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Asymmetric binding and metabolism of polyunsaturated fatty acids (PUFAs) by CYP2J2 epoxigenase

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Abstract

Cytochrome P450 (CYP) 2J2 is the primary epoxigenase in the heart and is responsible for the epoxidation of arachidonic acid (AA), an ω -6 polyunsaturated fatty acid (PUFA), into anti-inflammatory epoxide metabolites. It also epoxidizes other PUFAs such as docosahexaenoic acid (DHA), linoleic acid (LA), and eicosapentaenoic acid (EPA). Herein, we have performed detailed thermodynamic and kinetic analyses to determine how DHA, LA and EPA modulate AA metabolism by CYP2J2. We use the Nanodisc (ND) system to stabilize CYP2J2 and its redox partner CYP reductase (CPR). We observe that DHA strongly inhibits CYP2J2-mediated AA metabolism, while LA only moderately inhibits and EPA exhibits insignificant inhibition. We also characterized the binding of these molecules using ebastine competitive binding assays and show that DHA binds significantly tighter to CYP2J2 as compared to AA, EPA, or LA. Furthermore, we

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CONFLICTS OF INTEREST

Authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

WRA wrote the manuscript, designed and performed the experiments, made figures, and analyzed the data. JLB and ET performed MD simulations, and analyzed corresponding data and contributed to making figures and writing the manuscript. AD designed the experiments and wrote the manuscript. All authors analyzed the results and approved the final version of the manuscript.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. WRA wrote the manuscript, designed and performed the experiments, made figures, and analyzed the data. JLB and ET performed MD simulations, and analyzed corresponding data and contributed to making figures and writing the manuscript. AD designed the experiments and wrote the manuscript.

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Chemical structures, extra kinetic data, extra kinetic analyses, MD simulation energies, MD simulation movies, MD simulation graphics, mutagenesis primers

utilize a combined approach of molecular dynamics (MD) simulations and docking to predict key residues mediating the tight binding of DHA. We show that although all the tested fatty acids form similar contacts to the active site residues, the affinity of DHA binding to CYP2J2 is tighter due to the interaction of DHA with residues Arg-321, Thr-318 and Ser-493. To demonstrate the importance of these residues in binding, we mutated these residues to make two mutant variants—CYP2J2-T318A and CYP2J2-T318V/S493A. Both of these variants showed weaker binding affinity to DHA and AA compared to the WT and the stronger inhibition of AA by DHA in the WT is mitigated in these mutants. Therefore, using a combined experimental and MD simulations approach, we establish that CYP2J2 inhibition of AA metabolism by DHA, EPA and LA is asymmetric due to tighter binding of DHA to select residues in the active site.

Graphical Abstract

Keywords

Eicosanoids; linoleic acid; docosahexaenoic acid; eicosapentaenoic acid; ω -3 fatty acids; enzyme kinetics; cytochrome P450; molecular dynamics; ω -6 fatty acids; lipids

INTRODUCTION

The ω -3 and ω -6 polyunsaturated fatty acids (PUFAs) are converted to lipid metabolites by three classes of eicosanoid-synthesizing enzymes: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP) epoxygenase. Collectively, these eicosanoids are responsible for producing several physiological responses related to inflammation, and their imbalance has been observed in several diseases and cancer.^{1–9}

CYP epoxygenases belong to the hemoprotein superfamily of CYPs that are primarily involved in xenobiotic metabolism and steroid biosynthesis. The CYP-mediated metabolism of ω -3 and ω -6 PUFAs has gained scientific interest due to their implications in cardiovascular health.^{1, 10–15} It was demonstrated that dietary ω -3 and ω -6 PUFAs are metabolized by the enzymes in the CYP-epoxygenase pathway into predominantly epoxide metabolites^{16–22} (Figure 1) that are biologically active and are generally anti-inflammatory. The primary CYP epoxygenase in human cardiomyocytes is CYP2J2.²³ CYP2J2 epoxidizes AA into four anti-inflammatory epoxyeicosatrienoic acids (EETs)^{17, 19, 23} and hydroxylates it into two hydroxyeicosatetraenoic acids (HETEs)^{3, 17, 24} (Figure 1).

CYP2J2 has also been shown to epoxidize another ω -6 PUFA, linoleic acid (LA), into two epoxyoctadecenoic acids (EpOMEs).²⁵ It also metabolizes the ω -3 PUFA eicosapentaenoic acid (EPA) into epoxyeicosatetraenoic acids (EEQs) and hydroxyeicosapentaenoic acids (HEPEs).^{17, 26} Additionally, it metabolizes the ω -3 PUFA docosahexaenoic acid (DHA) into

epoxydocosapentaenoic acids (EDPs) and hydroxydocosaheptaenoic acids (HDHEs).^{17, 26} The ω -3 epoxide metabolites have been shown to elicit more potent anti-inflammatory and cardioprotective responses as compared to their AA-derived counterparts, EETs.^{16, 27, 28}

It is important to study how these different PUFAs compete for metabolism by eicosanoid-synthesizing enzymes. For instance, the metabolism of ω -6 PUFAs by COX and LOX is often inhibited by the presence of ω -3 PUFAs,^{29–31} owing to the structural similarity of these two classes of PUFAs.^{32, 33} However, the biochemistry of CYP-epoxygenase interactions with both ω -3 and ω -6 PUFAs remains largely unknown. CYPs generally possess a large and promiscuous active site leading to drug-drug interactions.^{34–37} Therefore, it becomes imperative to establish the complex kinetics of PUFA-PUFA interactions at the CYP2J2 active site.

Herein we have performed detailed kinetic analyses to determine the effect various PUFAs have on AA metabolism by CYP2J2. We use the Nanodisc (ND) system to stabilize CYP2J2 and its redox partner cytochrome P450 reductase (CPR).³⁸ Nanodiscs are nanoscale lipid bilayers that have been used extensively to stabilize various types of membrane proteins in aqueous solutions.^{39–45} Using CYP2J2-NDs, we first determined the metabolism of AA in the presence of DHA, LA, and EPA. We find that these PUFAs competitively inhibit each other with different potencies. Using competitive binding and inhibition studies, we determined that the binding affinities of these PUFAs to the active site of CYP2J2 are different from each other. Notably, it was found that DHA binds the tightest compared to AA, LA, or EPA. Furthermore, we utilize a combined approach of molecular dynamic (MD) simulations and molecular docking to predict key residues that mediate the binding of the PUFAs to the active site of CYP2J2.^{46–48} The studies revealed three key residues in the CYP2J2 active site that govern the binding strength of these PUFAs. Our mutational analyses confirmed the importance of these residues in directing the binding of these PUFA to the active site of CYP2J2. Taken together, our studies show that the binding and metabolism of PUFAs by CYP2J2 is asymmetric due to differential binding affinities of the PUFAs, despite their strong structural similarity to each other.

EXPERIMENTAL PROCEDURES

Materials

Human CYP2J2 cDNA was obtained from OriGene (Catalog No. SC321730) and modified as published before.⁴⁵ Ampicillin, arabinose, chloramphenicol, isopropyl β -D-1-thiogalactopyranoside (IPTG), and Ni-NTA resin were obtained from Gold Biotechnology. δ -aminolevulinic acid was obtained from Frontier Scientific. NADPH and NADP⁺ were obtained from P212121.com. 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-L serine (POPS) were purchased from Avanti Polar Lipids, Inc. EPA; DHA; AA; 9,10-EpOME; 12,13-EpOME; 14,15-EEQ; 17,18-EEQ; 16,17-EDP; and 19,20-EDP were obtained from Cayman Chemical. Restriction enzymes and molecular biology materials were purchased from New England Biolabs. All other materials and reagents used were purchased from Sigma-Aldrich and Fisher Scientific.

Recombinant expression of CYP102A1 (BM3) in *E. coli*

The plasmid for expressing BM3 was a generous gift from Dr. Stephen G. Sligar. It was transformed into BL21 Gold cells and the expression of the protein was executed using a published protocol.⁴⁹ Cells were flash frozen and stored at -80°C for less than a month.

Expression and purification of recombinant CYP2J2 in *E. coli*

Recombinant D34-CYP2J2 containing a His₅ tag was expressed and purified as previously performed.^{38, 45} The D34-CYP2J2 is a 34-residue N-terminal truncation (residues 3-37) of CYP2J2 with a substitution of Leu2 for an Ala residue. These modifications have been previously shown to increase protein yield without affecting activity.^{38, 45} CYP2J2-T318V and CYP2J2-T318V/S493A mutant variants of the D34-CYP2J2 construct were expressed and purified using the same WT protocol. WT and mutants were characterized using a previously published CO-binding assay^{40, 41, 45, 50} based on a modified version of Omura and Sato's method.⁵¹

Expression and purification of cytochrome P450 reductase

Expression of cytochrome P450 reductase (CPR) was performed as described previously.⁴⁵

CYP2J2-T318V and T318V/S493A-double-mutant mutagenesis

CYP2J2 mutant variants CYP2J2-T318V (T318V) and a T318V/S493A double variant (Double) were generated using overlap-extension PCR. Residue numbers refer to the WT sequence. Mutagenesis primers also contained silent mutations to introduce restriction enzyme sites for screening purposes (Table S4). For T318V an *AleI* site was introduced and for S493A a *BsiEI* site.

Incorporation of CYP2J2 into Nanodiscs

Nanodiscs containing CYP2J2 and CYP2J2 mutants were prepared as previously described.⁴⁵ MSP1E3D1 and a 20:80 POPS:POPC lipid composition were used for making the Nanodiscs.

CYP102A1 (BM3) NADPH kinetics

The rate of NADPH consumption by BM3 in the presence of AA or EPA was determined using UV-Vis spectroscopy as previously described.⁵² Briefly, 0.01 μM BM3 was incubated for 1 min in 50 mM MOPS buffer with 100 μM of AA or EPA (5 mM in ethanol stocks). 200 μM of NADPH was added to initiate the reaction. Rates of NADPH consumption were determined by measuring the decrease in the NADPH absorption at 340 nm.

CYP2J2-ND ebastine (EBS) kinetics

CYP2J2 metabolism of ebastine (EBS)⁵³ was studied as previously mentioned with a modified LC-MS/MS technique as described below.⁴⁵ The rates of ebastine hydroxylation were fitted to the Michaelis-Menten equation and agree with previously published data.⁵³ All fittings were done using OriginPro 9.1 (Origin Labs Inc., Northampton, MA).

CYP2J2-ND and BM3 PUFA metabolisms

PUFA metabolism incubations were conducted using the following procedure. PUFAs (5–100 μ M from a 5 mM stock in ethanol) were solubilized in a 0.1 M KPi buffer for 30 minutes at 37°C. 0.2 μ M CYP2J2-ND and 0.6 μ M CPR were added in a separate 0.1 M KPi mixture and allowed to incubate at 37°C for 10 min. The PUFAs and protein were mixed and were further incubated for additional 10 min. The reaction was initiated with the addition of 0.5 mM NADPH and allowed to proceed at 37°C for 30 minutes based upon our linearity measurements (reaction is linear up to 35 min). The reaction was terminated with the addition of 100 μ L of glacial acetic acid (pH<4) and the metabolites were extracted thrice with equivolume ethyl acetate. The organic layer was dried under a flow of N₂ gas and then reconstituted in ethanol for LC-MS/MS analysis. All single kinetic studies were fitted to the Michaelis-Menten equation using OriginPro 9.1. A standard of 14,15-EET was used to determine the extraction efficiency by treating 14,15-EET to the same conditions. The extraction efficiency was determined to be ~70–80%.

Reactions with BM3 were performed similarly, but with the following modifications. AA or EPA was solubilized for 30 min at 30° C in 50 mM MOPS buffer before adding 0.01 μ M BM3 for 1 min. The reaction was started using 0.5 mM NADPH, and terminated with acetic acid (pH<4) after 30 s. AA and EPA metabolites were extracted and analyzed as described above.

Co-kinetic assays measuring the products of AA with DHA, LA, or EPA metabolism by CYP2J2 were performed similarly. 15 μ M and 50 μ M of DHA, LA, or EPA were concurrently solubilized in separate mixtures alongside AA. CYP2J2 and CPR were added to the DHA, LA, or EPA mixtures and allowed to incubate for 10 min before the AA and PUFA/protein mixtures were combined and incubated for an additional 10 min. The rest of the reaction and extraction procedure was followed as stated above. The metabolites of AA and DHA, LA, or EPA (terminal two epoxides) were simultaneously measured using the methods described below. Mixed-substrate kinetic data fit to a competitive inhibition model (Equation 1) using OriginPro 9.1.

Liquid chromatography- tandem mass spectrometry for quantitation of fatty acid metabolites

Analyses were performed at the Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign, as previously described.^{40, 41} Metabolites were separated using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a flow rate was 0.4 mL/min. The linear gradient was as follows: 0–2min, 90% A; 8min, 55% A; 13–25min, 40% A; 30min, 30% A; 35min, 25% A; 40min, 20% A; 45–47min, 15% A; 48–54min, 90% A. The autosampler was set at 5°C. The injection volume was 10 μ L. Negative mass spectra were acquired with the ion spray voltage of –4500 V under electrospray ionization (ESI). The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 32 psi, 50 psi, and 55 psi, respectively. Multiple reaction monitoring (MRM) was used for quantitation: : 5,6-EET m/z 319.2 → m/z 191.0; 8,9-EET m/z 319.2 → m/z 155.1; 11,12-EET m/z 319.2 → m/z 167.0; 14,15-EET m/z 319.2 → m/z 219.1; 17,18-EEQ m/z 317.0 → m/z 299.0; 14,15-EEQ m/z

317.0 $\rightarrow$ m/z 207.0; 12,13-EpOME m/z 295.0 $\rightarrow$ m/z 195.0; 9,10-EpOME m/z 295.0 $\rightarrow$ m/z 277.0; 16,17-EDP m/z 343.0 $\rightarrow$ m/z 274.0; 19,20-EDP m/z 343.0 $\rightarrow$ m/z 299.0. Four methods were developed to analyze the different combinations of metabolites. 1.) EETs and HETEs alone were quantified using 16,17-EDP (m/z 343.0 $\rightarrow$ m/z 274.0) as the Internal standard. 2.) EETS, HETEs, and EEQs were quantified using 16,17-EDP as the internal standard. 3.) EETs, HETEs, and EDPs were quantified using 17,18-EEQ (m/z 317.0 $\rightarrow$ m/z 299.0) as the Internal standard. 4.) EETs, HETEs, and EpOMEs were quantified using 17,18-EEQ as the internal standard.

Liquid chromatography- tandem mass spectrometry for quantitation of ebastine (EBS) metabolites

Metabolites were separated using mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min. The linear gradient was as follows: 0–2 min, 90% A; 8 min, 55% A; 13–25 min, 40% A; 30 min, 30% A; 35 min, 25% A; 40 min, 20% A; 45–47min, 15% A; 48–54min, 90% A. The autosampler was set at 15°C. The injection volume was 1 μ L. Mass spectra were acquired under positive electrospray ionization (ESI) with ion spray voltage at 5500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 32, 50, and 65 psi, respectively. Multiple reaction monitoring (MRM) was used for quantitation: Ebastine m/z 470.4 $\rightarrow$ m/z 302.3; Hydroxyebastine m/z 486.4 $\rightarrow$ m/z 318.3; internal standard terfenadine m/z 472.4 $\rightarrow$ m/z 262.1.

Ebastine competitive binding

Ebastine binding near the heme was determined via monitoring the Soret shift from ~420 nm to ~390 nm. Absorbance spectra were taken using a Cary Bio 300 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA). Substrates were dissolved in DMSO as 10 mM, 1 mM, and 0.1 mM stocks, as DMSO had been previously shown to not affect the CYP2J2 heme absorption.^{40, 41} Titrated volumes remained < 2.5% the initial volume. 3 μ M of CYP2J2 was incubated at 37°C during the experiments and titrations were incubated for 5 min before taking the spectra. Concentrations of ebastine ranged from 0 to 155 μ M in the presence or absence of 30 μ M of AA, DHA, EPA, or LA pre-incubated with CYP2J2. The data was processed using Matlab R2014a (MathWorks Inc., Natick MA) by subtracting the titration spectra from the substrate-free spectrum. Difference spectra in the mixed systems were determined by subtracting the PUFA-bound spectrum from each ebastine spectrum. Ebastine binding was fitted to the Michaelis-Menten equation and the binding affinity of each PUFA inhibition was determined via the competitive binding equation (Equation 1) using OriginPro 9.1.

Competitive inhibition equation used in studies

All inhibition studies are best described by a competitive inhibition model (Equation 1)

$$B = \frac{B_{max}[S]}{K \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (\text{Equation 1})$$

where B_{max} and K are the Michaelis-Menten kinetic parameters of the substrate $[S]$ without inhibitor, $[I]$ is the concentration of inhibitor, and K_i is the inhibition constant. For kinetic experiments, B and K represent velocity and K_m , respectively; for Soret binding experiments, B and K represent A and K_D , respectively.

MD simulations of AA, EPA, and DHA binding to CYP2J2

Molecular docking of AA, DHA and EPA using AutoDock Vina,⁵⁴ was employed to probe its binding to CYP2J2. We employed our previously obtained 100 ns MD simulation of POPC membrane-bound CYP2J2⁴⁰ to include a large number of snapshots in the docking. This allows taking into the account the dynamics of the protein in the presence of the membrane and probing putative binding sites that are only transiently present during the simulation. Snapshots of membrane-bound CYP2J2 were taken from the MD trajectory at 100 ps intervals, resulting in 1000 different snapshots used for AA, EPA and DHA docking. A grid box with dimensions 22.5 Å in the x , y and z directions and with center in the active site of CYP2J2 was employed. For each ligand (AA, EPA or DHA), the top 10 docking poses for each snapshot were collected for further analysis, resulting in 10000 docked poses for each fatty acid. The resulting docked structures were then clustered by RMSD of the heavy atoms, with a maximal value distance between two poses of 4.0 Å. This approach identified four main conformations of the fatty acids in the active site (Figure 4). For each cluster, further filtering was performed on the basis of the distance of the carbons involved in the formation of the major epoxide experimentally observed (i.e., carbons 14,15 in AA; 17,18 in EPA and 19,20 in DHA) and the heme iron, with a cutoff distance of 6 Å, as well as docking score. For each cluster, the docked pose within the cutoff distance with the best docking score was selected as the initial structure for simulations, resulting in four initial conformations for each ligand (Figure 5). The resulting membrane-bound protein-ligand systems were minimized for 1000 steps and equilibrated for 100 ps while the heavy atoms of the protein backbone and the ligand were harmonically restrained ($k = 1 \text{ kcal mol}^{-1} \text{ Å}^{-2}$), then followed by unrestrained, production simulations for 100 ns for clusters 1,2 and 3, and 200 ns for cluster 4 for each fatty acid.

Simulation protocol

MD simulations were performed using NAMD2,⁵⁵ with the CHARMM36 force field for protein⁵⁶ and lipids.⁵⁷ Parameters for AA, EPA and DHA were obtained by analogy from the CHARMM General Force Field,⁵⁸ employing DHA force field parameters previously derived.⁵⁹ The TIP3P model was used for water.⁶⁰ Simulations were performed as an NPT ensemble at 1.0 atm and 310 K, with a time step of 2 fs. Constant pressure was maintained using the Nose–Hoover Langevin piston method.⁶¹ Constant temperature was maintained by Langevin dynamics with a damping coefficient of 0.5 ps^{-1} applied to all atoms.⁶² Nonbonded interactions were cut off after 12 Å with a smoothing function applied after 10 Å. Bond distances involving hydrogen atoms were constrained using the SHAKE

algorithm.⁶³ The particle mesh Ewald (PME) method was used for long-range electrostatic calculations with a grid density of $>1 \text{ \AA}^{-3}$.⁶⁴

Simulation analysis

To identify key CYP2J2 residues interacting with the fatty acids (AA, EPA or DHA), interaction energies were calculated for each simulation employing a generalized implicit solvation approach.⁶⁵

RESULTS

CYP2J2 epoxigenase was expressed in *E. coli* as described before^{38, 45} and was assembled into the nanoscale lipid bilayers of nanodiscs (NDs).^{39–42, 45} All the studies reported here are done with the CYP2J2-ND-CPR system (Figure 1).

Initial characterizations of PUFA metabolism analysis and CYP2J2 activity

We characterized the CYP102A1- (BM3)-mediated metabolism of AA and EPA to confirm the accuracy of our PUFA metabolism measurements. BM3 is a bacterial CYP that is known to metabolize AA and EPA with high turnover rates.^{49, 52, 66} Firstly, NADPH consumption rates as BM3 metabolizes AA and EPA were determined. We measured the k_{cat} of NADPH consumption to be $7.12 \pm 0.05 \text{ } \mu\text{mol/min/nmol}_{\text{BM3}}$ for AA and $5.49 \pm 0.18 \text{ } \mu\text{mol/min/nmol}_{\text{BM3}}$ for EPA. These values agree with previous measurements.^{52, 66} Subsequently, we measured the rate of 14,15-EET formation from AA and 17,18-EEQ formation from EPA. The rate of formation of 14,15-EET was determined to be $1.10 \pm 0.21 \text{ } \mu\text{mol/min/nmol}_{\text{BM3}}$, and for 17,18-EEQ to be $2.57 \pm 0.09 \text{ } \mu\text{mol/min/nmol}_{\text{BM3}}$, which correlates well to previous data.^{52, 66}

We then demonstrated functional CYP2J2 by measuring ebastine (EBS) metabolism kinetics (Figure 2A) (Table 1) and compared the results to previously published data.^{45, 53} The results agree with previously reported data for CYP2J2-mediated EBS metabolism.⁵³

Metabolism of AA, DHA, EPA and LA by CYP2J2-ND-CPR

DHA, EPA, and LA have been previously shown to be metabolized by CYP2J2^{17, 25, 26}. We developed quantitative LC-MS/MS methods to monitor the terminal two epoxides (the major metabolites) of these PUFAs (Figure 1). These metabolites correspond to 16,17- and 19,20-EDPs; 14,15- and 17,18-EEQs; and 9,10- and 12,13-EpOMEs for DHA, EPA, and LA, respectively. CYP2J2-ND-CPR were incubated with AA, DHA, EPA and LA separately. The rate of formation of the products is reported as a function of substrate concentration (Figure 2B–E) and is described by Michaelis-Menten kinetics (Table 1) with a constant ratio of metabolites. Due to the inherent insolubility of these substrates, these studies were performed below the critical micelle concentration (CMC) of each lipid substrate.

AA and LA have kinetic properties that are most similar to each other and therefore display similar catalytic efficiencies. The kinetic parameter values of DHA and EPA are most similar and have more similar catalytic efficiencies. As the soluble range of the PUFAs remained below the calculated K_m for each, in order to corroborate the calculated catalytic

efficiencies from these steady-state experiments, an estimate of the catalytic efficiency was obtained from the linear time measurements and fitting the data to Equation 2

$$[S] = [S_0]e^{-kt} \quad (\text{Equation 2})$$

where $[S_0]$ is the initial concentration of the substrate and $k = \frac{k_{cat}}{K_m} [E]$.⁶⁷ Analyzing the time-based kinetics data of the PUFA metabolism, we were able to obtain values for the catalytic efficiencies of these PUFAs (Table S1) that agree well with the catalytic efficiencies determined from our steady-state experiments (Table 1).

Co-substrate metabolism kinetics of AA in the presence of DHA, EPA, and LA by CYP2J2-ND-CPR

In order to determine PUFA-PUFA interactions, mixed-substrate kinetics were performed where the metabolism of AA was measured in the presence of low concentrations (15 μM) and high concentrations (50 μM) of DHA, LA, or EPA. In order to accomplish this, dual LC-MS/MS methods were developed for the concurrent analysis of the AA and the DHA, LA, or EPA metabolites within the same samples. Three different methods were developed to analyze the different combinations of metabolites. (1) An EPA epoxide method [EETS, HETEs, and EEQs]. (2) A DHA epoxide method [EETs, HETEs, and EDPs]. (3) An LA epoxide method [EETs, HETEs, and EpOMEs]. Experiments were conducted with the total fatty acid concentrations kept below CMC in order to avoid co-micellar formation between the PUFAs.

Co-metabolism of the PUFAs is best described using a competitive inhibition model (Equation 1, Table 2). The inhibition constant is given as $K_i^{\text{Inhib} \rightarrow \text{Sub}}$ in order to identify the inhibitor and substrate for each analysis. Treating AA as a substrate in the analysis reveals that DHA has the strongest inhibition of AA metabolism (Figure 3), with a $K_i^{\text{DHA} \rightarrow \text{AA}}$ of $16.5 \pm 2.2 \mu\text{M}$ (Table 2). This is about four-fold stronger than the inhibition of DHA metabolism by AA ($K_i^{\text{AA} \rightarrow \text{DHA}} = 65.2 \pm 15.8 \mu\text{M}$). In comparison, LA and AA inhibit each other with similar potencies (Figure S1), with a $K_i^{\text{LA} \rightarrow \text{AA}}$ value that is similar to the value of $K_i^{\text{AA} \rightarrow \text{LA}}$ (Table 2). Inhibition between AA and EPA (Figure S2) was the weakest and can be considered negligible. This may be due to the fact that out of all these pairings, these two substrates are the most similar structurally. To determine the mechanism behind these asymmetric inhibition strengths, we next sought to determine how these substrates bind to CYP2J2.

Spectral competitive binding studies of CYP2J2 with DHA, EPA, AA and LA to determine the origin of inhibition

Substrate binding to CYPs displaces a water molecule from the iron of the heme, producing a high-spin iron that concomitantly shifts the UV-Vis absorbance from $\sim 417 \text{ nm}$ to $\sim 390 \text{ nm}$. The magnitude of this change is then used to determine substrate binding to CYPs, and the spin-state equilibrium constant, K_S , is taken to approximate K_D . We performed binding

studies using UV-Vis spectral titrations of the PUFAs directly; however due to weak spin-state shift, an accurate binding constant could not be determined (Figure S3). Therefore, we determined the relative binding of the PUFAs by measuring their inhibition of EBS binding. EBS binds to CYP2J2 and produces ~90% spin-state shift, which is at least an order of magnitude greater spectral change as compared to the binding of PUFAs.^{41, 45} The binding of EBS (Figure S4) can be described by a Michaelis-Menten binding model with values for A_{max} and K_S of 0.218 ± 0.007 and $8.16 \pm 0.29 \mu\text{M}$, respectively. These titrations were then repeated by incubating the CYP2J2-ND system with $30 \mu\text{M}$ of each fatty acid substrate followed by titrations with EBS. At this concentration all PUFAs were soluble, bound tight enough to produce a measurable inhibition of EBS binding, and produced a relatively insignificant spin-state change compared to EBS.

The PUFA data were fitted to competitive binding equation (Equation 1) and the values are tabulated in Table 3. DHA had a K_i that is 53.4% ($p = 0.01$) the value of AA, which shows that DHA binds about two-fold more tightly to CYP2J2. For LA, the value of K_i was 160% the value for AA, but due to error is not significant. For EPA, the value was 168% ($p = 0.01$) of AA, signifying that EPA binds significantly weaker compared to AA. These data indicate that, similar to the metabolism data, DHA binds and inhibits CYP2J2 more strongly than AA. In comparison, LA binds similarly as AA and EPA binds weaker.

Molecular Dynamic Simulations of PUFA Binding to CYP2J2

To understand the atomistic details of fatty acid binding to CYP2J2, we employed a combined approach of molecular docking and MD simulations, as described in Experimental Procedures. We used the same CYP2J2 model that we had previously used to successfully predict key residues within the F-G loop that mediate CYP2J2 binding to the membrane.⁴⁰ Since the bound form of the fatty acids is not known, we employed different initial conformations of AA, EPA, and DHA in the active site of CYP2J2 in POPC membranes, obtained from molecular docking, as initial seeds to perform MD simulations. The initial conformations were selected from four identified clusters of conformations of docked poses resulting from ensemble molecular docking (Figure 4). For each cluster, a starting conformation was selected based on the minimum distance of the carbons involved in the formation of the major epoxidation product as shown in Figure 1 (carbons 14,15 in AA; 17,18 in EPA and 19,20 in DHA) to the heme and the best docking score.

Cluster analysis of the resulting docking poses revealed that AA, EPA, and DHA similarly adopt two major orientations in the active site of CYP2J2 (Figure 4A). The main difference between the two orientations is the location of the fatty acid carboxyl group within the active site. For clusters 1, 2, and 3, the fatty acids are extended along the active site cavity and the carboxyl group is pointing towards the F-G loop of the protein, formed by residues 224 to 240. In the simulations that started from these positions, the fatty acids adopt similar conformations, maintaining their overall orientation during the simulations. In contrast, in cluster 4 the carboxyl group is located close to helix I, composed of residues 297 to 330 (Figure 4A, Movies S1–S3), adopting a significantly different orientation and position compared to the three other clusters during simulation.

For DHA and EPA, the MD simulations showed that the alkene moieties of the fatty acid are close to heme for all starting conformations (i.e., carbon-to-heme distance $< 6 \text{ \AA}$ in Figure 4B and Figure S4), suggesting that either of the simulated orientations could lead to formation of metabolites shown in Figure 1. For AA, the carbon-to-heme distance distributions show an increase of about $4 \text{ \AA}$ compared to DHA and EPA, suggesting that this fatty acid is less restrained in active site than the other two. CYP2J2-substrate interaction energies (Table S2) revealed that fatty acids are stabilized mostly by establishing contacts with residues located in the active site of CYP2J2, mainly Ile-127, Phe-310, and Ile-489 (Figure 4A). In addition to these contacts, some important electrostatic interactions were identified for cluster 4.

DHA binding is stabilized by helix I interactions

For the three tested fatty acids, the conformation adopted in cluster 4 (Figure 4A) positions the carboxyl group close to Arg-321 located in helix I. Simulations revealed that fatty acids establish favorable interactions with the Arg-321 side-chain (Figure 5), and these interactions in turn facilitate formation of additional electrostatic interactions. AA, DHA, and EPA can transiently form hydrogen bonds with Thr-318 and Ser-493. As shown in Figure 4B, these interactions facilitate the accommodation of the epoxidation sites close to the heme (with a carbon-to-heme distance $< 6 \text{ \AA}$ for DHA and EPA, and $< 8 \text{ \AA}$ for AA. Of the three fatty acids, DHA showed the highest degree of interactions during the simulations, suggesting a tighter binding mode when compared to AA or EPA for this particular orientation of the fatty acid in the active site (Figure 5B). An additional transient interaction can be also formed with Arg-484 (Figure S5A), located in the vicinity of the other three side-chains; however when this interaction dominates, the distance of the epoxidation sites to the heme is significantly increased ($> 10 \text{ \AA}$), as observed for EPA after 150 ns of simulation (Figure S5B).

Characterizations of CYP2J2-T318V (T318V) and CYP2J2-T318V/S493A (Double) mutations

In order to verify the significance of residues Thr-318 and Ser-493 in mediating DHA binding, we made a single mutant (T318V) and a T318V/S493A double mutant (Double) of CYP2J2. These mutations are expected to disrupt the preferable interactions of DHA with the hydroxyl groups of Thr-318 and Ser-493. We did CO-binding measurements to confirm that these mutants were properly folded (Figure S6).

We determined the binding affinity of AA and DHA binding to these mutants using the EBS competitive binding assay (Table 4). The K_S of EBS binding to these mutants decreases as compared to the WT. Contrariwise, AA and DHA show weaker binding to T318V mutant and to the double mutant compared to WT as assessed by their K_i values. However, whereas the value of K_i for DHA is $\sim 50\%$ that of the value for AA with the WT or T318V variants, for the Double variant it is $\sim 80\%$ of AA (Table 4). This demonstrates that when the Thr-318 and Ser-493 residues are mutated to Val and Ala, DHA binds to CYP2J2 with a similar affinity as AA. This suggests that these residues are important in selectively directing the tighter binding of DHA to the active site of CYP2J2.

We next determined if the weaker binding of DHA to CYP2J2 active site will result in a weaker inhibition of AA metabolism by CYP2J2. The metabolism of AA in the presence of

an equal amount of DHA for the mutants showed poor inhibition (95.6% for T318V and 88.6% for Double) as compared to the inhibition of AA by DHA that was observed for the WT (31%) (Table 4). Likewise, the presence of AA has only a minor effect on the DHA metabolism by the CYP2J2 mutants (Table S3). Therefore, these binding and metabolism studies with the mutants indicate that the residues Thr-318V and Ser-493A are important in directing the selective inhibition of CYP2J2 mediated AA metabolism by DHA.

DISCUSSION

The enzyme pathways involved in PUFA metabolism are complex due to the multitude of PUFAs present in the cell. Herein, we report kinetic and thermodynamic analyses of the metabolism of PUFAs by the major cardiac enzyme, CYP2J2. *Ab initio*, we accurately measure the metabolism of AA and EPA by BM3, which confirms our accuracy in measuring the PUFA kinetics using LC-MS/MS. We next confirm the viability of the CYP2J2-ND-CPR system by measuring the metabolism of EBS that shows similar kinetics as previously reported.^{45, 53}

Sequentially, we further describe what happens to the rate of AA metabolism in the presence of other dietary lipids that are commonly present in the cardiovascular system, i.e. LA, EPA, and DHA. We perform global metabolism inhibition analyses at varying concentrations of AA and DHA that reveal a value of $K_i^{DHA \rightarrow AA}$ that is four-fold smaller than the $K_i^{DHA \rightarrow AA}$. This indicates that DHA is a stronger inhibitor of AA than AA is as an inhibitor of DHA. The EBS competitive binding assay shows that DHA binds almost two-fold more strongly to CYP2J2 than AA. Together, these data indicate that DHA exhibits preferential binding to CYP2J2 as compared to AA and the tighter binding of DHA likely contributes to its inhibitory properties.

LA shows a weaker competitive inhibition of AA metabolism by CYP2J2, i.e. the $K_i^{LA \rightarrow AA}$ is similar to the $K_i^{AA \rightarrow LA}$ value. Furthermore, the EBS binding inhibition reveals similar binding affinities of LA and AA. These data are supported by the fact that these two substrates also have similar values of V_{max} and K_m . Overall, the inhibitions of AA and LA against each other appear to be the result of similar binding and kinetic properties.

EPA and AA inhibition of each other is not significant, showing $K_i^{Inhib \rightarrow Sub}$ values for each other's metabolism that are high. This contradicts previously published data,¹⁷ which looked at a 1:1:1 ratio of AA:DHA:EPA metabolism with CYP2J2. EPA was shown to be preferentially metabolized by CYP2J2, with a four-fold greater metabolite formation. However, the previous experiments were conducted using supersomes, which are a different system compared to the homogenous CYP2J2-ND-CPR system. The discrepancies could arise from the different protein and lipid composition of the supersomes. EBS competitive binding shows that EPA binds with a K_i value that is 168% that of AA. Of all the substrates tested, EPA and AA are the most similar in structure with respect to the length of the carbon chain, different only in one extra double bond in EPA. Taken together, the kinetics and thermodynamics data reveal that DHA inhibits the most strongly compared to AA, LA, or EPA.

In order to understand how these lipid substrates with similar structures can have differential interactions with the active site of CYP2J2, we use MD simulations to investigate key residues that are involved in binding to the PUFAs. It was determined that PUFA binding is mainly governed by hydrophobic interactions with non-polar side chains including Ile-127, Phe-310, and Ile-489, located in the active site. In addition to these contacts, some additional electrostatic interactions appear to stabilize the binding of these PUFAs. We identified that the fatty acids in the binding site adopt two main orientations (Figure 4). In the main orientation (cluster 1, 2 and 3), the fatty acids are extended along the cavity connecting the active site and surface of the protein. In the second orientation (cluster 4), the carboxyl head of the molecule is located in the vicinity of Arg-321 of the I-helix. The three PUFAs can interact with Arg-321, but only DHA can form multiple additional electrostatic interactions with Thr-318 and Ser-493 at the same time, as observed in the calculated interaction energies. This can be due to the presence of one additional alkene moiety closer to carboxyl head in DHA (between carbons 4 and 5), not present in either AA or EPA. It has been shown that the polyunsaturation in DHA increases its flexibility, particularly by lowering rotational barriers about methylene bridges.⁵⁹ Therefore, DHA has increased flexibility closer to carboxyl group due to the additional double bond, which can facilitate interactions with surrounding residues, such as Thr-318 and Ser-493, in addition to Arg-321. In contrast, AA and EPA are less flexible around their carboxyl head due to the close saturated carbons. The interaction energy for AA and EPA with Arg-321 and Thr-318 in this conformation is similar (Figure 5), suggesting a similar binding mode for the two molecules, which can explain why there is no significant difference seen in our inhibition studies and subsequent binding analyses. Contrariwise, DHA binding establishes a key residue triad that could explain why the binding of DHA is stronger than AA or EPA. Interestingly, MD simulations of arachidonic acid binding to CYP2E1 also revealed its own unique triad of interacting residues separate from the triad seen in our simulations of CYP2J2 (His 107, Ala 108, and His 109 of CYP2E1).⁴⁷ This shows that the residues mediating fatty acid binding to CYPs may not be necessarily conserved, although it may involve a similar triadic theme. Further, since CYP2E1 mainly forms ω -1 and ω -2 hydroxylations, this could explain the differences in the preferred metabolism between CYP2J2 and CYP2E1.

In absence of a crystal structure, we have been able to use the homology model used in this study to successfully predict key membrane-interacting residues for CYP2J2.⁴⁰ Using homology models in MD simulations may not be as reliable as using crystal structures to predict protein-substrate interactions, which need to be verified experimentally. Therefore, to corroborate the MD data, mutants CYP2J2-T318V (T318V) and CYP2J2 T318V/S493A (Double) were made. Binding of EBS to these mutants was tighter than the WT; however, the binding of AA and DHA (as determined by EBS competitive binding assay) was weaker compared to WT. This demonstrates the importance of these residues in mediating PUFA binding specifically. The order of binding strength for both AA and DHA is WT > T318V > Double. However, the binding affinity of DHA with the Double mutant is more similar to AA when compared to WT or T318V. This supports the fact that without the preferential interactions offered by the hydroxyl groups of Thr-318 and Ser-493, AA and DHA bind similarly to the active site of CYP2J2.

Our kinetic data agrees well with kinetic values obtained with similar PUFA systems determined by other researchers. CYP4F11 hydroxylation of PUFAs showed V_{max} values between 1–2 nmol/min/mg and K_m values at ~50 μ M; and CYP2C enzymes showed PUFA metabolic rates between 100 and 1000 pmol/min/nmol.^{69, 70} Further, estimates of the catalytic efficiency based upon linear time measurements corroborates the catalytic efficiencies from our steady-state metabolism experiments. Therefore, we observe that PUFA kinetics with CYP2J2 and similar membrane-bound CYP systems are slow compared to soluble CYPs such as BM3.^{17, 69, 70} It is highly likely that CYP2J2 was evolutionarily selected to metabolize AA and other PUFAs into epoxide metabolites in order to selectively regulate cardiovascular physiology.

Our data also indicates that PUFA metabolism and inhibition by CYP2J2 is governed by the restricted conformation and length of the bound lipid substrate. For instance, EPA and AA showed similar flexibilities and adopt similar conformation when interacting the Arg-321, Thr-318 and Ser-493 triad. DHA, however, is an ω -3 PUFA which two extra carbons in the fatty acid chain length as compared to AA that adopts the conformation to favorably interact with this triad and therefore bind tighter to CYP2J2.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AA	Arachidonic Acid
CMC	critical micelle concentration
COX	cyclooxygenase
CYP	cytochrome P450
BM3	cytochrome P450 102A1
T318V	CYP2J2-T318V
Double	CYP2J2-T318V/S493A
CPR	cytochrome P450 reductase

DHA	docosahexaenoic acid
EBS	ebastine
EPA	eicosapentaenoic acid
EDP	epoxydocosapentaenoic acid
EEQ	epoxyeicosatetraenoic acid
EET	epoxyeicosatrienoic acid
EPOX	epoxygenase
EpOME	epoxyoctadecenoic acid
HDHE	hydroxydocosahexaenoic acid
HEPE	hydroxyeicosapentaenoic acid
HETE	hydroxyeicosatetraenoic acid
LA	linoleic acid
LOX	lipoxygenase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MD	molecular dynamics
ND	Nanodisc
PUFA	polyunsaturated fatty acid

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Figure 1.

(A) Metabolism of arachidonic acid (AA) by CYP2J2. Reaction schematic showing CYP2J2 and its redox partner CPR incorporated into Nanodiscs metabolizing PUFAs into products. Reaction steps are indicated: (1) CPR is reduced by two electrons from NADPH. (2) CPR transfers the electrons to CYP2J2 sequentially. (3) CYP2J2 utilizes the electrons in order to oxidize substrates (AA) into EETs and HETEs. (B) Structures and names of fatty acids used in study. [carbon length: degree of unsaturation (omega 3 or 6)]. Abbreviations given at the end. Metabolite structures and names also given. Numbering refers to the positions of the other regioisomers of epoxides and hydroxides, with the only the major metabolites shown for clarity.

Figure 2. CYP2J2-mediated metabolism of (A) ebastine (EBS), (B) arachidonic acid (AA), (C) docosahexaenoic acid (DHA), (D) eicosapentaenoic acid (EPA), and (E) linoleic acid (LA) forming the indicated metabolites. Structures of parent compounds shown. “Total” refers to total metabolites plotted. Total = black, filled squares and solid lines; epoxides = grey shapes and dashed lines; hydroxides = light grey shapes and dotted lines. Data fit to the Michaelis-Menten model for all substrates. Error bars represent $\pm$ SEM.

Figure 3.

AA and DHA mixed kinetics. (A) 2D plot of AA metabolism in the presence of indicated amounts of DHA. (B) 3D plot of AA metabolism in the presence of DHA. Here, DHA is treated as the inhibitor and its effects on AA calculated. (C) 3D plot of DHA metabolism in the presence of AA. Here, AA is treated as the inhibitor in the calculations. Total metabolite formation best fit to a competitive inhibition model. Rates are in units of $\text{pmol}_{\text{products}}/\text{min}/\text{nmol}_{\text{CYP2J2}}$. Error bars represent $\pm$ SEM.

Figure 4.

Conformation of AA, EPA, and DHA in the active site of CYP2J2. (A) Representative conformations of the four identified clusters identified from molecular docking of AA, DHA, and EPA. These conformations were employed as starting points for subsequent MD simulations. In each case, the ligand and the heme are shown in stick representation. The side-chain of residues interacting with the fatty acid during the simulations (based on the interaction energies, Table S2) are also shown in stick representation. (B) Distribution of distances between epoxidation sites of AA, DHA and EPA to the iron atom of the heme, obtained from MD simulations. For each fatty acid, data were obtained from 100 ns simulations for cluster 1, 2 and 3, and 200 ns simulations for cluster 4.

Figure 5. Interaction of fatty acids with helix I of CYP2J2. (A) Time series of the interaction energy of AA, DHA, and EPA with residues Thr-318, Arg-321, and Ser-493 (B) Representative conformations of AA, DHA, and EPA in the active site, observed in the simulations. Residues Thr-318 and Arg-321 are located in helix I, shown in cartoon representation with purple color. The fatty acids are shown in stick representation, AA in green, DHA in black, and EPA in magenta. Side chains and heme are also shown in stick representation.

Table 1

Kinetic parameters of single-substrate studies. Each substrate fit to the Michaelis-Menten model. Error represents the SEM.

Substrate	V_{max} (pmol/min/nmol)	K_m (μ M)	k_{cat}/K_m ($\text{min}^{-1} \text{nM}^{-1}$)
EBS	$6,170 \pm 150$	19.4 ± 0.8	318
AA	189 ± 9	131 ± 11	1.44
LA	146 ± 17	118 ± 20	1.24
EPA	259 ± 77	251 ± 19	1.03
DHA	254 ± 11	542 ± 34	0.469

Table 2

3D mixed-inhibition fits of AA/DHA, AA/EPA, and AA/LA. Kinetic metabolism measurements of CYP2J2 with AA and either LA, EPA, or DHA were performed at varying concentrations of each substrate. Each analyte was treated as either a substrate or inhibitor for each fit. Data fit best to a competitive inhibition model with a measured inhibition constant $K_i^{Inhib \rightarrow Sub}$. Error represents the error of the fits.

AA as a Substrate	
Inhibitor	$K_i^{PUFA \rightarrow AA}$ (μ M)
DHA	16.5 ± 2.23
EPA	260 ± 1573
LA	46.4 ± 9.93
AA as an Inhibitor	
Substrate	$K_i^{AA \rightarrow PUFA}$ (μ M)
DHA	65.2 ± 15.8
EPA	∞
LA	78.4 ± 31.9

Table 3

Ebastine binding inhibition by FAs. Ebastine inhibition by these FAs was determined by Soret spectral titrations. The data fit best to a competitive model (Equation 1) The binding affinity of the inhibitors (PUFAs) are then compared to AA as a percentage of the AA value. Error represents the SEM.

<u>EBS Competitive Inhibition</u>		
	K_i (μM)	% K_i (AA)
AA	66.4 $\pm$ 1.8	100%
DHA	35.5 $\pm$ 2.4	53.4%
LA	106 $\pm$ 26	160%
EPA	111 $\pm$ 5	168%

Table 4

Effects of CYP2J2-T318V (T318V) and CYP2J2-T318V/S493A (Double) on AA and DHA binding and inhibition. Binding of AA and DHA to T318V and Double were determined by using competitive ebastine (EBS) binding assay. % K_i (AA) compares the K_i of DHA as a percentage of the value of AA for each construct. The inhibition of AA by DHA was also determined for each construct using 50 μ M of AA or 50 μ M of DHA. Rates are in pmol/min/nmolCYP and the inhibition by DHA (DHA Inhib) is shown as the percent of the AA metabolism rate. Error represents $\pm$ SEM.

	EBS competitive binding			AA inhibition		
	EBS	AA	DHA	AA	AA + DHA	DHA Inhib
	$K_S(\mu$ M)	$K_i(\mu$ M)	$K_i(\mu$ M)	% K_i (AA)	Rate (AA)	% (AA)
WT	8.16 $\pm$ 0.29	66.4 $\pm$ 1.8	35.5 $\pm$ 2.4	53.4%	50.0 $\pm$ 4.1	15.5 $\pm$ 3.6
T318V	3.72 $\pm$ 0.06	115 $\pm$ 8	60.5 $\pm$ 3.9	52.6%	42.8 $\pm$ 1.3	40.9 $\pm$ 0.7
Double	5.60 $\pm$ 0.76	230 $\pm$ 126	180 $\pm$ 80	78.3%	46.3 $\pm$ 1.5	41.0 $\pm$ 0.9
						88.6%