuppressive, have anti-inflammatory activities, and lack psychoac-

tive properties, they are considered to be potential therapeutics for chronic pain and inflammation, including inflammation associated with neurodegenerative disease (Cabral et al., 2008). However, a much better understanding of the complex roles that the CB2 receptors play in the regulation of immune responses is needed before further progress can be made in the development of CB2 receptor agonists as therapeutic agents (Mackie, 2008).

C. Anandamide and the Therapeutic Potential of Fatty Acid Amide Hydrolase Inhibition

Anandamide exerts neuroprotective and immunosuppressive properties that are mediated by cannabinoid receptor-dependent and -independent pathways. The latter include the modulation of ion channel activity, such as the transient receptor potential vanilloid type-1 receptor (TRPV1), and activation of the nuclear peroxisome proliferator-activated receptors (PPARs) (Rockwell and Kaminski, 2004; van der Stelt and Di Marzo, 2005; Eljaschewitsch et al., 2006; O'Sullivan, 2007; Hegde et al., 2008). Therefore, enhancing and/or prolonging the effects of anandamide by increasing its endogenous concentration is a potential therapeutic alternative to the direct activation of cannabinoid receptors. Anandamide is produced in a stimulus-dependent manner, acts locally because of its lipophilic character, and is inactivated by the enzyme FAAH, which leads to the formation of arachidonic acid and ethanolamine (Liu et al., 2006, 2008). Numerous inhibitors of FAAH have been synthesized, and some are currently being developed as therapeutic agents for various applications, including the management of pain, inflammation, and neurological disorders (Clapper et al., 2009). The prototype small molecules that have been used most extensively in animal models are the irreversible FAAH inhibitor cyclohexylcarbamic acid 3′-carbamoylbiphenyl-3-yl ester (URB597) and the reversible competitive FAAH inhibitor 1-oxo-1-[5-(2-pyridyl)-2-yl]-7-phenylheptane (OL-135). The antinociceptive and anti-inflammatory properties of these and similar molecules have been clearly demonstrated in various animal models of acute and chronic pain, cholestasis, and gastrointestinal inflammation, with varying involvement of the CB1 and CB2 receptors as well as TRPV1 and PPAR underlying their functional mechanisms (Lichtman et al., 2004; Piomelli et al., 2006). For example, the antiallodynic effects of OL-135 and URB597 in mice are mediated by both CB1 and CB2 receptors in the von Frey and acetone-induced flinching assays but not TRPV1 receptors (Kinsey et al., 2009). On the other hand, in a rat model of carrageenan-induced inflammatory pain, URB597 inhibited receptive field expansions of spinal neurons in a PPARα-dependent, CB1 receptor-independent, manner based on pharmacological inhibition experiments (Sagar et al., 2008). However, another study using the spinal nerve-ligated (SNL) rat model of neuropathic pain demonstrated that although intraplantar injection of URB597 elevated anandamide levels and significantly reduced mechanically evoked responses of dorsal horn neurons in sham-operated rats, it did not have that same effect in the SNL rats, suggesting that peripheral anandamide metabolism is altered in neuropathy (Jhaveri et al., 2006). Upon administration of a higher dose of URB597, CB1 receptor-dependent attenuated responses of spinal neurons were observed in the SNL rats, but there was no increase in anandamide levels in the ipsilateral hindpaw, suggesting alternative routes of anandamide metabolism are likely to be responsible for the nociceptive processing in this setting.

A significant problem with many of the older FAAH inhibitors is their apparent lack of specificity because they also inhibit other serine hydrolases such as carboxylesterases (Zhang et al., 2007a). However, progress is being made in improving that aspect of FAAH inhibitors, as evidenced by the recent demonstration that selective covalent inhibition of FAAH by the piperidine urea inhibitor 4-(3-(5-(trifluoromethyl)pyridin-2-yloxy)benzyl)-*N*-(pyridin-3-yl)piperidine-1-carboxamide (PF-3845) has been shown to be efficacious in alleviating experimental inflammatory pain in a CB1/CB2-dependent manner (Ahn et al., 2009).

III. The Regulation of Cellular and Tissue Concentrations of Anandamide: Synthesis

A. Synthesis of the Anandamide Precursor C20:4-N-Arachidonoyl-phosphatidylethanolamine

The formation of C20:4-NAPE is the first step in the biosynthetic pathway leading to the formation of anandamide (Fig. 1). This reaction is catalyzed by a calcium-dependent *N*-acyltransferase (NAT) enzyme, which transfers an arachidonoyl group from the *sn*-1 position of a phospholipid such as phosphatidylcholine to the amino group of phosphatidylethanolamine (Ueda, 2009). The NAT-mediated pathway is not unique for anandamide synthesis and is also known to be involved in the formation of a number of other fatty acid ethanolamines containing both saturated and unsaturated acyl chains of varying chain lengths (C16:0–C22:6) (Natarajan et al., 1982; Schmid et al., 1983). In addition to the calcium-dependent NAT, the cDNA of which has not yet been cloned, another, calcium-independent enzyme with NAT activity was recently identified in rats (Jin et al., 2009). Although the calcium-independent NAT (termed iNAT), which is most abundantly expressed in rat testis, was able to generate the anandamide precursor in an in vitro overexpression system, its physiological relevance in the context of anandamide biosynthesis remains to be determined.

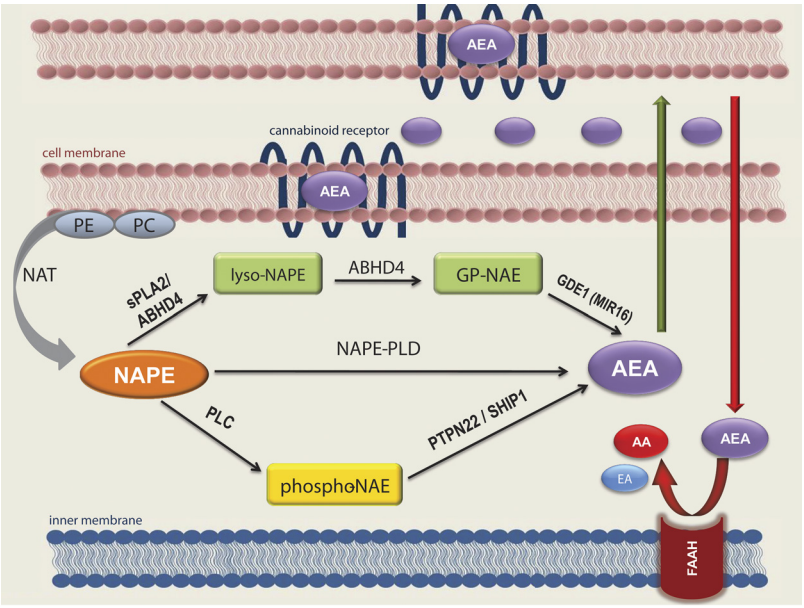

FIG. 1. Biosynthesis and degradation of anandamide. The formation of the anandamide precursor C20:4-NAPE is catalyzed by NAT, which transfers an arachidonoyl group from the *sn*-1 position of a phospholipid, such as phosphatidylcholine (PC), to the amino group of phosphatidylethanolamine (PE). Several pathways act upon C20:4-NAPE to produce anandamide, including 1) direct conversion by NAPE-PLD; 2) PLC-catalyzed formation of a phospho-NAE species, which is subsequently converted to anandamide via the action of phosphatases, including PTPN22 and SH2-containing inositol-5-phosphatase (SHIP1); and 3) sPLA2 or ABHD4-catalyzed formation of lyso-NAPE, followed by ABHD4-catalyzed formation of a GP-NAE species that is subsequently converted to anandamide by the phosphodiesterase GDE1. The enzymatic inactivation of anandamide is carried out by membrane-bound FAAH, which forms arachidonic acid (AA) and EA.

B. Synthesis of Anandamide from C20:4-N-Arachidonoyl-phosphatidylethanolamine by the N-Arachidonoyl-phosphatidylethanolamine-Phospholipase D Pathway

With respect to the formation of anandamide from C20:4-NAPE there are multiple potential pathways that could be involved and that may predominate in a cell- and tissue-dependent manner (Fig. 1). The original model proposed the involvement of a NAPE-specific phospholipase D (NAPE-PLD) as the major step in the direct conversion of C20:4-NAPE to anandamide (Okamoto et al., 2004). The NAPE-PLD enzyme was purified initially from rat heart, and its sequence was found to be highly conserved from rodents to human. It was determined that this enzyme does not share sequence homology with the other known PLDs, and it is also catalytically distinct. Classified as a zinc metallohydrolase with β-lactamase fold, recombinant NAPE-PLD is able to use C20:4-NAPE as a substrate to form anandamide with a K_m of 2.8 μM and a V_{max} of 73.2 nmol/min/mg protein. The highest levels of NAPE-PLD mRNA and protein were detected in mouse brain (cerebrum and cerebellum), kidney, and testis, whereas the liver and the spleen exhibited the lowest levels of expression (Okamoto et al., 2004).

However, the relative importance of NAPE-PLD in the synthesis of anandamide came into question after the generation and characterization of mice with a targeted deletion of the NAPE-PLD gene [NAPE-PLD(−/−) mice] (Leung et al., 2006). The NAPE-PLD(−/−) mice were generated by removal of exon 4, which encodes the majority of the protein sequence, including the zinc-binding domain that is required for catalysis. These animals were reported to be viable, healthy, and behaviorally indistinguishable from their wild-type littermates. It is noteworthy that liquid chromatography/mass spectrometry (LC/MS) measurements of the endogenous brain levels of anandamide and C20:4-NAPE revealed no difference between the wild-type [NAPE-PLD(+/+)] and NAPE-PLD(−/−) mice, suggesting that NAPE-PLD does not play a significant role in controlling the endogenous levels of anandamide. This was in contrast to the significant decreases (5- to 10-fold) observed in the levels of long-chain saturated (C20:0-C24:0) *N*-acyl ethanolamines (NAEs) in the NAPE-PLD(−/−) mice (Leung et al., 2006). Therefore, anandamide biosynthesis seems to differ from the biosynthesis of some of the other NAEs with regard to the dependence on NAPE-PLD, raising the possibility for selective pharmacological manipulation of anandamide levels in vivo at the level of its biosynthesis.

Another important finding from the study by Leung et al. (2006) was the observed difference in the rates of C20:4-NAPE hydrolysis when mouse brain homogenates were incubated in the presence or absence of calcium. Incubation of C20:4-NAPE in the presence of calcium in homogenized brain tissue from the NAPE-PLD(−/−) mice resulted in an approximately 3-fold reduction in the rate of hydrolysis of C20:4-NAPE compared with the NAPE-PLD(+/+) brain homogenates. However, when the same experiment was performed in the absence of calcium, the C20:4-NAPE hydrolysis activities were similar for the homogenates obtained from the NAPE-PLD(−/−) and those from the NAPE-PLD(+/+) mice. In

addition, the presence of calcium was found to cause a significant inhibition (2-fold) of C20:4-NAPE hydrolysis in the wild-type brain homogenates. The last two findings reveal a calcium-independent pathway for C20:4-NAPE hydrolysis that may be obscured, depending upon the assay conditions; thus, its contribution to anandamide synthesis might be underestimated.

C. Synthesis of Anandamide from C20:4-N-Arachidonoyl-phosphatidylethanolamine by the Soluble Phospholipase A2 Pathway

In addition to the NAPE-PLD pathway, anandamide formation can also proceed through two intermediates, C20:4-lyso-NAPE and C20:4-glycerophospho-*N*-acyl ethanolamine (GP-NAE), which are formed via deacylation of the *sn*-1 or *sn*-2 *O*-acyl chains, respectively (Simon and Cravatt, 2006) (Fig. 1). It had previously been demonstrated that the first step in the reaction, the formation of C20:4-lyso-NAPE, can be catalyzed by a soluble form of phospholipase A2 (sPLA2) (Sun et al., 2004). The highest specific activity of sPLA2 (which was based on metabolism of C16:0-NAPE rather than C20:4-NAPE as a substrate) was observed in the mouse stomach (49 nmol/min/mg protein) and was much lower in other tissues, including the brain, liver, and kidney (<0.1 nmol/min/mg protein). Purified sPLA from rat stomach was also able to use the anandamide precursor C20:4-NAPE as a substrate, and this was tested using 100 and 200 μM concentrations of C20:4-NAPE (Sun et al., 2004).

D. Synthesis of Anandamide from C20:4-N-Arachidonoyl-phosphatidylethanolamine by the α/β-Hydrolase 4 Pathway

Using C16:0-NAPE as a substrate, Simon and Cravatt reported the potential involvement of the serine hydrolase α/β-hydrolase 4 (ABHD4) in the formation of a lyso-NAPE species and a GP-NAE species as intermediates in NAE synthesis (Simon and Cravatt, 2006) (Fig. 1). In a subsequent study, the detection of endogenous levels of C20:4-GP-NAE was also reported, and this species was found in mouse brain at a concentration of 0.16 ± 0.07 pmol/g of wet tissue (Simon and Cravatt, 2008). To identify the enzyme responsible for the hydrolysis of the phosphodiester bond to release the NAE species, the Cravatt group pursued the glycerophosphodiester phosphodiesterase class of enzymes, which, in mammalian genomes, are encoded by seven genes (Simon and Cravatt, 2008). Based on several findings that led to the conclusion that the GP-NAE phosphodiesterase in question was an integral membrane protein, the pool of possible candidate enzymes was narrowed down to five that were predicted to have a transmembrane domain and that were expressed in brain. Only one of these five enzymes, GDE1, exhibited significant GP-phosphodiesterase activity, as measured by the formation of C16:0-NAE in incubations containing membrane fractions of COS-7 cells expressing GDE1 and 100 μM C16:0-GP-NAE as a substrate. It was subsequently determined that GDE1 also possesses significant C20:4-GP-NAE phosphodiesterase activity (approximately 100 nmol/min/mg when using 100 μM substrate). The highest levels of GP-NAE phosphodiesterase activity, which correlated well with GDE1 expression, were detected in mouse brain, spinal cord, kidney, liver, and testis, and the lowest levels were detected in the heart and spleen.

E. Synthesis of Anandamide from C20:4-N-Arachidonoyl-phosphatidylethanolamine by the Protein Tyrosine Phosphatase 22/SH2-Containing Inositol-5-phosphatase Pathway

Liu et al. (2006) identified an alternative pathway to the NAPE-PLD and ABHD4/GDE1 routes of anandamide synthesis after cellular stimulation by the bacterial cell wall component lipopolysaccharide (LPS) (Fig. 1). This study first ruled out the involvement of NAPE-PLD in the LPS-stimulated formation of anandamide by RAW264.7 macrophages, where an inverse relationship was observed between the production of anandamide by the LPS-stimulated macrophages and the expression of NAPE-PLD mRNA. By performing a polymerase chain reaction-select method of cDNA subtraction, the authors identified differentially expressed cDNAs in LPS-stimulated versus control RAW264.7 macrophages. Upon transfecting the full-length cDNAs of the potential targets, the levels of anandamide were measured by LC/MS and were found to increase (>50%) after transfection of 21 of the LPS-induced genes. One of the genes that resulted in a 2-fold elevation of anandamide in the macrophages was identified to be the one encoding PTPN22, a tyrosine phosphatase that is predominantly expressed in lymphoid tissues. The identification of a phosphatase as a contributor to anandamide synthesis in LPS-stimulated macrophages raised the possibility of the existence of a phospho-anandamide precursor. In fact, in the presence of LPS and sodium orthovanadate ($NaVO_3$), a nonselective tyrosine phosphatase inhibitor, the phospho-anandamide precursor was detected in both RAW264.7 macrophages and mouse brain extracts. In brain tissue, the levels of phospho-anandamide increased 15-fold in the presence of $NaVO_3$. A synthetic phospho-anandamide precursor was converted to anandamide by the RAW264.7 cells, and this conversion significantly increased in the presence of LPS, suggesting the possibility of PTPN22 involvement in this process. There was an approximately 30% decrease in the rate of conversion of phospho-anandamide to anandamide in brain homogenates from mice lacking PTPN22 relative to wild-type mice, implicating PTPN22 as a phosphatase involved in the formation of anandamide from phospho-anandamide, although it is not the only such phosphatase, and it was further shown that SH2-containing inositol-5-phosphatase is another phosphatase that may also be involved in this pathway (Liu et al., 2008).

IV. The Regulation of Cellular and Tissue Concentrations of Anandamide: Uptake

The mechanism whereby anandamide is taken up into cells has been a matter of considerable debate in the field, and there are currently several hypotheses regarding this mechanism, as detailed in several reviews (Glaser et al., 2005; Felder et al., 2006). We will outline the most recent developments in favor of and against each hypothesis.

A. Facilitated Diffusion of Anandamide Mediated by a Specific Transporter

Although the existence of a plasma membrane protein that translocates anandamide into the cytoplasm has been hypothesized for some time, this hypothesis has been weakened by the inability to clone the putative transporter. Numerous compounds have been synthesized as potential inhibitors of the anandamide transport process, but the vast majority of them are also potent inhibitors of FAAH, making it difficult to distinguish anandamide uptake and hydrolysis. Progress in the development of new small molecules, such as the 1,5- and 2,5-disubstituted tetrazoles, some of which potently and selectively inhibit anandamide uptake without inhibiting FAAH, should yield more information about the validity of this hypothesis (Ortar et al., 2008). Thus far, the strongest evidence in favor of the existence of an anandamide transporter is the identification of a high affinity anandamide binding site (K_d, 7.62 ± 1.18 nM; B_{max}, 31.6 ± 1.80 fmol/mg protein) using the small molecule LY2318912, which inhibits anandamide uptake (IC_{50}, 7.27 ± 0.510 nM), regardless of the presence of FAAH (Moore et al., 2005). However, it was recently demonstrated that anandamide undergoes carrier protein-mediated cytosolic trafficking from the plasma membrane to FAAH-containing intracellular membranes (Kaczocha et al., 2009; Oddi et al., 2009). Candidate proteins implicated in this mechanism are fatty acid binding proteins (FABPs), specifically FABP5 and FABP7, the 70-kDa heat shock protein, and albumin. Therefore, the possibility that LY2318912 and other uptake inhibitors that do not possess appreciable activity toward FAAH are able to alter the intracellular trafficking of anandamide remains to be investigated.

B. Passive Diffusion of Anandamide Facilitated by a Fatty Acid Amide Hydrolase-Driven Gradient

Owing to its high lipophilicity (XLogP3 = 5.4), it is likely that anandamide may cross the plasma membrane by simple diffusion. In support of this hypothesis, the inhibitors of anandamide transport are unable to inhibit the uptake process rapidly (<30 s), and many of them are also potent inhibitors of FAAH (Alexander and Cravatt, 2006). Furthermore, a recent study demonstrated that, by being able to adopt an extended conformation, anandamide is able to bind to cholesterol specifically and with high affinity, and their interaction is stabilized by both van der Waals interactions and hydrogen bonding (Di Pasquale et al., 2009). At low physiologically relevant concentrations (50 nM), anandamide was able to interact specifically with synthetic cholesterol monolayers in a time-dependent manner and did not display an appreciable affinity for monolayers composed of palmitoyl-oleyl-phosphatidylcholine. In addition, the interaction with cholesterol was strongest for anandamide, relative to arachidonic acid and oleic acid, and anandamide was also able to translocate through a cholesterol bilayer that was free of protein (ensured by the construction of the bilayers using synthetic lipids), ruling out any possible effects of a carrier protein. Whether these physicochemical data reflect a physiological phenomenon remains to be investigated.

C. Endocytosis-Mediated Anandamide Uptake

Based on demonstrations that pharmacological inhibition of endocytosis disrupts anandamide accumulation, the endocytotic pathway has also been proposed as a potential mechanism of cellular anandamide uptake. Preincubation of rat basophilic RBL-2H3 cells with inhibitors of caveolae-related endocytosis, such as nystatin/progesterone (cholesterol synthesis/transport inhibitors), in addition to alternative inhibitors of caveola-related endocytosis (genistein or *N*-ethylmaleimide), or an 18°C temperature block, all reduce anandamide uptake by approximately 50% (McFarland et al., 2004). This process is unaffected by preincubation with chlorpromazine or potassium-free buffer, suggesting that clathrin-dependent endocytosis is not a likely mechanism of anandamide uptake. The same study demonstrated that FAAH-derived metabolites of anandamide, but not intact anandamide, localize within caveolae-rich domains. These observations are not cell-type specific because they are also present when using the neuronal CAD cells. Furthermore, down-regulation of dynamin 2, a small GTPase involved in endocytic internalization, in CAD cells leads to a decrease in the uptake of a fluorescently labeled anandamide analog and disrupts the trafficking of radiolabeled anandamide to FAAH (McFarland et al., 2008).

V. The Regulation of Cellular and Tissue Concentrations of Anandamide: Degradation

The enzymatic hydrolysis of several endogenous fatty acid amides, including anandamide, is carried out by FAAH, an integral membrane protein associated with microsomal, mitochondrial, and plasma membranes and expressed in multiple tissues and cell types (McKinney and Cravatt, 2005). The liver has the highest level of expression and activity of FAAH, followed by the brain, although other organs, such as the intestine, kidney, spleen, and lung, also possess significant FAAH enzymatic activity. Intracellular anandamide is broken down by FAAH to yield arachidonic acid and ethanolamine (Fig. 1). The effect of anand-

amide upon different cell types is largely dictated by the levels of FAAH. For example, hepatocytes, which express 70-fold higher mRNA levels for FAAH compared with hepatic stellate cells (HSCs), are resistant to anandamide-induced cell death, whereas HSCs, having undetectable levels of FAAH protein, rapidly undergo reactive oxygen species-dependent necrosis (40–80% cell death within 2–24 h) in the presence of 25 to 50 μM anandamide (Siegmund et al., 2006). Further evidence for the important role of FAAH in this mechanism includes the observation that pharmacological inhibition of FAAH activity makes hepatocytes sensitive to anandamide-induced death and the infection of HSCs with FAAH-expressing adenovirus leads to their resistance to the necrotic effect of anandamide. In addition, the in vivo relevance of the hepatocyte-protective effect of FAAH is reflected in findings that FAAH(−/−) mice exhibit significantly more hepatocellular injury after bile duct ligation relative to FAAH+/+ mice. Raising the endogenous levels of anandamide via pharmacological inhibition of FAAH is a possible therapeutic alternative to direct cannabinoid receptor agonists and may offer more specificity because of the "on-demand" mode of synthesis of anandamide (Schlosburg et al., 2009). Therefore, given the potential clinical importance of FAAH inhibitors, it is crucial to understand alternative routes of anandamide metabolism, such as oxidation.

VI. Oxidative Metabolism of Anandamide

A. Oxidation of Anandamide: Major Enzymes Involved

Obvious candidate enzymes that could carry out anandamide oxidation are the same fatty acid oxygenases that are known to act on endogenous arachidonic acid; namely, the members of the COX, LOX, and 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). Existing knowledge regarding the synthesis and action of the COX-2 and LOX metabolites of anandamide has been reviewed previously (Starowicz et al., 2007; Woodward et al., 2008). For example, some of the COX-2 products of anandamide seem to have potent ocular hypotensive properties mediated by novel receptors, distinct from both the cannabinoid or prostanoid receptor families (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, which are outlined in Fig. 2 and discussed below.

B. The Cytochrome 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 P450 genes are expressed constitutively in a tissue-, gender-, and age-dependent fashion, and others are 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 mammalian P450s are membrane-bound and are localized in the endoplasmic reticulum or the mitochondrial membrane of cells in the liver and most other tissues, including

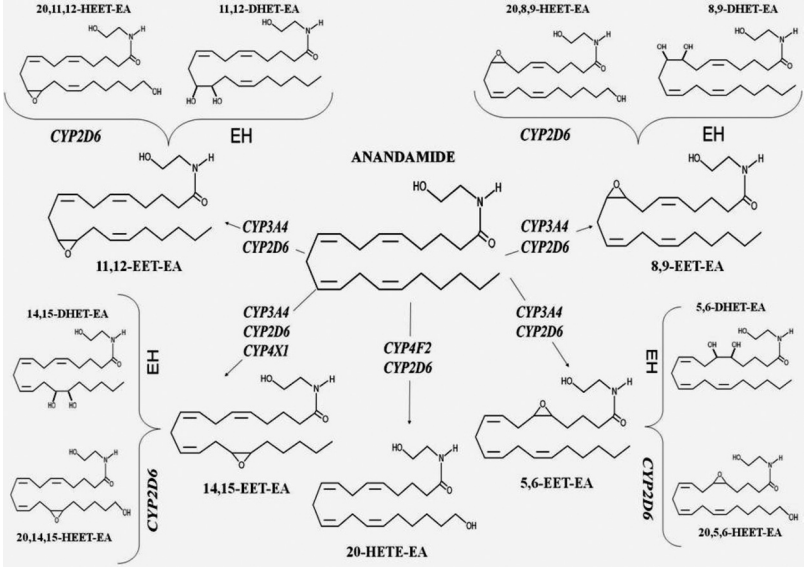

FIG. 2. Oxidative pathways for the metabolism of anandamide catalyzed by cytochrome P450s and epoxide hydrolases. Anandamide undergoes epoxidation by CYP3A4, CYP2D6, and CYP4X1 to form four EET-EAs. The hydroxylation of anandamide is carried out by CYP4F2 or CYP2D6 to form 20-HETE-EA. The EET-EAs can be further metabolized by microsomal and soluble epoxide hydrolase (EH) to form the corresponding DHET-EAs or by CYP2D6 to form the corresponding HEET-EAs.

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 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 other oxygen atom 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, approximately 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 are metabolized in the brain and kidney 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. Deficiencies in these enzymes can lead to serious diseases, such as congenital adrenal hyperplasia, which is caused by a deficiency of CYP21A2 (Harada et al., 1987; Tajima et al., 1993). Many members of P450 families 2 and 4 are promiscuous in the sense that they can metabolize a variety of 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).

C. Arachidonic Acid Metabolism by Cytochromes P450

The anandamide precursor arachidonic acid, an ω-6 essential fatty acid found in cells, is present primarily in an esterified form with 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 form 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, 2009). Decreases in the EET levels in the kidney and the vasculature have been associated with increases 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 the hydrolysis of epoxides in humans (Morisseau and Hammock, 2005). Inhibition of the soluble epoxide hydrolase leads to elevation of endogenous EET levels, and inhibitors are considered to be a potential novel treatment for renal, cardiovascular, and neurological disorders (Chiamvimonvat et al., 2007).

Arachidonic acid can also be hydroxylated at the terminal carbons by P450s 4A11, 4F2, and 4F3. The ω 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 pathological lesions, 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 a manner opposing that of the EETs, 20-HETE is a potent vasoconstrictor in the cerebral and renal microcirculation (Mayer et al., 2006). Polymorphisms in the *CYP4F2* gene have been associated with altered blood pressure, as well as myocardial and cerebral infarction (Fava et al., 2008; Ward et al., 2008; Fu et al., 2009). Inhibitors of 20-HETE synthesis have been developed and are being used to better understand the physiological 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).

VII. Anandamide Metabolism by Rodent Cytochrome P450 Enzymes

A. Anandamide Metabolism by Mouse Cytochromes P450

Because of its structural similarity to arachidonic acid, anandamide has also been thought to be a candidate to undergo oxygenation by P450s. Bornheim et al. (1995) were the first to report on the metabolism of

anandamide by mouse liver and brain microsomal P450s. 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 used 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 the CYP1A, CYP2B, CYP3A, and CYP4A isoforms, respectively. The most profound induction of anandamide metabolism was seen after 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, preincubation of the microsomes with an antibody against CYP3A significantly diminished production of all four metabolites, suggesting they were specifically formed by CYP3A. 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 no changes were observed in the metabolite profiles with microsomes from clofibrate-treated mice. Taken together, these data provided evidence that microsomal CYP3A, CYP2B and CYP1A (to a lesser extent), but not CYP4A contribute to anandamide metabolism in the mouse liver. Using LC/MS analysis, it was determined that the brain microsomal metabolites were mono-oxygenated products and that CYP3A was involved in the formation of one of the two products, based on antibody inhibition experiments. The actual structures of all of the anandamide products reported to be formed by the mouse microsomal P450s were not determined. The likelihood that all of these metabolites would be produced in vivo is not clear because the concentration of anandamide used in the microsomal incubation studies (720 μM) seems to be several orders of magnitude higher than its physiological levels. Although there are some discrepancies in the literature regarding the physiological levels of anandamide, its tissue and plasma levels seem to be in the low nanomolar range (Monteleone et al., 2005; Piomelli et al., 2006), but the levels are likely to be much higher within cells, where it is being produced in an activity-dependent manner. Nevertheless, the study by Bornheim et al. (1995) clearly demonstrated the possibility that P450s could play a role in the metabolism of anandamide.

B. Anandamide Metabolism by Rat Cytochromes P450

After the earlier work of Bornheim and colleagues, there were no further investigations into anandamide metabolism and P450s until a study published by Costa et al. (2002) examining the effect of short- and long-term 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 short- (one dose) and long-term (15 doses on consecutive days), caused a statistically significant induction in the expression of CYP3A and CYP2B isoforms in the rat liver microsomes. The total microsomal brain P450 content in rats treated with one dose, but not those treated with 15 doses, 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 CYP3A enzymes (Zhou, 2008). Assuming that is the case with anandamide, the results of this study suggest that, similar to the mouse, CYP3A and 2B isoforms are likely to be involved in anandamide metabolism in the rat.

VIII. Anandamide Metabolism by Human Cytochrome P450 Enzymes

The participation of human P450s in anandamide metabolism was recently reported by our laboratory (Snider et al., 2007, 2008). By using synthetically prepared authentic standards, the anandamide metabolites were structurally characterized (Fig. 2), and an 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, 2008). Although the metabolic reactions are mostly analogous to arachidonic acid metabolism, there are significant differences with respect to the identities of the specific P450 isoforms involved (Fig. 2).

A. Kidney Microsomal Anandamide Metabolism: Involvement of CYP4F2

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 upon preincubation of the microsomes with *N*-hydroxy-*N*′-(4-*n*-butyl-2-methylphenyl) formamidine (HET0016), an inhibitor of arachidonic acid omega hydroxylase; therefore CYP4F2 is the most likely predominant isoform involved in forming 20-HETE-EA (Fig. 2). In contrast to arachidonic acid metabolism, CYP4A11 is likely to play a relatively minor role in anandamide metabolism based on in vitro metabolism data (Snider et al., 2007). However, an important factor to consider when determining the relative roles of the CYP4A11 and CYP4F2 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 CYP4A11 and an inducer of CYP4F2 expression (Zhang and Hardwick, 2000; Zhang et al., 2000; Antoun et al., 2006). Comparison of the K_m values of CYP4F2 (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 an en-

dogenous substrate for CYP4F2. The results also suggest that anandamide is a higher affinity substrate for CYP4F2 relative to arachidonic acid, which is metabolized by CYP4F2 to 20-HETE with a K_m 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 CYP4A, suggesting a species similarity between mouse and human with regard to the apparent lack of 4A participation in the metabolism of anandamide. Whether there is a species similarity regarding the involvement of the CYP4F enzymes is difficult to assess without further studies, because there are a number of mouse 4F isoforms, and they are differentially regulated by clofibrate treatment (Cui et al., 2001).

B. Liver Microsomal Anandamide Metabolism: Involvement of CYP4F2 and CYP3A4

Human liver microsomal metabolism of anandamide leads to the formation of 20-HETE-EA, catalyzed by CYP4F2 in addition to the formation of 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acid ethanolamides (EET-EAs), which are the products of metabolism by CYP3A4 (Snider et al., 2007). The predominant role of CYP3A4 in anandamide epoxidation is a main distinction between anandamide and arachidonic acid metabolism, because the main arachidonic acid epoxygenases are CYP2C8, CYP2C9, and CYP2J2. The levels of liver microsomal EET-EAs formed are lower relative to 20-HETE-EA because they undergo secondary metabolism by epoxide hydrolase, which leads to the formation of the four corresponding dihydroxyeicosatrienoic acid ethanolamides (DHET-EAs). It is very 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, it could be speculated that the role of CYP3A in anandamide oxidation may be similar between mouse and human. However, unlike in the mouse, the human CYP2B (2B6) does not seem to be involved in liver microsomal anandamide metabolism, as evidenced by a lack of inhibition of anandamide metabolism in the presence of a CYP2B6 antibody, although the possibility that this enzyme could participate in extrahepatic anandamide metabolism cannot be excluded. The electrospray ionization-LC/tandem MS method that was developed for the separation and detection of the anandamide metabolites, along with the commercial 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. Additional insights into the importance of the oxygenated anandamide metabolites should also be gained through work generated by the LIPID Metabolites and Pathways Strategy (MAPS) (Fahy et al., 2007; Sud et al., 2007) and similar projects aimed at developing an integrated metabolomic system to characterize global changes in lipid metabolites.

C. Physiological and Pharmacological Relevance of Brain Cytochromes P450 in the Metabolism of Anandamide

Recent work has highlighted the importance of brain P450s in producing physiologically and pharmacologically relevant responses because of the metabolism of both endo- and xenobiotics (Liu et al., 2004; Meyer et al., 2007). Most known P450 isoforms are expressed in the human brain in a region-specific manner (Dutheil et al., 2009) and are metabolically active.

The presence of CYP3A at the blood-brain barrier in neurons, glial cells, and endothelial cells in humans and rodents has been reported (Woodland et al., 2008). In rat brain microsomes, CYP3A participates in the 6β-hydroxylation of testosterone (Rosenbrock et al., 1999), and both rat and human brain microsomes demethylate the therapeutic agent amitriptyline to form nortriptyline in a CYP3A-dependent manner (Voirol et al., 2000). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine is metabolized by monoamine oxidase B to 1-methyl-4-phenylpyridine, a neurotoxic metabolite that causes a Parkinson-like syndrome. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine is detoxified through *N*-demethylation catalyzed to a small extent by CYP3A4, but mostly by CYP2D6 (Coleman et al., 1996), which is in line with findings that CYP2D6 poor metabolizers have been reported to be more vulnerable to PD (Smith et al., 1992). Persons with alcoholism have a higher expression of CYP2D6 in 13 different brain regions compared with those who are not alcoholics (Miksys et al., 2002), and CYP2D6 is also involved in the hydroxylation of neurosteroids and progesterone (Hiroi et al., 2001; Kishimoto et al., 2004) as well as a large number of centrally acting drugs, including analgesics, antidementia drugs, tricyclic antidepressants, antipsychotics, and monoamine oxidase inhibitors (Zanger et al., 2004).

The metabolism of anandamide by human brain tissue microsomal and mitochondrial preparations (from nondiseased sections surrounding brain tumors) was recently reported (Snider et al., 2008). Anandamide is converted primarily to 20-HETE-EA or to the four EET-EAs by brain microsomal and mitochondrial preparations, respectively. Based on results from antibody and chemical inhibition studies, the P450 isoforms that participate in kidney and/or liver microsomal metabolism of anandamide (4F2 and 3A4) are also responsible for brain metabolism. In addition, based on the results of antibody inhibition experiments, mitochondrial CYP2D6 is involved in the epoxidation of anandamide in human brain. The mitochondrial targeting of 2D6 is of important interest to the role of this enzyme in both xeno- and endobiotic metabolism (Sangar et al., 2009). Because CYP2D6 is highly polymorphic (Ingelman-Sundberg, 2005), additional studies using a larger pool of brain

samples are needed to confidently establish the extent of its involvement in anandamide oxidation in the human brain.

D. Anandamide Metabolism by Recombinant CYP2D6

The finding that CYP2D6 participates in anandamide metabolism is somewhat unexpected, because CYP2D6 does not metabolize arachidonic acid, and its prototypical substrates (many cardiovascular and CNS-acting drugs) are structurally very different from anandamide. Recombinant CYP2D6 metabolizes anandamide to give 20-HETE-EA, the four EET-EAs, and several novel dioxygenated derivatives that are most likely 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 the CYP4A-derived ω and ω-1 hydroxylated metabolites of arachidonic acid-derived EETs that were found to be high-affinity ligands for PPARα (Cowart et al., 2002). The proposed ω-hydroxylated product of each of the EET-EAs, the 20-hydroxyepoxyeicosatrienoic acid ethanolamide (20-HEET-EA), seemed to be the predominant metabolite produced by CYP2D6 in each case (Fig. 2). Anandamide and the EET-EAs are the first eicosanoid-like molecules to be identified as CYP2D6 substrates, raising the possibility that CYP2D6 could be involved in the metabolism of other bioactive eicosanoids and fatty acid amides such as the sleep-inducing oleamide and the anti-inflammatory and antinociceptive palmitoylethanolamide (Farrell and Merkler, 2008). The highly polymorphic nature of CYP2D6 and the previously reported neurological and psychiatric differences among persons with various 2D6 phenotypes (Funae et al., 2003; Miksys and Tyndale, 2004; Yu et al., 2004; Dorado et al., 2007; Ingelman-Sundberg et al., 2007) suggest that these differences could be due to the involvement of this enzyme in the metabolism of anandamide as well as similar psychoactive substances.

E. Anandamide Metabolism by Recombinant "Orphan" CYP4X1

The functional significance of 25% of human P450s is still unknown, and they are referred to collectively as "orphan" P450s (Stark and Guengerich, 2007). Detection of mRNA for the orphan human CYP4X1 in several tissues was recently reported by Stark et al. (2008). The highest levels of expression were found in the prostate, the skin, and the amygdala, which had approximately 15-, 30-, and 30-fold higher levels of CYP4X1 mRNA, respectively, than the liver. The catalytic activity of recombinantly expressed CYP4X1 toward anandamide was tested, revealing the formation of a single product, 14,15-EET-EA, with a K_m of 65 μM and a catalytic rate of 65 pmol of product formed/min/nmol of P450. The high levels of mRNA for CYP4X1 detected in the amygdala, skin, and prostate suggest that anandamide may be epoxygenated to 14,15-EET-EA by CYP4X1 in those tissues (Stark et al., 2008).

IX. Physiological and Pharmacological Relevance of the Cytochrome P450-Mediated Oxidation of Anandamide

A. Biological Significance of Anandamide Oxidation

There are several possibilities with regard to the biological significance of the oxidative pathways for the metabolism of anandamide that were previously proposed (Kozak et al., 2004). The oxygenated anandamide metabolites could possess enhanced activity at the cannabinoid receptors or enhanced metabolic stability compared with anandamide, and, in that regard, oxygenation could represent an activation pathway. Alternatively, oxygenation of anandamide may represent an inactivation pathway, leading to the production of metabolites with decreased cannabimimetic activity. Last, because the oxidative metabolism of anandamide by COX-2, LOX, and P450s leads to the formation of a structurally diverse set of molecules, it is very likely that some of these novel lipids may possess potent biological activities that are distinct from their precursor molecules.

B. Anandamide Oxidation Leading to Bioactivation

With regard to the possibility of bioactivation, the P450-derived anandamide epoxide 5,6-EET-EA is a potent CB2 receptor-selective agonist (Snider et al., 2009). This metabolite competes with radiolabeled CP-55940, a nonselective cannabinoid agonist, for binding to membranes of Chinese hamster ovary cells expressing either the human CB1 or CB2 receptors with respective K_i values of 3.2 μM or 8.9 nM. In comparison, the K_i values obtained from parallel studies using anandamide as the competitor were 155 nM and 11.4 μM for CB1 and CB2, respectively. The 5,6-EET-EA 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 cells expressing the CB2 receptor. The level of CB2-mediated cAMP inhibition by 5,6-EET-EA (IC_{50}, 9.8 nM) is similar to that of the synthetic CB2-selective agonist (*R*)-3-(2-iodo-5-nitrobenzoyl)-1-(1-methyl-2-piperidinylmethyl)-1*H*-indole (AM1241) (Marriott and Huffman, 2008). In addition, 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 and never disappeared completely (exponential decay plateau at ~40%), whereas anandamide underwent almost complete degradation, with a half-life of 14 min (Snider et al., 2009). The finding that, relative to anandamide, 5,6-EET-EA is a more potent agonist at the CB2 receptor and more refractory to enzymatic hydrolysis suggests that P450-mediated formation of 5,6-EET-EA may represent an endocannabinoid bioactivation pathway that may be important in pathological conditions of the CNS as well as the liver (Fig. 3).

In addition to anandamide, there is evidence that epoxidation of the endocannabinoid 2-AG may also represent a bioactivation pathway. It was recently demonstrated that

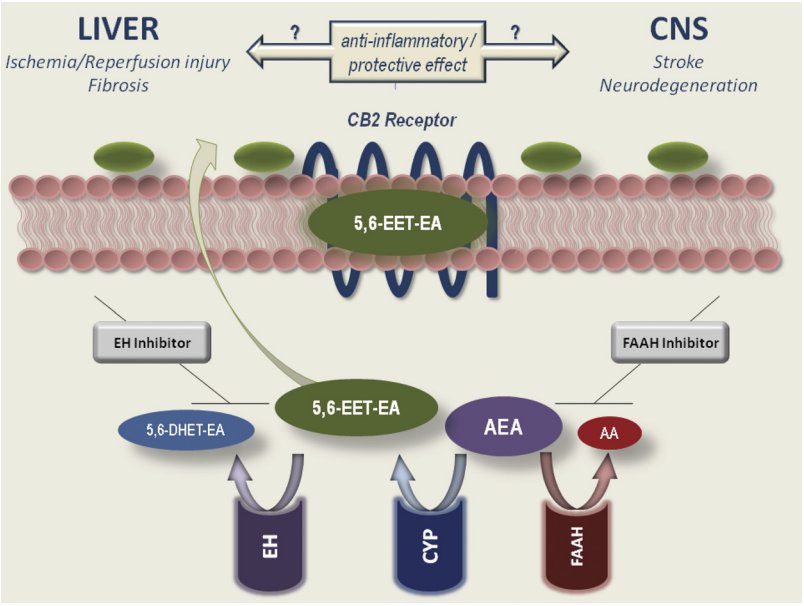

FIG. 3. Potential protective actions of 5,6-EET-EA in pathological conditions of the liver and CNS. Anandamide (AEA) is metabolized by FAAH to arachidonic acid (AA) and by cytochrome P450 (CYP) to 5,6-EET-EA, which is in turn metabolized by epoxide hydrolase (EH) to 5,6-DHET-EA. Administration of FAAH or EH inhibitors could lead to an increase in the formation of endogenously produced 5,6-EET-EA by affecting its synthesis or its degradation. 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 that could alter the pathological outcome in both acute and chronic conditions affecting the liver and the CNS.

the synthetically generated 2-AG epoxides 11,12- and 14,15-epoxyeicosatrienoyl glycerol (2-11,12-EG and 2-14,15-EG) bind to both CB1 and CB2 receptors with high affinity (Chen et al., 2008). In rat cerebellar membranes, which express CB1 receptors, 2-11,12-EG and 2-14,15-EG competed for binding with CP-55940 with K_i values of 23 and 40 nM, respectively, similar to the affinity of 2-AG (K_i = 45 nM). The K_i values obtained using spleen membrane preparations, which are enriched in CB2 receptors, were 75, 138, and 251 nM for 2-11,12-EG, 2-14,15-EG, and 2-AG, respectively, suggesting that the epoxides of 2-AG have a slightly higher affinity for CB2 receptors compared with the parent molecule. Using electrospray ionization/LC/tandem MS analysis, the presence of both 2-11,12-EG and 2-14,15-EG was detected in kidney and spleen at concentrations of 0.3 to 0.8 ng/g tissue, compared with the 7.4 to 11.4 ng/g levels of 2-AG detected in the same tissues. In contrast, only 2-11,12-EG was detected in brain tissue at a concentration of 0.2 ng/g, where 2-AG is also abundant (5.2 ng/g). In isolated renal microvessels, 2-11,12-EG elicits a CB1 receptor-dependent vasorelaxing response (Chen et al., 2008), whereas 2-14,15-EG produces a CB1/CB2-independent mitogenic response in renal proximal tubule cells (Chen et al., 2007). However, the source of the endogenously detected 2-11,12-EG and 2-14,15-EG is unclear, because the authors reported a complete lack of NADPH-dependent epoxidation or hydroxylation of [^{14}C]2-AG by rat liver and kidney microsomes or recombinant CYP2C8, CYP2C11, and CYP2C23 in the reconstituted system. It is also not currently known whether, like anandamide, 2-5,6-EG and 2-8,9-EG can also be formed from 2-AG.

C. Anandamide Oxidation Leading to Inactivation or the Generation of Bioactive Molecules Acting on Novel Targets

Anandamide metabolism by P450s can also be viewed as an endocannabinoid inactivation pathway in the context of the CB1 receptor, because 5,6-EET-EA does not seem to have an appreciable activity at the CB1 receptor. This is also the case with some of the other metabolites, such as 20-HETE-EA and 14,15-EET-EA, which have 3- to 5-fold lower affinity for the rat brain CB1 receptor relative to anandamide (N. T. Snider, unpublished data).

More work remains to be done in exploring the possibility that anandamide oxidation by P450s results in the generation of molecules with biological activity outside of the endocannabinoid system. However, in addition to its activity at the CB2 receptor, 5,6-EET-EA also activates the osmosensing transient receptor potential channel 4 (TRPV4) rapidly and at low nanomolar concentrations compared with the micromolar levels required for anandamide activation of TRPV4 (N. T. Snider, unpublished data). In that regard, 5,6-EET-EA acts in a manner similar to the arachidonic acid metabolite 5,6-EET, which was initially reported to be a potential endogenous agonist of the TRPV4 receptor (Watanabe et al., 2003).

D. Cytochrome P450-Mediated Anandamide Oxidation and Liver Pathophysiology

Given the abundance of P450 expression and activity in the liver, the interaction of the P450 enzymes with the endocannabinoid system is likely to play an important

physiological role there. A number of recent studies have demonstrated a protective role for the activation of the CB2 receptor in several pathological conditions of the liver: in liver fibrosis via reduction in the accumulation of fibrogenic cells; in hepatic ischemia/reperfusion injury via attenuation of the inflammatory response; in hepatic encephalopathy via stimulation of brain AMP-activated protein kinase; and in autoimmune hepatitis via up-regulation of regulatory T cells and down-regulation of inflammatory cytokines (Julien et al., 2005; Avraham et al., 2006; Bátkai et al., 2007; Mendez-Sanchez et al., 2007; Hegde et al., 2008; Caraceni et al., 2009b). Most of these studies have been reviewed previously (Caraceni et al., 2009a). Other recent developments in this field will be discussed in this section.

A key recent study revealed several novel findings regarding the contribution of hepatic CB1 receptors to various aspects of the metabolic syndrome (Osei-Hyiaman et al., 2008). Hepatocyte CB1 receptors are up-regulated under high-fat diet conditions, and liver-specific CB1 knockout mice [LCB1(−/−)] are not protected against the development of obesity. However, LCB1(−/−) mice are resistant to diet-induced steatosis to an extent that is similar to that in whole-body CB1-null mice [CB1(−/−)]. In addition, the high-fat-diet–induced hyperleptinemia, hyperinsulinemia, increased levels of triglycerides and LDL cholesterol observed in wild-type mice are either absent or attenuated to a similar extent in both CB1(−/−) and LCB1(−/−) mice. The high-fat-diet–induced increases in serum triglyceride and LDL cholesterol and decrease in HDL cholesterol levels in wild-type mice are also absent or attenuated in LCB1(−/−) or CB1(−/−) mice. These data, in addition to findings that hepatic CB1 receptors are involved in high-fat-diet–induced glucose intolerance and insulin resistance, suggest that peripherally acting CB1 antagonists might be a viable therapeutic option for the management of steatosis and cardiovascular risk factors associated with the metabolic syndrome. Similar to CB1 receptor-regulated pathways, CB2 receptor activation also seems to potentiate insulin resistance in high-fat-diet–fed mice and promotes the development of hepatic steatosis, possibly by enhancing adipose tissue inflammation (Deveaux et al., 2009).

In the context of liver fibrosis, CB1 and CB2 receptor activation results in pro- and antifibrogenic effects, respectively (Julien et al., 2005; Teixeira-Clerc et al., 2006; DeLeve et al., 2008). The expression of liver CB1 receptors during chronic liver diseases is up-regulated, and their antagonism by SR141716A decreases the accumulation of hepatic myofibroblasts and the expression of transforming growth factor β1 in an acute model of matrix remodeling using a single intraperitoneal injection of carbon tetrachloride (CCl_4). In addition, CB1 receptor antagonism reduces fibrosis associated with chronic liver injury in three models of fibrosis, including chronic CCl_4 intoxication, chronic thioacetamide intoxication, and bile duct ligation. Either genetic or pharmacological inactivation of CB1 receptors reduces accumulation of mouse hepatic myofibroblasts in vitro and in vivo by enhancement of apoptosis and/or attenuated proliferation of liver fibrogenic cells via phosphatidylinositol 3-kinase-Akt and extracellular signal-regulated kinase-regulated signaling. In contrast, CB2 receptor activation seems to play a protective role in liver fibrosis by exerting a proapoptotic effect upon hepatic stellate cells, the key profibrogenic cell type. Upon long-term intoxication with carbon tetrachloride, CB2-null mice develop more extensive liver fibrosis than their wild-type littermates. In addition, CB2 receptors are highly expressed on myofibroblastic cells of human cirrhotic livers, and cannabinoids inhibit DNA synthesis in cultured human hepatic myofibroblasts via a CB2 receptor-dependent pathway. Therefore, pharmacologic manipulation of the cannabinoid receptors in the potential management of liver disease, whether antagonism- or agonism-based, would need to be disease context-dependent.

It would be of interest to apply the knowledge on the identified anandamide metabolites, in particular 5,6-EET-EA, toward understanding the protective mechanism of CB2 receptor activation in liver disease, especially because many P540 isoforms, including the 5,6-EET-EA-generating CYP3A4 isoform have been found to be down-regulated 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 ultimately lead to a better understanding of its physiological role.

E. Cytochrome P450-Mediated Anandamide Oxidation and Central Nervous System Pathophysiology

Within the CNS, CB1 receptors are expressed on neurons whereas the CB2 receptors are 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). Use 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 CB2 receptor 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 disease and multiple sclerosis, as well as acute CNS injury such as

stroke and trauma (Carrier et al., 2004; Maresz et al., 2005; Ortega-Gutiérrez et al., 2005; Ramírez et al., 2005; Ashton and Glass, 2007; Ashton et al., 2007; Fernández-Ruiz et al., 2007; Sagredo et al., 2007). The anandamide product 5,6-EET-EA could be an endogenously produced mediator that can alter microglial activity. Upon stimulation with the cytokine interferon γ (IFNγ), murine microglial BV-2 cells have increased capacity for converting anandamide to 5,6-EET-EA, which correlates with an increase in the level of CYP3A expression and can be inhibited by treatment with ketoconazole, a selective inhibitor of CYP3A (Snider et al., 2009). In contrast to the IFNγ-stimulated induction of CYP3A protein expression observed in the microglia, previous reports by others have demonstrated an IFNγ-stimulated down-regulation of CYP3A 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 CYP3A by this cytokine and is in agreement with data demonstrating a differential regulation of 3A protein expression in liver and brain (Robertson et al., 2003).

F. Potential Involvement of Cytochrome P450-Mediated Anandamide Oxidation in the Effects of Epoxide Hydrolase Inhibitors

The existence of CNS-modulating properties of P450-derived anandamide metabolites, such as 5,6-EET-EA, may become more apparent in the presence of an epoxide hydrolase inhibitor. During ischemic stroke in laboratory animals as well as in human patients, there is an increased release of several fatty acid ethanolamides, including anandamide (Schäbitz 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 to 50% (Zhang et al., 2007b). This protective effect is almost completely reversed by coadministration of the P450 epoxygenase inhibitor 6-(2-propargyloxyphenyl) hexanoic acid. However, it was found that the mechanism of protection by AUDA-BE was not due to vascular effects that would be characteristic of arachidonic acid-derived EETs or altered ischemic severity, as determined by blood flow rates. This suggests an alternate mechanism for the protection by AUDA-BE and is in agreement with the findings of another recent study demonstrating that in transgenic mice with neuron-specific overexpression of soluble epoxide hydrolase there was no change in arterial blood pressure (Bianco et al., 2009). Given that AUDA inhibits epoxide hydrolase-mediated metabolism of 5,6-EET-EA (Snider et al., 2009), 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 endogenous 5,6-EET-EA at the CB2 receptor on microglia and/or infiltrating lymphocytes. Coadministration of a CB2-selective antagonist such as 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl(4-methoxyphenyl) methanone (AM630) with AUDA-BE in the experimental cerebral ischemia model could provide more insight into this mechanism.

In addition, a recent study demonstrated the neuroprotective properties of AUDA in normotensive Wistar-Kyoto and spontaneously hypertensive stroke-prone rats subjected to cerebral ischemia, which was determined to result from a differential modulation of genes involved in the apoptotic response, such as Bad, Bcl2, and Nfkb1 (Simpkins et al., 2009). Furthermore, another recent study provided evidence for the neuroprotective effect of selective CB2 receptor agonism in axotomized neurons via phosphatidylinositol 3-kinase-dependent regulation of Akt and JNK phosphorylation (Viscomi et al., 2009). Given that PI3/Akt activation is upstream of and regulates the expression of many of the genes implicated in the neuroprotective effects of AUDA, it is possible that the generation of more 5,6-EET-EA and subsequent CB2 receptor activation is an additional benefit of epoxide hydrolase inhibition. In addition, because 5,6-EET-EA is primarily generated by CYP3A4, and the 3A4 *4 polymorphism (I118V mutation) that produces an enzyme with decreased catalytic activity was recently reported to be strongly associated (P = 0.0006) with an increased risk for hemorrhagic stroke (Yamada et al., 2008), these observations may have potential clinical relevance. The other anandamide metabolites, such as those generated by CYP4F2, may potentially play a role in cerebral ischemia, as suggested by a recent report on the association between a V433M variant in CYP4F2 and ischemic stroke that is only partially explained by effects on blood pressure (Fava et al., 2008).

G. Potential Cross-Talk between Fatty Acid Amide Hydrolase and Cytochrome P450-Mediated Anandamide Metabolism in the Neuroprotective and Anxiolytic Effects of Anandamide

It was recently demonstrated in animal models of PD that endocannabinoid system-mediated long-term depression is absent but can be rescued by inhibition of FAAH via URB597 administration (Kreitzer and Malenka, 2007). The findings from this study, in combination with others demonstrating a neuroprotective effect of FAAH inhibitors (Gubellini et al., 2002), suggest that elevating brain anandamide levels is useful in treating neurodegenerative disease symptoms. It is noteworthy that there have been numerous studies linking the CYP2D6*4 poor metabolizer genotype with susceptibility to PD (Smith et al., 1992). Given the strong environmental effect on PD, this observation could be linked to deficits in the CYP2D6-mediated detoxification of PD-inducing xenobiotics. However, because of the involvement of CYP2D6 in the metabolism of endogenous substances, including anandamide, there is also a possibility that this effect could also be attributed to altered metabolism of endobiotics by CYP2D6.

In addition to neuroprotection, FAAH inhibitors also produce anxiolysis (Kathuria et al., 2003). Mice lacking

functional FAAH, and mice or rats treated with FAAH inhibitors exhibit anxiolytic properties (Kathuria et al., 2003; Moreira et al., 2008). For example, URB597 causes anxiolysis in rats subjected to the elevated zero-maze test and suppress isolation-induced vocalizations (Kathuria et al., 2003). This effect is CB1-dependent and is accompanied by elevated brain levels of anandamide. Several studies involving healthy subjects have linked the CYP2D6 poor metabolizer phenotype with increased anxiety (Smith et al., 1992; Llerena et al., 1993; González et al., 2008; Peñas-LLedó et al., 2009). With regard to the number of anandamide metabolites formed (5 monooxygenated and 13 dioxygenated; Fig. 2), CYP2D6 exceeds all other P450 isoforms tested, and it is likely that one or more of these products could be formed in vivo, especially when anandamide levels are elevated (in the presence of FAAH inhibition), potentially leading to neuroprotection and/or anxiolysis.

X. Future Directions

Backed by overwhelming scientific evidence for a critical role of the endocannabinoid system in human disease, the development of novel therapeutics that target this system is likely to stay an important focus of research in pharmacology. The significant progress that has been made in recent years in the area of lipidomics will aid in determining how the biotransformation products of endocannabinoids, such as anandamide, integrate with their functional mechanisms as signaling lipids in a cell-, tissue-, and disease context-dependent manner. There are many outstanding questions regarding the role of P450s in the metabolism and the termination or enhancement of the biological activity of endocannabinoids, ranging from the relatively simple, such as identification of the P450 isoforms involved in anandamide metabolism in other tissues, including the gastrointestinal and cardiovascular systems, to the more complex, such as determining the pharmacological activity profiles of the metabolites and their production in vivo.

Given the structural similarity of anandamide to arachidonic acid, a better understanding of the synthesis and action of its oxygenated products in the various tissues is necessary. Using microsomal preparations from rodent and/or human intestine, colon, spleen, heart, and lung, the NAPDH-dependent metabolism of anandamide can be investigated to determine the types of metabolites formed in these tissues and the involvement of specific P450 isoforms. Findings obtained from such studies can be complemented by additional investigations using individual membrane-expressed or purified P450s in the reconstituted system, including the many clinically relevant polymorphic P450s, such as the variants of CYP2D6 and CYP3A4. Comparisons of metabolite profiles between healthy and diseased tissues may provide important insights into the potential involvement of endocannabinoid oxidation in various disease processes.

The pharmacological properties of the EET-EAs and 20-HETE-EA need to be investigated in the context of various animal models of disease in which endocannabinoid system activation is thought to play a role, such as pain, inflammation, neurodegeneration, and cancer. Coadministration of inhibitors of FAAH and epoxide hydrolase will probably be necessary to achieve adequate levels of the anandamide metabolites in vivo upon systemic administration. It will also be important to determine the levels of endogenously produced oxygenated anandamide products under physiological and pathological conditions. This may represent a significant challenge, given the relatively low levels of endogenous anandamide and the likelihood that the oxygenated metabolites will undergo further transformation by other enzyme systems. Thus, defining additional metabolic pathways (e.g., cyclooxygenase-mediated) that could further transform the P450-derived anandamide products will also be necessary.

Although EET-EAs and 20-HETE-EA are available commercially, the dioxygenated anandamide metabolites HEET-EAs (derived via CYP2D6) and the DHET-EAs (derived via epoxide hydrolase) are currently not available in their pure forms. Generation of synthetic derivatives of these products and investigation of their pharmacological properties in vitro and in vivo will aid in determining whether these secondary metabolites are inactivation products or they possess pharmacological activity. In addition, and depending on their activity profile, these molecules could serve as templates for the design of new endocannabinoid-based drug molecules.

The results from these types of studies should provide a better understanding of the interaction between P450s and the endocannabinoid system, which in turn is going to be critical for the ultimate success of endocannabinoid-based therapies in the clinical setting.

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