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

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Abbreviations: P450, cytochrome P450; AEA, anandamide; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; DHET, dihydroxyeicosatrienoic acid; EA, ethanolamide; mEH, microsomal epoxide hydrolase; FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; LOX, lipoxygenase; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry; HKM, human kidney microsomes; HLM, human liver microsomes.

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Abstract

The endocannabinoid anandamide is an arachidonic acid derivative that is found in most tissues where it acts as an important signaling mediator in neurological, immune, cardiovascular and other functions. Cytochromes P450 (P450s) are known to oxidize arachidonic acid to the physiologically active molecules hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) which play important roles in blood pressure regulation and inflammation. To determine if anandamide can also be oxidized by P450s, its metabolism by human liver and kidney microsomes was investigated. The kidney microsomes metabolized anandamide to a single mono-oxygenated product, which was identified as 20-HETE ethanolamide (20-HETE-EA). Human liver microsomal incubations with anandamide also produced 20-HETE-EA in addition to 5,6-, 8,9-, 11-12, and 14,15-EET ethanolamide (EET-EA). The EET-EAs produced by the liver microsomal P450s were converted to their corresponding dihydroxy derivatives by microsomal epoxide hydrolase (mEH). P450 4F2 was identified as the isoform that is most likely responsible for the formation of 20-HETE-EA in both human kidney and human liver with an apparent K_m of 0.7 μM. The apparent K_m values of the human liver microsomes for the formation of the EET-EAs were between 2-5 μM and P450 3A4 was identified as the primary P450 in the liver responsible for epoxidation of anandamide. The *in vivo* formation and biological relevance of the P450-derived HETE and EET ethanolamides remains to be determined.

Introduction

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

The discovery of anandamide, an ethanolamide of arachidonic acid, as the first endocannabinoid (Devane et al., 1992) was followed by a great number of studies designed to understand the factors that regulate its bioavailability *in vivo*. The enzyme fatty acid amide hydrolase (FAAH) catalyzes the hydrolysis of anandamide to produce arachidonic acid and ethanolamine, and is known to be the chief enzyme that terminates the activity of anandamide *in vivo* (Giang and Cravatt, 1997). In addition to this hydrolytic mode of metabolism, it is possible that anandamide can also be subjected to oxidative metabolism by a number of fatty acid oxygenases that are known to metabolize endogenous arachidonic acid. These include the cyclooxygenases (COX), lipoxygenases (LOX) and cytochromes P450 (P450). Studies involving cell-free systems and cellular assays have revealed that anandamide can be converted into several prostaglandin ethanolamides by COX-2 (Yu et al., 1997; Burstein et al., 2000; Kozak et al., 2002) and 12- and 15- hydroperoxyeicosatetraenoic acid ethanolamides by 12-LOX and 15-LOX, respectively (Hampson et al., 1995; Ueda et al., 1995; Edgemond et al., 1998). However, the biological significance of these metabolic pathways remains to be elucidated.

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

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

hydrolase (mEH) to form the corresponding dihydroxyeicosatrienoic acid ethanolamides (DHET-EAs).

Methods

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

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

Anandamide metabolism assays. For the initial studies, the conversion of anandamide to oxygenated metabolites was assessed in incubation mixtures (0.5 mL) containing 100 mM KPO_4 buffer (pH 7.4), anandamide (20 or 100 μM), as specified in the legends to the figures), 1 mM NADPH and one of the following enzyme sources: human kidney or human liver microsomes (100 μg protein) or P450 4F2, 4F3b or 4A11 supersomes (25 pmol). Catalase (500 U) was also added to reaction mixtures using supersomes. The P450 3A4 reconstituted system consisted of 25 pmol of purified P450 3A4, 50 pmol of P450 reductase, 10 μg mixture of L-α-dilauroyl-phosphocholine, L-α-dioleyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidylserine (1:1:1), 50 U catalase, and 50 mM HEPES in a final reaction volume of 0.5 mL. For the reaction mixtures that contained microsomal

epoxide hydrolase (mEH), P450 3A4 (25 pmol) was reconstituted as above with the addition of mEH (25 pmol). All reactions were initiated by the addition of NADPH. For the initial studies the samples were incubated at 37 ºC for 45 min. The reactions were terminated by the addition of 2 mL of nitrogen-purged ethyl acetate and the samples were vortexed for 1-2 min. The samples were then centrifuged for 5-10 min at 1200 rpm to separate the organic layer, which was extracted and dried down under a constant stream of nitrogen gas. The dried samples were re-suspended in 200 μL methanol and 10 μL fractions were subjected to electrospray ionization-liquid chromatography/mass spectrometry (ESI-LC/MS) analysis as described below.

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

For the determination of the K_m and the V_{max} values, the incubation conditions were optimized for time and protein concentration and performed within the linear range of metabolite formation. The reaction mixture compositions were similar to those outlined above. For these studies, 75 μg of human kidney microsomal protein was incubated for 8 min and 25 μg of liver microsomal protein was incubated for 15 min. In experiments using the P450 4F2 supersomes, the reaction mixtures were incubated for 8 min with 2 pmol of enzyme. Following extraction and drying of the extracts, the samples were re-suspended in 100 μL of methanol and 10 μL aliquots were injected for analysis by ESI-LC/MS. Standard curves for 5,6-EET-EA, 14,15-EET-EA and 20-HETE-EA

were generated by extracting varying known amounts of the authentic standards from a 0.5 mL reaction mixture that did not contain anandamide and NADPH. Rates of formation for 8,9- and 11,12-EET-EA were determined based on the standard curves for 5,6- and 14,15-EET-EA, respectively.

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

Chemistry. 5,6-Epoxyeicosatrienoic acid (5,6-EET) was synthesized by iodolactonization of arachidonic acid, as described previously (Corey et al., 1980). 14,15-Epoxyeicosatrienoic acic (14,15-EET) was prepared using an arachidonic acid selective epoxidation (Corey et al., 1979). 20-Hydroxyeicosatetraenoic acid (20-HETE) was

prepared from 14,15-EET with a known 3-step procedure using the Wittig reaction as previously described (Manna et al., 1983). All three compounds were transformed into the corresponding ethanolamides using a standard procedure involving the intermediate NHS/DCC activation of the carboxylic moiety followed by the reaction with ethanolamine (Sehgal and Vijay, 1994). All of the products were purified by HPLC and characterized by NMR and mass spectrometry.

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

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

Results

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

Next, the identity of the P450 that is primarily responsible for the production of M1 in the human kidney was investigated. Members of the family 2 P450s, such as 2C8, 2C19 and 2J2 are known to be the predominant arachidonic acid epoxygenases in humans; whereas, hydroxylations of arachidonic acid are mainly carried out by P450s belonging to family 4, with 4A11, 4F2 and 4F3b playing the major roles in humans (Lasker et al., 2000; Capdevila and Falck, 2001, Christmas et al., 2001). In order to investigate the identity of the P450 responsible for the formation of M1, HKMs were pre-incubated with the antibodies that have been previously described (Gelboin and Krausz, 2006), and that possess inhibitory activity against specific P450s, to test for their effect on the formation of M1 following the initiation of metabolism by the addition of NADPH. Pre-incubation with antibodies against P450s 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 had no significant effect on the formation of M1 (data not shown), suggesting that these P450s did not play a role in the formation of M1. Because inhibitory antibodies specific for human P450s 4A and 4F were unavailable, the highly

selective arachidonic acid omega hydroxylase inhibitor, HET0016 (N-hydroxy-N'-(4-butyl-2-methylphenyl)-formamidine) was used to test inhibition of M1 formation. HET0016 has been shown to inhibit 20-HETE formation by 4A11 and 4F2 with a reported IC_{50} value of 8.9 nM in human kidney microsomes (Miyata et al., 2001). As shown in Figure 1B (inset), pre-incubation of the HKMs with 10 nM HET0016 (solid line) for 5 min prior to the addition of anandamide and NADPH resulted in a greater than 90% reduction in the formation of M1 compared to control (dotted line). Furthermore, as shown in Figure 1B, the effect of HET0016 was dose-dependent, exhibiting an IC_{50} value of 2.5 nM. This result was not surprising because human kidneys have been found to express P450s belonging to family 4 at much higher levels than most other P450s investigated. In fact, the kidney levels of P450 4F2 and 4A11 are similar to the levels found in human liver (Nishimura et al., 2003).

To determine whether the profiles of metabolite formation by the human P450s 4F2, 4F3b or 4A11 resembled those seen with HKMs, anandamide was incubated with supersomes containing these P450s. As shown in Figure 1C, M1 formation differed significantly among the three enzymes tested when using equal protein amounts, substrate concentration and incubation conditions, as described in the Methods section. The ratios of formation of M1, as determined by the corresponding peak heights, were approximately 58:7:1 (4F2:4F3b:4A11). To rule out significant differences in protein activity as a reason for the observed results, a parallel experiment was performed with arachidonic acid as a substrate (data not shown). The ratios of 20-HETE formation were 2.3:2:1 (4F2:4F3b:4A11), suggesting that the protein activity in the supersomes is

representative of the established role of each of the enzymes in the omega hydroxylation of arachidonic acid and is not a factor in the results seen with anandamide.

Although metabolism by the individual P450s in supersomes may not exactly reflect metabolism by those P450s in the kidney microsomes, these data suggest that the main P450 involved in the formation of M1 by the HKMs is P450 4F2. In addition to having the same retention time as M1, the metabolite of anandamide formed by P450 4F2 also exhibited MS/MS fragmentation identical to that seen with human microsomes (Figure 1D).

In combination, these results suggest that the human kidney microsomes metabolize anandamide to give a single mono-oxygenated product, probably a hydroxylated metabolite, and that this reaction is most likely catalyzed by P450 4F2.

Metabolism of anandamide by human liver microsomes (HLMs). In addition to forming product M1, HLM incubations led to the formation of several minor monooxygenated (*m/z* 364) metabolites eluting between 16 and 21 minutes (M2-M6), as shown in Figure 2A. Incubation of the HLMs in the presence of anandamide also resulted in the formation of four additional metabolites (M7-M10) with retention times between 10 and 18 minutes and having *m/z* values of 382 (Figure 2B). The mass to charge ratio of these products corresponded to that of one monooxygenated metabolite (*m/z* 364) plus a water molecule (M+18). Therefore, the possibility that metabolites M7-M10 may represent secondary products of the monooxygenated metabolites was considered because, in addition to P450s, the HLMs also contain other phase I and II metabolizing enzymes including microsomal epoxide hydrolase (mEH), which catalyzes the addition

of water to epoxides (Morisseau and Hammock, 2005). Because exactly four possible secondary products were seen and since there are four possible sites for epoxidation on the anandamide structure, these data suggest the possibility that some of the minor monooxygenated metabolites that were observed could be anandamide epoxides that are subsequently converted to their corresponding dihydroxy derivatives.

To determine the involvement of specific P450 isoforms in the formation of the HLM anandamide metabolites, a panel of monoclonal inhibitory antibodies and the chemical inhibitor HET0016 were used again. Once again, the formation of M1 was inhibited by HET0016 in a dose-dependent manner with an IC_{50} of approximately 93 nM (data not shown). As shown in Figure 3A, pre-incubation of the HLMs with the P450 3A4 antibody significantly inhibited the formation of metabolites M7-M10. The level of inhibition shown in Figure 3A was the maximum inhibition observed with respect to the amount of antibody added. Inhibition of metabolism was not observed for incubations in the presence of inhibitory antibodies against P450s 1A1, 1A2, 2A6, 2B6, 2D6, 2C8, 2C9 and 2E1, whereas antibody against P450 2C19 produced modest inhibition of M7-M10 ranging between 5-16% for the 6 HLMs tested (data not shown).

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

anandamide and NADPH. Figures 3B and C (dotted lines) depict the metabolic profile of the anandamide metabolites generated by P450 3A4 in the presence of mEH. This profile was similar to the one observed with the liver microsomes (Figure 2 A and B). A significant decrease in the formation of M3-M6 (*m/z* 364) was seen while at the same time the appearance of four new products that correspond to M7-M10 (*m/z* 382) was observed. This experiment confirms that products M3-M6 are epoxygenated metabolites of anandamide that undergo secondary metabolism by microsomal epoxide hydrolase in the liver microsomes.

The incomplete inhibition of M7-M10 formation by the antibody against P450 3A4 (Figure 3A) could be indicative of the participation of another hepatic P450 in the formation of these products, or the inability of the antibody to completely inhibit the activity of P450 3A4. To determine if the latter holds true, the effectiveness of the same antibody to inhibit the activity of reconstituted P450 3A4 was measured in the context of anandamide metabolism as well as the O-debenzylation of 7-benzyloxy-4-(trifluoromethyl-) coumarin (a probe substrate for P450 3A4). In both cases, the level of inhibition was similar (approximately 50%) to that observed with the HLM incubations (data not shown), suggesting that P450 3A4 is the primary enzyme involved in the human liver epoxidation of anandamide and the incomplete inhibition reflects the fact that the antibody is not fully inhibitory in these experiments.

Structural confirmation of anandamide metabolites. Chemical standards of 20-hydroxyeicosatetraenoic acid ethanolamide (20-HETE-EA), 14,15-epoxyeicosatrienoic acid ethanolamide (14,15-EET-EA) and 5,6- epoxyeicosatrienoic acid ethanolamide (5,6-

EET-EA) were synthesized for structural confirmation, as described in the Materials and Methods section.

Data from the ESI-LC-MS analysis of the chemical standards are shown in Figure 4 (A, C, E) and reveal that 20-HETE-EA, 14,15-EET-EA, and 5,6-EET-EA have the same retention times as M1 (15 min), M3 (17 min) and M6 (20 min), respectively. The MS/MS spectra of each of the standards, which were identical to the MS/MS spectra of the corresponding products obtained through the metabolic reaction, are also shown in Figure 4 (B, D, F).

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

Standards for 19-HETE-EA, 11,12- and 8,9-EET-EA are currently unavailable; however, they most likely correspond to M2, M4 and M5, respectively because the location of the functional group on the acyl chain would lead to that order of elution observed from the C18 column used in the study (Nithipatikom et al., 2001).

Kinetic analysis of anandamide hydroxylation by human kidney and liver microsomes and P450 4F2 supersomes. The human kidney and liver microsomes

converted anandamide to 20-HETE-EA in a time- and protein-dependent manner which was linear for at least 12 min of reaction time at 37 °C and 75 μg of kidney microsomal protein, and 15 min and 25 μg of liver microsomal protein (data not shown).

Pooled kidney microsomes from three subjects were used in the incubation reactions for the kinetic analysis of renal 20-HETE-EA formation. Over the range of substrate concentrations used (0.25-10 μM), anandamide metabolism to 20-HETE-EA exhibited simple Michaelis-Menten kinetics (Fig. 5A, open circles) which were consistent with reaction catalysis by a single enzyme. Nonlinear regression analysis was used to analyze the data and derive an apparent K_m of 2.6 ± 0.4 μM and a V_{max} of 122 ± 7.4 pmol of 20-HETE-EA formed/min/mg of kidney microsomal protein.

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

The kinetics of 20-HETE-EA formation by P450 4F2 supersomes are shown in Figure 5B. The V_{max} for omega hydroxylation of anandamide by P450 4F2 was 11.5 ± 0.4 pmol 20-HETE-EA/min/pmol P450 4F2 protein and the apparent K_m was 0.7 ± 0.07 μM. In comparison, P450 4F3b supersomes exhibited an apparent K_m of 3.4 ± 0.79 μM and a V_{max} of 0.1 ± 0.01 pmol 20-HETE-EA/min/pmol P450 4F3b protein (data not shown).

Combined, these data suggest that anandamide is a high affinity substrate for P450 4F2 and that this P450 may be the one which leads primarily to the formation of 20-HETE-EA in human kidney and liver microsomes.

Kinetic analysis of anandamide epoxidation by human liver microsomes. The human liver microsomes converted anandamide to 5,6-, 8,9-, 11,12-, and 14,15-EET-EA in a time- and protein-dependent manner which was linear for all products for 15 min of reaction time at 37 °C and 25 μg of microsomal protein (data not shown). Pooled liver microsomes were used for the kinetic analysis of hepatic EET-EA formation from anandamide at concentrations ranging from 1-25 μM. Non-linear regression analysis of the data was used to derive apparent K_m values ranging from 4-5 μM (Table 1).

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

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

Discussion

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

A number of novel oxygenated products of anandamide produced by human liver and kidney microsomal P450s were identified here. The data presented show that anandamide can undergo hydroxylation by human kidney microsomes and epoxygenation and hydroxylation by human liver microsomes. Lack of detection of epoxygenated metabolites in the reactions carried out with human kidney microsomes is in agreement with previously published studies on the lack of detection of EET production from arachidonic acid in this tissue (Lasker et al., 2000). This also points to the possibility of tissue-dependent production of anandamide metabolites in accordance with the presence of specific P450 isoforms in various tissues.

The finding that P450 4A11 exhibited negligible anandamide omega hydroxylase activity when compared to P450 4F2, although somewhat surprising, has precedent from at least one other example of a similar phenomenon in the literature. A study by Jin et al.

found that CYP4F2 converted leukotriene B4 (LTB4) to 20-OH LTB4 at a turnover rate of 392 pmol/min/nmol P450, whereas CYP4A11 exhibited negligible LTB4 omega-hydroxylase activity (Jin et al., 1998).

The studies presented here indicate a specific role for P450 4F2 in the formation of 20-HETE-EA in both the kidney and the liver and a role for P450 3A4 in the formation of EET-EAs in the liver. Although P450 2C and 2J families are known to be the major arachidonic acid epoxygenases in humans, it has previously been reported that monkey endothelial P450 3A4 can also act as an arachidonic acid epoxygenase to produce the vasorelaxant EETs (Ayajiki et al., 2003). EETs are known to play an important function in the vascular and renal systems and are also produced by the endothelium and can function as the endothelium-derived hyperpolarizing factors (EDHFs) in the coronary circulation (Spector and Norris, 2006). The vasorelaxant properties of anandamide have been well-documented and one mechanism involves the transient receptor potential channel TRPV4, which is also known to be activated by EETs (Watanabe et al., 2003). 20-HETE is an inhibitor of renal tubular Na+/K+-ATPase activity and a potent constrictor of kidney microvessels (Miyata and Roman, 2005). Anandamide has also been reported to have vasoconstrictor properties in renal vascular beds through a mechanism that is independent of its action at the cannabinoid receptor (Gardiner et al., 2002).

Conversion of anandamide to arachidonic acid via hydrolysis by FAAH and subsequent metabolism of the free fatty acid by P450s to vasoactive eicosanoids is one mechanism to explain the vascular properties of anandamide and this mechanism has been proposed previously (Watanabe et al., 2003). However, the possibility that P450s

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

samples. Because of the wide-ranging effects of anandamide on many organ systems, its metabolism by P450s from various tissues such as the brain and the spleen needs to be examined in detail and this is the focus of current and future investigations.

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Footnotes

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Legends for Figures

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

Figure 2. Metabolism of anandamide by human liver microsomes. Human liver microsomes (100 μg) were incubated in the presence of 100 μM anandamide and NADPH for 45 min. The selected ion chromatogram (panel A) shows several mono-oxygenated products (M1-M6, *m/z* 364) and the anandamide ion (AEA, *m/z* 348). Four

additional products (M7-M10) were detected from the same incubation having *m/z* values of 382 (panel B).

Figure 3. Involvement of P450 3A4 in the metabolism of anandamide by human liver microsomes and the effect of microsomal epoxide hydrolase. Human liver microsomes were pre-incubated in the presence of control antibody (hen egg lysozyme, HEL) or P450 3A4 inhibitory antibody (panel A). The peak heights for metabolites M7-M10, expressed as a percent of control (no antibody) are plotted on the y-axis. The data show the mean and standard deviation of six experiments. Human recombinant P450 3A4 (25 pmol) was reconstituted in the presence (……) or absence (___) of microsomal epoxide hydrolase, and was incubated with anandamide (100 μM) for 45 min (panels B and C). The selected ion chromatograms for the ions at *m/z* 364 (B) and *m/z* 382 (C) are shown.

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

Figure 5. Kinetics of 20-HETE-EA formation by human kidney and liver microsomes and P450 4F2 supersomes. 75 μg of human kidney microsomes (○) or 25 μg of human liver microsomes (●) were incubated with various concentrations of anandamide (0.1-10 μM) for 8 min and 15 min, respectively (panel A). P450 4F2

supersomes (2 pmol) were incubated in the presence of anandamide (0.1-5 μM) for 8 min (panel B). The amount of 20-HETE-EA formed was determined from a standard curve and the rate data (average of four experiments) were fitted to a one-enzyme Michaelis-Menten model using Prizm software.

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Table 1. Anandamide epoxidation by human liver microsomes.

Km and *Vmax* values were assessed from 15 min. incubations at 37 °C of 0.5 mL reaction mixtures containing 25 μg microsomal protein, 100 mM KPO4 buffer, pH 7.4, anandamide (1-25 μM) and NADPH (1 mM) and represent the average of five experiments. Rates of formation were determined as described under Methods.

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

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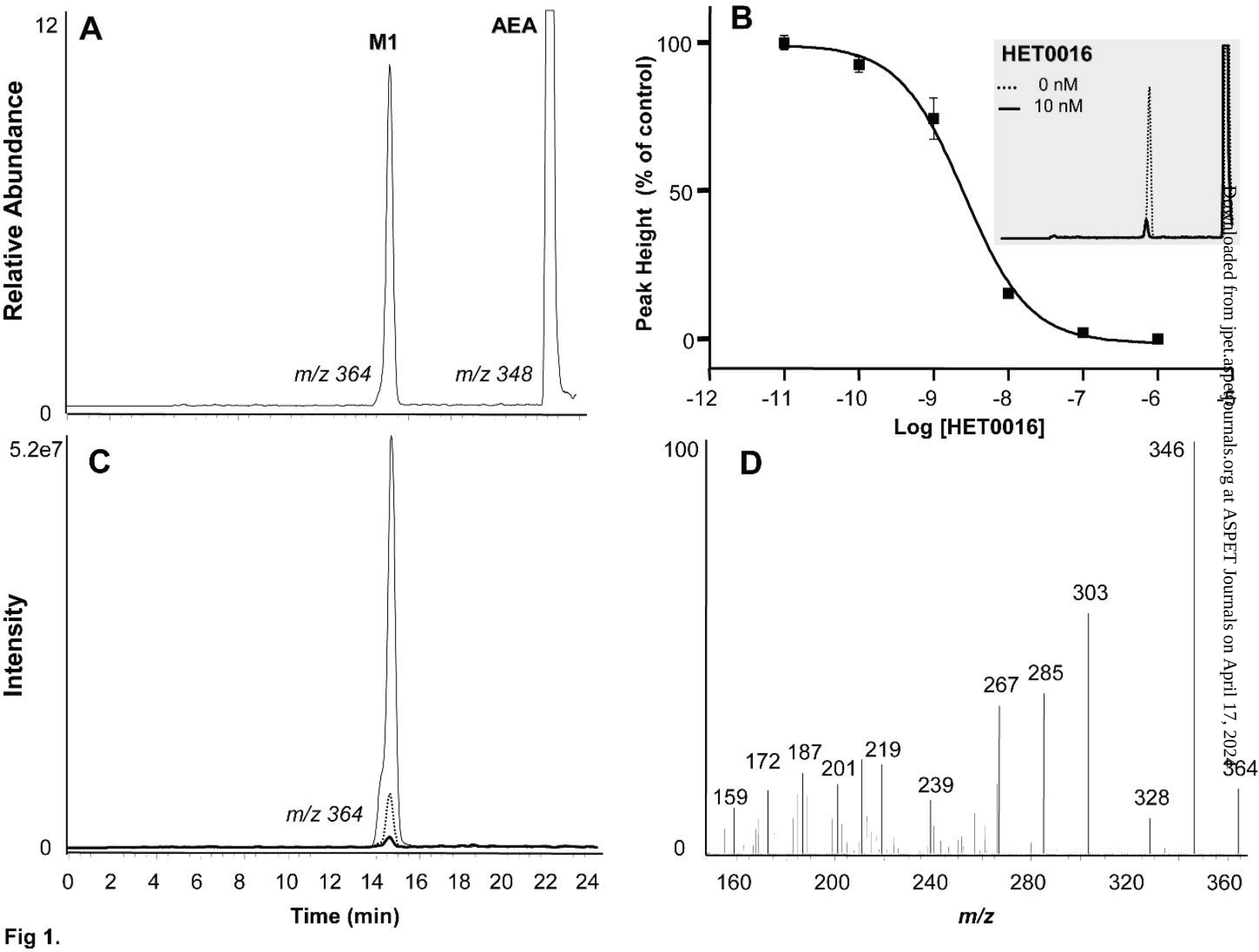

Fig 1.

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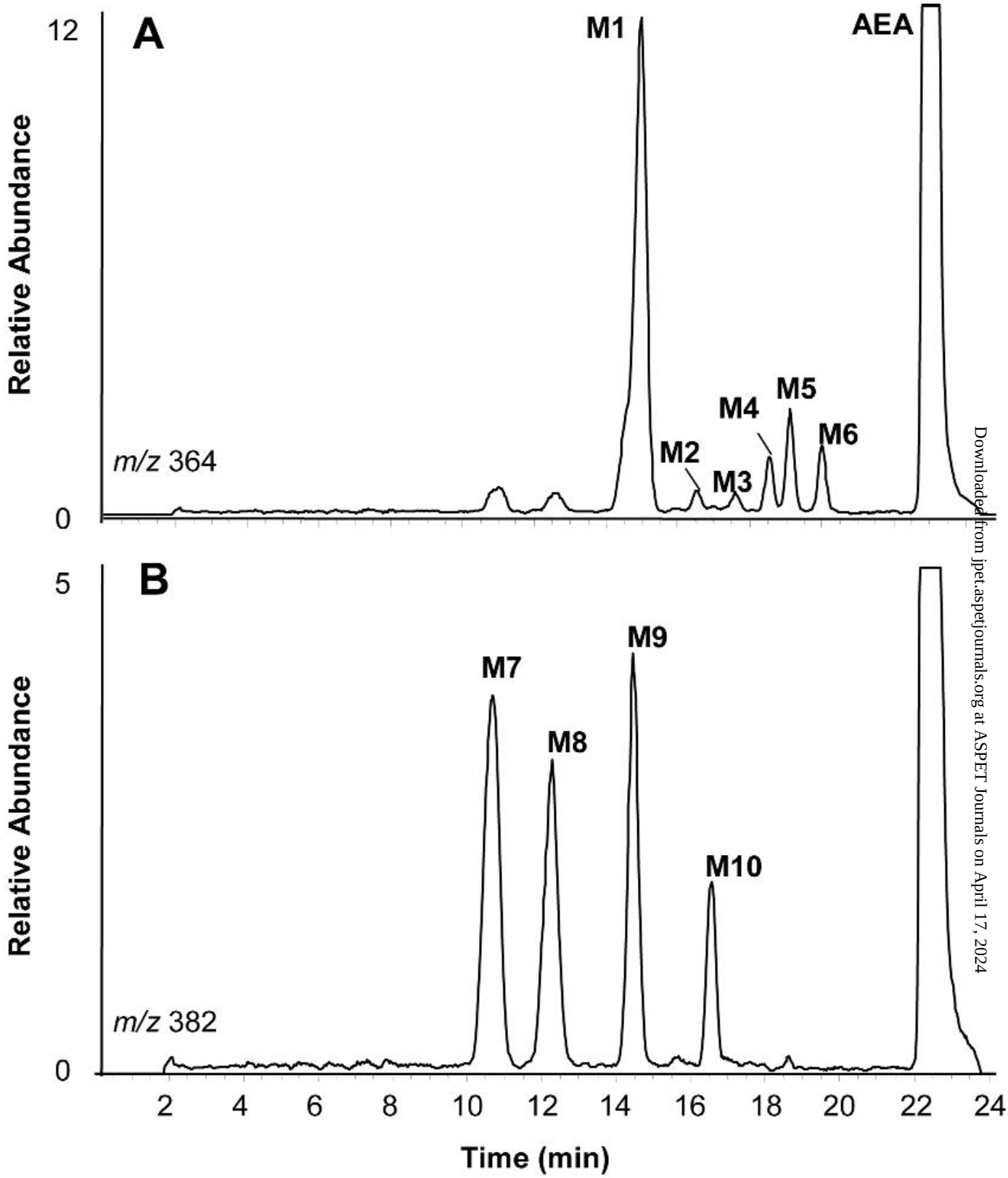

Fig. 2

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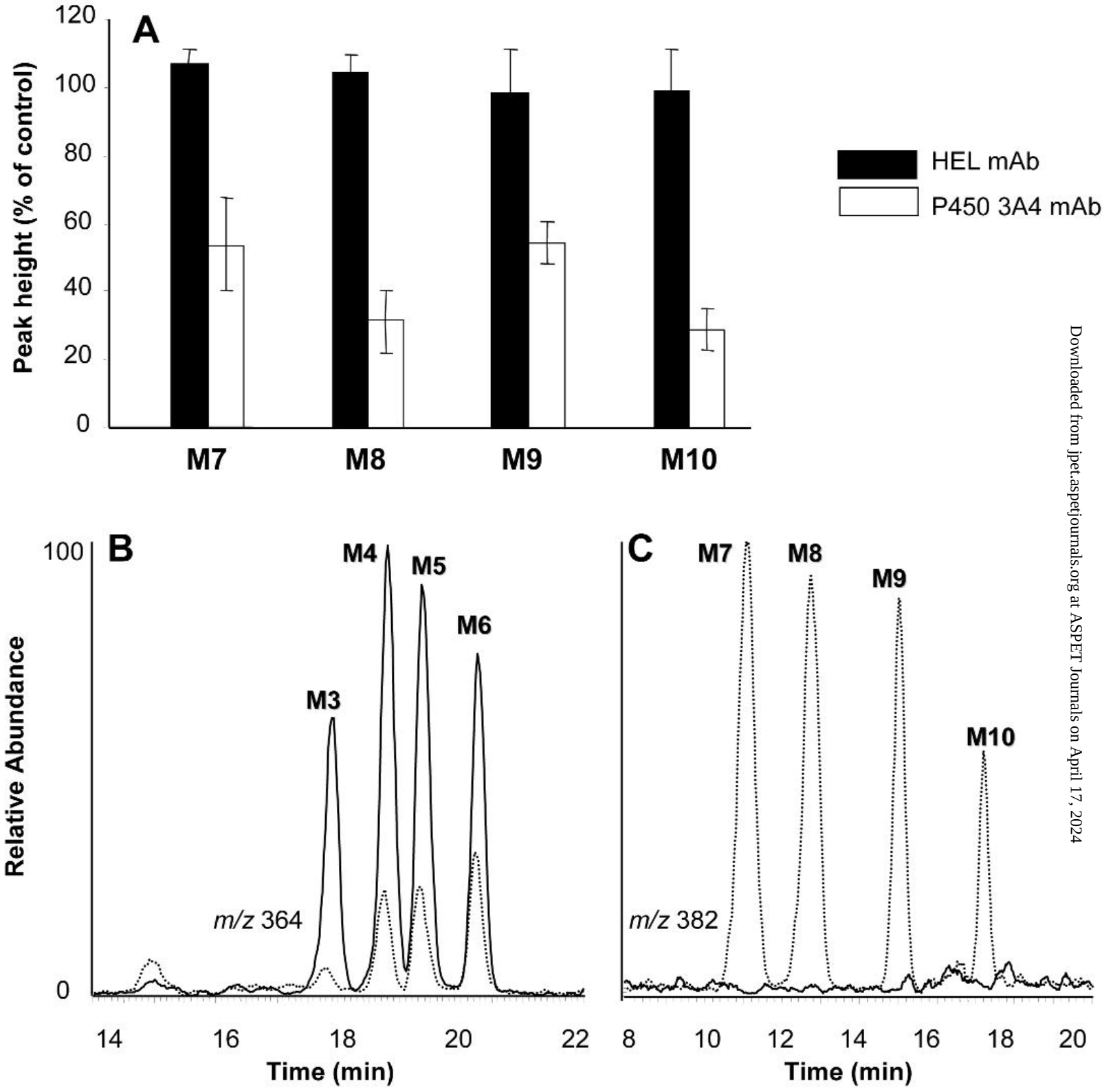

Fig. 3

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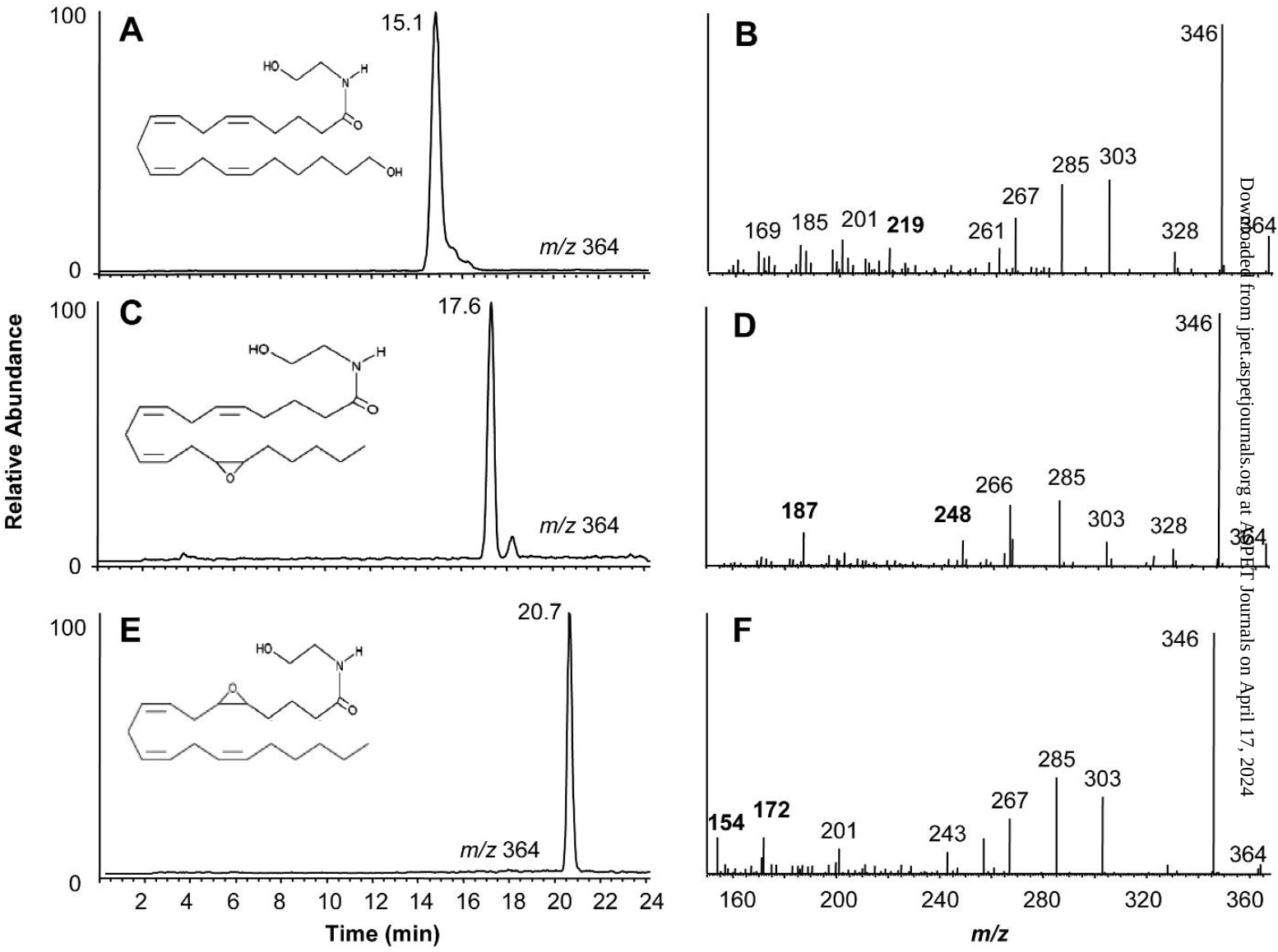

Fig. 4

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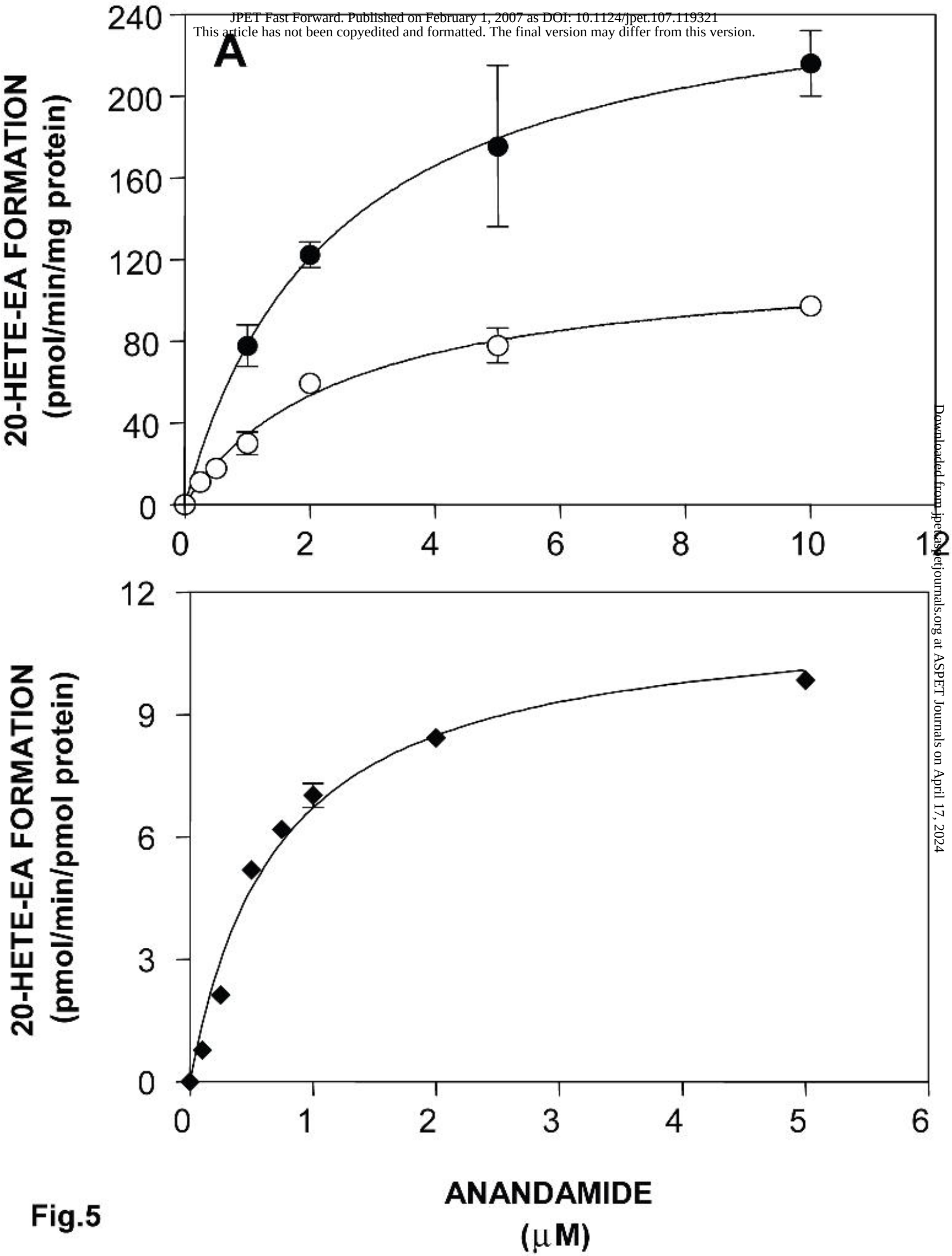

Fig.5