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The Endocannabinoid 2-Arachidonoyl-Glycerol Activates Human Neutrophils: Critical Role of Its Hydrolysis and De Novo Leukotriene B_4 Biosynthesis

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Although endocannabinoids are important players in nociception and obesity, their roles as immunomodulators remain elusive. The main endocannabinoids described to date, namely 2-arachidonoyl-glycerol (2-AG) and arachidonyl-ethanolamide (AEA), induce an intriguing profile of pro- and anti-inflammatory effects. This could relate to cell-specific cannabinoid receptor expression and/or the action of endocannabinoid-derived metabolites. Importantly, 2-AG and AEA comprise a molecule of arachidonic acid (AA) in their structure and are hydrolyzed rapidly. We postulated the following: 1) the released AA from endocannabinoid hydrolysis would be metabolized into eicosanoids; and 2) these eicosanoids would mediate some of the effects of endocannabinoids. To confirm these hypotheses, experiments were performed in which freshly isolated human neutrophils were treated with endocannabinoids. Unlike AEA, 2-AG stimulated myeloperoxidase release, kinase activation, and calcium mobilization by neutrophils. Although 2-AG did not induce the migration of neutrophils, it induced the release of a migrating activity for neutrophils. 2-AG also rapidly (1 min) induced a robust biosynthesis of leukotrienes, similar to that observed with AA. The effects of 2-AG were not mimicked nor prevented by cannabinoid receptor agonists or antagonists, respectively. Finally, the blockade of either 2-AG hydrolysis, leukotriene (LT) B_4 biosynthesis, or LTB_4 receptor 1 activation prevented all the effects of 2-AG on neutrophil functions. In conclusion, we demonstrated that 2-AG potently activates human neutrophils. This is the consequence of 2-AG hydrolysis, de novo LTB_4 biosynthesis, and an autocrine activation loop involving LTB_4 receptor 1. ***The Journal of Immunology*****, 2011, 186: 3188–3196.**

The (−)-Δ^9-tetrahydrocannabinol (THC), the core bioactive substance of cannabis, mediates most of its biological effects by activating at least two cannabinoid receptors (CB_1 and CB_2) and possibly GPR55 (1–4). Endocannabinoids are endogenous bioactive lipids mimicking the effect of cannabis, arachidonyl-ethanolamide (AEA) and 2-arachidonoyl-glycerol (2-AG) being the main endocannabinoids described to date. They are involved in neurologic disorders, obesity, and nociception (5, 6). Whereas the expression of CB_1 is largely localized in the brain, CB_2 is primarily expressed in the periphery and on myeloid cells (1, 2), suggesting a role for the cannabinoid system in inflammation. Moreover, elevated endocannabinoid levels have been observed in the synovium and synovial fluid of individuals suffering from arthritic diseases (7), hinting that endocannabinoids might play an important role in inflammatory diseases.

The genomic deletion of endocannabinoid-hydrolyzing enzymes (increased levels of endocannabinoids in tissues) or of the CB_2 receptor (decreased endocannabinoid signaling) supports an anti-inflammatory role of endocannabinoids in animal models of inflammation (8–11). However, other in vivo studies showed a proinflammatory role of endocannabinoids (12–14). Consistent with their anti-inflammatory role observed in CB_2 receptor-deficient mice, endocannabinoids can inhibit chemotaxis and the production of cytokines ex vivo (15–20). However, an increasing body of evidence also demonstrates that like eicosanoids, endocannabinoids can stimulate proinflammatory functions of myeloid cells, such as cell adhesion, chemotaxis, phagocytosis, and the release of cytokines (21–33).

One exciting possibility to explain the complex effects of endocannabinoids on immune cell functions might be that endocannabinoids also serve as precursors for the biosynthesis of other lipid mediators with either proinflammatory and/or anti-inflammatory effects (34, 35). In support of this hypothesis, a recent study demonstrated that the inhibition of IL-2 production by 2-AG is the consequence of its transformation into glyceryl-PGs (36). Thus, the complex biological profile of endocannabinoids most likely reflects the contribution of multiple mechanisms of action, including the direct interaction of endocannabinoids with their specific receptors and/or the metabolism of endocannabinoids into various lipid mediators of inflammation, notably into eicosanoids (Fig. 1).

In the current study, we investigated whether endocannabinoids could modulate the functions of human neutrophils as well as the cellular and molecular mechanisms involved in such a modulation.

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Abbreviations used in this article: AA, arachidonic acid; AEA, arachidonyl-ethanolamide; 2-AG, 2-arachidonoyl-glycerol; BLT_1, LTB_4 receptor 1; COX-II, cyclooxygenase-II; $cPLA_2\alpha$, cytosolic phospholipase $A_2\alpha$; FAAH, fatty acid amide hydrolase; LO, lipoxygenase; LT, leukotriene; MAFP, methyl arachidonyl fluorophosphonate; MAG, monoacylglycerol; MPO, myeloperoxidase; NAM, *N*-arachidonyl-maleimide; RP, reversed phase; THC, (−)-Δ^9-tetrahydrocannabinol.

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Materials and Methods

Materials

The 19-OH-PG B_2, 2-AG, D_8-2-AG, arachidonic acid (AA), D_8-AA, AEA, CP 55,940, L-759,633, leukotriene (LT) B_4, D_4-LTB_4, methyl arachidonyl fluorophosphonate (MAFP), MK-476, *N*-arachidonyl-maleimide (NAM), PGB_2, and URB-602 were purchased from Cayman Chemical (Ann Arbor, MI). AM 281, AM 630, O-1602, and thapsigargin were purchased from Tocris Bioscience (Ellisville, MO). A23187, fMLP, cytochalasin B, and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). The mouse phospho-p44/42 ERK-1/2 (Thr^{202}/Tyr^{204}) and the rabbit polyclonal p44/42 MAPK ERK-1/2 mAbs were obtained from Cell Signaling Technology (Beverly, MA). The magnetic bead-conjugated anti-CD16 mAb and MACS were purchased from Miltenyi Biotec (Auburn, CA). HBSS and Ficoll–Paque were obtained from Wisent Laboratories (St-Bruno, Quebec, Canada). JZL-184 was provided by B. Cravatt (The Scripps Research Institute, La Jolla, CA). Adenosine deaminase was obtained from Roche (Laval, Quebec, Canada). The ECL detection kit was purchased from Amersham. Pyrrophenone was a donation of Dr. K. Seno (Shionogi Research Laboratories, Osaka, Japan). The 5-lipoxygenase (LO) inhibitor L 739,010 and the 5-LO–activating protein antagonist MK-0591 were provided by Dr. D. Riendeau from Merck Frosst (Kirkland, Quebec, Canada).

Isolation of human neutrophils and eosinophils

Human venous blood was collected in 9-ml polystyrene tubes containing K_3EDTA as anticoagulant, and granulocytes were isolated, as described previously (37), with slight modifications. In brief, the platelet-rich plasma and the erythrocytes were discarded from blood samples by centrifugation and dextran sedimentation, respectively. Mononuclear cells then were separated from the granulocytes by centrifugation on Ficoll–Paque cushions, and a hypotonic lysis was performed on the granulocyte pellets to remove the residual erythrocytes. Eosinophils were purified from the granulocyte suspensions by negative selection using anti-CD16–coated magnetic beads, according to the manufacturer's instructions. The purity of the resulting eosinophil and neutrophil suspensions was always ≥97.5%, and the viability was ≥98%.

Removal of endogenous adenosine

It is well known that, as opposed to circulating and tissue neutrophils, isolated human neutrophil suspensions are very sensitive to adenosine, which inhibits most of their functional responses by activating the Gαs-coupled receptor A_{2A}. Indeed, adenosine is usually cleared from the tissues or blood by resident cells and erythrocytes, respectively. This does not occur in neutrophil suspensions in which a buildup of adenosine is observed. Consequently, and to better mimic the fate of human neutrophils, all experiments involving this granulocyte were performed in the presence of adenosine deaminase (0.1 U/ml), which was added 10 min before the addition of the stimuli (38, 39).

Cell stimulations

Neutrophil suspensions in HBSS containing 1.6 mM $CaCl_2$ were warmed at 37°C for 30 min and then stimulated with 2-AG ether, 2-AG, D_8-2-AG, AA, D_8-AA, AEA, LTB_4, fMLP, thapsigargin, or A23187 at the indicated times and concentrations (see figure legends). Incubations were stopped by the addition of 1 vol cold (4°C) incubation buffer and immediately centrifuged (700 × *g*). Supernatants were harvested for the analysis of lipid mediator biosynthesis, myeloperoxidase (MPO) release, and migration assays. Cell pellets were processed for immunoblot or RT-PCR analyses, as described below. To maximize the impact of agonists on MPO release, neutrophils were incubated in the presence of 10 μM cytochalasin B 20 min before the addition of the stimuli.

Analysis of MPO release

Following cell stimulation, samples were rapidly centrifuged; the supernatants were collected and stored at −80°C until further analysis. Quantitation of MPO in the supernatants was performed using a commercially available ELISA kit (Hycult, Uden, The Netherlands), according to the manufacturer's instructions.

Transmigration assay

Transmigration assays were performed with 3 μm–pore inserts (BD Biosciences), as recommended by the manufacturer. In brief, 700 μl prewarmed (37°C) HBSS containing 1.6 mM $CaCl_2$ and the agonist of interest was put in the lower chambers, and 200 μl prewarmed neutrophil suspensions (37°C, 2.5 × 10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ and 5% (w/v) FBS was added in the upper chamber of the transmigration apparatus. Neutrophils were allowed to migrate for 2 h at 37°C. The upper chambers then were removed, and cells that had migrated in the lower chambers of the transmigration apparatus were counted using a hemocytometer. In experiments in which the supernatants of endocannabinoid-treated human neutrophils were used, cells were stimulated with endocannabinoids for 1 min, stopped by the addition of 1 vol of cold (4°C) incubation buffer, and immediately centrifuged. Supernatants were collected, warmed at 37°C, and placed in the lower chambers, whereas prewarmed resting neutrophil suspensions (37°C, 2.5 × 10^6 cells/ml) were placed in the upper chamber of the transmigration apparatus. Transmigration studies were performed for 2 h, and migrated cells were counted using a hemocytometer. In experiments in which the LTB_4 receptor 1 (BLT_1) antagonist CP 105,696 was used, the antagonist (100 nM) was added to both the neutrophil suspensions and the lower chamber for 5 min before the transmigration assay.

Reversed phase–HPLC analysis of 5-LO product biosynthesis

After centrifugation, 0.5 ml of a cold (−20°C) stop solution (MeOH/MeCN; 1/1; v/v) containing 12.5 ng 19-OH-PGB_2 and PGB_2 was added to the harvested supernatants, which were then placed at −20°C overnight to maximize protein denaturation. Samples were centrifuged (750 × *g*; 10 min), and the supernatants were analyzed by reversed phase (RP)-HPLC using an online extraction procedure, as described previously (40). LTB_4, 20-COOH-LTB_4, 20-OH-LTB_4, 6(*E*)-LTB_4, 6(*E*)-12-epi-LTB_4, and 5(*S*)-HETE are referred to as 5-LO products. Quantitation of the various metabolites was done using the internal standard PGB_2.

Analysis of LTB_4 biosynthesis by liquid chromatography coupled to tandem mass spectrometry

In experiments using 2-AG, AA, and their deuterated derivatives (labeled at C5, C6, C8, C9, C11, C12, C14, and C15 of the arachidonoyl moiety), incubations were stopped by the addition of 0.5 vol of a cold (−20°C) stop solution (MeOH) containing 5 ng D_4-LTB_4 as internal standard. Samples were stored at −20°C to maximize protein denaturation and then centrifuged (750 × *g*; 10 min) to remove the denatured proteins. Eicosanoids were extracted by solid-phase extraction using StrataX (60 mg; 3 ml) columns. In brief, supernatants were diluted with 1 vol H_2O, loaded on the cartridges, and washed with 1.5 ml MeOH/H_2O (10/90 v/v), 1.5 ml hexane, 1.5 ml acidified MeOH/H_2O (30/70 v/v; 2% AcOH), and 1.5 ml MeOH/H_2O (10/90 v/v). Samples then were eluted from the cartridges with 2 ml MeOH, evaporated to dryness using a speed-vac evaporator, washed with 200 μl EtOH, evaporated to dryness with a stream of nitrogen, finally suspended in 50 μl mobile phase (MeCN/MeOH/H2O; 40/30/30; v/v/v), and analyzed by liquid chromatography coupled to tandem mass spectrometry in a negative ion mode. Analysis of LTB_4 was performed by establishing the LTB_4/D_4-LTB_4 ratio (*m/z* 335→195/*m/z* 339→197). Analysis of D_8-LTB_4 was performed by establishing the D_8-LTB_4/D_4-LTB_4 ratio (*m/z* 343→200/*m*/z 339→197). Quantitation was achieved using standard curves generated by analysis (ratio determination) of solutions containing increasing amounts of LTB_4 or D_8-LTB_4 and a fixed amount of D_4-LTB_4.

Analysis of mRNA expression by RT-PCR

For the analysis of mRNA expression, total RNA extracts were prepared with TRIzol, according to the manufacturer's instructions. RT-PCRs were performed using 50 ng RNA, the SuperScript III One-Step RT-PCR kit, and the Platinum Taq DNA polymerase (Invitrogen Canada). Amplifications were performed on a Peltier Thermal Cycler (PTC-200; MJ Research, Watertown, MA) for 28 cycles with the following settings: denaturation (94°C, 1 min [2 min for the first cycle]), annealing (60°C, 30 s), and extension (72°C, 1 min [5 min for the last cycle]). Amplicons then were separated on ethidium bromide–containing agarose gels and visualized under UV light. Images of the gels were captured with the Chemigenius software (Syngene, Frederick, MD), and densitometry analyses were performed with National Institutes of Health Image software. Primers used were as follows: CB_1 forward, 5′-TCCTAGATGGCCTTGCAGAT-3′; CB_1 reverse, 5′-GCCGATGAAGTGGTAGGAAG-3′; CB_2 forward, 5′-CCACAACACAACCCAAAGC-3′; CB_2 reverse, 5′-GCAGAGGTATCGGTCAATGG-3′; GPR55 forward, 5′-TCTACATGATCAACCTGGCAGTCT-3′; GPR55 reverse, 5′-CTGGGACAGGACCATCTTGAA-3′; monoacylglycerol (MAG) lipase forward, 5′-TGCCTACCATGTTCTCCACA-3′; MAG lipase reverse, 5′-CCTCCAGTTATTGCAGTCTGG-3′; fatty acid amide hydrolase (FAAH) forward, 5′-GAGGCCCAGATGGAACATTA-3′; FAAH reverse, 5′-GGAGGGATCAGGACGAAGA-3′. In experiments in which FAAH, CB_1, and GPR55 were investigated, mRNA from human eosinophils and/or mRNA

extract from human brain (provided by A. Parent, Université Laval, Québec City, Quebec, Canada) were used as positive control.

Measurement of calcium mobilization

Calcium mobilization assays were performed, as described previously (41), with minor modification. In brief, human neutrophil suspensions (5×10^6 cells/ml) were incubated with 1 μM fura-2-AM in HBSS containing 1.6 mM $CaCl_2$ (37°C, 30 min), washed twice, and then transferred in the thermally controlled (37°C) and magnetically stirred cuvette of the spectrofluorometer (Aminco-Bowman series 2, SLM-Aminco). LTB_4 (10 nM), CP 55,940 (100 nM), 2-AG (3 μM), or fMLP (100 nM) was added 10 s after data acquisition was started. The serine hydrolase inhibitor MAFP (100 nM), the BLT_1 receptor antagonist CP 105,696 (100 nM), or the CB_2 receptor antagonist AM 630 (100 nM) was added 5 min before starting data acquisition. Fluorescence was monitored at excitation and emission wavelengths of 340 and 510 nm, respectively. Raw data were transformed with the following formula: $224([y - \text{fluorescence}_{min}]/[\text{fluorescence}_{max} - y])$. $\text{Fluorescence}_{max}$ was obtained by disrupting the neutrophil suspensions with 1% Triton X-100, and $\text{fluorescence}_{min}$ was obtained by the addition of EGTA (5 mM) and NaOH (5 mM) in the lysed neutrophil suspensions.

Analysis of proteins by immunoblot

For the analysis of proteins, cell pellets were disrupted with 400 μl cold (4°C) Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 3 mM diisopropylfluorophosphate). Lysates were vortexed for 15 s, denatured by adding 100 μl of 5× Laemmli sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM DTT, 10% glycerol, 0.01% bromphenol blue, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF), and boiled for 10 min. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Transfer efficiency and equal loading were visualized by Ponceau Red staining. For the determination of phospho–ERK-1/2 and ERK-1/2, membranes were soaked in TBS (25 mM Tris-HCl [pH 7.6], 0.2 M NaCl, 0.15% Tween 20) containing 5% dried milk (w/v) (25°C, 30 min), probed with the primary Ab (4°C, overnight), and revealed by chemiluminescence using a HRP-coupled Ab and the ECL detection kit.

Results

2-AG activates neutrophil functions

Given the reported pro- and anti-inflammatory effects of endocannabinoids on inflammatory cells (32, 42), we initially tested whether endocannabinoids would modulate the degranulation of human neutrophils. Experiments were consequently performed in which neutrophils were treated with endocannabinoids alone or followed by 100 nM fMLP. As shown in Fig. 2*A*, 2-AG, but not AEA, induced MPO release by neutrophils, to a similar extent as fMLP. The effect of 2-AG was maximal at 1 μM and occurred within minutes (Fig. 2*B*, 2*C*). Importantly, the preincubation of neutrophils with either AEA or 2-AG did not inhibit or enhance the fMLP-induced MPO release. This indicated that in our experimental model, 2-AG was stimulatory rather than inhibitory, and that AEA was ineffective. Another series of experiments was therefore undertaken in which the impact of endocannabinoids on neutrophil migration was assessed using a Transwell assay. Neither 2-AG nor AEA induced a significant neutrophil migration, in sharp contrast to 30 nM LTB_4 (Fig. 3*A*). Because 2-AG potently induced neutrophil degranulation (Fig. 2) and their granules are a source of preformed neutrophil chemotaxins (43), we performed other experiments in which the supernatant of endocannabinoid-treated neutrophils was placed in the lower chamber of the Transwell system. The supernatants of 2-AG–treated neutrophils strikingly induced the transmigration of neutrophils. 2-AG was as potent as 30 nM LTB_4, and its effect was observed at concentrations similar to those detected in the aorta of apolipoprotein E–deficient mice (33). Supernatants from AEA-treated neutrophils did not elicit the transmigration of human neutrophils (data not shown).

2-AG induces a robust biosynthesis of LTB_4

We next performed experiments to assess whether endocannabinoids could also stimulate the biosynthesis of LTB_4. As shown in Fig. 4*A*, 2-AG, but not AEA, induced a robust biosynthesis of leukotrienes (LTs). The 2-AG–induced LT biosynthesis was similar to that observed with AA. It occurred very rapidly, although somewhat delayed compared with AA, and was done within 2 min (Fig. 4*B*). Given 2-AG has an AA molecule in its structure and is highly susceptible to hydrolysis, additional experiments were performed to elucidate whether LTs originated from 2-AG or were biosynthesized by a mechanism involving endogenous AA release by phospholipases A_2. This was done with D_8-2-AG by assessing the biosynthesis of both LTB_4 and D_8-LTB_4. More than 97% of the LTB_4 found in the incubation media of human neutrophils stimulated with D_8-2-AG was octadeuterated (Fig. 4*C*), clearly demonstrating that the 2-AG–induced LT biosynthesis is the consequence of its hydrolysis into AA.

We next investigated the expression and the impact of endocannabinoid hydrolase inhibition in neutrophils. As shown in Fig. 4*D*, RT-PCR experiments indicated that human neutrophils did not express MAG lipase, in sharp contrast with human eosinophils.

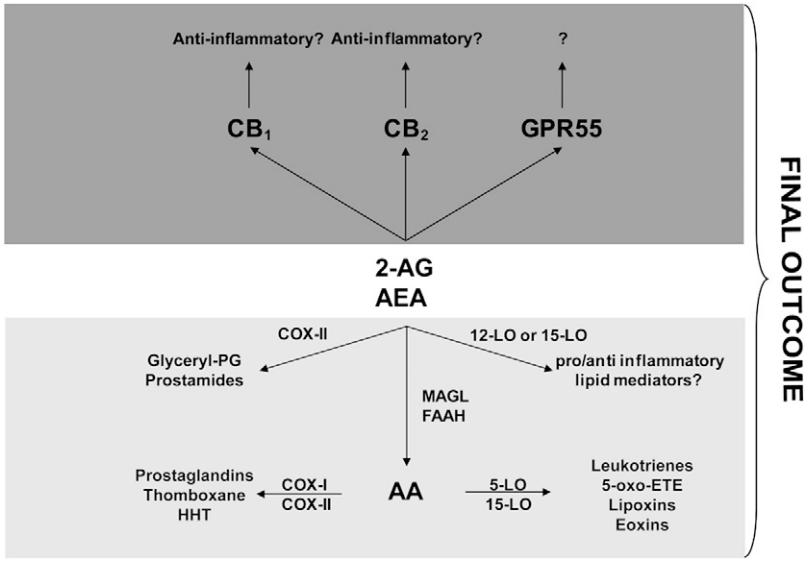

FIGURE 1. Putative mechanisms for the regulation of inflammation by the endocannabinoids 2-AG and AEA. 2-AG and AEA activate cannabinoid receptors. AEA is rapidly and almost exclusively hydrolyzed to AA and ethanolamine by the FAAH (65). AEA can also be oxidized to prostamides, 12-OH-, and 15-OH-AEA by the COX-II, 12-LO, and 15-LO pathways, respectively (66–69). The hydrolysis of 2-AG to glycerol and AA is mainly catalyzed by the MAG lipase (70–75). 2-AG can also be oxidized into other bioactive lipids by COX-II, 12-LO, and 15-LO, generating glyceryl-PG, 12-, and 15-OH-eicosatetraenoylglycerols, respectively (61, 76–78). 2-AG is not oxidized by the 5-LO (61). Thus, the complexity of the endocannabinoid system is increased by the formation of other bioactive lipids originating from their catabolism, including the putative link between endocannabinoid hydrolysis, and the biosynthesis of eicosanoids.

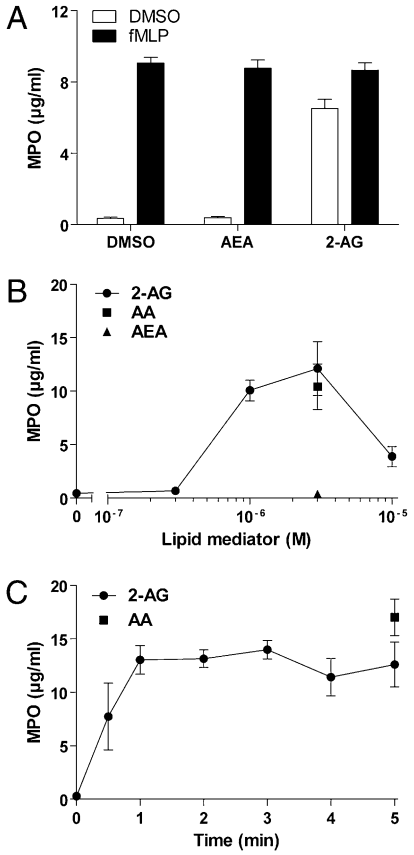

FIGURE 2. Effect of endocannabinoids on MPO release by human neutrophils. *A*, Human neutrophil suspensions (37°C; 5×10^6 cells/ml) were stimulated with vehicle (DMSO), 100 nM fMLP, 3 μM AEA, or 2-AG for 5 min. When neutrophils were stimulated with the combination of fMLP and endocannabinoids, 3 μM of either 2-AG or AEA was added 5 min before fMLP. *B*, Human neutrophil suspensions (37°C; 5×10^6 cells/ml) were stimulated with either AA, AEA, or 2-AG at the indicated concentrations for 5 min; *C*, Human neutrophil suspensions (37°C; 5×10^6 cells/ml) were stimulated with 3 μM of either AA or 2-AG for the indicated times. *A–C*, Incubations were stopped by the addition of 1 vol cold incubation medium, and MPO release was analyzed, as described in *Materials and Methods*. Results are the mean (±SEM) of three independent experiments, each performed in duplicate.

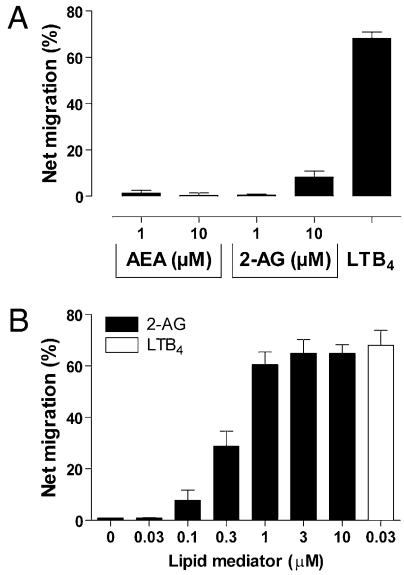

FIGURE 3. Impact of endocannabinoids on human neutrophil recruitment. A total of 200 μl human neutrophil suspensions (37°C; 2.5×10^6 cells/ml) in HBSS containing 5% FBS was added to the upper chambers of the transmigration apparatus, and neutrophils were allowed to migrate (37°C, 5% CO_2) for 2 h into the lower chambers containing 700 μl HBSS and the indicated concentration of either AEA, 2-AG, or 30 nM LTB_4 (*A*) or 700 μl supernatants of 2-AG–treated neutrophils (*B*), as indicated in *Materials and Methods*. Migrated cells were counted using a hemocytometer. Results are the mean (±SEM) of three independent experiments, each performed in duplicate.

This result was very surprising, given the potent inhibitory effect of two structurally distinct serine hydrolase inhibitors that inhibit MAG lipase (44). Indeed, MAFP and JZL-184 potently inhibited the 2-AG– but not the AA-induced LT biosynthesis (Fig. 4*E*, 4*F*). We also found that the well-known MAG lipase inhibitors NAM and URB-602 were equipotent as inhibiting the 2-AG–, the AA-, the thapsigargin-, and the A23187-induced LT biosynthesis in neutrophils (Fig. 5), indicating that NAM and URB-602 were also inhibiting an enzyme involved in LT biosynthesis. Importantly, the effect of 100 nM MAFP on A23187-induced LT biosynthesis was minimal and in line with our previous observations, showing its inhibitory effect on cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) occurred in the low micromolar range (45).

Role of CB receptors in neutrophil activation

To define the mechanisms by which 2-AG stimulates the proinflammatory functions of human neutrophils, another series of experiments was performed to assess the role of CB receptors. In contrast to human eosinophils, highly purified neutrophils did not express significant levels of CB_2 receptor mRNA (Fig. 6*A*, 6*B*). Neither CB_1 nor GPR55 receptors were found in neutrophils (data not shown). Moreover, the THC analog CP-55,940 (which activates CB_1 and CB_2) did not induce calcium mobilization (Fig. 6*C*) nor ERK-1/2 phosphorylation (Fig. 6*D*). Given 2-AG activated these two signaling events (Fig. 6*D*, 6*E*), a subset of experiments was performed in which neutrophils were pretreated with either the CB_1 antagonist AM 281 or the CB_2 antagonist AM 630. In agreement with a lack of CB receptor expression by human neutrophils, the CB receptor antagonists used in the current study did not prevent the effects of 2-AG on both ERK phosphorylation and calcium mobilization (Fig. 6*E*, 6*F*). Altogether, these results provide strong evidence that CB receptors are not involved in the stimulatory effects of 2-AG on human neutrophil functions.

Critical role of LTB_4 for the stimulation of neutrophils by 2-AG

The lack of functional CB receptors on human neutrophils, evidenced by our mRNA and pharmacological data (Fig. 6), was somewhat puzzling. This suggested that either another mediator and/or a receptor not activated by AEA were most likely involved in the stimulatory effects of 2-AG on human neutrophil functions. As reviewed earlier (34, 35) and pictured in Fig. 1, endocannabinoid metabolism can lead to several other bioactive lipids, including eicosanoids. Although they do not express MAG lipase, human neutrophils have a MAG lipase activity and are sensitive to MAG lipase inhibitors (Fig. 4) (46). We thus postulated that the hydrolysis of 2-AG to AA and its subsequent metabolism into LTB_4 were involved in its stimulatory effects on human neutrophil functions. A series of experiments were consequently performed to rule out this hypothesis. As shown in Fig. 7*A* and 7*B*, preventing 2-AG hydrolysis with MAFP or selectively blocking BLT_1 with CP 105,696 inhibited the 2-AG–, but not the fMLP-induced Ca^{2+} mobilization in human neutrophils. Moreover, MAFP inhibited the 2-AG–, but not the AA-induced ERK-1/2 phosphorylation (Fig.

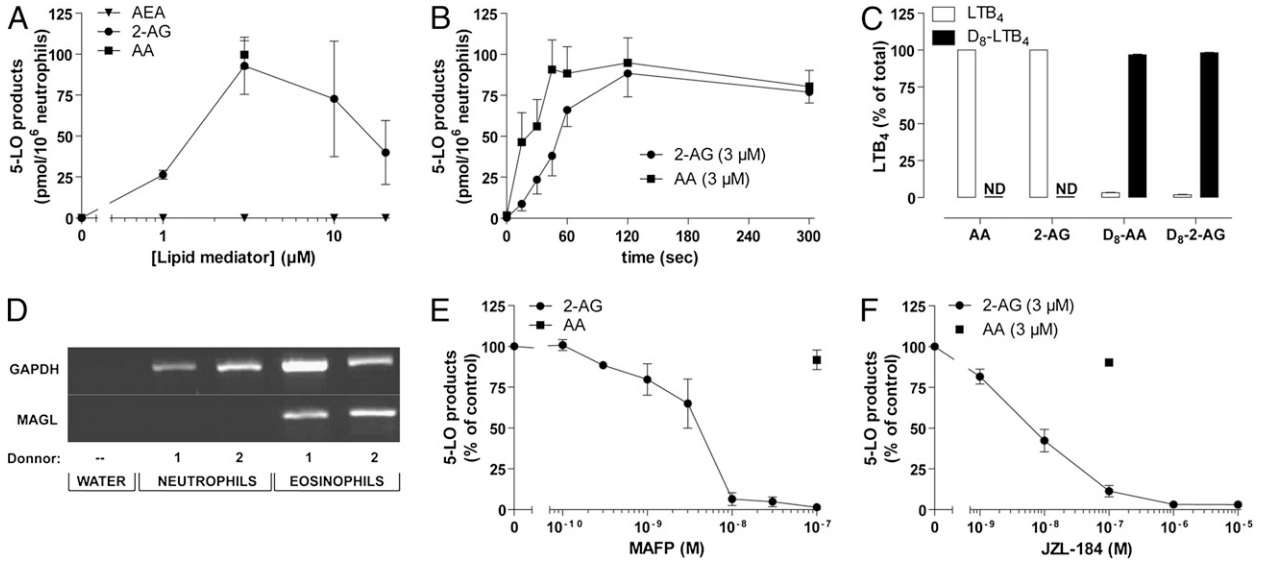

FIGURE 4. Impact of endocannabinoids on 5-LO product biosynthesis by human neutrophils. Human neutrophil suspensions (37°C; 5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were stimulated with increasing concentrations of either AA, 1-AG, 2-AG, or AEA for 5 min (*A, E, F*), or with 3 μM of either AA or 2-AG for the indicated times (*B*). MAFP (*E*) or JZL-184 (*F*) was added at the indicated concentrations 5 min prior to the addition of either 2-AG or AA. All incubations were stopped by adding 0.5 vol stop solution (MeOH/MeCN, 1/1, v/v) containing 12.5 ng 19-OH-PGB_2 and PGB_2 as internal standards and analyzed by RP-HPLC, as described in *Materials and Methods*. *C*, Prewarmed human neutrophil suspensions (37°C, 5×10^6 cells/ml) were incubated with either AA, 2-AG, D_8-AA, or D_8-2-AG for 5 min. Incubations were stopped by adding 0.5 vol cold (4°C) stop solution containing 5 ng D_4-LTB_4 as internal standard. Samples were processed and analyzed of both LTB_4 and D_8-LTB_4, as described in *Materials and Methods*. ND, Not detected. *A–C*, *E*, and *F*, The results are the mean (±SEM) of three independent experiments, each performed in duplicate. *D*, Total RNA was isolated from freshly isolated human neutrophils and eosinophils, and RT-PCR for MAG lipase expression was performed, as described in *Materials and Methods*. ND, not detected.

7*C*, 7*D*), again demonstrating the importance of 2-AG hydrolysis to induce intracellular signaling in human neutrophils. Additionally, both the AA- and the 2-AG–induced ERK-1/2 phosphorylation were inhibited by LT biosynthesis inhibitors, such as the 5-LO–activating protein antagonist MK-0591, the 5-LO inhibitor L-739,010, and the LTA_4 hydrolase inhibitor SC57461A. Finally, preventing BLT_1 activation with CP 105,696 also impaired the effect of AA and 2-AG on ERK-1/2 phosphorylation, whereas the $CysLT_1$ receptor antagonist MK-476 had no effect. Importantly, all the inhibitors/antagonists used did not modulate the fMLP-induced ERK-1/2 phosphorylation (Fig. 7*E*).

The striking effect of LTB_4 modifiers on the signaling events induced by 2-AG and AA led us to investigate whether the stimulation of human neutrophil functions by 2-AG was the consequence of its hydrolysis to AA, rapidly followed by de novo LTB_4 biosynthesis. BLT_1 blockade with CP 105,696 strongly limited the migration potential of 2-AG–stimulated neutrophil supernatants (~75%) while barely affecting that of fMLP-treated neutrophil supernatants, confirming that most of the biological activity inducing the migration of neutrophils found in the supernatant of 2-AG–stimulated neutrophils was LTB_4 (Fig. 7*F*). Finally, we undertook another series of experiments in which we addressed whether the release of MPO induced by 2-AG could be prevented by CP 105,696. As shown in Fig. 7*G*, the BLT_1 antagonist prevented the effect of 2-AG on MPO release while having no effect on the fMLP-induced MPO release. This indicates that MPO release triggered by 2-AG is, as opposed to fMLP, dependent on de novo LTB_4 biosynthesis.

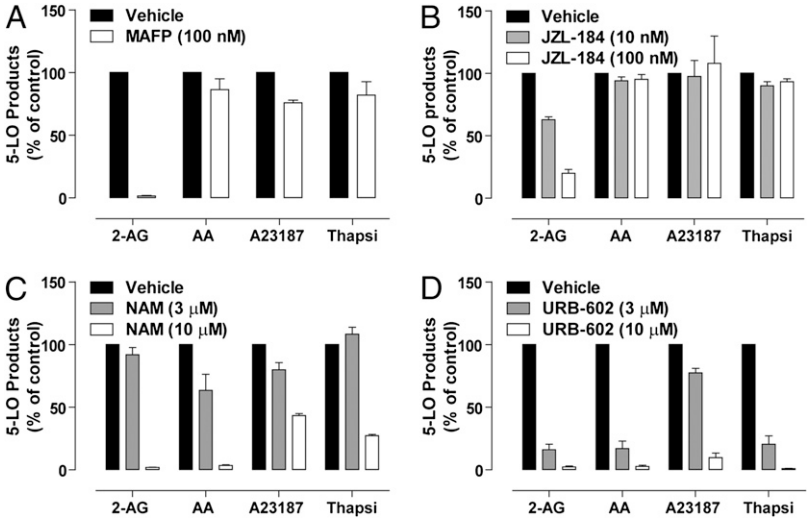

FIGURE 5. Impact of endocannabinoid hydrolysis inhibitors on 5-LO product biosynthesis by activated human neutrophils. Prewarmed suspensions of freshly isolated human neutrophils (37°C; 5×10^6 cells/ml) in HBSS containing 1.6 mM $CaCl_2$ were stimulated with either 3 μM 2-AG, 3 μM AA, 100 nM A23187 for 5 min or 100 nM thapsigargin (Thapsi) for 10 min. MAFP (*A*), JZL-184 (*B*), NAM (*C*), or URB-602 were added 5 min before the addition of the stimuli. Incubations were stopped by the addition of 0.5 vol stop solution (MeOH/MeCN, 1/1, v/v) containing 12.5 ng 19-OH-PGB_2 and PGB_2 as internal standards and analyzed for LT biosynthesis by RP-HPLC, as described in *Materials and Methods*. The results are the mean (± SEM) of at least two independent experiments, each performed in duplicate.

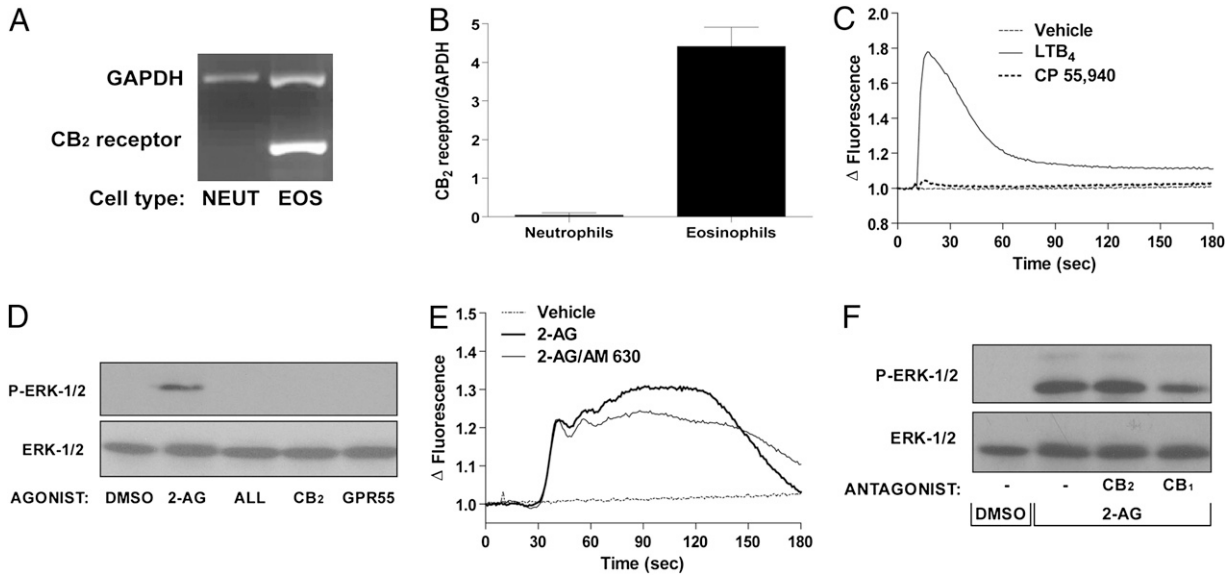

FIGURE 6. Role of cannabinoid receptors in the regulation of human neutrophil functions by 2-AG. *A* and *B*, RT-PCR for CB_2 receptor expression was performed, as described in *Materials and Methods*. *A*, Represents a typical result; *B*, represents the mean (±SEM) of at least three independent experiments. *C* and *E*, Freshly isolated human neutrophil suspensions (5×10^6 cells/ml in HBSS containing 1.6 mM $CaCl_2$) loaded with fura-2-AM were stimulated with either vehicle (DMSO), 30 nM LTB_4, 100 nM CP 55,940, or 3 μM 2-AG, and fluorescence was monitored for 3 min. AM 630 (1 μM) was added 5 min before the addition of 2-AG. The data are from a single experiment representative of three. *D* and *F*, Prewarmed neutrophil suspensions (5×10^6 cells/ml in HBSS containing 1.6 mM $CaCl_2$) were stimulated with 3 μM 2-AG, and 100 nM of either CP 55,940 (ALL), L-759,633 (CB_2), or O-1602 (GPR55) for 2 min. Incubations were stopped by the addition of 1 vol cold (4°C) incubation buffer, and samples were processed and analyzed, as described in *Materials and Methods*. Addition of 1 μM of either the CB_1 receptor antagonist AM 281 or the CB_2 receptor antagonist AM 630 was performed 5 min before the addition of 2-AG. The results are from one experiment representative of three.

Discussion

Although its biosynthesis was documented in human platelets back in 1983 (47), the role of 2-AG as immunomodulator has been underappreciated for almost 20 years, until CB_1 and CB_2 receptors were cloned and deficient mice were engineered. The finding that inflammation was enhanced in CB_2-deficient mice classified endocannabinoids as anti-inflammatory lipids. However, a growing body of evidence suggests the roles of endocannabinoids in the regulation of inflammation might be more sophisticated than anticipated. Indeed, 2-AG and/or CB_2 receptor agonists have been reported to stimulate proinflammatory functions of leukocytes such as chemotaxis and the production of proinflammatory cytokines. This might be related, at least in part, to the complex metabolism of endocannabinoids leading to a wide array of pro- and anti-inflammatory bioactive lipids (Fig. 1). We indeed document an important stimulatory effect of 2-AG in the regulation of human neutrophil functions, which necessitates its hydrolysis to AA, its metabolism to LTB_4, and the activation of BLT_1.

Previous reports investigating the impact of endocannabinoids as regulators of human neutrophil functions are limited. In fact, it is unclear whether human neutrophils express either CB_1, CB_2, or GPR55. The data presented in this work in which we were unable to observe the expression of CB_2 (Fig. 6) were generated using eosinophil-depleted neutrophils. We favored this approach because eosinophils express the CB_2 receptor (26). Given the strong mRNA expression of CB_2 receptor in human eosinophils, it is possible that they were involved in the previously observed CB_2 expression by neutrophils (48–50). Additionally, it is noteworthy that the use of CB receptor Abs can be misleading (50, 51). Our data show that freshly isolated human neutrophils do not significantly express CB receptors (mRNA) and are not activated by the CB receptor agonists used in the current study, and the effects of 2-AG are not prevented by CB receptor blockade. This is in agreement with the inefficacy of THC and CP 55,940 to stimulate isolated neutrophils (52, 53) and the CB receptor-independent inhibitory effect of cannabinoids, phytocannabinoids, and/or endocannabinoids on neutrophil functional responses (54, 55). Based on these results, we consequently conclude that isolated human neutrophils do not respond to endocannabinoids through their classic receptors (CB_1 and CB_2) nor GPR55.

Interestingly, endocannabinoids were reported to inhibit the fMLP-induced migration and polarization (54, 56) of neutrophils, in contrast to our results on MPO release (Fig. 2). This could be related to the use of adenosine deaminase in our experiments. Indeed, adenosine inhibits several functions of human neutrophils, including the biosynthesis of LTs, which is a critical player in the stimulatory effects of 2-AG we observed. Adenosine removal is important when studying human neutrophil functions to mimic both blood and tissues where adenosine is removed from the extracellular space or blood by resident cells and erythrocytes, respectively (38, 39). Additionally, adenosine promotes cyclooxygenase-II (COX-II) expression in human neutrophils (57). Experiments using endocannabinoids in human neutrophil suspensions in which adenosine is present might therefore result in COX-II expression and the biosynthesis of PG-ethanolamides and/or glyceryl-PGs. These endocannabinoid-derived lipids could modulate human neutrophil functions, possibly through the prostanoid receptor EP_2 (58, 59), which inhibits LT biosynthesis in neutrophils (60). This would not be observed in freshly-isolated, adenosine-depleted neutrophils because they do not express significant levels of COX-II (57).

In this study, we provide evidence that unlike AEA, 2-AG was hydrolyzed into AA, followed by a rapid biosynthesis of LTB_4. Although our data are consistent with a previous study showing neutrophils can hydrolyze arachidonoyl-glycerol (46), we were not able to detect MAG lipase by RT-PCR (Fig. 4). Using fluorophosphonate-biotin, Blankman et al. (44) demonstrated that MAG lipase, α/β-hydrolase-6, and α/β-hydrolase-12 were responsible for 98% of 2-AG hydrolytic activity in the mouse brain even if other enzymes

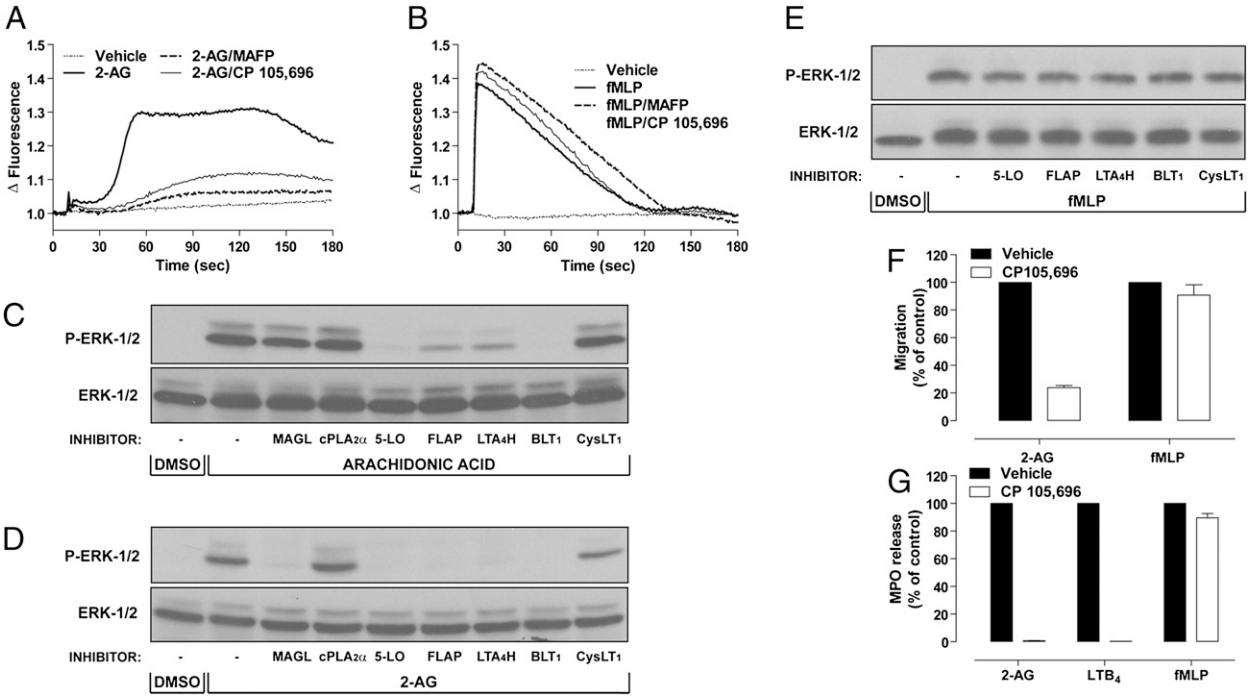

FIGURE 7. Involvement of 2-AG hydrolysis and de novo LTB_4 biosynthesis in the regulation of human neutrophil functions by 2-AG. *A* and *B*, Prewarmed, fura 2–loaded neutrophil suspensions (37°C, 5×10^6 cells/ml in HBSS containing 1.6 mM $CaCl_2$) were stimulated with either vehicle (DMSO), 100 nM fMLP, or 3 μM 2-AG, and fluorescence was monitored for 3 min. MAFP (100 nM) or CP 105,696 (100 nM) was added 5 min before addition of 2-AG or fMLP. Data acquisition was started 10 s before the agonists. The data presented are from a single experiment representative of three. Prewarmed freshly isolated neutrophil suspensions (37°C, 5×10^6 cells/ml in HBSS containing 1.6 mM $CaCl_2$) were stimulated with 100 nM fMLP (*E*) or 3 μM of either AA (*C*) or 2-AG (*D*) for 2 min. Incubations were stopped by the addition of 1 vol cold (4°C) incubation buffer, and samples were processed and analyzed by immunoblot, as described in *Materials and Methods*. All inhibitors and antagonists were added 5 min before the addition of fMLP, AA, or 2-AG. Inhibitors were 100 nM MAFP (MAGL), 100 nM pyrrophenone (cPLA$_2$α), 30 nM L-739,010 (5-LO), 100 nM MK-0591 (FLAP), and 10 μM SC 57461A (LTA_4H). Antagonists were 100 nM CP 105,696 (BLT_1) and 100 nM MK-476 ($CysLT_1$). The results are from one experiment representative of three. *F*, A total of 200 μl prewarmed suspensions of freshly isolated human neutrophils (37°C; 2.5×10^6 cells/ml) in HBSS containing 5% FBS was transferred to the upper chambers of the migration device, and neutrophils were allowed to migrate (37°C, 5% CO_2) for 2 h into the lower chambers containing 700 μl supernatants of 2-AG– or fMLP-treated neutrophils, as indicated in *Materials and Methods*. Migrated cells were counted using a hemocytometer at the end of the assays. CP 105,696 (100 nM) was added to both the upper and the lower chambers 5 min prior to the assays. Results are the mean (±SEM) of three independent experiments, each performed in duplicate. *G*, Prewarmed neutrophil suspensions (37°C; 5×10^6 cells/ml) were stimulated with either 3 μM or 100 nM fMLP for 5 min in the presence and absence of 100 nM CP 105,606. Incubations were stopped by the addition of 1 vol cold incubation buffer, and MPO release was analyzed, as described in *Materials and Methods*. CP 105,696 (100 nM) was added 5 min prior to the addition of the stimuli. Results are the mean (±SEM) of three independent experiments, each performed in duplicate.

capable of hydrolyzing 2-AG (FAAH, platelet-activating factor acetyl hydrolase 1Bγ, hormone-sensitive lipase, and neuropathy target esterase) were expressed. Other experiments are therefore needed to unravel the enzyme responsible for the hydrolysis of 2-AG in our neutrophil preparations.

Our data also support that stimulation of human neutrophil functions by 2-AG was linked to its hydrolysis into AA, a subsequent biosynthesis of LTB_4 and the activation of BLT_1 (Fig. 7). The kinetics of AA and 2-AG–induced LT biosynthesis are somewhat different, and a small delay is observed for the 2-AG–induced LT biosynthesis, compared with AA (Fig. 4). We believe this is related to 2-AG hydrolysis, which must occur before 5-LO activation because 5-LO cannot oxidize 2-AG (61). Human neutrophils are important players in the biosynthesis of LTB_4, which is a well-known activator of leukocyte functions. Interestingly, AA itself can trigger LTB_4 biosynthesis (62), and our data are in line with this. In fact, 2-AG induces the biosynthesis of LTB_4 as potently and efficiently as AA (Fig. 4), and both 2-AG and AA activate the ERK-1/2 phosphorylation through an autocrine loop involving LTB_4. The only difference we observed between 2-AG and AA was the impact of the serine hydrolase inhibitor MAFP, which blocked the effect of 2-AG, but not that of AA, providing evidence that hydrolysis to AA was a key step in the regulation of neutrophil functions by 2-AG.

It is well known that PLA_2 enzymes, cPLA$_2$α in particular, are important players of eicosanoid biosynthesis in activated cells. However, the data presented in this work suggest that AA coming from other sources, endocannabinoid hydrolysis for example, might play an important role in the regulation of cell functions through its metabolism into eicosanoids. In fact, preventing 2-AG hydrolysis with JZL-184 led to a 50% decrease of unesterified AA in the mouse brain (63). Given the reported proinflammatory and pronociceptive effects of several eicosanoids, it is conceivable that some of the anti-inflammatory effects of specific endocannabinoid hydrolysis inhibitors might be the consequence of decreased AA availability and eicosanoid biosynthesis. The novel functional link between endocannabinoids and LT biosynthesis that we have unraveled fosters further investigation of endocannabinoid hydrolysis inhibitors and their impact on eicosanoid biosynthetic enzymes.

In conclusion, we have demonstrated that 2-AG activates human neutrophils. The molecular mechanisms involved are as follows: 1) the hydrolysis of 2-AG to AA; 2) AA metabolism into LTB_4; and 3) activation of BLT_1. The important role of LTB_4 in the regulation

of neutrophil functions by 2-AG that we have unraveled in this study also underscores the importance endocannabinoid metabolites can have in the regulation of cell functions by endocannabinoids (Fig. 1). Given endocannabinoid levels found in sepsis or inflamed tissues can range from 0.45 to 1.5 μM (33, 64), this metabolic pathway may play an important role in the regulation of pain and inflammation in vivo.

Disclosures

The authors have no financial conflicts of interest.

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