15-Lipoxygenase Metabolism of 2-Arachidonyleglycerol: Generation of a PPARα Agonist

Kevin R. Kozak‡, Rajnish A. Gupta§, John S. Moody§, Chuan Ji‡, William E. Boeglin§, Raymond N. DuBois§, Alan R. Brash§, and Lawrence J. Marnett††

From the ‡ Departments of Biochemistry and Chemistry, Vanderbilt-Ingram Cancer Center and Center in Molecular Toxicology, § Departments of Medicine and Cell Biology, and †† Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

Corresponding author: Lawrence J. Marnett, Ph.D.
Department of Biochemistry
Vanderbilt University School of Medicine
Nashville, TN 37232-0146
Telephone: (615)-343-7328
Fax: (615)-343-7534
Email: marnett@toxicology.mc.vanderbilt.edu

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Abbreviations: COX, cyclooxygenase; LOX, lipoxygenase; AG, arachidonyleglycerol; PG, prostaglandin; HETE, hydroxyeicosatetraenoic acid; HpETE, hydroperoxyeicosatetraenoic acid; G, glyceryl ester; MS, mass spectrometry; LC/MS, liquid chromatography/mass spectrometry; RP-HPLC, reversed phase high performance liquid chromatography; PPAR, peroxisome proliferator-activated receptor

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Summary

The recent demonstrations that cyclooxygenase-2 and leukocyte-type 12-lipoxygenase (LOX) efficiently oxygenate 2-arachidonylglycerol (2-AG) prompted an investigation into related oxygenases capable of metabolizing this endogenous cannabinoid receptor ligand. We evaluated the ability of six LOXs to catalyze the hydroperoxidation of 2-AG. Soybean 15-LOX, rabbit reticulocyte 15-LOX, human 15-LOX-1, and human 15-LOX-2 oxygenate 2-AG providing 15-(S)-hydroperoxyeicosatetraenoic acid glyceryl ester. In contrast, potato and human 5-LOXs do not efficiently metabolize this endocannabinoid. Among a series of structurally related arachidonyl esters, arachidonylglycerols serve as the preferred substrates for 15-LOXs. Steady-state kinetic analysis demonstrates that both 15-LOX-1 and 15-LOX-2 oxygenate 2-AG comparably or preferably to arachidonic acid. Furthermore, 2-AG treatment of COS-7 cells transiently transfected with human 15-LOX expression vectors or normal human epidermal keratinocytes results in the production and extracellular release of 15-hydroxyeicosatetraenoic acid glyceryl ester (15-HETE-G) establishing that lipoxygenase metabolism of 2-AG occurs in an eukaryotic cellular environment. Investigations into the potential biological actions of 15-HETE-G indicate that this lipid, in contrast to its free-acid counterpart, acts as a peroxisome proliferator-activated receptor α agonist. The results demonstrate that 15-LOXs are capable of acting on 2-AG to provide 15-HETE-G and elucidate a potential role for endocannabinoid oxygenation in the generation of peroxisome proliferator-activated receptor α agonists.
Introduction

In 1995, 2-arachidonylglycerol (2-AG) was isolated from rat brain and canine gut and shown to bind both the central (CB1) and peripheral (CB2) cannabinoid receptors (1,2). Subsequently, 2-AG was shown to be present in vivo at levels several orders of magnitude higher than the other known endocannabinoid, anandamide (2-4). Accumulating evidence supports assertions that 2-AG serves as a physiologically relevant cannabinoid receptor ligand occupying a central role within the endogenous cannabinoid system (5,6). Therefore, the identification and characterization of enzymes capable of metabolizing this lipid mediator should aid in the elucidation of mechanisms by which cannabinoid tone is modulated in vivo. We are particularly interested in the role of fatty acid oxygenases, such as cyclooxygenases (COXs) and lipoxygenases (LOXs), in 2-AG metabolism and have previously shown that 2-AG is an excellent substrate for COX-2 and leukocyte-type 12-LOX (7,8).

LOXs are a diverse family of nonheme ferroproteins that catalyze the hydroperoxidation of polyunsaturated fatty acids both regio- and stereospecifically (9-14). Six LOXs have been identified in humans: platelet-type 12-LOX, 12(\(R\))-LOX, 15-LOX-1, 15-LOX-2, e-LOX-3, and 5-LOX (9,15). The ability of leukocyte 12-LOX, but not platelet 12-LOX, to oxidize 2-AG, and the ability of some lipoxygenases to oxidize the endocannabinoid anandamide, prompted us to evaluate additional possible lipoxygenase metabolic pathways for 2-AG (8,16-20). In the present study, we investigated the ability of two plant and four animal LOXs to catalyze the hydroperoxidation of 2-AG. 5-LOX catalyzes the hydroperoxidation of arachidonic acid providing 5-hydroperoxyeicosatetraenoic acid (HpETE), the precursor to the leukotrienes. The possibility that 5-LOX might oxygenate 2-AG to generate 5-HpETE glyceryl ester (HpETE-G) and, subsequently, leukotriene glyceryl esters in a manner similar to the ability of cyclooxygenase-2 to generate prostaglandin glycerol esters was investigated using two 5-LOX enzymes. The endocannabinoid oxygenase activities of four 15-LOX enzymes were also rigorously characterized. Lipoxygenase metabolism of 2-AG in an eukaryotic cellular environment was examined in two distinct cell systems. The results suggest that 15-LOX
enzymes, but not 5-LOX enzymes, may play a role in endogenous cannabinoid signaling and should be included in the growing family of oxygenases capable of 2-AG metabolism.

Various oxygenated derivatives of arachidonic and linoleic acids have been found to be ligands for peroxisome proliferator-activated receptors (PPARs) (21,22). Of particular interest, 15-HETE is reported to be a ligand for the PPARγ receptor (21,23). Characterization of the ability of 15-HETE-G to transactivate various PPAR receptors revealed that it is a specific agonist for the PPARα receptor. This is the first reported biological activity of a glyceryl eicosanoid and suggests that fatty acid oxygenase metabolism of 2-AG might represent a pathway for the generation of ligands for nuclear receptors.
**Experimental Procedures**

**Materials.** Arachidonic acid and arachidonyl ethyl ester were purchased from Nu-Chek Prep (Elysian, MN). Arachidonylglycerols (1- and 2-), HETEs (15(R), 15(S), and (±)-15-HETE), soybean 15-LOX (P1), potato 5-LOX, human 5-LOX, and 15-LOX (rabbit reticulocyte) polyclonal antiserum were purchased from Cayman Chemical (Ann Arbor, MI). Soybean 15-LOX and potato 5-LOX were obtained at ≥ 98% purity. Human, recombinant 5-LOX was obtained as a 16,000 x g supernatant from baculovirus infected, Sf-21 cells overexpressing the enzyme. Human 15-LOX-2 polyclonal antiserum was generated and characterized as previously described (24). Rabbit reticulocyte 15-LOX was obtained from Calbiochem (La Jolla, CA) as a partially purified preparation from rabbit reticulocytes generated essentially as previously described (25). L-α-Phosphatidylcholine (egg) as purchased from Avanti Polar Lipids. All other chemicals and solvents were purchased from Aldrich (Milwaukee, WI) unless otherwise noted. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). The vectors pcDNA3 and pCR2.1 were purchased from Invitrogen (Carlsbad, CA) and pET3a was obtained from Stratagene (La Jolla, CA). The Bac-to-Bac baculovirus expression system, including the pFastBac HT vector, was purchased from Life Technologies (Rockville, MD). The cDNA for wild-type human 15-LOX-1 in a pcDNA3 vector was a generous gift of Professor C. D. Funk (University of Pennsylvania, Philadelphia). The cDNA for wild-type human 15-LOX-2 was subcloned into the pcDNA3 vector as previously described (26). The baculovirus vector containing hexahistidine-tagged human 15-LOX-2 was prepared by subcloning human 15-LOX-2 into pFastBac HT using XbaI and BamH1. The pET3a vector containing hexahistidine-tagged human 15-LOX-2 was prepared by PCR amplification of 15-LOX-2 in pcDNA3 using an upstream primer encoding six histidines. The PCR product was cloned into pCR2.1 and amplified. The 15-LOX-2 fragment was then subcloned into the pET3a vector using BamH1 and EcoR1. All vectors were sequenced to ensure no mutations had been incorporated. COS-7 cells were obtained from American Type Culture Collection (Rockville, MD). Undifferentiated human keratinocytes were a generous gift from Professor D. S. Keeney (Veterans Administration
Hospital, Nashville, TN). Lipofectamine was purchased from Life Technologies (Gaithersburg, MD). Immobilon-P transfer membranes were obtained from Millipore (Bedford, MA). Enhanced chemiluminescence reagent was purchased from Amersham (Arlington Heights, IL).

**Chemistry.** Eicosa-5,8,11,14-tetraenoic acid 2-hydroxyethyl ester and eicosa-5,8,11,14-tetraenoic acid 2-methoxyethyl ester were prepared as described (8). 11- and 15-HETE-G were generated by incubating purified murine COX-2 with 2-AG and 12-HETE-G was obtained by incubating purified leukocyte 12-lipoxygenase with 2-AG as described (8,27). Large-scale 15-HETE-G synthesis was effected enzymatically using purified soybean 15-LOX. To 300 mL 100 mM Na₂B₄O₇ containing 10 mM sodium deoxycholate (pH 9.0) was added approximately 15 mg purified soybean 15-LOX. The solution was stirred to homogeneity at room temperature followed by the addition of 2-AG (10 mg, 26 µmol). A reaction mixture aliquot was followed by UV spectroscopy (240 nm) until no further increase in absorbance was detected (≈ 5 min). The reaction was quenched with 300 mL EtOAc containing 25 mg triphenylphosphine to reduce 15-HpETE-G to 15-HETE-G. Organics were removed and the aqueous layer was reextracted with 300 mL EtOAc. The combined organics were washed with saturated NaHCO₃ and H₂O, dried (MgSO₄), filtered, and concentrated *in vacuo*. The resultant residue was resuspended in EtOAc, filtered over glass wool, and purified by silica gel chromatography (EtOAc:hexanes, 1:3 then 1:1; fractions containing desired product were concentrated and rechromatographed CHCl₃ then CHCl₃:MeOH, 98:2) to provide the desired ester as a colorless oil (6.1 mg, 58%). *R*₇ = 0.44 (EtOAc:hexanes, 7:3); UV (MeCN) λₘₐₓ 196, 238 nm; ¹H NMR (CDCl₃) δ 6.50-6.57 (dd, 1H, *J* = 11.1, 15.1 Hz, CH), 5.98-6.03 (t, 1H, *J* = 10.9 Hz, CH), 5.68-5.74 (dd, 1H, *J* = 6.6, 15.1 Hz, CH), 5.33-5.45 (m, 5H, 5 x CH), 4.10-4.26 (m, 2H, CH₂), 3.89-3.95 (m, 1H, CH), 3.81-3.83 (t, 1H, *J* = 5.1 Hz, CH), 3.66-3.75 (m, 1H, CH), 3.56-3.62 (m, 1H, CH), 2.95-3.02 (m, 2H, CH₂), 2.78-2.83 (m, 2H, CH₂), 2.31-2.42 (m, 2H, CH₂), 2.10-2.16 (m, 2H, CH₂), 1.68-1.76 (m, 2H, CH₂), 1.51-1.59 (m, 2H, CH₂), 1.15-1.41 (m, 6H, 3 x CH₂), 0.85-0.91 (t, 3H, *J* = 6.7 Hz, CH₃); ESI-MS *m/z* calculated for C₂₃H₃₈O₄ (M+ Na⁺) 417.3, found 417.3.
Enzyme Preparation. Hexahistidine-tagged human 15-LOX-2 was expressed in Sf-9 insect cells using the Bac-to-Bac baculovirus expression system according to manufacturer’s instructions and in E. coli from a pET3a vector as previously described (28). The lipoxygenase product was then purified on Ni-NTA agarose (Qiagen, Valencia, CA) according to manufacturer’s instructions. Fractions containing 15-LOX-2 were pooled and dialyzed against PBS containing 5% glycerol to remove imidazole. E. coli-expressed human 15-LOX-2 was further purified by anion exchange chromatography.

Lipoxygenase Assays. Lipoxygenase activity, except in the case of human 5-LOX, was detected by monitoring the absorbance of the conjugated diene product at 236 nm as previously described (8). Briefly, UV assays were monitored using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a water-jacketed cuvette. The enzyme reactions included reaction buffer (50 mM Tris-Cl, 0.03% Tween 20, pH 7.4), arachidonic acid or arachidonylester, and enzyme. The reaction temperature was 30 °C and the final reaction volume was 1 mL. Potato 5-LOX reactions were conducted at 30 °C and included arachidonic acid or 2-AG (10 µM) and enzyme (200 U) in 1 mL buffer (50 mM Tris-Cl, pH 7.4). The commercially obtained, human 5-LOX preparation displayed considerable background absorbance at 236 nm preventing spectrophotometric quantitation of enzyme activity. Consequently, human 5-LOX activity was assessed by measuring oxygen consumption according to manufacturer’s instructions. Briefly, oxygen consumption was measured with a Gilson model 5/6 oxygraph (Gilson Medical Electronics, Middleton, WI) equipped with a Clark electrode and a thermostatted cuvette (37 °C). Enzyme aliquots (10 U) were added to 50 mM Tris-Cl, 2 mM CaCl₂, and 1 mM ATP, pH 7.4 containing either 0.03% Tween 20 or 0.015% Tween 20 and 100 µg/mL phosphatidylcholine in a final volume of 1 mL. Oxygen uptake was initiated by the addition of 100 µM arachidonic acid or 2-AG.

Enzyme kinetics. Enzyme kinetics were assessed using the computer program Enzyme Kinetics 1.5 (Trinity Software, Campton, NH). Kinetic values were determined using non-linear regression analysis. Velocity data were obtained by taking the slope of the reaction curve at the
point of maximal reaction velocity. Due to the characteristic lag phase of lipoxygenases in some reactions, this rate was not necessarily the initial rate.

**Lipoxygenase Product Characterization.** HpETE-G regiochemistry was established by MS. Incubations of enzyme and 2-AG in 25 mM Tris, 0.015% Tween 20, pH 7.4 (37 °C, 10 min) were extracted with EtOAc and dried under Ar. The residue was redissolved in 1:1 MeCN:H₂O and infused into the mass spectrometer. Regiochemistry was established by diagnostic, collision-induced hydroperoxide cleavage. HpETE-Gs were reduced with triphenylphosphine to the corresponding HETE esters. Saponification with 1 N NaOH followed by RP-HPLC (Supelcosil LC-18, 250 x 4.6 mm, 5 μm, 80:20:0.01 MeOH:H₂O:HOAc, 1.4 mL/min) provided purified HETEs for chiral analysis and confirmed regiochemical assignment established by mass spectrometry (MS). Following methylation with diazomethane, HETE methyl esters were analyzed by chiral-phase HPLC (Chiralpak AD, 250 x 4.6 mm, 1.5 mL/min, hexanes:EtOH 100:1) (29). Enantiomerically pure HETE methyl ester standards were well resolved on the chiral column. LC effluents were routinely monitored by UV at 235 nm.

**Cell Culture.** COS-7 cells were maintained at 37 °C in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum. Human 15-LOX cDNAs in pcDNA3 or vector without insert were transfected into COS-7 cells using LipofectAMINE according to manufacturer’s instructions. Following transfection, media was replaced with HBSS and cells were treated with 2-AG (20 μM) or DMSO vehicle for 30 min (15-LOX-1) or 45 min (15-LOX-2) at 37 °C. After treatment, HBSS was removed and extracted twice with an equal volume of 2:1 CHCl₃:MeOH. The organic extract was dried under argon and the residue was analyzed by LC/MS. Cells were harvested for immunoblotting by scraping, washed twice with PBS, and lysed in 50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 4 mM EDTA, 50 mM NaF, 0.1 mM Na₃VO₄, 1 mM DTT and protease inhibitors for 30 min at 4 °C. Cell lysates were cleared by centrifugation at 15000 x g for 15 min and resulting supernatant was collected. Cellular proteins were separated by SDS-polyacrylamide gel electrophoresis (8%) then electrophoretically transferred to Immobilon-P
transfer membrane. The membrane was probed with goat polyclonal antiserum to rabbit reticulocyte 15-LOX (cross-reactive with human 15-LOX-1) or rabbit polyclonal antiserum to human 15-LOX-2 followed by anti-goat or anti-rabbit horseradish peroxidase-conjugate. Immunoreactive bands were visualized by enhanced chemiluminescence.

Undifferentiated human epidermal keratinocyte production of 15-HETE-G was evaluated by treating cells in HBSS (50-70% confluence) with vehicle or 2-AG (50 µM). Following incubation at 37 °C for 45 min, HBSS was removed and extracted twice with an equal volume of CHCl₃:MeOH (2:1). The organic extract was dried under argon and the residue was analyzed by LC/MS. Human epidermal keratinocyte expression of 15-LOX enzymes was examined by immunoblotting as described above.

Transfections and Luciferase Assays. UAS-tk-luciferase, PPARδ-pCMX, pGAL4, PPARα-GAL4, PPARγ-GAL4, and PPARδ-GAL4 plasmids have been described previously (30). NIH 3T3 cells (5.0 X 10⁵ cells per well using 24 well plates) were transfected using FUGENE 6 at a lipid:DNA ratio of 3:1. Cells were exposed to a mix containing 300 ng/ml UAS-tk-luc (GAL4 reporter plasmid expressing Firefly Luciferase), 300 ng/ml of the indicated PPAR-GAL4 plasmid, and 1.0 ng/ml of pRL-SV40 (control plasmid expressing Renilla Luciferase) in Opti-MEM (GIBCO-BRL, Rockville, MD). The transfection mix was replaced after 4-5 h with 10% charcoal stripped FBS containing media supplemented with either 0.1% vehicle or the indicated compound. After 24-36 h, cells were harvested in 1 X luciferase lysis buffer. Relative light units from firefly luciferase activity were determined using a luminometer (MGM Instruments, Hamden, CT) and normalized to the relative light units from renilla luciferase using the Dual Luciferase kit (Promega, Madison, WI).
Results

2-AG Oxygenation by 5-Lipoxygenases. Human recombinant 5-LOX and purified potato 5-LOX were used to assess the ability of 5-LOXs to oxygenate 2-AG. Potato 5-LOX was incubated with substrate (10 μM arachidonate or 2-AG) in 50 mM Tris-HCl, pH 7.4, and the reaction mixture was monitored by spectrophotometry. A marked increase in absorbance at 236 nm demonstrated that the enzyme was catalytically active with arachidonic acid as substrate. However, no change in absorbance was detected upon incubation with 2-AG (data not shown).

To evaluate the ability of human 5-LOX to metabolize 2-AG, enzymatic hydroperoxidation was monitored by oxygen uptake. When human 5-LOX was incubated with substrate (100 μM arachidonate or 2-AG) in 50 mM Tris-Cl, 0.03% Tween 20, pH 7.4 containing 2 mM CaCl₂ and 1 mM ATP, robust oxygen uptake was detected with arachidonic acid but not with 2-AG (Fig. 1). The maximal rate of 2-AG oxygenation catalyzed by 5-LOX was 6.6 ± 0.9% that observed with arachidonic acid (mean ± S.E., n = 3). In addition, direct liquid infusion MS of organic extracts of human 5-LOX incubations with 2-AG failed to reveal a mass ion consistent with a HpETE, HETE, dihydroxyeicosatetraenoic acid, or leukotriene A₄ glycerol ester (data not shown).

The inclusion of phosphatidylcholine in human 5-LOX assays has been shown, under some conditions, to enhance enzyme activity (31). To confirm our findings with human 5-LOX, we repeated activity assays with arachidonic acid and 2-AG using a phospholipid-containing buffer (50 mM Tris-Cl, 0.015% Tween 20, 100 μg/mL phosphatidylcholine, pH 7.4 containing 2 mM CaCl₂ and 1 mM ATP). Similar results were obtained in the absence or presence of exogenous phosphatidylcholine (data not shown).

2-AG Oxygenation by Soybean 15-Lipoxygenase. Purified soybean 15-LOX was incubated with substrate (50 μM arachidonate or 2-AG) in 50 mM Tris-HCl, 0.03% Tween 20, pH 7.4 and the reaction course was followed spectrophotometrically. Increases in absorbance at 236 nm demonstrated that soybean 15-LOX converted both arachidonic acid and 2-AG into a conjugated diene (Fig. 2A,B). Although the maximal rate of arachidonic acid oxygenation was
markedly higher than that observed with 2-AG, total product formation after 5 min was only slightly lower with the endocannabinoid substrate (approximately 80%).

To assess the substrate structural requirements for soybean 15-LOX oxygenation, arachidonic acid and a series of related arachidonylesters (50 µM) were evaluated as substrates. The maximal rate of 2-AG oxygenation by soybean 15-LOX under these screening conditions was only 15% that observed with arachidonic acid. However, 2-AG proved to be the preferred arachidonylester substrate (Fig. 3A).

Having established that soybean 15-LOX oxygenates 2-AG, we determined the steady-state kinetic values for both 2-AG and arachidonate metabolism (Table 1). The enzyme displayed $K_m$ values in the low micromolar range for both substrates ($7 \pm 2$ µM for 2-AG versus $13 \pm 4$ µM for arachidonic acid). Consistent with the initial observations of more rapid oxygenation of arachidonic acid by soybean 15-LOX, the $V_{max}$ value with the free acid substrate was approximately 9-fold higher than that observed with 2-AG yielding a 4-5-fold higher $V_{max}/K_m$ ratio for arachidonic acid (Table 1). Soybean 15-LOX therefore appears capable of more rapidly metabolizing arachidonic acid, but the affinities of the enzyme towards both substrates and total product synthesis appear similar.

Characterization of the product of 2-AG metabolism by soybean 15-LOX was achieved by chromatography, UV spectroscopy, and MS. As described above, incubations of 2-AG with soybean 15-LOX resulted in an increase in absorbance at 236 nm suggesting the formation of a conjugated diene. Direct infusion of the organic extract of 2-AG/soybean 15-LOX incubations into the mass spectrometer revealed a single predominant product with a mass-to-charge ratio of 433 consistent with a sodiated HpETE-G (Fig. 4A). Collision-induced dissociation of this metabolite produced the expected hydroperoxide cleavage and established the C15 regiochemistry of 2-AG oxygenation by 15-LOX (Fig. 4B). In addition to the major ion at $m/z$ 433, minor ions were detected with mass-to-charge ratios of 449 and 465. The former ($m/z$ 449) represents the potassiated HpETE-G species. The latter ($m/z$ 465) is consistent with a sodiated bisdioxygenation product ($M + 2O_2 + Na^+$). Both soybean and rabbit reticulocyte 15-LOX
enzymes have been reported to carry out bisdioxygenations of arachidonic acid under some conditions and thus the appearance of an ion at $m/z$ 465 is not surprising (32,33). However, under the conditions employed, this species represented only a minor product and unambiguous characterization was not pursued. Reduction of the HpETE-G product of 2-AG oxygenation by 15-LOX with triphenylphosphine followed by saponification afforded a product that coeluted on RP-HPLC with a 15-HETE standard, confirming the regiochemical assignment provided by MS. RP-HPLC-purified HETEs were methylated with diazomethane and analyzed by chiral chromatography to establish the stereochemistry of enzymatic hydroperoxidation. Soybean 15-LOX produced the expected 15(S) enantiomer almost exclusively (95.3 ± 0.2% S, (mean ± S.E., n = 3)).

**2-AG Oxygenation by Rabbit Reticulocyte 15-Lipoxygenase.** Investigations into mammalian 15-LOX metabolism of 2-AG began with partially purified rabbit reticulocyte 15-LOX (15-LOX-1). As with the soybean enzyme, rabbit reticulocyte 15-LOX catalyzed the formation of conjugated diene products when incubated with either 50 µM arachidonate or 2-AG (Fig. 2C,D). The maximal rate of arachidonic acid oxygenation was higher than that observed with 2-AG. However, the difference in rates was not as dramatic as seen with the soybean enzyme. In addition, rabbit 15-LOX total product formation after 5 min was more markedly reduced with the endocannabinoid substrate (40-50%) than was seen with the soybean enzyme (80%).

To assess the substrate structural requirements for rabbit 15-LOX, arachidonic acid and a series of related arachidonylesters (50 µM) were tested as substrates. The maximal rate of 2-AG oxygenation by rabbit 15-LOX under these screening conditions was 40% that observed with arachidonic acid. However, 2-AG proved to be the preferred arachidonylester substrate (Fig. 3B).

We next determined steady-state kinetic values for both 2-AG and arachidonate oxygenation (Table 2). Rabbit reticulocyte 15-LOX displayed a relatively high $K_m$ (28 ± 11 µM) for arachidonic acid. In contrast, this enzyme displayed a low micromolar $K_m$ for 2-AG (9 ± 3
µM) similar to the soybean enzyme. The $V_{max}$ value obtained with the free acid substrate was approximately threefold higher than that for 2-AG. In total, arachidonic acid and 2-AG appear to be comparable substrates for the rabbit enzyme with nearly identical $V_{max}/K_m$ ratios (Table 2).

The product of 2-AG metabolism by rabbit 15-LOX was established in a manner similar to the characterization discussed above for the soybean enzyme. MS identified the major product of 2-AG oxygenation by the rabbit enzyme as 15-HpETE-G. Reduction of the HpETE-G product with triphenylphosphine followed by saponification afforded a major product that coeluted by RP-HPLC with a 15-HETE standard, confirming the regiochemical assignment provided by MS (data not shown). In addition, a small amount of 12-HETE was detected following reduction and saponification (9.3 ± 0.1% (mean ± S.E., $n = 3$)). 15-HETE, purified by RP-HPLC, was methylated with diazomethane and analyzed by chiral chromatography to establish the stereochemistry of enzymatic hydroperoxidation. Again, rabbit 15-LOX produced the expected 15(S) enantiomer (95.4 ± 0.1% S, (mean ± S.E., $n = 3$)).

2-AG Oxygenation by Human 15-Lipoxygenase-2. To assess the ability of the recently discovered human 15-LOX-2 to effect endocannabinoid metabolism, we incubated partially purified hexahistidine-tagged 15-LOX-2 with 50 µM arachidonic acid or 2-AG. Both substrates were metabolized by the enzyme as evidenced by the increase in absorbance at 236 nm (Fig. 2E,F).

To characterize the substrate specificity of 15-LOX-2, we evaluated the capacity of this enzyme to oxygenate arachidonic acid, arachidonylesters, and anandamide (50 µM). Anandamide was included in this investigation because, in contrast to soybean 15-LOX and 15-LOX-1, no information is available regarding the ability of 15-LOX-2 to oxygenate this endocannabinoid. Under these screening conditions, both endocannabinoids, as well as the more stable regioisomer of 2-AG, 1-AG, were rapidly metabolized by 15-LOX-2 when compared to arachidonic acid (Fig. 3C).

Steady-state kinetic analysis of 15-LOX-2 revealed that both endocannabinoids are superior to arachidonic acid as substrates for this enzyme primarily due to markedly reduced $K_m$
values (Table 3). Due to the poor specific activity of insect cell-expressed 15-LOX-2, steady-state kinetic parameters also were determined for E. coli-expressed 15-LOX-2 (Table 3). Both enzyme preparations displayed similar kinetic properties.

Not surprisingly, mass spectrometric analysis identified the product of 15-LOX-2 action on 2-AG as 15-HpETE-G. In contrast to 15-LOX-1, however, 15-LOX-2 catalyzed C15 hydroperoxidation very regiospecifically; no significant 12-HETE was detected following product reduction and saponification (data not shown). In addition to high regiospecificity, 15-LOX-2 demonstrated high stereospecificity producing, almost exclusively the 15(\(S\)) enantiomer (98± 2\% \(S\), (mean ± S.E., \(n = 3\))).

**Cellular Metabolism of 2-AG by Human 15-Lipoxygenases.** Having demonstrated that both mammalian 15-LOX-1 and 15-LOX-2 effectively oxygenate 2-AG \textit{in vitro}, we evaluated the ability of these enzymes to metabolize exogenous 2-AG in a mammalian cellular environment. Human 15-LOX-1 or 15-LOX-2 cDNA or empty vectors were transfected into COS-7 cells. Enzyme expression was confirmed by Western blotting. 15-LOX-transfected COS-7 cells produced a major metabolite with a mass-to-charge ratio of 417, consistent with a sodiated HETE-G species, upon treatment with 20 µM 2-AG (Fig. 5A and B, top panels). Metabolite production required both 2-AG treatment and 15-LOX (Fig. 5A and B, middle and bottom panels, respectively). In addition, the major cellular metabolite coeluted with the triphenylphosphine reduction product with \(m/z\) 417 of \textit{in vitro} incubations of 15-LOX and 2-AG as well as the minor HETE-G product generated by incubation of 2-AG with COX-2. Taken together, these results identify the cellular metabolite produced by 2-AG-treated, 15-LOX-transfected COS-7 cells as 15-HETE-G. Finally, as seen with \textit{in vitro} reactions with rabbit reticulocyte 15-LOX (15-LOX-1), COS-7 cells transfected with human 15-LOX-1 also generated a small amount of the C12 oxygenated product when treated with 2-AG (Fig. 5A, top panel). In contrast, human 15-LOX-2 appears to oxygenate 2-AG regiospecifically both \textit{in vitro} and in cells (Fig. 5B, top panel).
**Transactivation of PPARs by 15-HETE-G.** PPAR subtypes (-α, -δ, and -γ) constitute a family of ligand-activated nuclear hormone receptors with diverse roles in fatty acid metabolism, cellular differentiation, and inflammation (34). Putative natural ligands for PPARs include a broad range of fatty acids and certain fatty acid metabolites. The arachidonate-derived, 15-LOX product 15(S)-HETE is a PPARγ agonist (21,23). To determine if 15(S)-HETE-G retains the capacity to transactivate PPARγ, milligram quantities of this lipid were generated by treatment of 2-AG with purified soybean 15-LOX. Following purification, 15(S)-HETE-G was assayed for its ability to transactivate all three PPAR subtypes using the PPAR-GAL4 transactivation assay. In this assay, a chimeric receptor is used which contains the ligand binding domain (LBD) of a PPAR subtype fused with the DNA binding domain of the yeast GAL4 transcription factor. Transactivation of the chimeric receptor is detected by co-transfection with a reporter gene containing GAL4 response elements (UAS-tk-luc). As has been reported earlier, 15(S)-HETE is a specific agonist for the PPARγ subtype (Fig. 6). In contrast, 15(S)-HETE-G exhibited no agonism for PPARγ or -δ but was capable of transactivating PPARα in a dose-dependent manner (Fig. 6).

**Normal Human Epidermal Keratinocyte (NHEK) Metabolism of 2-AG.** PPARα agonists induce the differentiation of murine, rat, and human keratinocytes (35-40). Under some conditions, cultured NHEKs generate 15-HETE upon incubation with exogenous arachidonic acid (41-43). When undifferentiated NHEKs were incubated with 2-AG (50 µM), a prominent product was detected with m/z 417 (Fig. 7). This product could not be detected in vehicle-treated NHEKs. This product was identified by coelution with 15-HETE-G standards generated by soybean 15-LOX or acetylated COX-2 action on 2-AG (Fig. 7). The enzyme responsible for 15-HETE-G synthesis was identified by Western blot analysis of keratinocyte proteins; 15-LOX-2 was detected in NHEKs whereas no 15-LOX-1 was observed (data not shown).
Discussion

Despite its relatively recent identification, the endocannabinoid, 2-AG, has been implicated in an impressive array of central and peripheral signaling pathways (44). Consequently, enzymes capable of 2-AG metabolism have received considerable attention. The demonstrations that both COX-2 and leukocyte 12-LOX efficiently catalyze the oxygenation of 2-AG suggests that fatty acid oxygenases may play a role in the endogenous cannabinoid system (7,8). To develop a more complete understanding of potential interactions between fatty acid oxygenases and the endogenous cannabinoid system, multiple LOXs were rigorously examined for their abilities to oxygenate 2-AG.

Our results indicate that 2-AG is a poor substrate for 5-LOX enzymes; neither potato nor human 5-LOX catalyzed significant oxygenation of 2-AG (Fig. 1). Neither 5-LOX enzyme was available in a highly purified form, so it is possible that the low extent of oxygenation of 2-AG reflected the quality of the enzyme preparation. Nevertheless, glycerol esters of leukotrienes and other 5-LOX-generated metabolites appear unlikely to be produced in significant amounts from 2-AG. These results are consistent with the observation that porcine leukocyte 5-LOX is inactive with anandamide as substrate (16,17). In contrast, our results suggest that 15-LOXs efficiently metabolize 2-AG. Although the soybean enzyme displays a $V_{max}/K_m$ with 2-AG as substrate that is only 20% that observed with arachidonic acid, rabbit reticulocyte 15-LOX-1 and human 15-LOX-2 catalyze the oxygenation of 2-AG at least as efficiently as the oxygenation of arachidonic acid (Tables 1-4). These results are consistent with observations that soybean, human polymorphonuclear leukocyte, and rabbit reticulocyte 15-LOX enzymes can act on anandamide (16-19).

15-LOX enzymes shared the same stereo- and regiospecificity with arachidonic acid and 2-AG (Figs. 4). Furthermore, among arachidonylesters, arachidonylglycerols were the preferred substrates (Figs. 3). Coupled with the high efficiency of 15-LOX oxygenation of 2-AG, these in vitro results suggest that 15-LOX enzymes have evolved to utilize 2-AG as a substrate.
To extend these in vitro results into a cellular environment, COS-7 cells were transiently transfected with either human 15-LOX-1 or 15-LOX-2. Treatment of these cells with 20 μM 2-AG led to the appearance of 15-HETE-G in the medium (Fig. 5). The low, micromolar concentration of 2-AG was employed to approximate physiological conditions. Typically, 2-AG is found in whole rat brain at concentrations ranging from 2-5 nmol/g of wet tissue (2,3,45). In addition, 2-AG levels exceed 10 nmol/g in multiple rat brain regions including the hippocampus, striatum, brainstem, and medulla (4). Mouse hypothalamic 2-AG levels range from approximately 5 nmol/g to greater than 30 nmol/g depending on the species used (46). In addition, mouse cerebral 2-AG levels have been shown to increase nearly 10-fold following mechanical trauma to levels exceeding 100 nmol/g wet tissue (47). Finally, although much less data is available concerning nonnervous tissue, significant levels of 2-AG are found in multiple organs and this endocannabinoid is generated by endothelial cells and several blood cell types (45,48,49). Although a direct translation of 2-AG levels in whole tissue homogenates to concentrations available for enzymatic action by LOX enzymes is difficult, these data suggest that low, micromolar concentrations of endocannabinoid are likely present in vivo under some circumstances. Furthermore, the oxygenation of 2-AG by 15-LOX-expressing cells in the absence of esterase inhibition indicates that this reaction can compete with alternative 2-AG-metabolic pathways such as hydrolysis and that the product, 15-HETE-G, may be sufficiently stable to serve as an intra- or intercellular mediator. Extending this observation into primary cell preparations, NHEKs treated with 2-AG produced 15-HETE-G (Fig. 7). Evaluation of the cellular proteins expressed in NHEKs demonstrates that their 15-lipoxygenase capacity arises from the expression of 15-LOX-2, the human homologue of the murine 8-LOX.

In mouse epidermis, a lipoxygenase-dependent pro-differentiation pathway has been elegantly dissected. Following phospholipase liberation of arachidonic acid in murine keratinocytes, a resident 8-LOX catalyzes the formation of 8(S)-HpETE which is subsequently reduced to 8(S)-HETE (50). The 8(S)-HETE then binds to and activates PPARα. Activation or repression of PPARα downstream target genes leads to cell differentiation. Strong experimental
support for this pathway can be found in transgenic mice that overexpress 8-LOX. In these animals, the epidermis is stretched thin, highly differentiated, and characterized by marked keratosis (40). In addition, 8(S)-HETE treatment of wild-type keratinocytes induces PPARα-dependent keratin-1 expression, a marker for differentiation (40). Finally, PPARα-deficient animals possess a hypodifferentiated epidermis (38).

Although PPARα agonists also induce differentiation in human keratinocytes, a similar signaling pathway as observed in mouse does not account for NHEK differentiation. Humans do not express an 8-LOX nor are 8-LOX products found in human epidermis. The human 15-LOX-2 enzyme is considered the human homologue of the murine 8-LOX. These enzymes share 78% identity at both the DNA and protein levels (51). Interestingly, the human 15-LOX-1 and 15-LOX-2 only share 35% amino acid identity (52). Human 15-LOX-2 was originally cloned from human hair roots and has recently been reported to be expressed in the basal cell layer of human skin (26,53). The possibility that 15-LOX-2 may serve a similar biological function as murine 8-LOX in the epidermis appeared to be precluded by the observation that the 15-LOX-2 product of arachidonic acid oxygenation, 15(S)-HETE, displays no PPARα activity. In fact, 15(S)-HETE is a modest PPARγ agonist (21,23). With the demonstration that 2-AG is a superior substrate for 15-LOX-2 relative to arachidonic acid, the possibility exists that 15(S)-HETE-G is the human equivalent of 8(S)-HETE in epidermis. Employing PPAR reporter assays and enzymatically-generated, purified 15(S)-HETE-G, we demonstrated that, in marked contrast to the free acid, 15(S)-HETE-G is capable of transactivating PPARα but not PPARγ. These results conclusively demonstrate that eicosanoid glycerol esters can possess strikingly different biological activities than their free acid counterparts. In addition, although extensive further experimentation will be required to establish the physiological significance of 2-AG metabolism by 15-LOX-2 in human keratinocytes, the present studies support the theoretical possibility that, in humans, 15-LOX-2 and 2-AG serve the same functions that 8-LOX and arachidonic acid serve in mice. Thus, despite catalyzing different reactions and generating different products, 15-LOX-2 and 8-LOX may represent both genetic and functional homologues.
The present study suggests that 15-LOX enzymes may play a role in endogenous cannabinoid signaling and demonstrates that these enzymes should be included in the growing family of oxygenases capable of acting on 2-AG. Furthermore, this study provides a more complete understanding of mammalian oxidative pathways for 2-AG metabolism (Scheme 1). Finally, the present results identify the first biological activity for a glyceryl eicosanoid and point to the possibility that fatty acid oxygenase-mediated endocannabinoid metabolism may provide a diverse set of biological mediators with activities distinct from the corresponding arachidonate metabolites.
Acknowledgements

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Figure Legends

Figure 1. Time courses of oxidation of arachidonic acid and 2-AG by human 5-LOX. The reaction conditions were as described in Experimental Procedures. Reactions contained 5-LOX (10 U) in reaction buffer (50 mM Tris-Cl, 0.03% Tween 20, pH 7.4 containing 2 mM CaCl₂ and 1 mM ATP). Oxygen uptake was initiated by the addition of (A) 100 µM arachidonic acid or (B) 100 µM 2-AG. (vertical bar, 10 µM O₂; horizontal bar, 20 s)

Figure 2. Time courses of oxygenation of arachidonic acid and 2-AG by 15-LOX enzymes. The reaction conditions were as described in Experimental Procedures. Reactions contained (A, B) purified soybean 15-LOX (12 µg), (C, D) partially purified rabbit reticulocyte 15-LOX (4 µg), or (E, F) partially purified human 15-LOX-2 (88 µg) and (A, C, E) 50 µM arachidonic acid or (B, D, F) 50 µM 2-AG in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20, pH 7.4].

Figure 3. Arachidonylester oxygenation by 15-LOX enzymes. Reaction conditions were as described in Experimental Procedures. Reactions contained (A) purified soybean 15-LOX (12 µg), (B) partially purified rabbit reticulocyte 15-LOX (5 µg), or (C) partially purified human 15-LOX-2 (88 µg) and 50 µM substrate in reaction buffer [50 mM Tris-HCl, 0.03% Tween 20, pH 7.4]. Data were obtained by taking the slope of the reaction curve at the point of maximal velocity and have been normalized to the maximal rate of arachidonic acid oxygenation. The results represent the mean of four determinations ± S.E.
Figure 4. **Mass spectrometry of oxygenated 2-AG products.** (A) Representative direct liquid infusion, positive ion, electrospray ionization mass spectrum of 2-AG metabolites produced by treating 10 µg endocannabinoid with 10 µg soybean 15-LOX (37 °C, 10 min) in 100 µL 25 mM Tris, 0.015% Tween 20, pH 7.4. (B) Collision-induced dissociation spectrum of primary metabolite shown in (A).

Figure 5. **2-AG metabolism by human 15-LOX expressing mammalian cells.** COS-7 cells were transfected with expression vectors containing (A) human 15-LOX-1 or (B) human 15-LOX-2. Selected ion monitoring chromatograms (m/z = 417.3) depicting 15-HETE-G production and extracellular release by 15-LOX transfected cells treated with 20 µM 2-AG (*top panels*), 15-LOX transfected cells treated with DMSO vehicle (*middle panels*), and vector transfected cells treated with 20 µM 2-AG (*bottom panels*). Chromatograms have been normalized to the total ion current in the *top panels*. Products were eluted with MeCN in H₂O containing 0.001% NaOAc (40 to 70% MeCN gradient in 5 min followed by 70% MeCN isocratically). Results are a representative example of one of three separate experiments.

Figure 6. **15(S)-HETE-G is a PPARα agonist.** NIH3T3 cells were transiently transfected with UAS-tk-luc, pRL-TK, and the indicated PPAR-GAL4 subtype. Cells were then treated with vehicle or the indicated dose of 15(S)-HETE or 15(S)-HETE-G for 24-36 h. Cells were harvested and the dual-luciferase assay was performed. Data are presented as fold induction in luciferase activity of treated samples over vehicle and represent the mean from three independent transfections ± SE.
Figure 7. **2-AG metabolism by NHEKs.** Selected ion mass chromatograms (m/z 417.3) depicting 15-HETE-G production by undifferentiated NHEKs treated with vehicle (*top panel*) or 50 µM 2-AG (*middle panels*) and acetylated COX-2 treated with 2-AG (*bottom panel*). Chromatograms have been normalized to the total ion current in the *middle panel*. Products were eluted with acetonitrile in H$_2$O containing 0.001% sodium acetate (40-70% acetonitrile gradient in 5 min followed by 70% acetonitrile isocratically). Results are a representative example of three independent experiments.

Scheme 1. **Oxidative metabolism of 2-AG.**
Table 1: Steady-state kinetic values of soybean 15-LOX<sup>a</sup>

<table>
<thead>
<tr>
<th>Kinetic value</th>
<th>AA</th>
<th>2-AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (µM)</td>
<td>13 ± 4</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>$V_{max}$ (nmol s&lt;sup&gt;-1&lt;/sup&gt; mg&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>76 ± 9</td>
<td>8.9 ± 0.8</td>
</tr>
<tr>
<td>$V_{max}/K_M$</td>
<td>5.8</td>
<td>1.3</td>
</tr>
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</table>

<sup>a</sup>Kinetic values were determined using the UV assay as described in Materials and Methods. The maximum rates of reaction were obtained at least in triplicate with substrate concentrations varying from 5 to 60 µM. Substrate stocks were prepared in acetonitrile and diluted 500-fold to yield final concentrations. Values given are the mean ± S.E.
Table 2: Steady-state kinetic values of rabbit reticulocyte 15-LOX

<table>
<thead>
<tr>
<th>Kinetic value</th>
<th>AA</th>
<th>2-AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M$ (µM)</td>
<td>28 ± 11</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>$V_{max}$ (nmol s$^{-1}$ mg$^{-1}$)</td>
<td>54 ± 8</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>$V_{max}/K_M$</td>
<td>1.9</td>
<td>1.9</td>
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</table>

*Kinetic values were determined using the UV assay as described in Materials and Methods. The maximum rates of reaction were obtained at least in triplicate with substrate concentrations varying from 2 to 120 µM. Substrate stocks were prepared in acetonitrile and diluted 500-fold to yield final concentrations. Values given are the mean ± S.E.*
Table 3: Steady-state kinetic values of human 15-LOX-2

<table>
<thead>
<tr>
<th>Kinetic value</th>
<th>Kinetic value</th>
<th>Sf-9-expressed enzyme:</th>
<th>E. coli-expressed enzyme:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>2-AG</td>
<td>AEA</td>
</tr>
<tr>
<td></td>
<td>KM (µM)</td>
<td>23 ± 3</td>
<td>9 ± 2</td>
</tr>
<tr>
<td></td>
<td>V_max (nmol s⁻¹ mg⁻¹)</td>
<td>0.82 ± 0.04</td>
<td>0.51 ± 0.04</td>
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<tr>
<td></td>
<td>V_max/K_M</td>
<td>0.036</td>
<td>0.057</td>
</tr>
</tbody>
</table>

*Kinetic values were determined using the UV assay as described in Materials and Methods. The maximum rates of reaction were obtained at least in quadruplicate with substrate concentrations varying from 5 to 120 µM. Substrate stocks were prepared in acetonitrile and diluted 500-fold to yield final concentrations. Values given are the mean ± S.E.*
Figure 1
Figure 2
Figure 3
Figure 4

A.

Relative Abundance

300 400 500 600

M + Na⁺ = 433.26
Found = 433.15

M + Na⁺ = 401.27
Found = 401.25

B.

Relative Abundance

100 200 300 400

M + Na⁺ = 345.17
Found = 345.18

15-HPETE-G
m/z = 433.28
Figure 5
Figure 6
Figure 7
Scheme 1

- COX-2
- 15-LOX-1
- 15-LOX-2
- Leukocyte 12-LOX

Glyceryl Prostaglandins
15-lipoxygenase metabolism of 2-arachidonylglycerol: Generation of a PPARα agonist


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