Anandamide Oxidation by Wild-Type and Polymorphically Expressed CYP2B6 and CYP2D6

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ABSTRACT:

Anandamide is an arachidonic acid-derived endogenous cannabinoi

d that regulates normal physiological functions and pathophys-

iological responses within the central nervous system and in the

periphery. Several cytochrome P450 (P450) isoforms metabolize

anandamide to form hydroxylated and epoxigenated products. Human CYP2B6 and CYP2D6, which are expressed heteroge-

neously throughout the brain, exhibit clinically significant polymor-

phisms and are regulated by external factors, such as alcohol and

smoking. Oxidative metabolism of anandamide by these two P450s

may have important functional consequences for endocannabinoid

system signaling. In this study, we investigated the metabolism of

anandamide by wild-type CYP2B6 (2B6.1) and CYP2D6 (2D6.1) and

by their common polymorphic mutants 2B6.4, 2B6.6, 2B6.9, and

2D6.34. Major differences in anandamide metabolism by the two

isoforms and their mutants were found in vitro with respect to the

formation of 20-hydroxyeicosatetraenoic acid ethanolamide (20-

HETE-EA) and 14,15-epoxyeicosatetraenoic acid ethanolamide

(14,15-EET-EA). Pharmacological studies showed that both 20-

HETE-EA and 14,15-EET-EA bind to the rat brain cannabinoid CB1

receptor with lower affinities relative to that of anandamide. In

addition, both products are degraded more rapidly than anand-

amide in rat brain homogenates. Their degradation occurs via

different mechanisms involving either fatty acid amide hydrolase

(FAAH), the major anandamide-degrading enzyme, or epoxide hy-

drolase (EH). Thus, the current findings provide potential new

insights into the actions of inhibitors FAAH and EH, which are being

developed as novel therapeutic agents, as well as a better under-

standing of the interactions between the cytochrome P450 mono-

oxgenases and the endocannabinoid system.

Introduction

The endogenous lipid arachidonoyl ethanolamide (anandamide) is a

naturally occurring amide of arachidonic acid that binds reversibly

and activates the cannabinoid receptors CB1 and CB2, and, therefore,

it is referred to as an endocannabinoid. It is estimated that the whole

brain basal levels of anandamide are approximately 19 pmol/g (Buc-

zynski and Parsons, 2010). Anandamide binding to its receptors leads
to antinociceptive, anti-inflammatory, and neuroprotective effects,

which makes the development of pharmacological agents that can

selectively elevate the endogenous levels of anandamide, a promising

therapeutic approach (Di Marzo, 2008). Such drug candidates include

inhibitors of fatty acid amide hydrolase (FAAH), the enzyme that

primarily inactivates anandamide, which are being developed for pain,

anxiety, and inflammatory disorders (Schlosburg et al., 2009). A

better understanding of other metabolic pathways that can exert con-

trol over the endogenous levels of anandamide is essential for further

progress in this area.

In addition to hydrolysis by FAAH, anandamide is oxygenated by

several human cytochrome P450 enzymes, including 3A4, 4F2, 4X1,

and the highly polymorphic 2D6, forming a number of metabolites

that are likely to have important physiological roles (Snider et al.,

2010). For example, the epoxide of anandamide at position C5–C6
formed by hepatic CYP3A4 is a potent agonist at the CB2 receptor

(Snider et al., 2009, 2010). Human CYP2D6.1 metabolizes anand-

amide to produce five monoxygenated metabolites, including a hy-

droxylated product, the 20-hydroxyeicosatetraenoic acid ethanol-

amide (HETE-EA), and four epoxides, the 5,6-, 8,9-, 11,12-, and

14,15-epoxyeicosatetraenoic acid ethanolamides (Snider et al., 2008).

Because CYP2D6 is expressed and functional in human brain and

there are neuropsychiatric differences among individuals with differ-

ent CYP2D6 genotypes (Funae et al., 2003; Miksys and Tyndale,
2004; Ingelman-Sundberg et al., 2007), it is plausible that CYP2D6-mediated biotransformation of endogenous psychoactive substrates, such as anandamide, results in the formation of metabolites with important activity. The amino acid substitution R296C is present in a large number of CYP2D6 alleles (http://www.cypalleles.ki.se/cyp2d6.htm) either alone (CYP2D6*34) or in conjunction with other mutations that are commonly observed (Marez et al., 1997). Furthermore, CYP2D6 variants containing this mutation have been shown to have an effect on the disposition of neuroactive steroids and tyramine in the brain (Niwa et al., 2004).

As for CYP2D6, CYP2B6 is also expressed in brain and is one of the most polymorphic P450 genes in humans (Miksys et al., 2003; Zanger et al., 2007). Several widely used drugs are metabolized by CYP2B6, including the antidepressant and smoking cessation agent bupropion (Faucette et al., 2000; Hesse et al., 2000), the anticancer agent cyclophosphamide (Chang et al., 1993), and the non-nucleoside reverse transcriptase inhibitor efavirenz (Miksys et al., 2003). Genetic polymorphisms seem to be contributing to changes in the expression and activity of the drug-metabolizing enzymes and thus to drug metabolism reactions. In the brain, CYP2B6 is expressed in neurons and astrocytes in a region-specific manner, and the levels vary depending on an individual’s exposure to smoking and alcohol (Miksys et al., 2003). Several common single nucleotide polymorphisms have been identified in the human CYP2B6 gene (Lang et al., 2001; Klein et al., 2005). The frequency of the 2B6.4 allele has been shown to be approximately 50% in Ghanians and close to 30% in African Americans and whites (Klein et al., 2005). The polymorphic 2B6.9 has an allelic frequency of 20% in Japanese populations as reported by Aiyoshi et al. (2001). The frequency of the K262R (*4) and Q172H (*9) CYP2B6 alleles was found to be 0.29 and 0.28, respectively, when screened across a panel of human livers derived from an ethnically diverse population (Hesse et al., 2004), with the CYP2B6*6 haplotype (Q172H/K262R) exhibiting a similar frequency. The objective of the current study was to investigate the major differences in the metabolism of anandamide between wild-type and mutant forms of CYP2D6 and CYP2B6 and to examine some of the pharmacological properties of the differentially generated products.

Materials and Methods

Materials. Oligonucleotide primers were obtained from the University of Michigan Core facility. Anandamide and anandamide metabolites were purchased from Cayman Chemical (Ann Arbor, MI). Cat- Michigan Core facility. Anandamide and anandamide metabolites used for enzymes were supplemented with catalase (500 U) and 50 mM potassium with 0.2 nmol of reductase individually at 4°C for 45 min. The reconstituted and purified according to protocols published previously (Hanna et al., 1998, 2000). The expression plasmid for His-tagged P450s 2B6 and 2D6 was a generous gift of the current study was to investigate the major differences in the metabolism of anandamide between wild-type and mutant forms of CYP2D6 and CYP2B6 and to examine some of the pharmacological properties of the differentially generated products.

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extracted with 3 volumes of ethyl acetate, dried down, and resuspended in 100 μl of methanol followed by ESI-LC-MS analysis as described above. Control experiments contained the same components with the exception that the brain homogenate was first heat-inactivated by boiling for 10 min.

Testosterone Metabolism. 2B6.1 (25 μM) or the mutant enzymes were reconstituted with 0.5 μM reductase in an ice bucket for 45 min. The reconstituted enzymes were then supplemented with catalase (100 units/ml) and diluted with 50 mM potassium phosphate buffer (pH 7.4) to a final volume of 130 μl. The mixture was allowed to equilibrate at 37°C for 10 min, and 100 μl of the volume was transferred into testosterone assay buffer (0.2 mM testosterone and 2 mM NADPH in 50 mM HEPES buffer). The final concentrations of the testosterone used in the buffer were 200, 100, 50, 25, 10, and 0 μM in methanol or methanol in the control sample. Reactions were allowed to proceed at 37°C for 30 min and were terminated with two 2-ml portions of ethyl acetate and extracted. The organic phases were pooled together and dried under nitrogen. The dried extracts were resuspended in 125 μl of 65% methanol, and 100 μl was resolved using high-performance liquid chromatography under isocratic conditions with 65% methanol-35% water and a flow rate of 0.85 ml/min on a Microsorb-MV C18 column (5 μm, 4.6 × 150 mm; Varian, Palo Alto, CA). Metabolites were detected using UV absorption at 254 nm. The area under the peaks for the major metabolites of testosterone, 16α-hydroxytestosterone, and 16β-hydroxytestosterone were integrated to compare the quantities that were formed by each enzyme.

Data Analysis. Nonlinear regression and statistical analyses of the data were performed using Prism.

Results

Metabolism of Anandamide by Wild-Type CYP2B6 and CYP2D6. We initially compared the ability of purified human CYP2B6 and CYP2D6 to form 20-HETE-EA and the EET-EAs when incubated with anandamide in the reconstituted system with NADPH. As shown in Fig. 1 and as we have reported previously (Snider et al., 2008), anandamide is metabolized by CYP2D6 to yield five monooxygenated metabolites in the following order of abundance: 20-HETE-EA > 14,15-EET-EA > 8,9-EET-EA ≈ 11,12-EET-EA > 5,6-EET-EA. The incubation of anandamide with CYP2B6 in the presence of NADPH resulted in the same products as CYP2D6, with m/z ratios of 364, 16 mass units higher than that of the parent anandamide (m/z 348). However, the relative ratios of the metabolites formed were very different, as can be seen in Fig. 1. The major difference was the formation of 20-HETE-EA, which was the major metabolite of CYP2D6, but only a minor product of CYP2B6. No changes in the amounts or ratios of product formed were observed when anandamide was metabolized by CYP2B6 in the presence or absence of cytochrome b5 in the reconstituted system (data not shown). Unlike CYP2D6, which is able to further hydroxylate the EET-EAs to yield dioxygenated derivatives (Snider et al., 2008), no such products were formed when anandamide was incubated with CYP2B6 for up to 90 min (data not shown).

Table 1: Comparison of Anandamide Metabolism by Wild-Type CYP2B6 and Its Three Polymorphic Mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>16α-Hydroxytestosterone</th>
<th>16β-Hydroxytestosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt;</td>
</tr>
<tr>
<td>WT</td>
<td>13.3 ± 3</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>2B6.4</td>
<td>99 ± 17</td>
<td>31 ± 4</td>
</tr>
<tr>
<td>2B6.6</td>
<td>20.7 ± 6</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>2B6.9</td>
<td>10 ± 2</td>
<td>24 ± 4</td>
</tr>
</tbody>
</table>

WT, wild-type.

Comparison of Anandamide Metabolism by Wild-Type CYP2B6 and CYP2D6 R296C (CYP2D6.34). Because there are at least 40 different alleles of the CYP2D6 gene that contain the nucleotide...
substitution 2850C>T, which results in the substitution of the amino acid cysteine for arginine at position 296 (http://www.cypalleles.ki.se/cyp2d6.htm), we investigated the effect of this single mutation (CYP2D6.34) on the metabolism of anandamide. As shown in Fig. 3, CYP2D6.34, formed all of the same products as the wild-type enzyme, with the exception of 5,6-EET-EA. However, the formation of all four metabolites was significantly lower compared with that for the wild-type enzyme. We were unable to determine the kinetics for the formation of these products because higher concentrations of anandamide resulted in an inhibitory effect on the enzyme activity (data not shown). The formation of 20-HETE decreased the most by far. Another key difference between wild-type CYP2D6 and CYP2D6.34 was that the major product formed by CYP2D6.34 was 14,15-EET-EA, and not 20-HETE-EA, as in wild-type CYP2D6.

Binding of 20-HETE-EA and 14,15-EET-EA to the Brain Cannabinoid CB1 Receptor. We selected 20-HETE-EA and 14,15-EET-EA for further examination of their pharmacological properties because their formation was significantly different between the two wild-type proteins and also between wild-type CYP2B6 and the CYP2B6.4 and CYP2B6.6 variants.

TABLE 2  
Comparison of anandamide metabolism by wild-type and mutant CYP2B6 enzymes

| CYP2B6 enzymes were reconstituted with reductase, and the kinetic data were determined as described under Materials and Methods. The concentrations of anandamide ranged from 0.75 to 10 μM. Metabolites were extracted and analyzed by ESI-LC-MS. The data represent the mean of experiments done in triplicate. The data were fitted to the Michaelis-Menten model using Prism software to derive the kinetic constants. |
| 20-HETE-EA | 14,15-EET-EA | 11,12-EET-EA | 8,9-EET-EA | 5,6-EET-EA |
| K_m (μM) | V_max (pmol · min⁻¹ · pmol⁻¹) |
| CYP2B6.1 (WT) | N.D. | 3.6 | 1.320 | 1.210 | 1.320 |
| CYP2B6.4 | 0.362 | 1.237 | 1.380 | 1.510 | 0.591 |
| CYP2B6.6 | N.D. | 1.501 | 1.341 | 1.211 | N.D. |
| CYP2B6.9 | N.D. | 3.089 | N.D. | N.D. | N.D. |
| CYP2B6.9 | N.D. | 3.089 | N.D. | N.D. | N.D. |

Ligand binding experiments to the CB1 receptor were performed to compare the affinities of 20-HETE-EA and 14,15-EET-EA for the CB1 receptor with that of anandamide. This assay examined the ability of the three compounds to compete with the radiolabeled cannabinoid agonist CP-55940 for binding to the CB1 receptor in rat brain preparations. Rat CB1 shares 97.3% identity with the human receptor (Matsuda et al., 1990). As shown in Fig. 4, anandamide competed with CP-55940 for binding to CB1 with a K_i of 275 nM, which is consistent with what has previously been reported in the literature (Pertwee, 2005). Although both 20-HETE-EA and 14,15-EET-EA were also able to compete with CP-55940 for binding to CB1, as evidenced by the dose-dependent decrease in CP-55940 binding, their affinities were significantly lower than that of anandamide.
amidine. Thus, from the competition curves, the $K_I$ values were determined to be 985 nM and 1.56 μM for 20-HETE-EA and 14,15-EET-EA, respectively.

Decreased Biological Stability of 20-HETE-EA and 14,15-EET-EA Relative to Anandamide. Anandamide is extensively degraded by the enzyme FAAH, which is abundantly expressed in the brain (Giang and Cravatt, 1997). The P450-derived epoxide of anandamide, 5,6-EET-EA, is significantly more stable than anandamide when incubated with mouse brain homogenate (Snider et al., 2009). To compare the relative stabilities of anandamide with those of 20-HETE-EA and 14,15-EET-EA, we incubated each compound in rat brain homogenates for 0 to 64 min and monitored the amounts of 20-HETE-EA and 14,15-EET-EA. We also investigated the primary routes for the degradative metabolism of the two P450-derived products of anandamide by incubating them in rat brain homogenates in the presence of 50 μM PMSF, a serine protease inhibitor that inhibits FAAH activity (Deutsch and Chin, 1993). As shown in Fig. 5B, there was minimal loss of 20-HETE-EA under these conditions (less than 20% after 90 min of incubation). In contrast, 14,15-EET-EA disappeared fairly rapidly over time in the presence of PMSF with a half-life of 9.4 min, which was slightly higher than the $t_{1/2}$ of 6.1 min that was observed in the absence of PMSF (Fig. 5A). The EET-EAs can be further metabolized by epoxide hydrolase to their corresponding dihydroxyeicosatetraenoic acid ethanolamides (DHET-EAs) (Snider et al., 2007, 2010). To determine the relative contribution of brain epoxide hydrolase activity to the disappearance of 14,15-EET-EA, we measured the amount of 14,15-DHET-EA formed in the presence and absence of PMSF. As shown in Fig. 5C, the ratios of 14,15-DHET-EA to 14,15-EET-EA increased significantly over time and were determined to be approximately 18 and 53 in the absence and presence of PMSF, respectively, after 45 min of incubation time, indicating that metabolism of the 14,15-EET-EA by epoxide hydrolase is the predominant metabolic pathway when FAAH is not inhibited and is significantly augmented upon FAAH inhibition.

Discussion

Understanding the outcomes of the metabolism of various endogenous substrates by brain P450s has physiological and pharmacological significance. For example, it was recently demonstrated that brain P450 epoxygenase activity is essential for achieving pain-relieving effects upon administration of μ opioids (Conroy et al., 2010), raising the possibility that this could be the case for cannabinoids as well. The unique regulation of brain CYP2B6 and CYP2D6 by drugs and genetics (Miksys and Tyndale, 2004) requires a much better understanding of their contribution to the metabolism of endogenous psychoactive substrates. The endocannabinoid anandamide has been shown to exert neuroprotective effects that may be mediated either by cannabinoid receptors or independently of them. The latter include activation of nuclear peroxisome proliferator-activated receptors or the modulation of ion channels, including the transient receptor potential vanilloid type 1 receptor (Rockwell and Kaminski, 2004; van der Stelt and Di Marzo, 2005; Hegde et al., 2008). The CB1 receptor, like CYP2B6 and CYP2D6, is expressed heterogeneously within the brain, where its activation leads to the impairment of cognition and memory, alterations in motor function, and antinociception, among other effects (Mackie, 2008).

P450 enzymes are known to oxidize arachidonic acid to form the physiologically active 5,6-, 8,9-, 11,12-, and 14,15-EET-EAs and several hydroxyeicosatetraenoic acids including 20-HETE (Capdevila et al., 1992). Purified rabbit CYP2B4 and rat CYP2B1 have previously been shown to metabolize arachidonic acid, producing primarily EETs (Zeldin et al., 1995). In the study reported here, anandamide, a derivative of arachidonic acid, is shown to be oxidized by both CYP2B6 and CYP2D6 to yield different EET-EAs and HETEs. We have also investigated the major differences in the metabolism of anandamide by the human wild-type and some of the major polymorphically expressed mutants of CYP2B6 and CYP2D6 in vitro in the reconstituted system and have examined some of the pharmacological properties of the products resulting from anandamide metabolism by these P340s. We found that the main difference between wild-type CYP2B6 and CYP2D6 is the formation of the hydroxylated anandamide product 20-HETE-EA, which is the major metabolite formed by CYP2D6, and it is not formed in significant amounts by CYP2B6. In addition, there was increased formation of 20-HETE-EA by 2B6.4 compared with that by the wild type. It has previously been shown that the 2B6.4 allelic protein has higher 7-ethoxy-4-trifluromethyl coumarin O-deethylase activity and a higher bupropion clearance rate (Kirchheiner et al., 2003), whereas the 2B6.6 allele has decreased protein expression (Lang et al., 2001) and decreased efavirenz clearance. The mutant CYP2B6 G516T (2B6.9) metabolized anandamide to form only 11,12-EET-EA. This mutation has previously been shown to be associated with a reduction in the catalytic function of this enzyme (Rotger et al., 2005). Thus, the effects of CYP2B6 polymorphisms on the metabolism of anandamide appear to be similar to the influence of the polymorphic alleles on substrates such as bupropion, efavirenz, and cyclophosphamide.

Although CYP2D6 accounts for only 2 to 9% of the total hepatic P450 content, it is involved in the metabolism of many clinically used drugs, including ones that affect the central nervous system. Many
variants of CYP2D6 carry the mutation R296C, and individuals with this variant have been characterized as poor metabolizers.

Intrinsic clearance ($V_{\text{max}}/K_m$) values that can be used to compare the abilities of CYP2B6 and CYP2D6 to metabolize anandamide and the various metabolites are shown in Table 3. The intrinsic clearance ($C_{\text{int}}$) based on 11,12-EET-EA formation was approximately 13-fold greater for CYP2B6 compared with that for CYP2D6. In comparison, only a slight increase was observed for the $C_{\text{int}}$ to form 14,15-EET-EA and a 3-fold increase for 8,9-EET-EA. Overall, lower intrinsic clearance values were found for all the EET-EA metabolites formed by CYP2D6, whereas $C_{\text{int}}$ for the formation of 20-HETE-EA was much greater for CYP2B6 than for CYP2D6. Major differences were also seen between the wild-type enzymes and their mutants that involved some significant changes in the product formation ratios and $K_m$ values. For example, both 2B6.4 and 2B6.6 exhibit decreased $K_m$ values for anandamide when formation of 14,15-EET-EA was catalyzed. In addition, 14,15-EET-EA is the major product formed by 2D6.34.

We also found that 20-HETE-EA binds to the CB1 receptor with a 3.6-fold lower affinity relative to anandamide and is more rapidly degraded in the brain than anandamide in a PMSF-sensitive manner, most likely by FAAH. Like 20-HETE-EA, 14,15-EET-EA also binds to the CB1 receptor, but with a 5.7-fold lower affinity relative that of anandamide. In addition, 14,15-EET-EA is degraded more rapidly than anandamide, in an epoxide hydrolase-dependent manner, based on the appearance of the 14,15-DHET-EA product (Snider et al. 2007). The current findings may contribute to a better understanding of the potential pharmacological effects of inhibitors of both FAAH and epoxide hydrolase in the brain. A study reported that administration of the soluble epoxide hydrolase inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester either before or after experimental ischemic stroke reduced the infarct size by 40 to 50% (Zhang et al., 2007). This protective effect was almost completely reversed by coadministration of the P450 epoxygenase inhibitor 6-(2 proparglyoxyphenyl) hexanoic acid, and this occurred apparently independent of P450-derived arachidonic acid metabolites. During ischemic stroke in laboratory animals and in human patients, there is an increased synthesis of anandamide (Muthian et al., 2004), which might be expected to lead to an increase in the generation of P450-derived anandamide products. It remains to be investigated whether some of the metabolites generated by CYP2B6 and CYP2D6 are involved in the neuroprotective effects of epoxide hydrolase inhibitors. Although these metabolites bind more weakly to the CB1 receptor, it is possible that they have other molecular targets.
In summary, the metabolism of anandamide by CYP2B6 and CYP2D6 and their polymorphically expressed mutants leads to the
formation of different profiles of metabolites, with the major changes being primarily the formation of 20-HETE-EA and 14,15-EET-EA.
Although these two products are CB1 receptor ligands, they exhibit decreased stability in the brain. Future functional characterization of
the P450-derived metabolites of anandamide and their secondary products will yield additional information regarding the biological
significance of these metabolic pathways in the brain.

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Authorship Contributions
Participated in research design: Sridar, Snider, and Hollenberg.
Conducted experiments: Sridar and Snider.
Performed data analysis: Sridar and Snider.
Wrote or contributed to the writing of the manuscript: Sridar, Snider, and Hollenberg.

References

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Anandamide oxidation by wild type and polymorphically expressed CYP2B6 and CYP2D6

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Drug Metabolism and Disposition
Legend to Supplemental Figure 1. Profile for the metabolism of anandamide by CYP2B6 in the absence of NADPH. CYP2B6 was reconstituted with reductase in the presence of 2 µM anandamide. No NADPH was added. Subsequent analysis was done as described in Materials and Methods. The top panel shows the total ion chromatogram of the control sample and the bottom panel shows the extracted ion chromatogram at m/z 348. The extracted ion chromatogram for m/z 364 did not show any peaks (data not shown) indicating the absence of hydroxylated products.
Supplemental figure 1.
**Legend to Supplemental Figure 2.** Enzymatic degradation of 20-HETE-EA and 14,15-EET-EA by rat brain homogenate. A. Selected ion chromatograms at m/z 364 showing the peaks for 20-HETE-EA (RT = 14.9 min) and 14,15-EET-EA (RT= 17.5 min) at times 0 and 15 min after addition to brain homogenates in the presence and absence of PMSF. Note that amount of 20-HETE-EA decreases significantly after 15 min in the absence, but not in the presence of PMSF. In contrast, the peak for 14,15-EET-EA shows a significant decrease after 15 min in the presence or absence of PMSF (compare red to blue and green to black chromatograms). The relative intensity units for the highest peak shown are 1.12x10^7. The MS spectra of the respective peaks are also shown. B. Selected ion chromatograms for the epoxide hydrolase-derived 14,15-DHET-EA product formed from 14,15-EET-EA. Note the significant increase in 14,15-DHET-EA after 15 min of reaction time compared to 0 min in the presence or absence of PMSF. The MS spectrum for 14,15-DHET-EA is also shown. The relative intensity units for the highest peak shown are 2.59^6.
Supplemental Figure 2.