Supporting Information Appendix

Anti-inflammatory ω-3 Endocannabinoid Epoxides

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SUPPORTING FIGURES AND TABLES — PAGES

MATERIALS AND METHOD

	Rat Brain (pmol/gm)	Rat Heart (pmol/gm)	Rat Kidney (pmol/gm)	Rat Spleen (pmol/gm)	Rat Liver (pmol/gm)	Pig Brain (pmol/gm)	Human Plasma (pg/mL)
17,18-EEQ-EA	97.4 ± 42.2	50.2 ± 14.9	93.2 ± 90.4	65.3 ± 42.7	60.6 ± 24.7	105.7 ± 99.1	N.D.
14,15-EEQ-EA	67 ± 62	52.1 ± 33.1	12.8 ± 12.2	10.8 ± 6.5	161.2 ± 65.7	42.4 ± 41.4	N.D.
11,12-EEQ-EA	17.3 ± 14	10.5 ± 7.8	N.D.	3.8 ± 2.2	36.6 ± 14.9	31 ± 29.9	N.D.
8,9-EEQ-EA	N.D.	N.D.	N.D.	2.6 ± 1.7	N.D.	1.3 ± 0.6	N.D.
5,6-EEQ-EA	N.D.	N.D.	N.D.	0.1 ± 0.05	N.D.	N.D.	N.D.
EPEA	6.2 ± 1.6	9.4 ± 6.0	37.1 ± 21	9.2 ± 4.1	80.8 ± 20.1	2.2 ± 0.4	N.D.
19,20-EDP-EA	142.7 ± 58.7	87.3 ± 62.1	420.7 ± 265	72.4 ± 43.9	148 ± 60.4	136.9 ± 110.7	167.3 ± 11.8
16,17-EDP-EA	10.4 ± 5.2	4.2 ± 2.8	15.7 ± 9.5	N.D.	N.D.	2.2 ± 1.1	N.D.
13,14-EDP-EA	41.1 ± 22.7	13 ± 9.7	61.7 ± 55	35.4 ± 22	37 ± 15.1	0.5 ± 0.5	N.D.
10,11-EDP-EA	36.5 ± 20.1	10.2 ± 8.3	56.7 ± 54.9	31.6 ± 19.2	33.5 ± 13.6	N.D.	N.D.
7,8-EDP-EA	20.5 ± 11.4	10.2 ± 8.3	34.4 ± 42.1	20.5 ± 12.7	24.3 ± 9.9	N.D.	N.D.
DHEA	128.9 ± 27.9	57.7 ± 24.4	49.6 ± 22.2	22.9 ± 1.3	195.1 ± 48.7	265.5 ± 100	13.3 ± 0.4
Anandamide	154.5 ± 15.8	65.6 ± 25.0	565.2 ± 306	162.4 ± 87.1	567.7 ± 141.9	2825 ± 975	20.6 ± 2.6

N.D. = not detected

Table ST1. **Summary of the average levels of detected EEQ-EA, EDP-EA, EPEA, DHEA and AEA levels in rat brain, rat heart, rat kidney, rat spleen, rat liver, porcine brain and human plasma.** Sprague-Dawley rat tissues, pig brain and human pooled plasma were analyzed for EEQ-EA, EDP-EA, EPEA, DHEA and AEA metabolites as described in the materials and methods in the main text.

	BV-2 Microglia post-LPS stimulation		
	1 hr	4 hr	12 hr
Total EEQ-EA (pmol / 10^6 cells)	1.8 ± 0.1	4.1 ± 0.5	30.1 ± 8.4
Total EDP-EA (pmol / 10^6 cells)	5.9 ± 0.5	182.5 ± 15.9	172.8 ± 70.3
Total EPEA (pmol / 10^6 cells)	17.3 ± 2	483.4 ± 8.7	2042 ± 939.2
Total DHEA (pmol / 10^6 cells)	30.3 ± 8.4	324.3 ± 20.2	6.7 ± 4.6
Total AEA (pmol / 10^6 cells)	7.4 ± 1.1	215.6 ± 33.1	4895 ± 540.9

Table ST2. **Time course of EEQ-EA, EDP-EA, EPEA, DHEA and AEA production by LPS stimulated BV-2 cells.** 6-well plates were seeded with 500,000 BV-2 cells and grown to 80-90% confluence. Microglia were stimulated with LPS (100 ng/mL) and placed back in the incubator (37°C and 5% CO_2) for each specified period of time. At each time point, cells were scraped in the supernatant, lysed and extracted three times with ethyl acetate/hexane (9:1). The organic layer was dried down and re-suspended in ethanol for targeted lipidomic analysis using LC-MS/MS as described in the main text.

Table ST3. **EC_{50} values (nM) of different lipid species as determined using PRESTO-Tango assay of cannabinoid receptor 1 (CNR1) and 2 (CNR2) in figure 4E-G in the main text.**

	CNR1	**CNR2**
Compound	**EC_{50} (nM)**	**EC_{50} (nM)**
CP 55 940	**9.9**	**8.0**
2-AG	**0.4**	**0.3**
AEA	**0.7**	**1352**
DHEA	**1044**	**305**
EPEA	**0.1**	**2.1**
19,20 EDP-EA	**108**	**280**
17,18 EEQ-EA	**18.5**	**1.4**

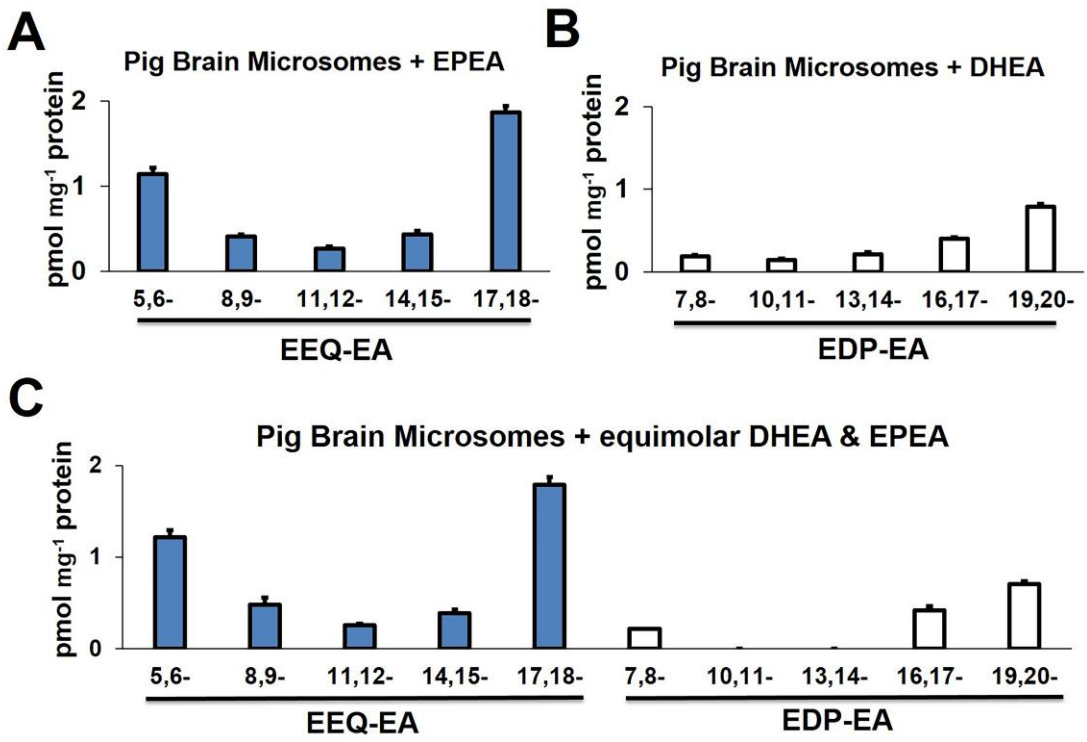

Figure S1. **Incubations of EPEA and DHEA with pig brain microsomes.** Microsomes were prepared from pooled (n=2) homogenized whole porcine brain to measure the capacity of endoplasmic reticulum epoxygenases to directly epoxygenate **(A)** EPEA (40 µM), **(B)** DHEA (40 µM) and **(C)** Equimolar mixture of EPEA (40 µM) and DHEA (40 µM).

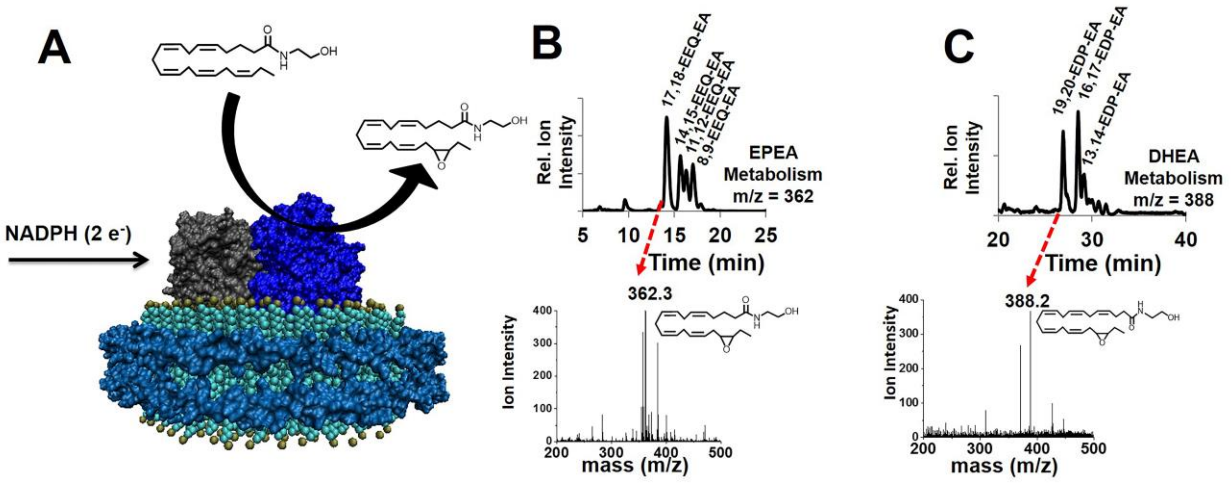

Figure S2. **LC-ESI-MS global analysis of the CYP2J2-ND mediated metabolism of EPEA and DHEA.** LC-ESI-MS was employed to qualitatively characterize CYP2J2 nanodisc metabolism of EPEA and DHEA. **(A)** CYP2J2-CPR was incorporated into nanodiscs. The membrane scaffold protein (cyan) surrounds a lipid bilayer (aqua marine with gold phospholipid head groups) in which both CYP2J2 (dark blue) and CPR (grey) are incorporated. The addition NADPH provides the electron source that drives the P450 cycle resulting in the olefin epoxidation of EPEA to EEQ-EA. The CYP2J2-ND metabolism of **(B)** EPEA produced the four detectable epoxide products 17,18-, 14,15-, 11,12-, and 8,9-EEQ-EA whereas **(C)** DHEA produced the three detectable epoxide products 19,20-, 16,17-, and 13,14-EDP-EA.

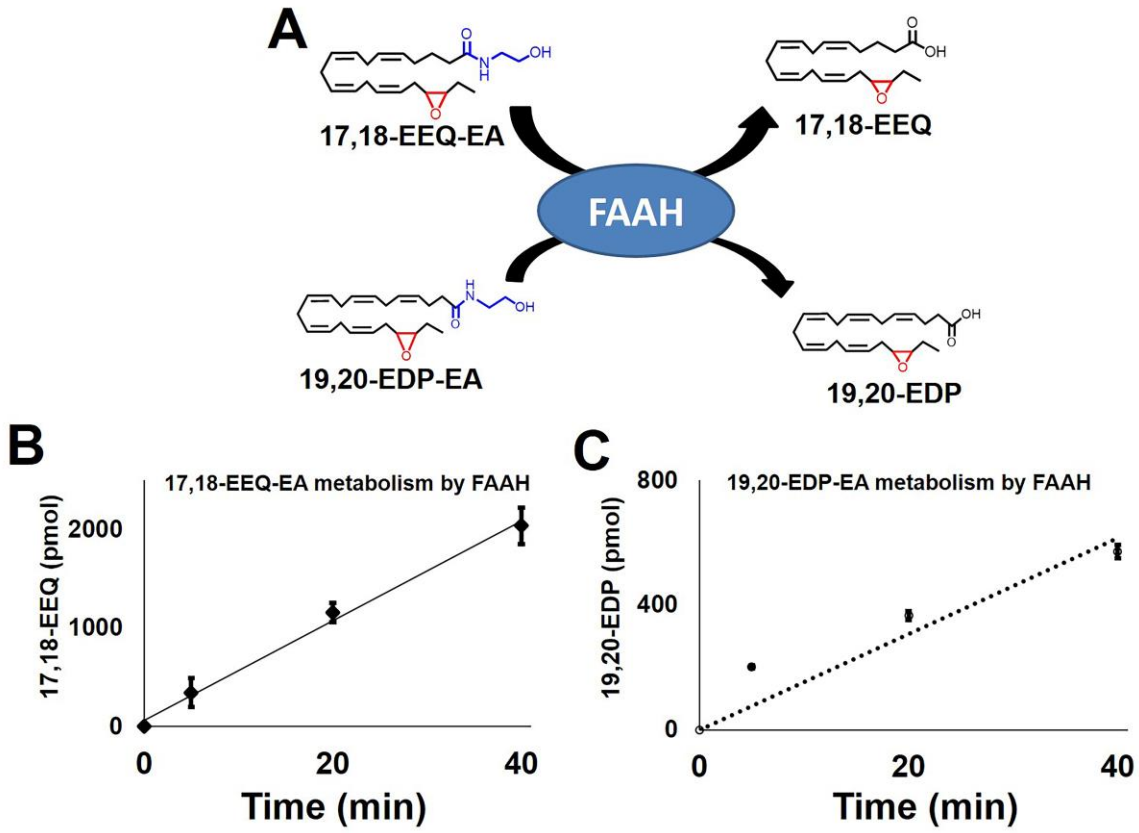

Figure S3. **Time-dependent hydrolysis of 17,18-EEQ-EA and 19,20-EDP-EA hydrolysis by rat forebrain membranes.** Rat forebrain membranes containing fatty acid amide hydrolysis (FAAH) were isolated and protein content was determined using a BCA assay as described in the supplemental methods. Incubations were performed with 100 µg of forebrain protein, 50 µM of substrate (17,18-EEQ-EA or 19,20-EDP-EA) in the presence of 50 mM Tris (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$ at 37°C as previously described (1). Samples were quenched with methanol containing 1 mM PMSF at 5, 20 and 40 minutes and analyzed via LC-MS/MS.

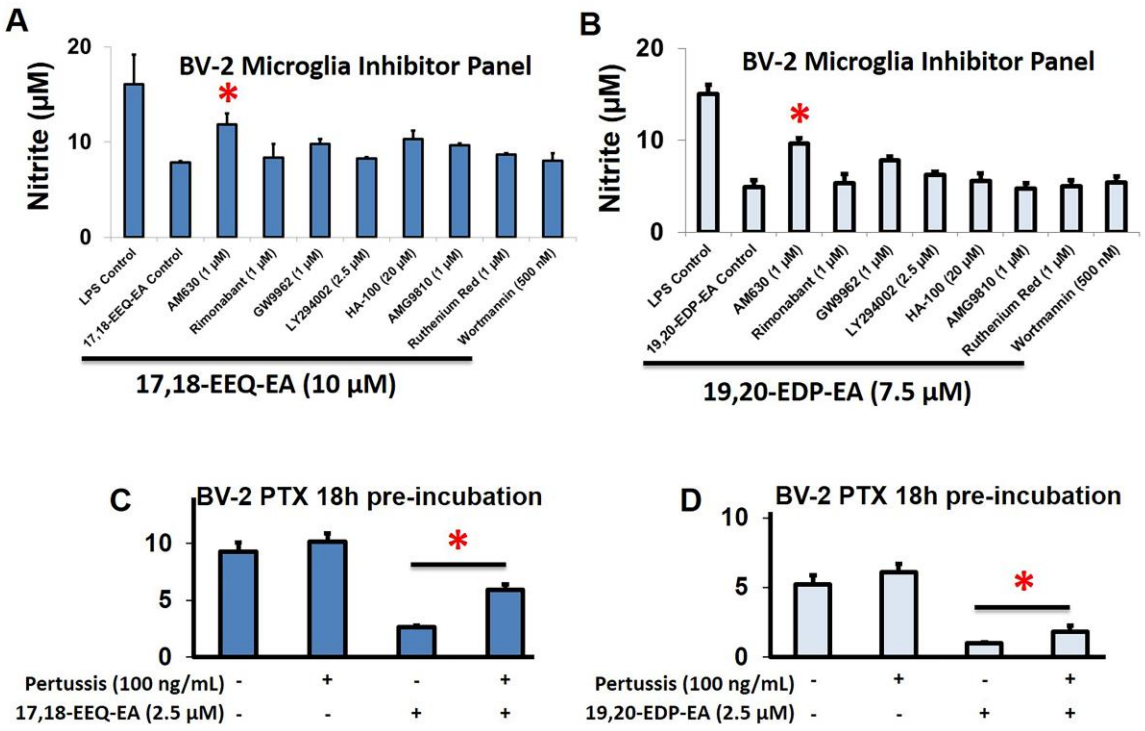

Figure S4. **Effects of inhibitors on 17,18-EEQ-EA and 19,20-EDP-EA mediated anti-inflammatory effects in BV-2 microglia cells.** Both epoxy-eicosanoids and endocannabinoids function through multiple receptors and confer complex anti-inflammatory actions. A panel of inhibitors were screened for their ability to reverse **(A)** 17,18-EEQ-EA and **(B)** 19,20-EDP-EA mediated anti-inflammatory effects in BV-2 microglia cells. Inhibitor concentrations were chosen based on reported EC_{50} values and lack of inhibitor effect in the assay. **(C-D)** BV-2 cells were pre-incubated with vehicle or pertussis toxin (100 ng/mL) in serum free-media for 18 hours prior to LPS stimulation in the absence or presence of **(C)** 17,18-EEQ-EA and **(D)** 19,20-EDP-EA

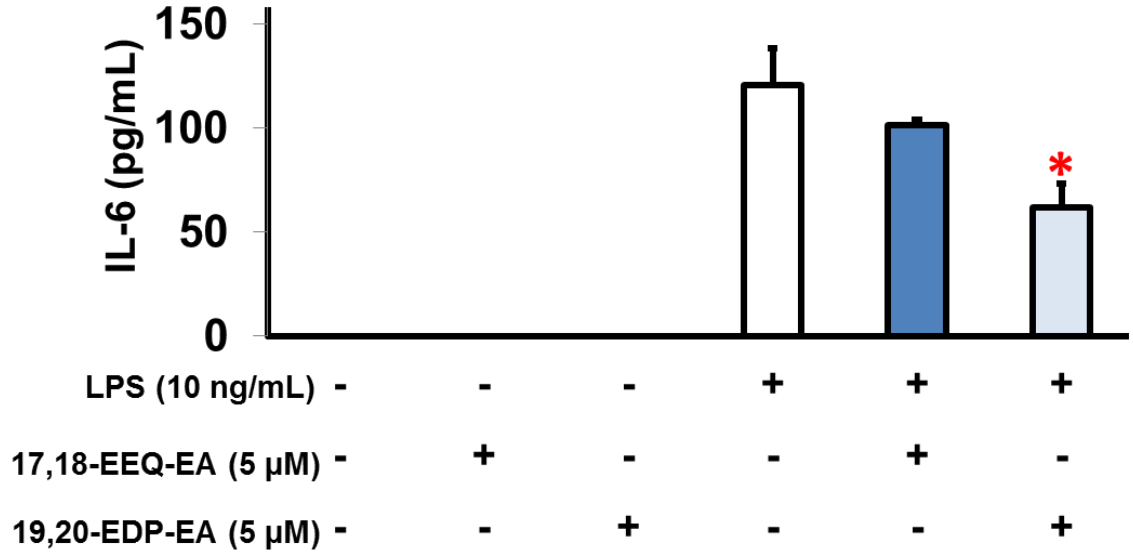

Figure S5. **In vitro primary piglet microglial neuroinflammation model.** Isolated piglet microglia cells were pre-treated with either 17,18-EEQ-EA (5 µM) or 19,20-EDP-EA (5 µM) for 1 hour before stimulation with LPS (10 ng/mL). After 12 hours, the supernatant was collected and IL-6 content was measured via ELISA. The final vehicle (DMSO) concentration was 0.04% and each group represents an n = 3 or greater.

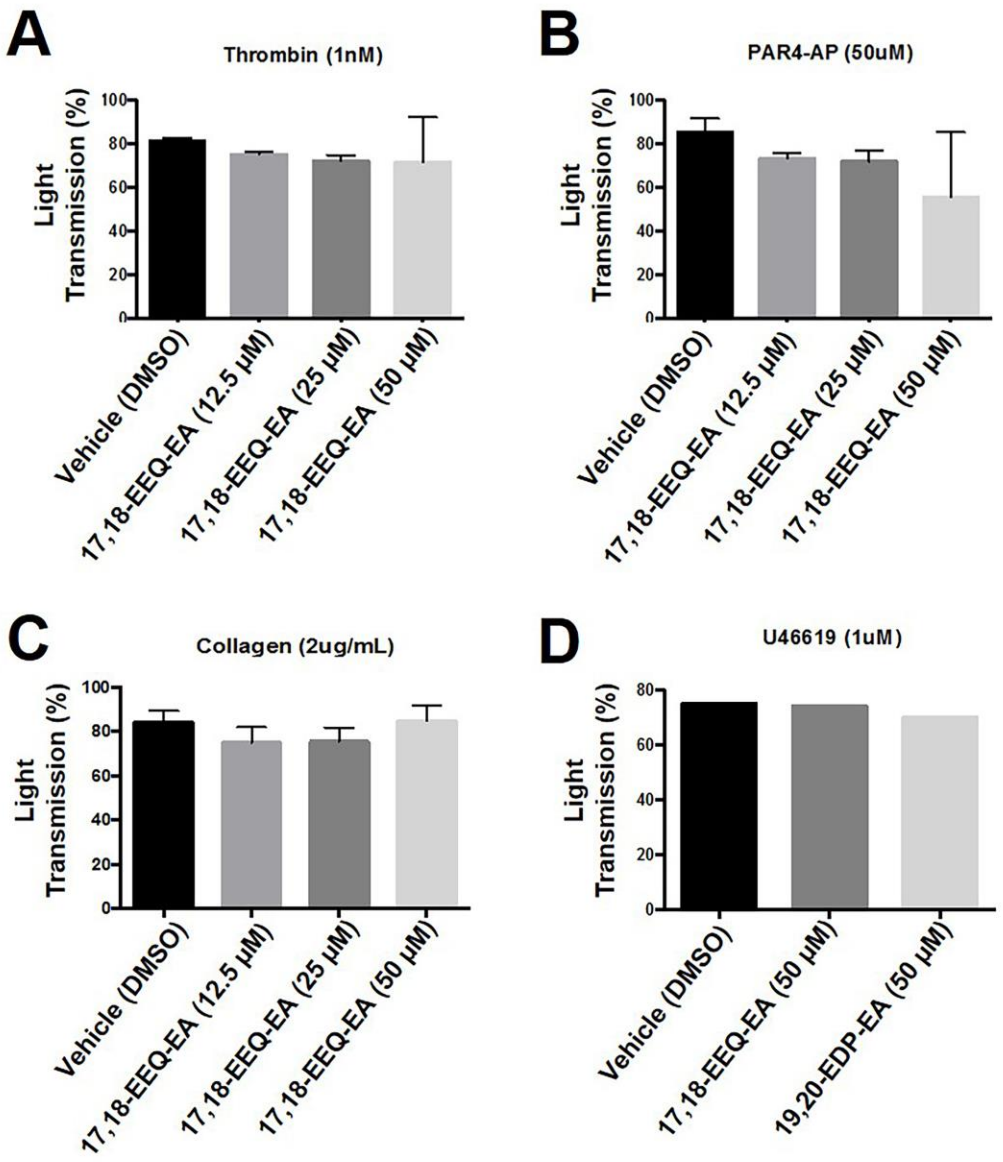

Figure S6. **Effects of 17,18-EEQ-EA on thrombin, PAR4-AP, collagen or U44619-induced aggregation in washed human platelets.** Washed human platelets were activated with either **(A)** 1 nM thrombin **(B)** 50 µM PAR-AP **(C)** 2 µg/mL collagen in the presence of 12.5, 25 and 50 µM 17,18-EEQ-EA (n = 3) which had no significant effect on platelet aggregation as determined by light transmission (%). **(D)** Similarly, washed human platelets activated with 1 µM U46619 (n = 1) did not exhibit any change in the presence of either 50 µM 17,18-EEQ-EA or 19,20-EDP-EA as estimated by light transmission (%).

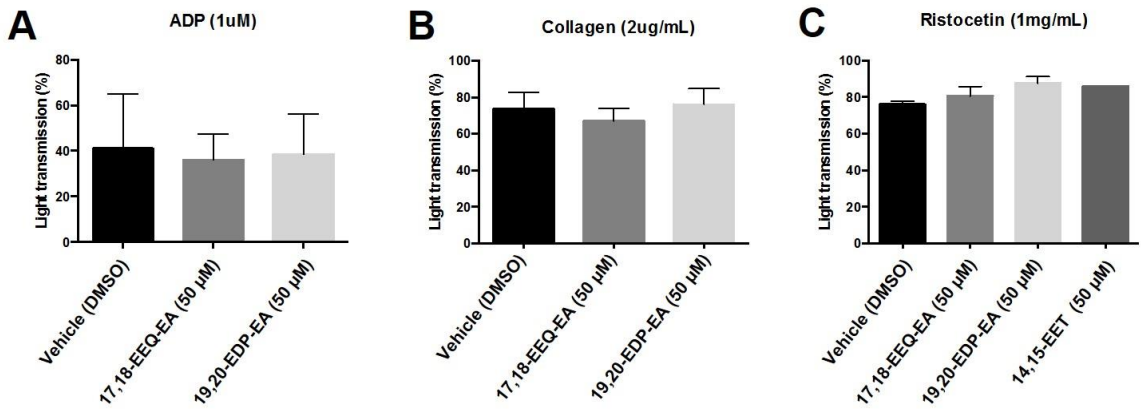

Figure S7. **17,18-EEQ-EA and 19,20-EDP-EA effects on ADP, collagen or ristocetin-induced platelet aggregation in platelet rich plasma.** The effects of 17,18-EEQ-EA, 19,20-EDP-EA at 50 µM relative to vehicle (DMSO) in were investigated in platelet rich plasma clotting via **(A)** ADP (1 µM), **(B)** collagen (2 µg/mL) and **(C)** ristocetin (1 mg/mL).

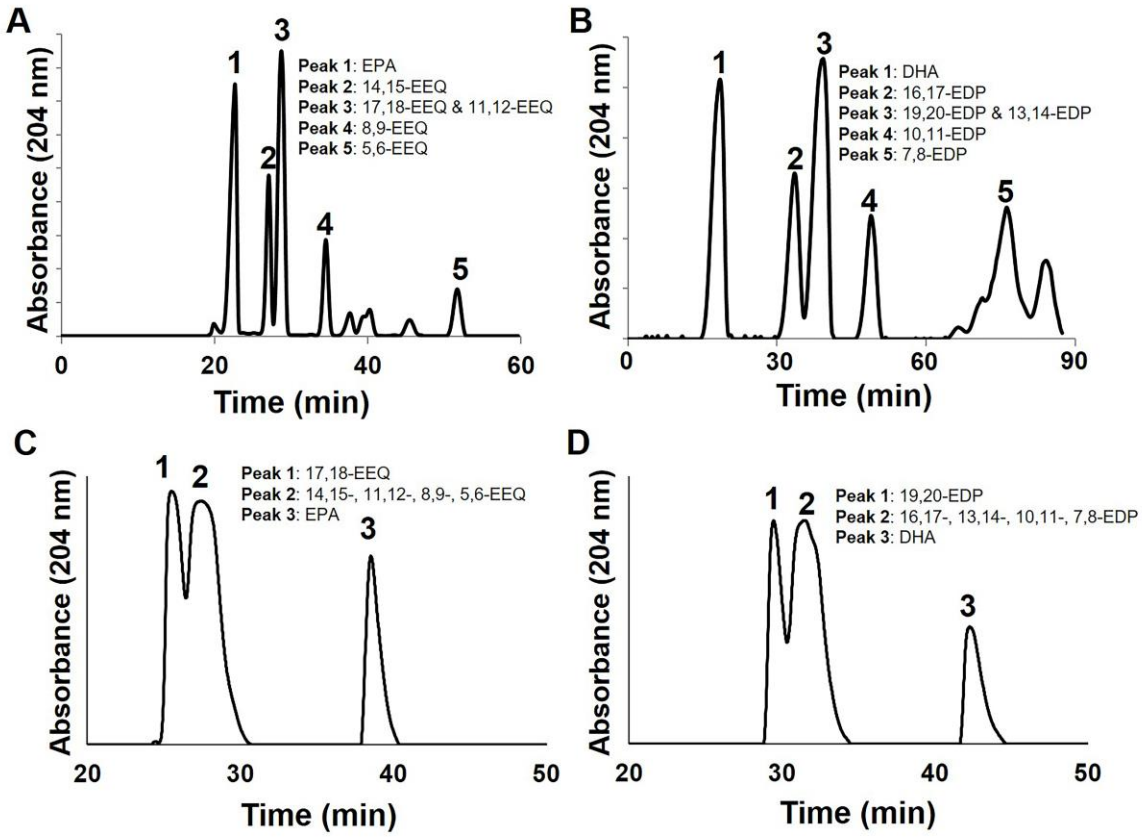

Figure S8. **Purification of EDP and EEQ regio-isomers from crude reaction mixture by normal- and reversed-phase HPLC.** Synthesis reactions detailing the non-specific olefin epoxidation of EPA or DHA are detailed in the materials and methods. A combination of both normal phase-high performance liquid chromatography (NP-HPLC) and reversed phase-high performance chromatography (RP-HPLC) were necessary for isolation epoxides. NP-HPLC was employed for separation of (A) EEQ and (B) EDP regioisomers from the crude reaction mixture. RP-HPLC was employed due to the alternative elution profile of (C) EEQ and (D) EDP regioisomers which elutes with the terminal epoxide first (17,18-EEQ or 19,20-EDP-EA) followed by the sequential elution of the other metabolites. The isolation of specific EEQ (17,18-, 14,15-, 11,12-, 8,9-, and 5-6-EEQ) and EDP (19,20-, 16,17-, 13,14-, 10,11-, and 7,8-EDP) regioisomers were confirmed using high resolution mass spectrometry and commercially available authentic standards for use in analytical RP-HPLC.

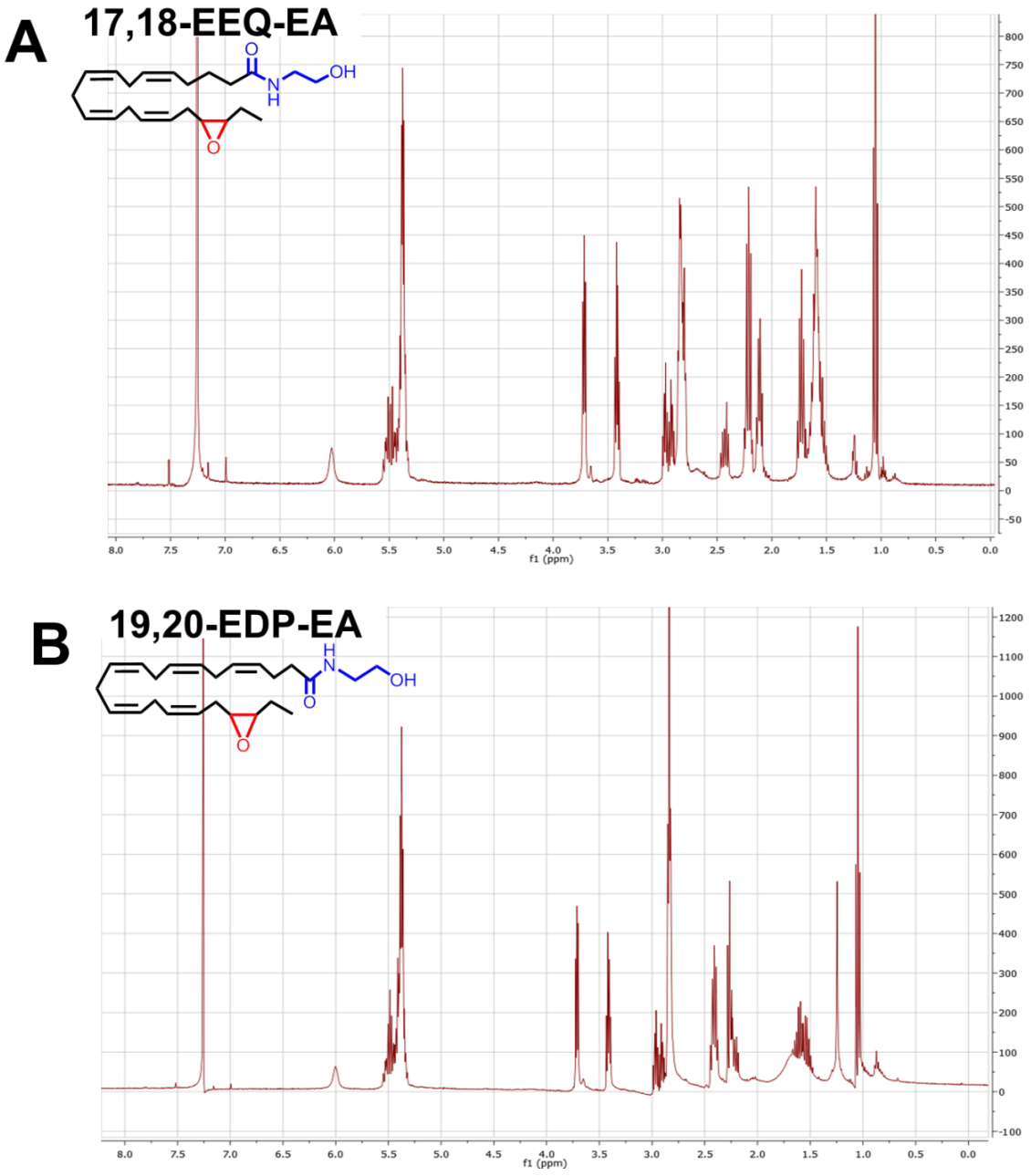

Figure S9. **Spectral Data of purified 17,18-EEQ-EA and 19,20-EDP-EA. (A)** ^{1}H NMR (400 MHz, $CDCl_3$) δ6.03 (s, 1H), 5.55 to 5.33 (m, 10H), 3.72 (t, 2H, J = 4Hz), 3.42 (quartet, 2H, J= 4 Hz), 3.03 to 2.79 (m, 6H), 2.43 (dt, 1H, J = 2Hz), 2.25 to 2.05 (m, 4H), 1.73 (apparent quintet, 2H, J = 8Hz), 1.67 to 1.48 (m, 6H), 1.05 (t, 3H, J = 8Hz). HRMS (m/z) calc. mass = 362.2695; HRMS measured mass = 362.2693; atomic form = $C_{22}H_{36}NO_3$; UV-Vis λ_{max} = 191 nm; Purity > 98% **(B)** ^{1}H NMR (400 MHz, $CDCl_3$) δ6.03 (s, 1H), 5.55 to 5.33 (m, 8H), 3.72 (t, 2H, J = 4Hz), 3.42 (quartet, 2H, J= 4 Hz), 3.00 to 2.79 (m, 8H), 2.43 (apparent quintet, 1H, J = 2Hz), 2.25 to 2.05 (m, 4H), 1.73 (apparent quintet, 2H, J = 8Hz), 1.67 to 1.48 (m, 6H), 1.05 (t, 3H, 8Hz). HRMS calc. mass = 388.2828; HRMS (m/z) measured mass = 388.2852; atomic form = $C_{24}H_{38}NO_3$; UV-Vis λ_{max} = 191 nm; Purity > 98%.

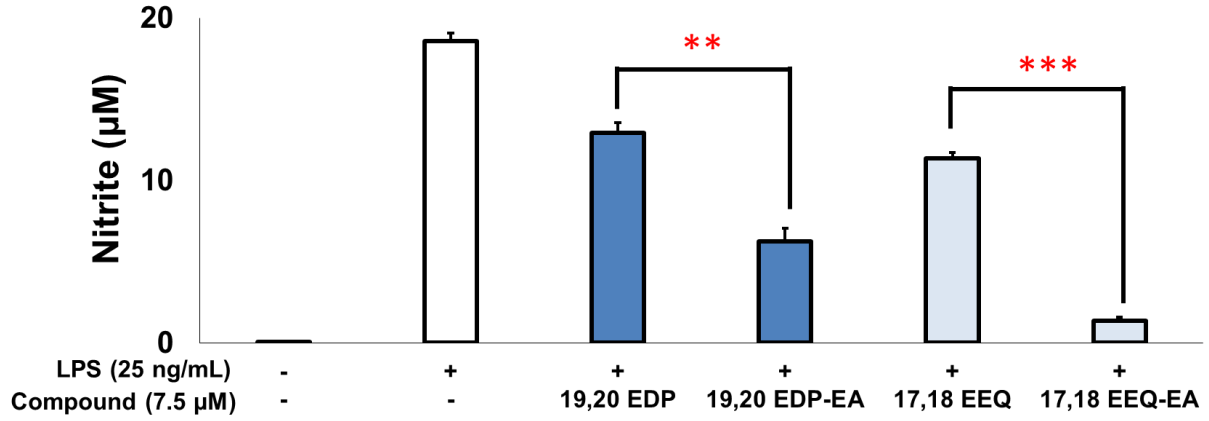

Figure S10. **Comparison of epoxide and epoxide-EA effects of on nitrite reduction.** BV-2 microglial cells were pretreated with 7.5 µM of compound for 4h before LPS (25ng/mL) stimulation. Culture media was collected after 24h and nitrite was measured as described in the materials and methods. The decrease of the nitrite inflammatory marker was compared to non-treated cells LPS for assessment of their anti-inflammatory effects.

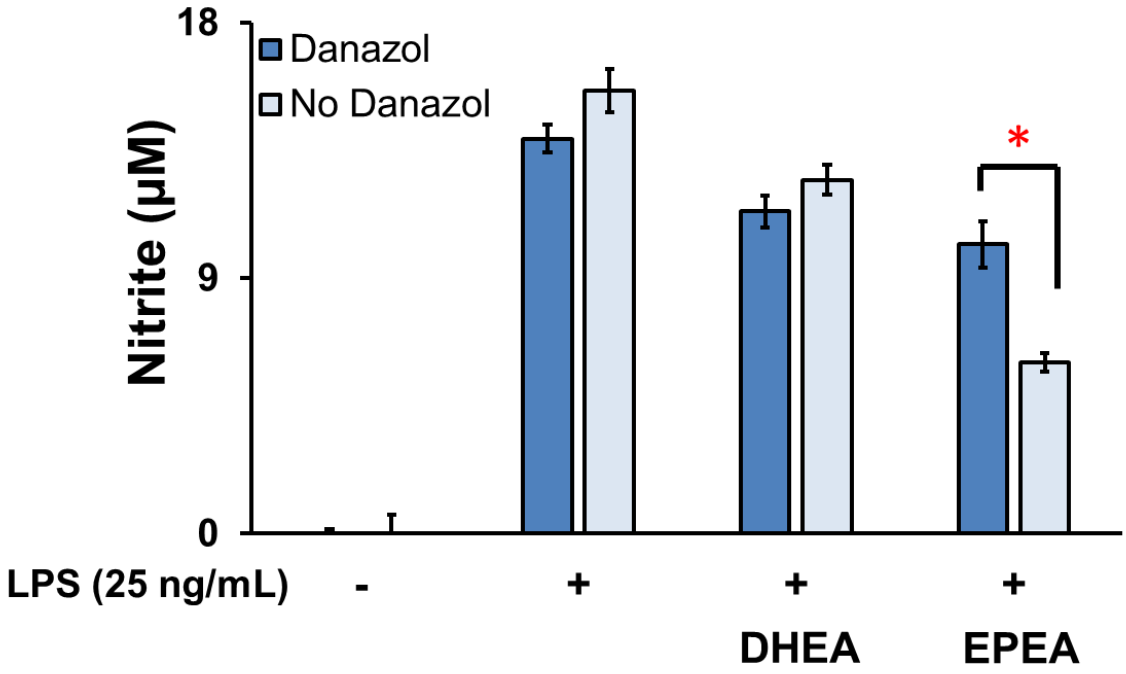

Figure S11. **Effect of CYP inhibitor, danazol, on LPS induced inflammation.** BV-2 microglial cells were pretreated with or without danazol (0.5μM) for 30min before the addition of DHEA and EPEA (2.5μM) for 1 hr followed by LPS (25ng/mL) stimulation. Culture media was collected after 24 hours and analyzed for pro-inflammatory cytokines NO.

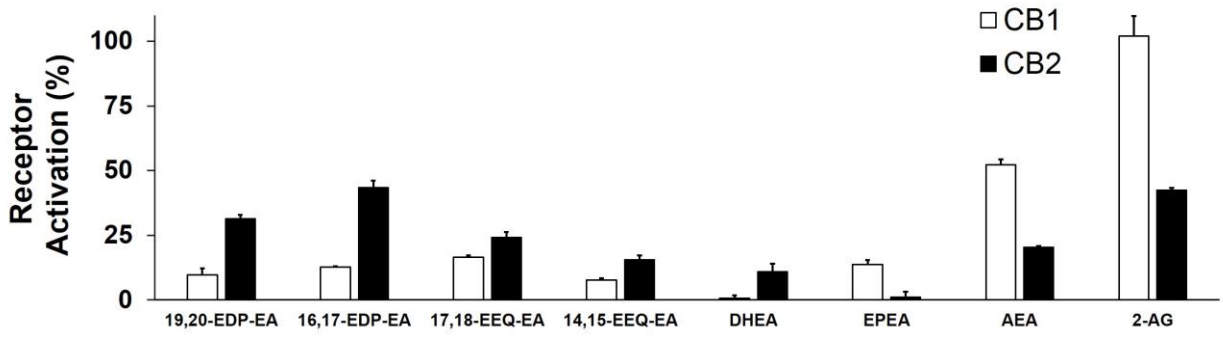

Figure S12. **Activation of CB1 and CB2 by different lipid species.** Functional activation of CB1 and CB2 was measured for EDP-EA, EEQ-EA, DHEA and EPEA at 10 μM as well as AEA and 2-AG (5 μM) using β-arrestin recruitment luciferase reporter assay in transiently transfected HEK293 cells. Values represent the percent activation of each ligand relative to saturating concentrations of the full CB1 and CB2 agonist CP 55940 (n = 3).

MATERIALS AND METHODS

MATERIALS

Tissue cell cultures: Dr. Robert H. McCusker Jr. (University of Illinois at Urbana-Champaign) generously provided the BV-2 cell line originally created by Blasi et. al.(2). Primary human microvascular endothelial cells (HMVEC) were purchased from cell systems (Certificate #CSC 2M1). Complete serum containing medium kit was purchased from cell systems (Cat. #4Z0-500). Fetal bovine serum (cat. #16140-071) and DMEM (cat. 10-013-CV) were obtained from Gibco. Pertussis toxin (cat. #181) was purchased from List Bio Labs. AM630 (cat. #10006974), rimonabant (cat. #9000484), GW9962 (cat. #70785), LY294002 (cat. #70920), HA-100 (cat. #14955), AMG9810 (cat. #14715), ruthenium red (cat. #14339), wortmannin (cat. #10010591) and 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) (cat. #10007927) were procured from Cayman Chemical.

Human plasma: University of Illinois at Urbana-Champaign approved studies and written informed consent was obtained from all participants prior to blood collection and subsequent plasma isolation.

Animal organs: Fresh bovine hearts were obtained on ice from the local slaughterhouse at time of slaughter. Fresh whole female porcine brains and hearts were obtained from the UIUC meat sciences laboratory. At the time of sacrifice, male Sprague-Dawley (250-300 g) rat carcasses were obtained from the Division of Animal Resource facility (University of Illinois at Urbana-Champaign) and immediately dissected for brain, heart, kidneys, spleen and livers organs.

Other chemicals and reagents: Technical grade meta-chloroperoxybenzoic acid (Cat #273031) and dichloromethane was obtained from Sigma-Aldrich. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) used for synthesis were purchased from Nu-Chek Prep Inc (Elysian, MN). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) HCl was purchased from Thermo Scientific (catalog #22980). N-hydroxysuccinimide (NHS) was purchase from Acros Organics (catalog #157270250). NADPH was obtained from P212121 (Ann Arbor, MI). POPC [1-hexadecanoyl-2-(9*Z*-octadecenoyl)-sn-glycero-3-phosphocholine],1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine], POPS [1-hexadecanoyl-2-(9*Z*-octadecenoyl)-sn-glycero-3-phospho-l serine] were purchased from Avanti Polar Lipids (Alabaster, AL). 19,20-EDP, 16,17-EDP, 17,18-EEQ, 14,15-EEQ, 11,12-EEQ, 8,9-EEQ, 5,6-EEQ and 14,15-EET were obtained from Cayman Chemical (Ann Arbor, MI). The antioxidant butylated hydroxytoluene (BHT) and was obtained from Sigma-Aldrich (St. Louis, MO). PAR4-AP (AYPGKF) was purchased from GL Biochem (Shanghai, China). Thrombin was purchased from Enzyme Research Laboratories (South Bend, IN), Collagen, ristocetin was purchased from Chorono LOG. ADP was purchased from Sigma. All other materials and reagents used were purchased from Sigma-Aldrich and Fisher Scientific.

METHODS

LC-ESI-MS for global analysis of CYP2J2-ND incubations with EPEA and DHEA (Figure S2). The reactions containing CYP2J2, CPR and NADPH with either EPEA or DHEA were resolved using a XTerra C18 column 2.1 × 150 mm, 3.5 μM (Waters), and a Waters Alliance 2695 reversed-phase high performance liquid chromatography (HPLC) coupled to an electrospray

ionization (ESI) source. The different regioisomers were separated with mobile phase A (acetonitrile/H_2O/formic acid, 95:5:0.1) and mobile phase B (acetonitrile/H_2O/formic acid, 5:95:0.1) over a linear gradient as follows: 0–1 minute, 40% B; 41 minutes, 80% A; 42 minutes, 100% B; and then returned to initial conditions. For ESI, a Q-TOF Ultima time-of-flight mass spectrometer was used in positive ion mode, with a spray voltage of 4.5 V and capillary temperature of 200°C. The mass and elution times of the metabolites were confirmed using authentic standards synthesized in-house.

LC-MS/MS analysis method for comparison of the predominant epoxy-eicosanoids, epoxy-endocannabinoids and endocannabinoids. Samples were analyzed with the 5500 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA) 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 x 150mm, 5μm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetontrile). The flow rate was 0.4 mL/min. The linear gradient was as follows: 0-2min, 90%A; 10-24min, 5%A; 25-32min, 90%A. The autosampler was set at 10°C. The injection volume was 10 μL. Mass spectra were acquired under electrospray ionization (ESI) in positive mode (with the ion spray voltage of 5500V) and negative mode (with the ion spray voltage of -4500V). The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 32 psi, 60 psi, and 60 psi, respectively. Multiple reaction monitoring (MRM) in both positive and negative ion mode was used for quantitation of the predominant metabolites from each class of lipids. Positive ion mode was used for detections AEA (m/z 348.3 → m/z 62.1), DHEA (m/z 372.4 → m/z 62.1), EPEA (m/z 346.3 → m/z 62.1), 14,15-EET-EA (m/z 364.2 → m/z 346.2), 17,18-EEQ-EA (m/z 362.1 → m/z 344.1), 19,20-EDP-EA (m/z 388.1 → m/z 370.1) and quantified using internal standards AEA-d4 (m/z 352.3 → m/z 66.1), DHEA-d4 (m/z 376.4 → m/z 66.1), EPEA-d4 (m/z 350.3 → m/z 66.1) and 14,15-EET-EA-d8 (m/z 372.1 → m/z 354.1). Negative ion mode was simultaneously employed for quantitation of 14,15-EET (m/z 319.2 → m/z 219.1), 17,18-EEQ (m/z 317.0 → m/z 299.0) and 19,20-EDP (m/z 343.1 → m/z 299.0) and quantified relative to 14,15-EET-d11 (m/z 330.2 → 219.1).

17,18-EEQ-EA and 19,20-EDP-EA hydrolysis by rat forebrain membranes containing FAAH. The preparation of rat forebrain membranes was achieved using dounce homogenization in buffer (50 mM Tris pH 7.4, 1 mM EDTA and 3 mM $MgCl_2$) and membrane pelleting as previously described (1). Incubations contained 100 μg forebrain protein in a 0.5 mL reaction containing 50 mM Tris (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$, and 17,18-EEQ-EA or 19,20-EDP-EA at 50 μM. Linearity of the reactions were confirmed at 5, 20 and 40 minutes. Reactions were quenched with methanol containing 1 mM PMSF and and centrifuged to pellet protein (10,000 g x 10 min). The supernatant was collected and analyzed via LC-MS/MS as outlined below.

LC-MS/MS method for quantitation of 17,18-EEQ and 19,20-EDP. Analyses were performed using the 5500 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) includes a degasser, an autosampler, and a binary pump, and this system was used to separate the metabolites. LC separation was performed on an Agilent Eclipse XDB-C18 (4.6 x 150mm, 5μm) with 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 µL. 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: 17,18-EEQ m/z 317.0 → m/z 299.0 and 19,20-EDP m/z 343.0 → m/z 299.0 using 14,15-EET as the internal standard.

Primary microglia neuroinflammation studies. Primary microglia cultures were isolated from freshly euthanized piglets following previously described methods (3). Isolated microglia cells were plated at 2 x 10^6 in 24-well plates and allowed to adhere for 3 hrs before a 1 hour compound pre-incubation step followed by stimulation with LPS (10 ng/mL) for 12 hours in presence or absence of compound. Supernatant was analyzed for IL-6 content using a porcine specific ELISA (R&D Systems Inc., Minneapolis, USA cat #P6000B). The final vehicle (DMSO) concentration was 0.04% (n = 3 or greater).

PRESTO-Tango β-arrestin recruitment assay. Functional CB1 and CB2 assays were performed by the NIMH PDSP as previously described (4). In short, each metabolite was screened in triplicate at 10 µM in transiently transfected CB1/CB2 HEK293 cells for their ability to functionally activate each respective receptor. Results reflect the percent receptor activation relative to CP55940 EC_{90}. Subsequent in-house PRESTO-Tango assays were developed for calculation of dose response curves as described below.

The PRESTO-Tango β-arrestin recruitment assay. The Presto-Tango β-arrestin recruitment assay was used for the measurement of CB1 and CB2 GPCR activation as previously described (4). HTLA cells, CNR1 (Addgene #66254) and CNR2 (Addgene #66255) plasmids were a generous gift from the Roth Lab (UNC Chapel Hill). HTLA cells were maintained in DMEM with 10% FBS containing 2 µg/mL of puromycin and 100 µg/mL of hygromycin B at 37 °C in a 5% CO_2 humidified air atmosphere and grown to 80-90% confluency. Cells were then seeded at 20,000 cells per 100 µL into a poly-L-lysine coated 96-well plate. After 18-24 hrs, cells were transfected with CNR1 or CNR2 plasmids (0.1 µg/well) using Calfectin (0.4 µL) as the transfection reagent in a 4:1 reagent to plasmid ratio (final well vol. 110 µL). Transfection media was replaced after 12-18 hours with fresh serum-media and maintained for 36-48 hours. On the day of the assay, serum-media was replaced with 100 µL media containing 1% dialyzed FBS for 4 hours, then compound was further diluted with media containing 1% dialyzed FBS and was added in a log dose manner (final well vol. 200 µL) and incubated for 8-14 hours. For epoxygenated metabolites, serum-media was replaced with 100 µL media containing 1% dialyzed FBS and 1uM AUDA for 30 min, then compound was further diluted with media containing 1% dialyzed FBS and was added in a log dose manner (final well vol. 200 µL including the media with AUDA) and incubated for 8-14 hours.The following day, media was removed and 40 µL of diluted Bright-Glo solution (Promega, Madison, WI) was added to each well and incubated in the dark for 20min at room temperature before luminescence recordings. Relative luminescence units (RLU) values were normalized to % receptor response, plotted as a function of compound concentration and analyzed using "DoseResp" in OriginPro.

Synthesis of 17,18-DiHETE-EA and 19,20-DiHDPA-EA authentic standards. The EPA and DHA terminal epoxides, 17,18-EEQ and 19,20-EDP, were synthesized and purified as described above. The conversion of 17,18-EEQ and 19,20-EDP (100 ug) to the corresponding diol was accomplished by first solubilizing the standard in 100 uL acetonitrile and adding 1 mL of glacial acetic acid and water (1:1). The hydration of the epoxide to the diol moiety was accomplished by purging the reaction with N_2 and incubating the reaction at 45°C for 12 hours. The resulting diols were extracted with ethyl acetate and purified by RP-HPLC. The ethanolamide coupling was performed with the same conditions as the epoxides as listed above. Successful synthesis of the standards was confirmed by high resolution mass spectrometry and RP-HPLC elution times.

REFERENCES

1. Hillard CJ, Wilkison DM, Edgemond WS, & Campbell WB (1995) Characterization of the Kinetics and Distribution of N-Arachidonylethanolamine (Anandamide) Hydrolysis by Rat-Brain. *Bba-Lipid Lipid Met* 1257(3):249-256.
2. Blasi E, Barluzzi R, Bocchini V, Mazzolla R, & Bistoni F (1990) Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *J Neuroimmunol* 27(2-3):229-237.
3. Ji P, Schachtschneider KM, Schook LB, Walker FR, & Johnson RW (2016) Peripheral viral infection induced microglial sensome genes and enhanced microglial cell activity in the hippocampus of neonatal piglets. *Brain Behav Immun* 54:243-251.
4. Kroeze WK, *et al.* (2015) PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nature Structural & Molecular Biology* 22(5):362-U328.