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 (5S)-hydroperoxyeicosatetraenoic acid glyceryl ester. In contrast, potato and human 5-LOXs do not efficiently metabolize this endocannabinoid. Among a series of structurally related arachidonyl glycerols, 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. 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.

In 1995, 2-arachidonylglycerol (2-AG)\(^1\) was isolated from rat brain and canine gut and shown to bind both the central and peripheral 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 endocannabinoids, AEA (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/\(\beta\)-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 lipoxigenases to oxidize the endocannabinoid AEA prompted us to evaluate additional possible lipoxigenase 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-hydroxyeicosatetraenoic acid (HpEET), the precursor to the leukotrienes. The possibility that 5-LOX might oxygenate 2-AG to generate 5-HpEET glycerol ester (HpEET-G) and, subsequently, leukotriene glycerol 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. Lipooxygenase 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 PPARα 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 glycerol 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). Arachidonoylglycerols (1- and 2-), HETEs (15R-, 15S-), 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. All other chemicals and solvents were purchased from Aldrich unless otherwise noted. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). The vectors pcDNA3 and pCR2.1 were purchased from Invitrogen and pET3a was obtained from Stratagene (La Jolla, CA). The Bac-to-Bac baculovirus expression system, including the pFastBac HT vector, was purchased from Invitrogen and pET3a was obtained from Stratagene as a partially purified preparation from rabbit reticulocyte lysates generated essentially as previously described (25). t-Phosphatidylcholine (egg) was purchased from Avanti Pclar Lipids. All other chemicals and solvents were purchased from Aldrich unless otherwise noted. The initial rate.

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 (9). Briefly, UV absorbance was monitored using a Spectronic 21 (Bausch &amp; Lomb) spectrophotometer equipped with a water-jacketed cuvette. The enzyme reactions included reaction buffer (50 mM Tris-hCL, 0.03% Tween 20, pH 7.4), arachidonic acid or arachidonyl ester, 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 (100 µM), 5-LOX (250 units) in 1 ml of a 100 µM reaction buffer (50 mM Tris-hCL, 0.03% Tween 20, 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 the manufacturer’s instructions. Briefly, oxygen consumption was measured with a Gilson model 5/6 oxigraph (Gilson Medical Electronics, Middleton, WI) equipped with a Clark electrode and a thermostatted cuvette (37 °C). Enzyme aliquots (10 units) were added to 50 mM Tris-hCL, 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, Campion, NH). 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. Because of the characteristic lag phase of lipoxygenases in some reactions, this rate was not necessarily the initial rate.

Lipoxygenase Product Characterization—HpETE-G regionology was established by mass spectrometry (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 argon. The residue was redisolved in 1:1 MeCN/H2O and infused into the mass spectrometer. Rejection was established by diagnostic, collision-induced hydroperoxide cleavage. HpETE-Gs were reduced with triphenylphosphine to the corresponding HETEs esters. Saponification with 1N NaOH followed by RP-HPLC (Supelcosil LC-18, 250 × 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 establishment of MS. Following methylation with diazomethane, HETE methyl esters were analyzed by chiral-phase HPLC (Chiralpak AD, 250 × 4.6 mm, 1.5 ml/min, hexanes: EtOH, 98:2). 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’s medium supplemented with 10% heat-inactivated fetal bovine serum. Human 15-LOX cDNA in pcDNA3 or vector without insert were transfected into COS-7 cells using LipofectAMINE according to the manufacturer’s instructions. Following transfection, medium was replaced with HBSS and cells were treated with 2-AG (20 µM) or MeSO 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 1:1 CHCl3:MeOH. The organic extract was dried under argon, and the residue was analyzed by LC/MS. Saponification with 1N NaOH followed by RP-HPLC (Supelcosil LC-18, 250 × 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 MS. Following methylation with diazomethane, HETE methyl esters were analyzed by chiral-phase HPLC (Chiralpak AD, 250 × 4.6 mm, 1.5 ml/min, hexanes: EtOH, 98:2). Enantiomerically pure HETE methyl ester standards were well resolved on the chiral column. LC effluents were routinely monitored by UV at 235 nm.

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Enzyme Kinetics—Human keratinocyte expression of 15-LOX-2 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, PPAR-
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% of 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, dihydroyeicosatetraenoic 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. 2, A and 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 (~80%).

To assess the substrate structural requirements for soybean 15-LOX oxygenation, arachidonic acid and a series of related arachidonoyl esters (50 μM) were evaluated as substrates. The maximal rate of 2-AG oxygenation by soybean 15-LOX under these screening conditions was only 15% of that observed with arachidonic acid. However, 2-AG proved to be the preferred arachidonoyl ester 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 I). The enzyme displayed $K_m$ values in the low micromolar range for both substrates (7 ± 2 μM for 2-AG versus 13 ± 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 ~9-fold higher than that observed with 2-AG, yielding a 4–5-fold higher $V_{max}/K_m$ ratio for arachidonic acid (Table I). Soybean 15-LOX therefore appears capable of more rapidly metabolizing arachidonic acid, but the affinities of the enzyme toward 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 HpETE-G sodium adduct (Fig. 4A). Collision-induced dissociation of this metabolite produced the expected hydroperoxide cleavage and established the C-15 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 potassium adduct of the HpETE-G species. The latter (\( m/z \) 465) is consistent with a sodium adduct of a bis-dioxygenation product (\( M + 2O_2 + Na^+ \)). Both soybean and rabbit reticulocyte 15-LOX enzymes have been reported to carry out bis-dioxygenations 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 co-eluted on RP-HPLC with a 15-HETE standard, confirming the regiochemical as-

**FIG. 3. Arachidonyl ester oxygenation by 15-LOX enzymes.** Reaction conditions were as described under “Experimental Procedures.” Reactions contained purified soybean 15-LOX (12 \( \mu \)g) (A), partially purified rabbit reticulocyte 15-LOX (5 \( \mu \)g) (B), or partially purified human 15-LOX-2 (88 \( \mu \)g) (C) and 50 \( \mu \)M 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.

**TABLE I**

| Kinetic value | AA       | 2-AG     |
|---------------|----------|----------|
| \( K_M (\mu M) \) | 13 ± 4   | 7 ± 2    |
| \( V_{max} (\text{nmmol s}^{-1} \text{mg}^{-1}) \) | 76 ± 9   | 8.9 ± 0.8 |
| \( V_{max}/K_M \) | 5.8      | 1.3      |

Steady-state kinetic values of soybean 15-LOX

Kinetic values were determined using the UV assay as described under “Experimental Procedures.” The maximum rates of reaction were obtained at least in triplicate with substrate concentrations varying from 5 to 60 \( \mu \)M. Substrate stocks were prepared in acetonitrile and diluted 500-fold to yield final concentrations. Values given are the mean ± S.E.
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 50 µM arachidonate or 2-AG (Fig. 2, C and 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 that 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 arachidonyl esters (50 µM) were tested as substrates. The maximal rate of 2-AG oxygenation by rabbit 15-LOX under these screening conditions was 40% of that observed with arachidonic acid. However, 2-AG proved to be the preferred arachidonyl ester substrate (80%).

We next determined steady-state kinetic values for both 2-AG and arachidonate oxygenation (Table II). 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 ~3-fold 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 II).

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 co-eluted 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—To assess the ability of the recently discovered human 15-LOX-2 to effect endocannabinoid metabolism, we incubated partially purified

### Table II

| Kinetic Value | AA | 2-AG |
|--------------|----|------|
| $K_m$ (µM)   | 28 ± 11 | 9 ± 3 |
| $V_{max}$ (nmol s$^{-1}$ mg$^{-1}$) | 54 ± 8 | 17 ± 2 |
| $V_{max}/K_m$ | 1.9 | 1.9 |

To assess the substrate structural requirements for rabbit 15-LOX, arachidonic acid and a series of related arachidonyl esters (50 µM) were tested as substrates. The maximal rate of 2-AG oxygenation by rabbit 15-LOX under these screening conditions was 40% of that observed with arachidonic acid. However, 2-AG proved to be the preferred arachidonyl ester substrate (80%).

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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. 2, E and F).

To characterize the substrate specificity of 15-LOX-2, we evaluated the capacity of this enzyme to oxygenate arachidonic acid, arachidonoyl esters, and AEA (50 µM). AEA 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 with 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 because of markedly reduced \( K_m \) values (Table III). Because of 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 III). 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 C-15 hydroperoxidation very regiospecifically; no significant 12-HETE product 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 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 sodium adduct of a HETE-G species, upon treatment with 20 µM 2-AG (Fig. 5, A and B, top panels). Metabolite production required both 2-AG treatment and 15-LOX (Fig. 5, A and B, middle and bottom panels, respectively). In addition, the major cellular metabolite co-eluted with the triphenylphosphine reduction product with \( m/z \) 417 of 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 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 C-12 oxygenated product when treated with 2-AG (Fig. 5A, top panel). In contrast, human 15-LOX-2 appears to oxygenate 2-AG regiospecifically both in vitro and in cells (Fig. 5B, top panel).

**Transactivation of PPARs by 15-HETE-G—PPAR subtypes (\( \alpha, \delta \), and \( \gamma \)) 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 PPAR\( \gamma \) agonist (21, 23). To determine whether 15(S)-HETE-G retains the capacity to transactivate PPAR\( \gamma \), 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 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-luciferase). As has been reported earlier, 15(S)-HETE is a specific agonist for the PPAR\( \gamma \) subtype (Fig. 6). In contrast, 15(S)-HETE-G exhibited no agonist activity for PPAR\( \alpha \) or -\( \delta \) but was capable of transactivating PPAR\( \delta \) in a dose-dependent manner (Fig. 6).

**Normal Human Epidermal Keratinocyte (NHEK) Metabolism of 2-AG**—PPAR\( \alpha \) 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 undiffer-
entiated 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 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 suggest 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 AEA as substrate (16, 17). In contrast, our results suggest that 15-LOX enzymes efficiently metabolize 2-AG. Although the soybean enzyme displays a $V_{\text{max}}/K_{\text{m}}$ with 2-AG as substrate that is only 20% of 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 I–III). These results are consistent with observations that soybean, human polymorphonuclear leukocyte, and rabbit reticulocyte 15-LOX enzymes can act on AEA (16–19).

15-LOX enzymes shared the same stereo- and regiospecificity with arachidonic acid and 2-AG (Fig. 4). Furthermore, among arachidonyl esters, arachidonylglycerols were the preferred substrates (Fig. 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 to 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 ~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 of wet tissue (47). Finally, although much fewer data are available concerning non-nervous 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 biological function similar to that of 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 PPARδ. These results conclusively demonstrate that eicosanoid glycerol esters can possess strikingly different biological activities from 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.

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