Selective Oxygenation of the Endocannabinoid 2-Arachidonylglycerol by Leukocyte-Type 12-Lipoxygenase†

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ABSTRACT: The endogenous cannabinoid system appears to serve vascular, neurological, immunological, and reproductive functions. The identification of 2-arachidonylglycerol (2-AG) as an endogenous ligand for the central (CB1) and peripheral (CB2) cannabinoid receptors has prompted interest in enzymes capable of modifying or inactivating this endocannabinoid. Porcine leukocyte 12-liopoxygenase (12-LOX) oxygenated 2-AG to the 2-glyceryl ester of 12(*S*)-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12-HPETE-G). The k_{cat}/K_M for oxygenation of 2-AG was 40% of the value for arachidonic acid. In contrast to the results with leukocyte 12-LOX, 2-AG oxygenation was not detected with platelet-type 12-LOX. Among a series of structurally related arachidonyl esters, 2-AG served as the preferential substrate for leukocyte 12-LOX. 12(*S*)-Hydroxyeicosa-5,8,10,14-tetraenoic acid glyceryl ester (12-HETE-G) was produced following addition of 2-AG to COS-7 cells transiently transfected with leukocyte 12-LOX. These results demonstrate that leukocyte-type 12-LOX efficiently oxidizes 2-AG in vitro and in intact cells, suggesting a role for this oxygenase in the endogenous cannabinoid system.

Arachidonylethanolamide (anandamide) and 2-arachidonylglycerol (2AG)[1] have been identified as endogenous ligands for the cannabinoid receptors. Accumulating evidence suggests that 2-AG may be particularly important in the immune and neurological functions associated with the endogenous cannabinoid system (*1*−*3*). Consequently, elucidation of the metabolism of this lipid mediator should provide insight into its regulation and shed light on mechanisms by which cannabinoid tone is modulated in vivo. Until recently, 2-AG metabolism studies have focused on hydrolytic and reacylation pathways. However, we recently reported that 2-AG is an excellent substrate for cyclooxygenase-2 but not cyclooxygenase-1 (*4*). This raises the possibility that other fatty acid oxygenases metabolize 2-AG.

LOXs are ubiquitous plant and animal enzymes that catalyze the dioxygenation of polyunsaturated fatty acids both regio- and stereospecifically (*5*−*9*). As some lipoxygenases oxidize the endocannabinoid anandamide, we tested the possibility that a lipoxidative metabolic pathway exists for 2-AG (*10*−*14*). We focused on the ability of 12-LOX to oxygenate 2-AG. Hydroxylation of anandamide at C12 renders this endocannabinoid resistant to hydrolysis by fatty acid amide hydrolase and the 12(*S*) enantiomer appears to have similar affinity for the CB1 and CB2 receptors as anandamide (*12*). In contrast, 5-, 8-, 9-, 11-, or 15 hydroxylated anandamide derivatives bind with significantly less affinity to the CB1 receptor (*21*).

In the present study, we investigated the ability of the two major types of mammalian 12-lipoxygenases (leukocyte-type and platelet-type) to catalyze the hydroperoxidation of 2-AG. Porcine leukocyte 12-LOX oxidized 2-AG whereas human platelet 12-LOX effected essentially no hydroperoxidation of this endocannabinoid. Structural requirements for C12 hydroperoxidation were probed and demonstrated that 2-AG was the preferred arachidonyl ester substrate for leukocyte 12-LOX. The hydroperoxyeicosatetraenoic acid glyceryl ester (HPETE-G) produced by leukocyte 12-LOX oxygenation of 2-AG was identified as the 12(*S*)-isomer. In addition, a steady-state kinetic analysis of 2-AG oxygenation by leukocyte 12-LOX demonstrated that 2-AG is metabolized 40% as efficiently as arachidonic acid. Finally, mammalian cells expressing leukocyte 12-LOX proved capable of producing 12-HETE glyceryl ester when treated with a physiologically relevant concentration of 2-AG even in the absence of hydrolase inhibitors (*18*). Taken together, these results suggest that leukocyte 12-LOX may play a role in 2-AG metabolism and thus in endogenous cannabinoid signaling.

MATERIALS AND METHODS

Arachidonic acid and arachidonyl ethyl ester were purchased from Nu-Chek Prep (Elysian, MN). 1- and 2-arachidonylglycerols, HETEs [12(*R*)-, 12(*S*)-, and (±)12], and 12-lipoxygenase (murine leukocyte) polyclonal antiserum were purchased from Cayman Chemical (Ann Arbor, MI). Bacterial Protein Extraction Reagent was purchased from Pierce (Rockford, IL). The QuickChange Site-Directed Mutagenesis

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[1] Abbreviations: 2-AG, 2-arachidonylglycerol; LOX, lipoxygenase; HPETE-G, hydroperoxyeicosatetraenoic acid glyceryl ester; HETE-G, hydroxyeicosatetraenoic acid glyceryl ester; HETE, hydroxyeicosatetraenoic acid; ETYA, eicosatetraynoic acid.

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Kit was from Stratagene (La Jolla, CA). All other chemicals and solvents were purchased from Aldrich (Milwaukee, WI) unless otherwise noted. The expression vector pCI was purchased from Promega (Madison, WI), the expression vector pET20b(+) was purchased from Novagen (Madison, WI), and the restriction enzymes *Nde*I, *Eco*RV, and *Xba*I were purchased from New England Biolabs (Beverly, MA). COS-7 cells were obtained from American Type Culture Collection (Rockville, MD). 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 by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride-mediated coupling of arachidonic acid with ethylene glycol and 2-methoxyethanol, respectively. Both esters were purified by silica gel chromatography and characterized by NMR (^{1}H and ^{13}C) and mass spectrometry. Reaction details and spectroscopic data are available in Supporting Information.

Enzyme Preparation. Recombinant porcine leukocyte-type 12-LOX was purified to homogeneity as previously described (*22*). Human platelet 12-LOX was expressed and partially purified using an additional protocol. The full-length cDNA for wild-type human platelet 12-LOX was originally present in the pBS+ plasmid. A unique *NdeI* site was added to the 5′ end of the cDNA using the QuikChange Site-Directed Mutagenesis Kit according to manufacturer's instructions and its incorporation was verified by sequencing. The platelet 12-LOX cDNA was removed from the pBS+ plasmid by a *NdeI/EcoRV* digest (with *EcoRV* yielding a blunt end 3′ of the cDNA). The pET-20b(+) was digested with *XbaI*, incubated with Klenow Fragment, heat-inactivated at 75 °C for 10 min then digested with *NdeI*. The platelet 12-LOX cDNA and the linearized pET20b(+) vector were then ligated together and the proper orientation of the 12-LOX cDNA was verified by sequencing. *E.coli* expressing leukocyte or platelet 12-LOX were grown and pelleted as described (*22*). The cell pellets were resuspended in lysis buffer (Bacterial Protein Extraction Reagent with 1 mM DTT, 20 μg/mL catalase, 60 μg/mL chicken egg white type II−O trypsin inhibitor) and shaken at room temperature for 15 min. The lysate was centifuged at 5000 g for 15 min at 4 °C, and the supernatant was then centrifuged at 100000 g for 1 h at 4 °C. This supernatant was treated with 40 μg/mL DnaseI for 15 min at room temperature. Ammonium sulfate was added at 4 °C to the lysate to yield 60% saturation and the solution was stirred for 30 min. The solution was then aliquoted into 15 mL conical tubes and centrifuged at maximum speed in a Dynac tabletop centrifuge for 20 min. The supernatant was removed and the pellets were stored at −20 °C. Immediately prior to use, the pellets were resuspended in assay buffer and maintained on ice during experiments.

Lipoxygenase Assays. Lipoxygenase activity was detected by monitoring the absorbance of the conjugated diene product at 236 nm. 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 arachidonyl ester, and enzyme (3−12 μg). Inhibitor was added to some assays by 500- or 1000-fold dilution from methanol stocks. The reaction was initiated by the addition of enzyme to the reaction cuvette followed by mixing. The reaction temperature was 25 °C and the final reaction volume was 1 mL.

Enzyme Kinetics. Enzyme kinetic results were assessed using the computer program Enzyme Kinetics 1.5 (Trinity Software). Kinetic values were determined using nonlinear 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 glyceryl ester regiochemistry was established by mass spectrometry. Incubations of 10 μg purified LOX and 10 μg 2-AG (37 °C, 10 min) in 100 μL 25 mM Tris, 0.015% Tween 20, pH 7.4 were extracted with EtOAc and dried under Ar. The residue was redissolved in 1:1 MeCN:H_2O and infused into the mass spectrometer. Regiochemistry was established by diagnostic, collision-induced hydroperoxide cleavage. Stereochemistry was established by chiral-phase HPLC. Incubations of 50 μg purified LOX and 20 μg 2-AG (37 °C, 10 min) in 100 μL 25 mM Tris, 0.015% Tween 20, pH 7.4 were extracted with EtOAc and dried under Ar. Residue was redissolved in 50 μL MeOH containing 1 mg/mL triphenylphosphine to reduce HPETE glyceryl esters to the corresponding HETE esters. Saponification with 1 N NaOH followed by RP-HPLC (Supelcosil LC-18, 250 × 4.6 mm, 5 μm, 80:20:0.01 MeOH:H_2O:HOAc, 1.4 mL/min) provided purified HETEs for chiral analysis and, by coelution with standards, confirmed regiochemical assignment obtained mass spectrometrically. Following methylation with diazomethane, HETE methyl esters were analyzed by chiral-phase HPLC (Chiralpak AD, 250 × 4.6 mm, 1.4 mL/min, hexanes:EtOH 100:0.9). 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. The full-length cDNA for wild-type porcine leukocyte 12-LOX was subcloned from the pBS+ plasmid into the mammalian expression vector pCI. A *Xba*I digest of both the pBS+/12LO plasmid and pCI vector was used to transfer the entire 12-lipoxygenase cDNA into the multiple cloning site of pCI. The presence and proper orientation of the 12-lipoxygenase cDNA was verified by restriction enzyme digests and by sequencing. COS-7 cells were maintained at 37 °C in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum. Leukocyte 12-LOX cDNA or pCI vector without insert were transfected into COS-7 cells using LipofectAMINE according to manufacturer's instructions. Following transfection, media was replaced with PBS and cells were treated with ETYA or DMSO vehicle for 20 min followed by 2-AG or DMSO vehicle for 30 min at 37 °C. The final DMSO concentration was 0.4% v/v. After treatment, PBS was removed and extracted twice with an equal volume of 2:1 $CHCl_3$:MeOH. Organics were dried under argon and the residue was analyzed by mass spectrometry.

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 Na_3VO_4,

1 mM DTT, and protease inhibitors for 30 min at 4 °C. Cell lysates were cleared by centrifugation at 15000*g* for 15 min and the resultant supernatant was collected. Cellular proteins (20 μg) were separated by SDS−polyacrylamide gel electrophoresis (8%) then electrophoretically transferred to Immobilon-P transfer membrane. The membrane was probed with rabbit polyclonal antiserum to leukocyte 12-LOX followed by anti-rabbit horseradish peroxidase-conjugate. Immunoreactive bands were visualized by enhanced chemiluminescence.

Mass Spectrometry. Liquid chromatography/mass spectrometry was conducted with a Waters 2690 Separations Module interfaced to a Finnigan TSQ-7000 triple quadrupole mass spectrometer using a Zorbax RX-C18 narrow bore column (15 cm × 2.1 mm, 5 μm). Analytes were eluted with increasing concentrations of MeCN in 0.001% aqueous NaOAc and detected as positive ions. Evaluation of 12-HETE-G production by transiently transfected COS-7 cells was conducted with selected ion monitoring at $m/z = 417.3$. Electrospray ionization was carried out using nitrogen as sheath gas (76 psi) and auxiliary gas (14 psi) to assist nebulization. A potential of 5.5 kV was applied to the electrospray ionization needle and the capillary temperature was maintained at 220 °C. Mass spectrometry parameters were optimized to maintain maximum sensitivity without sacrificing unit resolution. Direct liquid infusion mass spectrometry was conducted with solutions of 50 μg/mL (with respect to substrate) in MeCN:H_2O at an infusion rate of 10 μL/min. Collision-induced dissociation was accomplished with argon as the collision gas at a pressure of 2.5 × 10^{-3} Torr in the second quadrapole.

RESULTS

2-AG Oxygenation by Leukocyte 12-Lipoxygenase. Partially purified leukocyte- and platelet-12-LOXs were incubated with substrate (50 μM 2-AG or arachidonate) in 50 mM Tris-HCl, 0.03% Tween 20, pH 7.4 and followed spectrophotometrically. Both types of 12-LOX rapidly metabolized arachidonic acid (Figure 1). A marked increase in absorbance at 236 nm demonstrated that leukocyte 12-LOX also converted 2-AG into a product with a conjugated diene functionality (Figure 1A) whereas platelet 12-LOX did not display significant metabolism of 2-AG (Figure 1B). Ammonium sulfate pellets of porcine leukocyte 12-LOX and human platelet 12-LOX were prepared as described in Materials and Methods. Ammonium sulfate pellets of leukocyte 12-LOX and the purified leukocyte 12-LOX showed similar rates of metabolism of 2-AG when compared to the rates of metabolism of arachidonic acid (see below). Additionally, the ammonium sulfate pellets of leukocyte-type 12-LOX displayed neglible activity toward the ethyl ester of arachidonic acid, similar to the purified protein (data not shown). These results indicate that the 2-AG substrate is available to enzyme purified to the ammonium sulfate pellet stage and that arachidonylester hydrolysis did not occur to a significant extent in these assays. The extremely slow metabolism of 2-AG by ammonium sulfate pellets of human platelet 12-LOX purified in the exact same manner therefore indicates that this enzyme cannot efficiently metabolize 2-AG.

The ability of leukocyte 12-LOX to oxygenate 2-AG was further characterized using purified protein. Incubations of

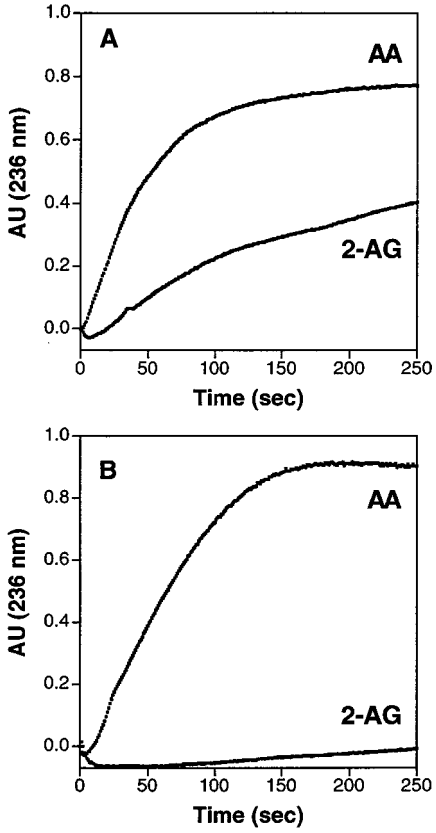

FIGURE 1: Time courses of oxygenation of arachidonic acid and 2-AG by leukocyte and platelet 12-LOXs. The reaction conditions and preparation of ammonium sulfate pellets were as described under Materials and Methods. Reactions contained 50 μM substrate (arachidonic acid or 2-AG) and (A) porcine leukocyte 12-LOX or (B) human platelet 12-LOX in reaction buffer (50 mM Tris-HCl, 0.03% Tween 20, pH 7.4).

enzyme (3.2 μg of leukocyte 12-LOX) and substrate (50 μM 2-AG or arachidonate) in 50 mM Tris-HCl, 0.03% Tween 20, pH 7.4, were again followed spectrophotometrically. A marked increase in absorbance at 236 nm demonstrated that purified leukocyte 12-LOX converted 2-AG into a conjugated diene (Figure 2B). Importantly, total product formation from both 2-AG and arachidonic acid was similar despite a slower rate of 2-AG metabolism (compare panel A in Figure 2 to panel B). The plateau in product formation is due to the well-documented self-inactivation of leukocyte 12-LOX (*9*). Additionally, the lipoxygenase inhibitor eicosa-5,8,11,14-tetraynoic acid (ETYA, 4 μM) greatly decreased metabolism of 2-AG by 12-LOX (Figure 2C). To assess the substrate structural requirements for leukocyte 12-LOX, arachidonic acid and a series of related arachidonyl esters (50 μM) were tested as LOX substrates. The maximal rate of 2-AG oxygenation by leukocyte 12-LOX under these screening conditions was approximately 50% that observed with arachidonic acid. In addition, 2-AG proved to be the preferred arachidonyl ester substrate (Figure 3). Similar results were obtained with substrate concentrations of 20 and 8 μM (data not shown).

Steady-State Kinetics of 12-Lipoxygenase Oxidation of 2-AG. We determined the steady-state kinetic values of both 2-AG and arachidonate using substrate concentrations varying from 5 to 120 μM (Table 1). Both arachidonic acid and 2-AG yielded K_M values in the low micromolar range (6 ± 2 μM for 2-AG versus 8 ± 2 μM for arachidonic acid).

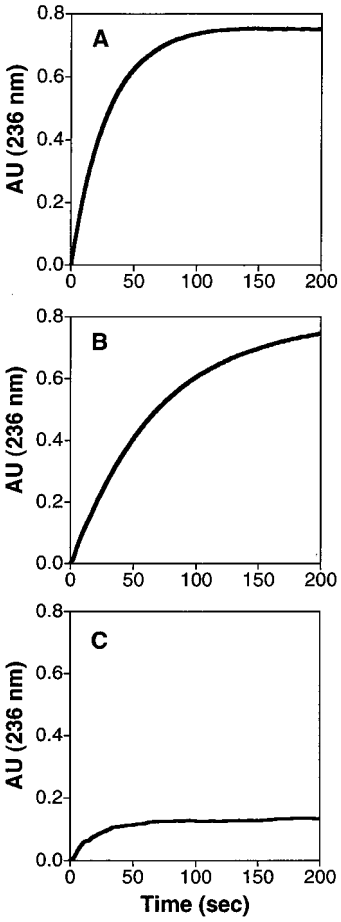

FIGURE 2: Time courses of oxygenation of arachidonic acid and 2-AG by purified leukocyte 12-LOX. The reaction conditions were as described for the LOX assay under Materials and Methods. Reactions contained leukocyte 12-LOX (3.2 μg) and (A) 50 μM arachidonic acid, (B) 50 μM 2-AG, and (C) 50 μM 2-AG and 4 μM ETYA in reaction buffer (50 mM Tris-HCl, 0.03% Tween 20, pH 7.4).

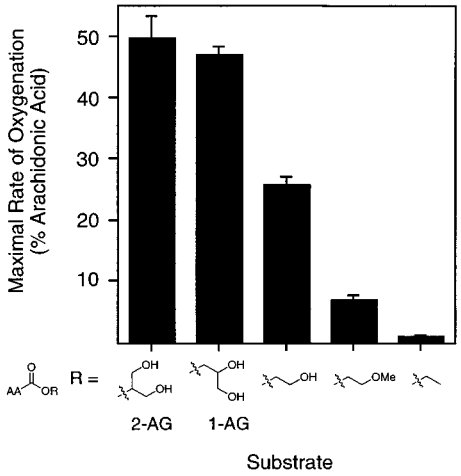

FIGURE 3: Endocannabinoid 2-AG is the preferred leukocyte 12-LOX arachidonyl ester substrate. Reaction conditions were as described for the LOX assay under Materials and Methods. Reactions contained leukocyte 12-LOX (3.2 μg) and 50 μM substrate in reaction buffer (50 mM Tris-HCl, 0.03% Tween 20, pH 7.4). Data were obtained by taking the slope of the reaction curve at the point of maximal velocity and were normalized to the maximal rate of arachidonic acid oxygenation. The results represent the mean of four determinations ± standard error.

Table 1: Steady-State Kinetic Values of Leukocyte 12-LOX[a]

kinetic value	AA	2-AG
K_M (μM)	8 ± 2	6 ± 2
k_{cat} (s^{-1})	13 ± 1	4.0 ± 0.4
k_{cat}/K_M ($M^{-1}s^{-1}$)	1.6×10^6	0.7×10^6

[a] 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 120 μM. Substrate stocks were prepared in acetonitrile and diluted 500-fold to yield final concentrations. Values given are the mean ± SEM.

Consistent with our initial observations of more rapid metabolism of arachidonic acid by leukocyte 12-LOX, the k_{cat} value of arachidonic acid was approximately 3-fold higher than that for 2-AG, whereas the k_{cat}/K_M ratio was 2.5-fold higher for arachidonic acid. Therefore, leukocyte 12-LOX appears capable of metabolizing arachidonic acid more rapidly, but the affinity of the enzyme toward both substrates and total product synthesis appear similar.

Enzyme Product Characterization. Rigorous characterization of the product of 2-AG metabolism by leukocyte 12-LOX was achieved by chromatography, UV spectroscopy, and mass spectrometry. Incubations of 2-AG with leukocyte 12-LOX resulted in a rapid increase in absorbance at 236 nm, suggesting the formation of a conjugated diene (Figures 1 and 2). Direct infusion of organic extracts of 2-AG/leukocyte 12-LOX incubations into the mass spectrometer revealed a single predominant product with a mass-to-charge ratio of 433.3 consistent with a sodiated HPETE glyceryl ester (Figure 4A). Collision-induced dissociation of this metabolite produced the expected fragmentation at the labile hydroperoxide bond and established the C12 regiochemistry of 2-AG oxygenation by 12-LOX (Figure 4B). Reduction of the HPETE-G product of 2-AG oxygenation by 12-LOX with triphenylphosphine followed by saponification afforded a product that coeluted with a 12-HETE standard by reversed-phase HPLC confirming the regiochemical assignments provided by mass spectrometry (data not shown). HPLC purified HETEs were methylated with diazomethane and analyzed by chiral chromatography to establish the stereochemistry of enzymatic hydroperoxidation. Leukocyte 12-LOX produced, almost exclusively, the expected *S* enantiomer [99.1 ± 0.1% *S*, respectively (mean ± SEM, $n = 3$)].

Cellular Metabolism of 2-AG by 12-Lipoxygenase. Having demonstrated effective 2-AG oxygenation by leukocyte 12-LOX in vitro, we evaluated the ability of this enzyme to metabolize exogenous 2-AG in a mammalian cellular environment. Leukocyte 12-LOX cDNA was transfected into COS-7 cells and enzyme expression was confirmed by Western blotting (Figure 5A). COS-7 cells do not express detectable levels of leukocyte 12-LOX when transfected with vector alone (Figure 5A). Leukocyte 12-LOX-transfected COS-7 cells produced a metabolite with a mass-to-charge ratio of 417.3, consistent with a sodiated HETE-G species, upon treatment with a physiologically relevant concentration of 2-AG (20 μM) (*18*). Metabolite production required 12-LOX expression and 2-AG treatment and was potently inhibited by the lipoxygenase inhibitor ETYA (Figure 5B). In addition, this metabolite coeluted with the triphenylphosphine reduction product of in vitro incubations of 12-LOX and 2-AG (data not shown). Taken together, these results

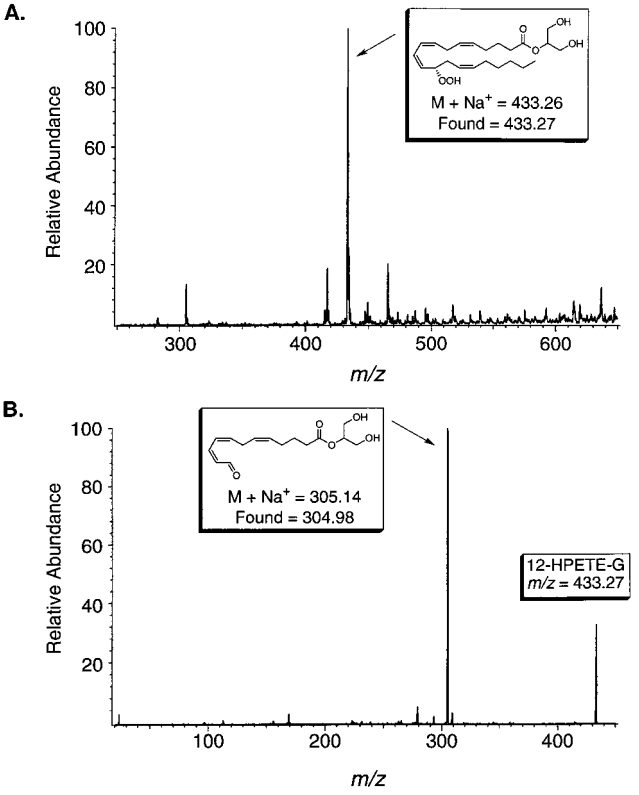

FIGURE 4: Mass spectrometry of oxygenated 2-AG products. (A) Representative direct liquid infusion, positive ion, electrospray ionization mass spectrum of 2-AG metabolites produced by treating 10 μg endocannabinoid with 10 μg of leukocyte 12-lipoxygenase (37 °C, 10 min) in 100 μL of 25 mM Tris, 0.015% Tween 20, pH 7.4. (B) Collision-induced dissociation spectrum of primary metabolite shown in panel A.

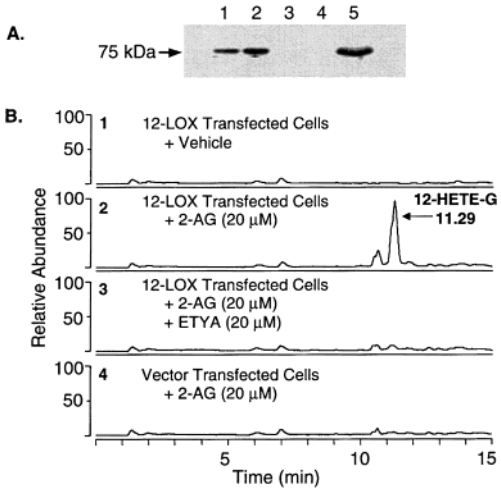

FIGURE 5: (A) Immunoblot of porcine leukocyte 12-LOX expressed in COS-7 cells. Lane 1, purified, recombinant porcine 12-LOX (32 ng); lane 2, purified, recombinant porcine 12-LOX (64 ng); lane 3, blank; lane 4, cellular protein (20 μg) from vector transfected COS-7 cells; lane 5, cellular protein (20 μg) from leukocyte 12-LOX- transfected COS-7 cells. (B) 2-AG metabolism by leukocyte 12-LOX-transfected COS-7 cells. Selected ion monitoring chromatograms (m/z = 417.3) depicting 12-HETE-G production and extracellular release by 12-LOX-transfected cells treated with (1) DMSO vehicle, (2) 2-AG (20 μM), and (3) ETYA (20 μM) and 2-AG (20 μM) and (4) vector-transfected cells treated with 2-AG (20 μM). Chromatograms were normalized to total ion current in chromatogram 2. Products were eluted with MeCN in H_2O containing 0.001% NaOAc (40−70% MeCN gradient in 5 min followed by isocratic elution with 70% MeCN). Results are a representative example of one of three separate experiments.

identify the cellular metabolite produced by 2-AG-treated, 12-LOX transfected COS-7 cells as 12-HETE-G.

DISCUSSION

Unambiguous experimental evidence for the existence of an endogenous ligand for cannabinoid receptors, anandamide, was not provided until 1992 (*23*). Three years later, a second endocannabinoid, 2-arachidonylglycerol, was isolated from canine gut and has since been shown to be present at significantly higher concentrations than anandamide in mammalian brain (*24*, *1*). Despite their recent discovery, endocannabinoids have already been implicated in a broad array of physiological and pathological responses (*25*). Not surprisingly, the diverse biological activities of endocannabinoids has spurred investigations into their regulation. Observations that prompted our interest in possible roles for LOXs in 2-AG metabolism include the demonstration that this endocannabinoid is efficiently and selectively oxidized by cyclooxygenase-2 and reports of LOX-mediated anandamide hydroperoxidation (*4*, *10*−*14*). Furthermore, similarities exist between the activation of some LOX pathways and endocannabinoid mobilization. For example, cellular 2-AG production and the activities of multiple LOXs are stimulated by calcium (*1*, *26*−*41*). Of particular interest, leukocyte 12-LOX is expressed in the central nervous and immune systems, two sites of 2-AG synthesis and action (*15*−*18*).

The current study indicates that leukocyte 12-LOX effectively metabolizes the endocannabinoid 2-AG (Figures 1A and 2). Using 50 μM substrate, 2-AG is metabolized by 12-LOX approximately 50% as rapidly as arachidonic acid. Screening a series of structurally related arachidonyl esters indicated that 2-AG was the preferred substrate (Figure 3). Rigorous product characterization studies indicated that the expected hydroperoxy-fatty acid glyceryl ester was produced: 12(*S*)-HPETE-G (Figure 4). Maintenance of both regio- and stereochemistry suggests that arachidonic acid and 2-AG adopt similar conformations within the active sites. Steady-state kinetic analysis of 2-AG oxygenation by leukocyte 12-LOX indicated that 2-AG is efficiently metabolized. The K_M for 2-AG dioxygenation was similar to arachidonic acid (6 ± 2 μM for 2-AG versus 8 ± 2 μM for arachidonic acid) and the turnover number was approximately 3-fold less for 2-AG (4.0 ± 0.4 s^{-1} for 2-AG versus 13 ± 1 s^{-1} for arachidonic acid) (Table 1). Due to the rapid self-inactivation of leukocyte 12-LOX, however, the amount of product formed after several minutes from either arachidonic acid or 2-AG was similar (Figure 2). Finally, we have shown that metabolism of 2-AG by leukocyte 12-LOX occurs in a cellular environment by transiently transfecting COS-7 cells with 12-LOX. Evaluation of products released extracellularly from these cells following treatment with 2-AG indicated the presence of 12-HETE-G, the reduction product of 12-HPETE-G (Figure 5B). The production and release of 12-HETE-G from leukocyte 12-LOX-expressing cells in the absence of hydrolase inhibition supports an assertion that this metabolite is sufficiently stable to serve as an intra- or intercellular mediator.

This work, in conjunction with previous observations, implies physiological relevance of lipoxygenase-mediated

2-AG oxygenation. The low K_M of leukocyte 12-LOX toward 2-AG, in light of 2-AG levels in excess of 10 μmol/kg tissue in several brain regions, suggests C12 hydroperoxidation of 2-AG may occur in vivo (*18*). Our results with transiently transfected mammalian cells represent an initial step in evaluating this possibility. In addition, 2-AG was the preferred arachidonyl ester substrate for this enzyme. Coupled with the exquisite stereo- and regiospecificity of 2-AG oxygenation by leukocyte 12-LOX, these data suggest that 12-LOX metabolism of 2-AG represents a purposeful enzymatic transformation. Our results suggest that the selective oxidation of 2-AG by leukocyte 12-LOX may be one reason for the evolutionary divergence of platelet- and leukocyte-type 12-lipoxygenases.

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SUPPORTING INFORMATION AVAILABLE

Reaction details and spectroscopic data for eicosa-5,8,11,14-tetraenoic acid 2-hydroxyethyl ester and eicosa-5,8,11,14-tetraenoic acid 2-methoxyethyl ester. This material is available free of charge via the Internet at http://pubs.acs.org.

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