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Endocannabinoid Virodhamine is an Endogenous Inhibitor of Human Cardiovascular CYP2J2 Epoxygenase

Lauren N. Carnevale¹, Andres S. Arango^{2,3}, William R. Arnold¹, Emad Tajkhorshid^{*,2,3,4}, and Aditi Das^{*,§,1,2,3}

[§]Department of Comparative Biosciences, Division of Nutritional Sciences, Neuroscience Program, University of Illinois Urbana-Champaign, Urbana IL 61801

¹Department of Biochemistry, Division of Nutritional Sciences, Neuroscience Program, University of Illinois Urbana-Champaign, Urbana IL 61801

²Center for Biophysics and Computational Biology, Division of Nutritional Sciences, Neuroscience Program, University of Illinois Urbana-Champaign, Urbana IL 61801

³Beckman Institute for Advanced Science and Technology, Division of Nutritional Sciences, Neuroscience Program, University of Illinois Urbana-Champaign, Urbana IL 61801

⁴Department of Bioengineering, Division of Nutritional Sciences, Neuroscience Program, University of Illinois Urbana-Champaign, Urbana IL 61801

Abstract

The human body contains endogenous cannabinoids (endocannabinoids) that elicit similar effects as ⁹-tetrahydrocannabinol, the principal bioactive component of cannabis. The endocannabinoid virodhamine (O-AEA) is the constitutional isomer of the well-characterized cardioprotective and anti-inflammatory endocannabinoid anandamide (AEA). The chemical structures of O-AEA and AEA contain arachidonic acid (AA) and ethanolamine, however AA in O-AEA is connected to ethanolamine via an ester linkage whereas AA in AEA is connected through an amide linkage. We show that O-AEA is found at 9.6 fold higher levels than AEA in porcine left ventricle and is involved in regulating blood pressure and cardiovascular function. On a separate note, the cytochrome P450 (CYP) epoxygenase CYP2J2 is the most abundant CYP in the heart where it catalyzes the metabolism of AA and AA-derived eCBs to bioactive epoxides that are involved in diverse cardiovascular functions. Herein, using competitive binding studies, kinetic metabolism

***Corresponding Author:** To whom correspondence should be addressed: Aditi Das, PhD., University of Illinois Urbana-Champaign, 3836 VMBSB, 2001 South Lincoln Avenue, Urbana IL 61802, Phone: 217-244-0630. aditidas@illinois.edu, Emad Tajkhorshid, PhD., University of Illinois Urbana-Champaign, 3009 Beckman Institute, 405 N. Mathews Avenue, Urbana IL 61801, Phone: 217-244-6914. emad@life.illinois.edu.

AUTHOR CONTRIBUTIONS

LC wrote the manuscript, designed and performed all experiments, made figures, and analyzed the data. WRA helped in designed the experiments and analyzed the data. AA performed MD simulations, and analyzed corresponding data and contributed to making figures. AD helped design the experiments and wrote the manuscript. ET wrote and edited the manuscript. All authors analyzed the results and approved the final version of the manuscript.

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

Interaction energy figures and tables as well as molecular docking of neutral O-AEA at CYP2J2 epoxygenase have been included in the supplemental material. This material is available free of charge via the Internet at <http://pubs.acs.org>.

CONFLICTS OF INTEREST

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

measurements, molecular dynamics and wound healing assays we have shown that O-AEA is an endogenous inhibitor of CYP2J2 epoxigenase. Together, the role of O-AEA as an endogenous eCB inhibitor of CYP2J2 may provide a new mode of regulation to control the activity of cardiovascular CYP2J2 *in vivo* and suggests a potential cross talk between the cardiovascular endocannabinoids and cytochrome P450 system.

Graphical Abstract

Keywords

endocannabinoid; virodhamine; cytochrome P450; epoxyeicosatrienoic acids

INTRODUCTION

The human body contains endogenous lipid signaling molecules that elicit similar effects as the exogenous cannabinoid, (–)-⁹ tetrahydrocannabinol (THC). These endogenous molecules, or endocannabinoids (eCBs), are defined by their ability to bind and activate the G-protein coupled receptors (GPCRs), cannabinoid receptors 1 and 2 (CB1/2).^{1,2} The CB1 receptor is primarily located in the brain and throughout the central nervous system (CNS) where it regulates synaptic transmission and psychoactive effects. On the other hand, the CB2 receptor is predominantly located in peripheral tissues and macrophages, where it plays key roles in inflammation.^{2–5}

The eCBs are derived from ω–3 and ω–6 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and arachidonic acid (AA), respectively.⁶ Due to their widespread biological activity, the dysregulation of eCBs have been implicated in a variety of pathophysiological states, such as neurodegeneration, cardiovascular disease, and obesity.^{7–12} To treat these conditions, major degradative enzymes have been targeted to modulate eCB tone. For instance, fatty acid amide hydrolase (FAAH), a membrane bound serine hydrolase that degrades the eCB anandamide (AEA) has been targeted for a variety of neurological conditions.^{13,14} We anticipate that a better understanding of eCB signaling will unveil new biological targets for the development of therapeutics.

AEA and 2-arachidonoylglycerol (2-AG) were the first eCBs to be discovered in the early 1990s. Recent reports have shown that cytochrome P450 (CYP) epoxigenases are, in part, responsible for the metabolism of PUFAs and eCBs yielding a variety of bioactive epoxide and endocannabinoid epoxide metabolites.^{15–19} These metabolites are collectively called the epoxyeicosanoids and consist of a variety of classes (e.g., epoxyeicosatrienoic acid, EET; epoxyeicosatrienoic acid ethanolamine, EET-EA; etc.).^{20–23} Previously, these molecules

have been shown to act as cardioprotective agents against atherosclerosis, ischemia reperfusion, and regulate blood pressure.^{9,17,20} Specifically, AEA and 2-AG interact with CYPs and undergo epoxidation to their more bioactive products, EET-EA and EET-G with varied biological activities.^{16,20,24–26} These metabolites are targets of both FAAH and soluble epoxide hydrolase (sEH) which degrades CYP epoxygenase-derived eCB metabolites to yield the corresponding diol product, which is a current target for the development of anti-inflammatory drugs.^{5,14,27,28}

In 2002, Porter and coworkers discovered a novel eCB O-AEA. This molecule was found during a lipidomics experiment to detect endocannabinoids in tissues. When they analyzed the LC-MS/MS spectra, they found another molecule with the same mass-to-charge (m/z) ratio as AEA. However, this molecule possessed a unique retention time ($t = 3.25$ min. versus $t = 5.75$ min of AEA, respectively). This was termed as O-AEA as it has an opposing structure to AEA and was identified using a synthetic standard.²⁹ While AEA and its CYP-derived EET-EAs have been studied, there remains a dearth of knowledge regarding the biological activity of O-AEA and its potential metabolites. To date, it has been determined that O-AEA acts as a full agonist at the CB2 receptor and a partial agonist at the CB1 receptor. However, the EC₅₀ is 1401 nM and 1906 nM at CB2 and CB1, respectively.³⁰ Moreover, it activates an unknown endothelial receptor thereby modulating vasodilatory actions in the mesenteric artery.³¹ Interestingly, the concentrations of O-AEA drastically surpasses that of AEA in rat ventricles (~9:1).³² With O-AEA's cardioprotective and anti-inflammatory roles, we sought to characterize its interactions with cardiovascular CYP epoxygenases. Specifically, we chose to focus on the biophysical interactions of O-AEA with CYP2J2 epoxygenase since this CYP isoform is the major cardiovascular CYP in the cardiomyocytes present in the ventricles.³³

CYP2J2 epoxygenase converts AA to EETs that are known to have beneficial cardiovascular effects within the vasculature. For example, CYP2J2 has been shown to mediate the wound repair process in human endothelial cells through the cross-talk of several signaling pathways.^{15,22,26} Wound healing is a tightly-regulated and highly-coordinated process regulated by an assembly of growth factors, inflammatory cytokines, and cellular signaling events that control inflammation and cell migration. Accumulating evidence show that the bioactive EETs contribute wound healing in a CYP-dependent manner through their vasodilatory, cardioprotective, and anti-inflammatory actions. The ability of the EETs to help repair damaged tissue suggest a source of protection during post-ischemia reperfusion, atherosclerosis, and diabetes.^{26,5} Therefore, we sought to confirm our findings in a model of CYP2J2-dependent wound healing in HUVEC cells.

Taken all of this together, we report the competitive binding and inhibition of eCB metabolism by CYP2J2 by O-AEA using spectral titrations, reaction kinetics, and molecular docking. Nanoscale lipid bilayers of nanodiscs (ND) were employed for the solubilization and stabilization of CYP2J2 and are model lipid bilayers which mimic the composition and native environment of plasma membranes both in solution and on surfaces.^{34–37} We further corroborated these findings in a CYP2J2-dependent wound healing model using HUVEC cells.

Materials

Materials.—O-AEA was obtained from Enzo Life Sciences (Farmingdale, NY). POPC and POPS were bought from Avanti Polar Lipids (Alabaster, AL). AEA was purchased from Cayman Chemical (Ann Arbor, MI). Chloramphenicol, ampicillin, arabinose, isopropyl β -D-1-thiogalactopyranoside (IPTG), and Ni-NTA resin were purchased from Gold Biotechnology. δ -Aminolevulinic acid was obtained from Frontier Scientific. All other reagents used were purchased from Sigma-Aldrich and Fisher Scientific, or otherwise specified.

Experimental Details

Recombinant Expression of CYP2J2 in *Escherichia coli*.—Recombinant D34-CYP2J2 containing a His-tag and truncated N-terminal was expressed along with the pTGro7 plasmid encoding for the expression of the GroEL-GroES chaperon system, and was purified as previously described.^{38,39,11} The residues 3–37 in D-34 CYP2J2 are truncated and have a substitution of Leu-2 for Ala. These modifications have been previously shown to significantly increase protein purification yield without effecting protein activity.^{39,38} The presence of CYP2J2 was confirmed with SDS-PAGE with a band at 52 kDa.

Recombinant Expression of Cytochrome P450 Reductase (CPR).—Expression of cytochrome P450 Reductase, the redox partner of CYP2J2, was purified as previously described.³⁹

Cytochrome P450 Reductase (CPR) NADPH Kinetics.—The enzymatic activity of CPR was determined through the NADPH consumption assay as previously described.⁴⁰ A Cary UV-vis spectrophotometer was used to determine the rate of NADPH consumption in the absence and presence of Ebastine, a known substrate of CPR and CYP2J2. 0.2 μ M CYP2J2, 0.6 μ M CPR, and 50 μ M ebastine was incubated for 5 minutes in 0.1M potassium phosphate buffer. The reaction was initiated using 200 mM NADPH. The NADPH consumption rate was determined by measuring the decrease in NADPH absorption at 340 nm in the linear range of the reaction.

Incorporation of CYP2J2 into Nanodiscs.—Nanodiscs (ND) containing CYP2J2 were prepared as previously described.^{39,25} The apolipoprotein MSP1E3D1 and POPS:POPC in a 20:80 ratio were utilized to make 13 nm protein nanodiscs.

Extraction of O-AEA and AEA in Porcine Left Ventricle.—Tissue extractions of O-AEA and AEA were performed similarly as previously reported for the quantitative analysis of the endocannabinoids.⁴¹ Porcine left ventricle was obtained through the University of Illinois at Urbana-Champaign Meat Science Laboratory, and was immediately dissected, weighed, and homogenized on ice with a BioHomogenizer (BioSpec). Homogenization took place in a 9:1 ratio of ethyl acetate: hexanes supplemented with PMSF (1 mM) until a homogenous slurry was formed. The resulting mixture was washed with 10% water, vortexed for 60 seconds, and centrifuged for separation. The organic layer was removed, and the aqueous layer was extracted twice with an equal volume of ethyl acetate/hexane (9:1).

The pooled organic supernatant was removed using a Buchi 120 rotary evaporator. The sample was purified using solid phase extraction (SPE). Specifically, 40 g of silica was equilibrated with 3 volumes of chloroform. Sample reconstituted in 10 mL chloroform was applied gently to the column and washed with 3 column volumes of chloroform. 4 column volumes of 9:1 chloroform: methanol was used to elute. The sample was dried down, and resuspended in 200 μ L 90% ethanol.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) for Quantification of O-AEA and AEA.—Samples were analyzed with the 5500 QTRAP LC/MS/MS system (Sciex, Framingham, MA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent Eclipse XDB-C18 (4.6 $\times$ 150mm, 5 μ m) with 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: 0–1min, 50%A; 5–15min, 0%A; 16–22min, 50%A. The autosampler was set at 10°C. The injection volume was 10 μ L. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage of +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, respectively. Multiple reaction monitoring (MRM) was used for quantitation: O-AEA m/z 348.2 $\rightarrow$ m/z 62.1; AEA m/z 348.2 $\rightarrow$ m/z 62.1; NADA m/z 440.2 $\rightarrow$ m/z 137.1. Internal standard AEA-d4 was monitored at m/z 352.3 $\rightarrow$ m/z 66.1.

Metabolism of O-AEA by CYP2J2-CPR-Nanodiscs.—The metabolism of O-AEA was performed using the following procedure. 1 mL reaction mixtures contained O-AEA (10–100 μ M) from a 5 mM stock in ethanol), CYP2J2-ND (0.2 μ M), CPR (0.6 μ M), and 0.1 M potassium phosphate (KPi) buffer pH 7, and were allowed to incubate at 37°C for 10 min. The reaction was initiated upon addition of 0.5 mM NADPH and based on linearity measurement, was allowed to progress for 30 minutes. The reaction was quenched with the addition of 100 μ L glacial acetic acid (pH <4), and the metabolites were subsequently extracted three times using an equal volume of ice-cold ethyl acetate. Samples were centrifuged at 3,000 rpm for 5 minutes, and the organic layers were combined and dried under a flow of N₂ gas, and resuspended in ethanol for LC-MS/MS analysis as described in *Methods*. For quantitative analysis of epoxidized O-AEA, extracted samples were split into two groups as described below in *Saponification of Epoxidized O-AEA Regioisomers and Quantification*.

Saponification of Epoxidized O-AEA Regioisomers and Quantification.—For quantification of O-AEA epoxides, methanolic potassium hydroxide (KOH) was used to cleave the ethanolamine group to produce epoxyeicosatrienoic acids (EETs), as previously described.¹¹ Briefly, extracted samples were treated with 1 mL of methanol containing 0.4 M KOH and were incubated for 1 hr at 50°C. The reaction was terminated with glacial acetic acid to reach a pH of 3–4. Samples were subsequently extracted thrice with equal volume ice-cold ethyl acetate and water, vortexed, and centrifuged at 3000 rpm for 5

minutes. The organic layer was dried under a flow of N₂ gas, and resuspended in ethanol for LC-MS/MS as described for detection of EETs.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) for Quantification of EET Metabolites.—Analyses were performed at the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center of the University of Illinois at Urbana-Champaign as previously described.⁴² 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: 90% A from 0 to 2 min, 55% A until 8 min, 40% A from 13 to 25 min, 30% A until 30 min, 25% A until 35 min, 20% A until 40 min, 15% A from 45 to 47 min, and 90% A from 48 to 54 min. The autosampler was set at 5°C and the injection volume was 10 µL. Negative mass spectra were recorded with an 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, 50, and 55 psi, respectively. Multiple-reaction monitoring (MRM) was used for quantitation: m/z 319.2 → m/z 191.0 for 5,6-EET. 16,17-EEQ was used as an internal standard. EETs were quantified using 16,17 EDP (m/z 343.0 → m/z 274.0) as the internal standard.

AEA Metabolism in the Presence of O-AEA by CYP2J2-ND-CPR System.—

Kinetic assays measuring the epoxyeicosatrienoic acid ethanolamine (EET-EA) regioisomers were performed in a similar manner as the O-AEA metabolism but without saponification. 10–100 µM AEA was added in the presence of 10 µM O-AEA, and were incubated at 37°C for 10 minutes before 0.5 mM NADPH was added. EET-EAs were extracted as stated above. Control samples without O-AEA were used to validate the metabolism of AEA by the nanodisc system, as well as to provide a comparison to the inhibition curve. Samples were analyzed using LC-MS/MS as described in *Methods*. Results were fitted to the following competitive inhibition equation:

$$V_o = \frac{V_{\max} [S]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [S]} \quad (\text{Eq. 1})$$

where V_o is the initial velocity of the reaction, $V_{\max}$ is the maximal velocity, $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, K_m is the Michaelis-Menton constant and K_i is the inhibition constant.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) for Quantification of EET-EA Metabolites.—Analyses were performed at the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center of the University of Illinois at Urbana-Champaign as previously described.¹¹ For ESI, a Q-TOF Ultima time-of-flight mass spectrometer was used in positive ion mode for analysis of AEA, with a spray voltage of 4.5 V and capillary temperature of 200°C. 14,15-EET-EA-d8 was used as an internal standard. LC-MS/MS was performed similarly to the previously described method.

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Molecular Docking and Molecular Dynamic Simulations.—To characterize binding of O-AEA to CYP2J2, ensemble molecular docking was done using Autodock Vina.⁴³ The structure of CYP2J2 was obtained from previous publication, which was determined using homology modeling of CYP3A4 and bound to a POPC membrane using an accelerated membrane binding mimetic. Once the constructed CYP2J2 system was bound to the membrane mimetic, an equilibrium simulation was done with full tail lipids.⁴⁶ The resulting MD simulation was used to fully sample the dynamics of CYP2J2, allowing for a myriad of snapshots to be used for the ensemble docking protocol.⁴⁴ Specifically, the last 20 ns of the simulation trajectory were used, where 200 unique protein snapshots were taken every 100 ps. To perform ensemble molecular docking, a $22.5 \times 22.5 \times 22.5$ Å grid box was used, positioned at the center of the catalytic site of CYP2J2, containing the heme group. O-AEA was then docked onto each snapshot, with the top 10 poses collected for further analysis, yielding a total of 2000 docked poses. The 2000 poses were then clustered using a hybrid K-centers and K-medoids clustering algorithm using root-mean-square deviation (RMSD) as the metric.⁴⁵ The cutoff distance used was 1.5 Å, using only the C6, C7, C9, C10, N, and the carbonyl oxygen atoms for clustering. The largest cluster was then selected for further filtering. We then narrowed down the possible poses using the distance between C6 carbon, due to its relatively low metabolic activity (Figure. 1C) and the iron on the heme as the filtering criteria. For each cluster, the poses that were closest to the heme, following the filtering, were used for further analysis and simulating.

The simulations, containing the membrane bound protein ligand system were minimized for 5000 steps and equilibrated for 10 ns. During the equilibration the protein backbone, specifically the alpha carbons, were harmonically restrained ($k=1 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$). Following the equilibration, a production run was done without any restraints for an additional 190 ns, for a total of 200 ns for each cluster.

Molecular Dynamics Simulations of CYP2J2 Protocol.—The simulation was performed using the molecular dynamics software package, NAMD⁴⁶, using the CHARMM36m force field for the protein⁴⁷ and lipids.⁴⁸ The parameters for O-AEA were obtained using the CHARMM General Force Field.⁴⁹ For water, the TIP3P model was utilized.⁵⁰ The simulation was done using an NPT ensemble at 1.0 atm at 310 K and a time step of 2 fs. Moreover, constant pressure was maintained using the Nose-Hoover Langevin piston method.⁵¹ The constant temperature was maintained using Langevin dynamics with a damping coefficient of 0.5 ps^{-1} .⁵¹ A 12 Å cutoff was used for nonbonded interactions with a smoothing function implemented after 10 Å. The bond distances of the hydrogen atoms were constrained using the SHAKE algorithm.⁵² For long range electrostatic calculations, the particle mesh Ewald (PME) method was used, with a grid density of less than $1 \text{ \AA}^{-3}$.⁵³ Visualization of the simulations was performed using Visual Molecular Dynamics (VMD).⁵⁴

Simulation Interaction Energy Analysis.—Key residues of CYP2J2 interacting with O-AEA were determined by identifying residues within 4 Å of O-AEA throughout the trajectory, as seen in Figure. 3B–C. The interaction energy between O-AEA and the identified residues was then determined using a generalized implicit solvation method.⁵⁵

Human Umbilical Vein Endothelial Cell (HUVEC) Viability.—The MTT colorimetric assay (Cayman Chemical, Ann Arbor, MI) was used to determine cell viability in HUVEC cells treated with EETs, O-AEA. The manufacturer's protocol was followed. Cells transfected with human CYP2J2 were also tested for viability. HUVEC cells were plated at 1×10^4 cells/well in a 96-well plate for 12 hours. At 12 hours, O-AEA (250 nM, 500 nM, 1 μ M, 5 μ M, 10 μ M, and 20 μ M), 11,12-EET (10 nM, 50 nM, and 250 nM), or 14,15-EET (10 nM, 50 nM, and 250 nM) were added to fresh media. Cells were allowed to incubate at 37°C and 5% CO₂. At 12 h, a second dose was administered to account for the reduction in EETs due to degradation. Absorbance in each well was measured at 570 nm using a microplate reader. The optical density of cells containing compounds at 570 nm was normalized to wells without compound. For transfected cells, the manufacturer's protocol was also used, however cells were transfected in a 96-well plate at 60–80% confluency using the JetPrime PolyPlus kit for 48 hours. Then, 20 μ M O-AEA was added and the assay was performed as previously described above.

Wound Healing Assay.—A wound healing or “scratch” assay assessed wound repair in HUVEC cells. HUVEC cells plated in a 12-well plate were grown to a confluent monolayer. A scratch was made in a straight line down the surface of each well using a pipette tip. After the scratch was made, media was replaced with fresh growth-factor free media and treated with compounds. Cells without treatment were used as a negative control. Throughout the assay, cells were maintained in an incubator at 37°C and 5% CO₂, and images were taken every 2 hours. Images were processed using ImageJ via the wound healing plugin and normalized to 100% area at time 0.

RESULTS

Endogenous O-AEA Levels Exceed AEA Levels in the Heart.

Previously, *N*-acyl amide lipids such as AEA have been successfully quantified via LC-MS/MS in the selected reaction monitoring (SRM) mode in rat brain, heart, and spleen, as well as in human hippocampus.³⁰ Previously, O-AEA levels were found to be 7-fold higher than AEA in rat hearts.³⁰ To confirm that the ratio of O-AEA to AEA ratio is consistent in higher-level organisms such as pig, we sought to quantify O-AEA levels in the left ventricle of porcine heart. In particular, we chose porcine hearts because they possess greater anatomical and physiological similarity to human hearts than rat hearts.^{56,57} In addition, CYP2J2 levels are highest within the left ventricle of the heart where they mediate left ventricular function following ischemia reperfusion.^{58,59}

O-AEA and AEA were extracted from heart tissues using a two-step extraction method as described in the materials and methods section. Briefly, the organic layer is extracted followed by solid-phase extraction. Then O-AEA and AEA detected and quantified by LC-MS/MS as described in the materials and methods section.^{60,20} Interestingly, O-AEA was found at 9.6-fold higher levels than AEA (~0.440 pmol/g of wet tissue for O-AEA and ~0.046 pmol/g of wet tissue of AEA) in the left ventricle of pig hearts. This is consistent with the previous studies on rat ventricles.³⁰ It is likely that the local effective concentration of

virodhamine is higher than the picomolar levels determined from tissue extractions as the endocannabinoids are locally produced signaling molecules and are short-lived.^{61,62}

CYP2J2-CPR Nanodiscs Formation.

CYP2J2 epoxigenase was expressed in *E.coli* and purified on a Nickel-NTA affinity column as described previously.^{63,38} CPR was similarly expressed in *E.coli* and purified on a ADP agarose column. Nanodiscs were assembled based on previously reported methods.^{11,44,64,65} Nanodiscs are used to increase solubility and stabilization of membrane proteins.^{42,35,66}

Metabolism of O-AEA by the CYP2J2.

We initially characterized the CYP2J2-mediated O-AEA metabolism in nanodiscs (Figure. 1A). In order to quantify O-AEA metabolites, samples were saponified to the corresponding EETs for quantification by LC-MS/MS. Due to the innate insolubility of these lipid compounds, each study was performed below the critical micelle concentration (CMC) of each lipid compound. CYP2J2 was not saturated with concentrations up to 100 μ M (Figure 1B). This is not surprising, given that no endogenous substrate saturates CYP2J2 due to reaching the solubility limits of the hydrophobic lipid substrates before the maximum rate of metabolism is achieved.⁴² Product analysis using saponification indicated that the CYP2J2-mediated O-AEA metabolism produces four main epoxides, 5,6-epoxy-O-AEA; 8,9-epoxy-O-AEA; 11,12-epoxy-O-AEA; and 14,15-epoxy-O-AEA at significantly slower rates (Figure. 1B and C, < 3 pmol metabolite per minute per nmol CYP2J2) than previously reported eCB substrates (391 pmol per minute per nmol CYP2J2 and 265 pmol per minute per nmol CYP2J2 for EET-EA and EET-G, respectively).¹¹ To validate CYP2J2-CPR-ND system's activity, AEA metabolism was performed and agreed with previously published values (Figure. 1E and F).^{11,67} Based on these findings, O-AEA is a poor substrate for CYP2J2 and might be a potential inhibitor of CYP2J2.

AEA Metabolism in the presence of O-AEA by CYP2J2-CPR-ND System.

To further assess if the binding of O-AEA inhibit CYP2J2 mediated metabolism of substrates, CYP2J2-mediated metabolism of AEA was performed in the presence and absence of O-AEA (Figure 1D–F). The metabolism of AEA was monitored in the presence of low concentrations (10 μ M) and high concentrations (50 μ M) of O-AEA. As shown in Figure. 1E–F, AEA metabolism is significantly inhibited by O-AEA. Although the levels of the AEA epoxides produced are apparent values, the curves representing O-AEA inhibition are most accurately described through a competitive inhibition model as described in the materials and methods section (Eq. 1). In addition, throughout our metabolism studies we do not see a rearrangement of O-AEA to AEA, which has been previously suggested in the literature.⁶⁸ Thus, the decrease in EET-EA formation was due to O-AEA inhibition of CYP2J2. To determine the mechanism underlying O-AEA inhibition at CYP2J2, we next sought to determine how O-AEA binds to CYP2J2 using molecular dynamics.⁵¹ Thus, the decrease in EET-EA formation was due to O-AEA inhibition of CYP2J2. These results may help explain the strong inhibition by O-AEA at CYP2J2. To determine the mechanism underlying O-AEA inhibition at CYP2J2, we next sought to determine how O-AEA binds to CYP2J2 using molecular dynamics.

Binding Studies of CYP2J2-ND with O-AEA.

To probe the binding of O-AEA to CYP2J2, we performed Soret spectral binding assay. When a water molecule is bound to the iron of the CYP2J2-ND heme, the Soret maximum absorbance is at 417 nm. Upon binding of a substrate, the Soret can be perturbed to lower or higher wavelengths by type I and type II ligands, respectively. To validate our CYP2J2-nanodisc system, ebastine (EBS), a known CYP2J2 type I ligand, was used as a control (Figure. 2A). Our results showed that ebastine induced a type-I shift (shift from ~417 nm to ~390 nm) with an apparent spectral binding constant (K_d) of $3.4 \pm 0.8 \mu\text{M}$ as reported previously (Figure. 2A and D).⁴² Titration of O-AEA results in a shift in the peak absorption from 417 nm to 422 nm, characteristic of a type II ligand, with a K_d $14 \pm 2.1 \mu\text{M}$ (Figure. 2A and B). To confirm that binding of O-AEA to CYP2J2 was not due to the solvent used (ethanol), an ethanol control was utilized. In the presence of ethanol, there was no type II shift. However, when O-AEA was added, a type II shift resulted with a pronounced spectroscopic change in wavelength from 417 nm to 422 nm. It is common that ligands containing amines, pyridines, and urea groups inhibit CYPs through direct coordination of the iron.^{69,70} Based on these findings, we believed that O-AEA could act as an inhibitor through ligation of the heme group by the free amine, thus favoring the heme's low spin state. To assess the ability of O-AEA to inhibit ligand binding, we performed spectral competitive binding studies with EBS in the presence of O-AEA. O-AEA attenuated the spin-state shift to 390 nm with an inhibition constant of K_i of $13 \pm 4.7 \mu\text{M}$ (Figure. 2C). To the best of our knowledge this represents the best eCB inhibitor of CYP2J2-EBS binding.^{42,67}

Molecular Dynamics Simulations of O-AEA Binding to CYP2J2.

The atomistic details of O-AEA binding to CYP2J2 were probed using a joint approach of molecular docking as well as MD simulations, where snapshots of a CYP2J2 MD simulation were used for docking of protonated O-AEA and neutral O-AEA (Supplemental Figure 2). The CYP2J2 model that was used, was previously developed using homology modeling as well as equilibrated via simulation, which allowed for determination of key residues as well as interacting energy of CYP2J2 to O-AEA.⁴⁶ Utilizing the last 20ns of the previously obtained MD simulation of CYP2J2, 200 snapshots were made for the purpose of molecular docking. Each CYP2J2 snapshot yielded 10 docked poses, for a total of 2000 O-AEA binding poses. These O-AEA poses were then clustered, and filtered using distance to the heme as the filtering criteria. For each cluster, the top pose was then simulated for 200 ns within the membrane bound CYP2J2, and residues within four angstroms of O-AEA were calculated every 100 ps. The interactions between O-AEA and the residues of CYP2J2 were counted, normalized, and ordered by frequency of interaction (Figure 3C). This interaction analysis showed several residues more frequently interacting with O-AEA than others, mainly glutamate 314, phenylalanine 310, and threonine 219. To further characterize the role of these top three interacting residues, interaction energy between O-AEA and the top residues observed were calculated for time step (2 fs) for the last 100 ns of each simulation trajectory (Figure. 3C).⁴⁴ This analysis showed an average interaction energy of $-86.2 \pm 14.8 \text{ kcal/mol}$ for E314, $-8.0 \pm 2.9 \text{ kcal/mol}$ for F310, and $-1.7 \pm 2.4 \text{ kcal/mol}$ for T219 with O-AEA. These results demonstrate that E314 plays a key role in stabilization of O-AEA, binding almost completely throughout the simulation with some transient unbinding,

while being stabilized by nearby hydrophobic residues. This allows it to occupy the catalytic site, which may contribute to its inhibitory effects of CYP2J2.

O-AEA Inhibits CYP2J2-Mediated Wound Healing in HUVEC cells.

We next determined if O-AEA would inhibit CYP2J2 in a cell culture model. In order to execute this, we investigated the inhibition of wound healing by O-AEA in HUVEC cells. Wound healing is a tightly regulated process, in part, mediated by the production of CYP2J2-derived EETs in endothelial cells.^{22,17} We determined the effect of O-AEA on the ability of endogenous CYP2J2 and transfected human CYP2J2 to facilitate wound closure through a variety of experimental approaches. An initial MTT Assay performed over a 24-hour period confirmed that neither O-AEA nor overexpressed hCYP2J2 affected HUVEC cells' viability as shown Figure. 4A.

Next, we sought to test whether O-AEA could inhibit endogenous CYP2J2 and decrease the percent of wound closure over time compared to HUVEC without O-AEA treatment. For this assay, a scratch was made over a confluent monolayer of HUVEC cells grown in a 12-well plate. O-AEA was then added to appropriate treatment wells. As shown in Figure. 4B, O-AEA had a lower percent wound closure compared to untreated HUVEC cells (control). To further support this finding, human CYP2J2 was transfected into HUVEC cells and a similar experiment was performed to assess O-AEA's ability to decrease CYP2J2-mediated wound closure. CYP2J2 transfected cells showed an increase in the percent wound closed compared to untransfected HUVEC cells (control) (Figure. 4D). This is consistent with previous reports that show increased wound repair in response to an increase in EET production by CYPs within damaged tissue.^{17,22,71,18} From this experiment, we can also see in the images (Figure. 4C) that in the absence of O-AEA in CYP2J2-transfected HUVEC cells the wound becomes fully closed by the end of the experiment (Figure. 4C). However, in the presence of O-AEA, a significant portion of the wound remained open at 12 h. More specifically, nearly 60% of the wound was still opened at the end of the 12 h compared to the untransfected HUVEC cells (control) and the CYP2J2-transfected. Based on these findings we propose that O-AEA is capable of acting as an inhibitor of CYP2J2 and potentially other CYPs that produce EETs in HUVEC cells.

DISCUSSION

Extrahepatic CYP2J2 epoxygenase is the most abundant CYP epoxygenase expressed in the cardiomyocytes where it metabolizes AA and AEA to form bioactive EETs and EET-EAs, respectively. The EET products play a key role in the regulation of vascular tone and blood pressure. Moreover, AA-derived eCBs, such as AEA, play an essential role in cardiovascular function and tissue repair during ischemia-reperfusion.^{72,73,74} In our studies with pig left ventricles, we find that the molecule O-AEA is 9.6-fold higher concentrations than the well-characterized endocannabinoid AEA. Kozłowska and coworkers have demonstrated that O-AEA is implicated in vasodilation and regulation of the cardiovascular system.^{75,31} Therefore, we were interested in elucidating the interplay between O-AEA and the CYP epoxygenases within the heart, namely with CYP2J2.

We found that O-AEA metabolism by CYP2J2 is considerably slower than AEA metabolism. The results indicate that while the rate of AEA metabolism is 80 ± 9 pmol metabolite min^{-1} nmol CYP2J2 $^{-1}$, O-AEA metabolism proceeds at 3 ± 0.2 pmol metabolite min^{-1} nmol CYP2J2 $^{-1}$. O-AEA was not saturated under any experimental conditions (Figure. 1B and C) suggesting minimal metabolism.

Since O-AEA is a poor substrate for CYP2J2, we evaluated the ability of O-AEA to inhibit AEA metabolism. The product analysis of CYP2J2, AEA and O-AEA incubations by LC-MS/MS revealed a decreased formation of the epoxide regioisomers at the 5,6-, 8,9-, 11,12- and 14,15-alkene positions, and a K_i value of 8 μM (Figure. 2B and C). O-AEA displayed 10- to 100-fold higher CYP2J2 inhibition than previously reported endogenous PUFAs (AA, DHA, and EPA).⁴² Therefore, O-AEA is the strongest endogenous inhibitor of CYP2J2 reported and can be of potential therapeutic use.

To probe the molecular interactions between CYP2J2, spectral titration studies were performed to determine O-AEA binding to CYP2J2. These studies revealed that O-AEA is a strong type II ligand, where the titration of O-AEA to CYP2J2-ND resulted in a robust shift in the absorbance from 417 to 422 nm. This bathochromic shift is likely due to the primary amine ligating the heme.⁷⁶ This is consistent with past reports of amine-based type II ligands (aniline, benzylamine, *n*-butylamine, etc.).^{76,77} We further characterized O-AEA binding by determining its inhibition of EBS binding to CYP2J2. Compared to other endogenous ligands (e.g., AA, DHA, and EPA) of CYP2J2, O-AEA produced the lowest K_d and K_i with values of 12 μM and 13 μM compared to AA and DHA (K_i : 66 μM and K_i : 36 μM , respectively).⁴² Taken together, these data indicate that O-AEA ligates the iron-heme of CYP2J2 and acts as a competitive inhibitor.

The molecular understanding of the inhibition of O-AEA through binding to the active site of the CYP2J2 was explained using MD simulations, where the conformational state of O-AEA with CYP2J2's heme was monitored, as well as key residues that are involved in the binding of O-AEA were demonstrated. As shown in Figure 3, the molecular docking and MD simulations of O-AEA and CYP2J2 showed that glutamate 314 greatly stabilizes O-AEA binding within the catalytic site of CYP2J2 more strongly than other nearby interacting residues such as F310 and T219. Furthermore, it should be noted that glutamate 314 is on Helix I, residues 297–330, which has been shown to stabilize binding of polyunsaturated fatty acids including, arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA).⁴⁸ In addition, the interaction energy calculated between O-AEA and E314 was -86.2 ± 14.8 , which is nearly four times the reported values between interactions between and Arg-321, Thr-318, and Ser-493 with DHA.⁴² The interaction between O-AEA and E314 was predominantly driven by ionic interactions, as the protonated form of O-AEA was used for the molecular docking and the MD simulation, this likely the reason for the energetically favorable binding that is observed, which likely causes O-AEA to occupy the catalytic site of CYP2J2, giving rise to its inhibitory effects.

Furthermore, the neutral form of O-AEA was also studied via molecular docking, however we observed very few poses demonstrating a coordination of the amine group with the iron on the heme group (Supplemental Figure 2). Although amine-iron coordination can lead to a

type II ligand, it is not readily visible via the molecular dynamics simulations as the electrostatic interaction between the amine and E314 is present in three out of four of the simulations performed. The atomistic resolution provided by the MD simulations allows us to predict how O-AEA inhibits and interacts with CYP2J2. The inhibitory nature of O-AEA is likely due to O-AEA sterically blocking the access channel of CYP2J2 but may, in part, be due to coordination of O-AEA to the heme. Therefore both modes of inhibition are possible. In addition, the electrostatic interactions between O-AEA and E314 might be the precursor to ligation of the heme. Specifically, the glutamate could deprotonate the amine allowing ligation to occur more readily. Therefore both modes of inhibition are possible, and together may contribute to the inhibition.

Previously, O-AEA was determined to be an eCB due to its presence in tissues highly expressing cannabinoid receptors and its ability to bind and activate cannabinoid receptors. In peripheral tissues, which express the CB2 receptor, the concentration of O-AEA is higher than AEA. Correspondingly, O-AEA is a full agonist at the CB2 receptor, which may provide a means for its regulation of cardiovascular function and anti-inflammatory activity. (EC₅₀ is 1401 nM and 1906 nM at CB2 and CB1, receptively).³⁰

Increasing evidence supports the concept that AA- and AA-derived endocannabinoid epoxides, such as the EET-EAs, possess a multitude of activities within the cardiovascular system, such as blood pressure regulation, angiogenesis, and wound repair.^{5,78} With the high abundance of CYP2J2 in cardiomyocytes and endothelial cells, we were able to show that the EETs, which have previously been shown to increase the rate of wound healing, are functionally regulated through a feedback loop involving O-AEA, as an inhibitor of CYP2J2. Through our wound healing assay, we demonstrated that the overexpression of CYP2J2, an AA-epoxygenase, stimulated migration of endothelial cells. When the CYP2J2-inhibitor O-AEA was present, this process is attenuated, thus showing the ability of O-AEA to inhibit CYP2J2. The same was observed in wild type cells indicated that basal CYP2J2 and EET concentrations could be inhibitory to produce the same response (Figure. 4C). Furthermore, a dose-dependent wound healing experiment revealed O-AEA dose-dependently inhibits wound healing in hCYP2J2-transfected cells (Figure. 4E) with an IC₅₀ value of 0.61 μ M.

Our data provides a new mode of regulation of cardiovascular CYP2J2 with an endogenous eCB. While downstream degradative enzymes such as sEH regulate the EETs and EET-EA levels by hydrolyzing the epoxides to diols, O-AEA may serve as a more upstream regulator of AEA and lipid metabolism by CYP2J2 thereby controlling the local environment of tissues and cellular compartments. In addition, this is the first study to our knowledge that identified an eCB acting as an inhibitor of another eCB's metabolism, let alone, an endogenous inhibitor in the cytochrome P450-eCB system cross talk pathway. Also there is a continuous search for CYP inhibitors that are non-toxic and O-AEA can be such as inhibitor naturally expressed in the body whose degradative products will be non-toxic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

O-AEA	Virodhamine
AEA	anandamide
AA	arachidonic acid
CYP	cytochrome P450
CPR	cytochrome P450 reductase
EBS	ebastine
EET	epoxyeicosatrienoic acid
(EET-EA)	epoxyeicosatrienoic acid ethanolamine
(EEQ-EA)	epoxyeicosapentaenoic acid ethanolamine
EBS-OH	hydroxyebastine
LC/MS-MS	liquid chromatography-tandem mass spectrometry
MD	molecular dynamics
PUFA	polyunsaturated fatty acid
POPC	phosphatidyl choline
POPS	phosphatidyl serine

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Figure 1. Metabolism of O-AEA and AEA by CYP2J2-CPR Nanodiscs

(A) Schematic of CYP2J2-CPR-nanodisc system showing metabolism of O-AEA to O-AEA epoxides (CPR, cyan; CYP2J2, ice blue) (B) Total metabolism of O-AEA by CYP2J2-CPR-Nanodiscs. (C) O-AEA structure (red, closest interactions to heme group; blue, furthest interactions from heme group) metabolism of O-AEA by CYP2J2 showing the rate of reaction for each regioisomer. The part of O-AEA containing the 5,6- and 8,9-alkenes as well as the ester bond and amine was shown to interact more closely with the heme of CYP2J2 than the 11,12-alkene to the terminal methyl. (D) Metabolism of AEA by CYP2J2-CPR nanodiscs in the absence and presence of O-AEA. (E) Plot of AEA metabolism to the EET-EAs alone (black curve) and in the presence of 10 μM (solid blue curve) and 50 μM (dotted blue curve). Total metabolism fits best to a competitive inhibition model. Rates are in units of pmol of product per minute per nmol of CYP2J2. (F) Table of kinetic parameters for AEA metabolism alone and in the presence of O-AEA.

Figure. 2. Spectral Interactions of O-AEA at CYP2J2.

(A) Structure of Virodhamine (O-AEA) and Ebastine (EBS). (B) Binding of O-AEA to CYP2J2 Nanodiscs (0.2 μM CYP2J2). (C) Binding of EBS to CYP2J2 Nanodiscs (0.2 μM CYP2J2) and competitive binding of O-AEA (30 μM) to CYP2J2 Nanodiscs (0.2 μM CYP2J2) in the presence of EBS (0–155 μM).

Figure 3. Interactions of O-AEA to CYP2J2.

(A) MD simulations of O-AEA show a strong and consistent interaction between O-AEA and glutamate 314. (B) To identify the key residues, an MD contact analysis was performed where the y-axis corresponds to the normalized interaction counts throughout the trajectory. The results show E314 as the main contributor to binding for O-AEA, as well as some contributions from nearby residues, mainly F310 and T219, which likely aids in stabilizing binding. (C) Time series of the interaction energy between O-AEA and selected residues for the last 100 ns of the three molecular dynamics trajectories that see binding. The average interaction energy of E314 was calculated to be -86.2 ± 14.8 kcal/mol, with additional interaction energies for every other residue listed in the supplemental material.

Figure 4. Inhibition of CYP2J2-mediated wound healing by O-AEA.

(A) Cell viability of HUVECs incubated alone (control) or with O-AEA (20 μ M) and/or hCYP2J2 as determined by MTT assays over a 24 h period. (B) Wound healing assay shown in hCYP2J2-transfected cells at 0 h and 24 h in the absence or presence of O-AEA (20 μ M). (C) Percent wound closure in wild type HUVECs in the absence (control) and presence of O-AEA (20 μ M). Cellular wound healing was observed using a microscope and images were processed using the Image J plugin. (D) Wound healing in transfected HUVEC is represented as a percentage of wound closed in treatment relative to control HUVECs without hCYP2J2. (E) Dose-dependent inhibition of wound healing via O-AEA in hCYP2J2-transfected HUVEC cells. *p 0.05, **p 0.01, ***p 0.001 ****p 0.0001 calculated using two-way ANOVA with Tukey's multiple comparisons test (GraphPad Prism version 6.00); n (sample size) =3.