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The Endocannabinoid 2-Arachidonoyl-Glycerol Activates Human Neutrophils: Critical Role of Its Hydrolysis and De Novo Leukotriene B₄ Biosynthesis

François Chouinard,*† Julie S. Lefebvre,‡ Pauline Navarro,*† Line Bouchard,*† Claudine Ferland,*† Mélanie Lalancette-Hébert,‡ David Marsolais,*† Michel Laviolette,*† and Nicolas Flamand*‡

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 arachidonoyl-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) B₄ synthesis, or LTₐ 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 LTₐ biosynthesis, and an autocrine activation loop involving LTₐ receptor 1. The Journal of Immunology, 2011, 186: 3188–3196.

The (-)-Δ⁹-tetrahydrocannabinol (THC), the core bioactive substance of cannabis, mediates most of its biological effects by activating at least two cannabinoid receptors (CB₁ and CB₂) 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 CB₁ is largely localized in the brain, CB₂ is primarily expressed in the periphery and on myeloid cells (1, 2), suggesting a role for the cannabinoid system in inflammation.

Abbreviations used in this article: AA, arachidonic acid; AEA, arachidonyl-ethanolamide; 2-AG, 2-arachidonoyl-glycerol; BLT₁, LTₐ receptor 1; COX-II, cyclooxygenase-II; cPLA₂, cytosolic phospholipase A₂; FAAH, fatty acid amide hydrolase; LO, lipoxigenase; LT, leukotriene; MAPP, methyl arachidonyl fluorophosphonate; MAG, monoacylglycerol; MPO, myeloperoxidase; NAM, N-arachidonoyl-maleimide; RP, reversed phase; THC, (-)-Δ⁹-tetrahydrocannabinol.

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Materials and Methods

Materials

The 19-OH-PG B$_2$, 2-AG, D$_2$-2-AG, arachidonic acid (AA), D$_2$-AA, AEA, CP 55,940, L-759,633, leukotriene (LT) B$_4$, D$_8$-LTB$_4$, methyl arachidonyl fluorophosphonate (MAFP), MK-476, N-arachidonylemaleimide (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 Toyo Soda (Hamamatsu, MO). A23187, IMLP, cytochalasin B, and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). The mouse phospho-p44/p42 ERK-1/2 (Th$_{235}/$Th$_{236}$) and the rabbit polyclonal p44/p42 MAPK ERK-1/2 mAbs were obtained from Cell Signaling Technology (Beverly, MA). The monoclonal anti-CD16–coated antibody 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. R. Rendeau from Merck Frosst (Kirkland, Quebec, Canada). The 19-OH-PG B$_2$, 2-AG, D$_2$-2-AG, arachidonic acid (AA), D$_2$-AA, AEA, CP 55,940, L-759,633, leukotriene (LT) B$_4$, D$_8$-LTB$_4$, methyl arachidonyl fluorophosphonate (MAFP), MK-476, and the viability was determined with a standard Trypan Blue exclusion technique. The viability was determined with a standard Trypan Blue exclusion technique. The viability was determined with a standard Trypan Blue exclusion technique.

Isolation of human neutrophils and eosinophils

Human venous blood was collected in 9-ml polystyrene tubes containing K$_3$-EDTA 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 $\geq$97.5%, and the viability was $\geq$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 Gs-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

Neutral lipids were stored at $-80^\circ$C and used in all experiments up to 5 mo. Lipid mediator biosynthesis by liquid chromatography coupled to tandem mass spectrometry.

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extract from human brain (provided by A. Parent, Université Laval, Québec City, Québec, 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 × 10⁶ cells/ml) were incubated with 1 μM fura-2-AM in HBSS containing 1.6 mM CaCl₂ (37°C, 30 min), washed twice, and then transferred in the thermally controlled (37°C) and magnetically stirred cuvette of the spectrophotometer (Aminco-Bowman series 2, SLM-Aminco). LTB₄ (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 receptor antagonist CP 105,696 (100 nM), or the CB₂ 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( [fluorescence_{min}] / [fluorescence_{max}] ). Fluorescence_{max} was obtained by disrupting the neutrophil suspensions with 1% Triton X-100, and 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 MgCl₂, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 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, 140 mM NaCl, 0.1% Tween 20) containing 5% dried milk, 0.1% Triton X-100, 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.

**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. 2A, 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. 2B, 2C). 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 LTB₄ (Fig. 3A). 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 LTB₄, 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 LTB₄**

We next performed experiments to assess whether endocannabinoids could also stimulate the biosynthesis of LTB₄. As shown in Fig. 4A, 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. 4B). 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 A₂. This was done with D₂-2-AG by assessing the biosynthesis of both LTB₄ and D₂-LTB₄. More than 97% of the LTB₄ found in the incubation media of human neutrophils stimulated with D₂-2-AG was octadeuterated (Fig. 4C), 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. 4D, RT-PCR experiments indicated that human neutrophils did not express MAG lipase, in sharp contrast with human eosinophils.
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. 4E, 4F). 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 that 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. 6A, 6B). 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. 6C) nor ERK-1/2 phosphorylation (Fig. 6D). Given 2-AG activated these two signaling events (Fig. 6D, 6E), 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. 6E, 6F). 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 neutrophils (Fig. 7A, 7B, 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\(_{\text{L}}\) mobilization in human neutrophils. Moreover, MAFP inhibited the 2-AG–, but not the AA-induced ERK-1/2 phosphorylation (Fig.
C), 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 LTA₄ hydrolase inhibitor SC57461A. Finally, preventing BLT₁ activation with CP 105,696 also impaired the effect of AA and 2-AG on ERK-1/2 phosphorylation, whereas the CysLT₁ receptor antagonist MK-476 had no effect. Importantly, all the inhibitors/antagonists used did not modulate the fMLP-induced ERK-1/2 phosphorylation (Fig. 7E).

The striking effect of LTB₄ 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 LTB₄ biosynthesis. BLT₁ 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 LTB₄ (Fig. 7F). 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. 7G, the BLT₁ 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 LTB₄ biosynthesis.

**FIGURE 5.** Impact of endocannabinoids on 5-LO product biosynthesis by human neutrophils. Human neutrophil suspensions (37°C; 5 × 10⁶ cells/ml) in HBSS containing 1.6 mM CaCl₂ 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). MAAP (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-PGB₂ and PGB₂ as internal standards and analyzed by RP-HPLC, as described in Materials and Methods. C, Prewarmed human neutrophil suspensions (37°C, 5 × 10⁶ cells/ml) were incubated with either AA, 2-AG, D₈-AA, or D₈-2-AG for 5 min. Incubations were stopped by adding 0.5 vol cold (4°C) stop solution containing 5 ng D₈-LTB₄ as internal standard. Samples were processed and analyzed of both LTB₄ and D₈-LTB₄, 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.

**FIGURE 4.** Impact of endocannabinoids on 5-LO product biosynthesis by human neutrophils. Human neutrophil suspensions (37°C; 5 × 10⁶ cells/ml) in HBSS containing 1.6 mM CaCl₂ 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-PGB₂ and PGB₂ as internal standards and analyzed by RP-HPLC, as described in Materials and Methods. C, Prewarmed human neutrophil suspensions (37°C, 5 × 10⁶ cells/ml) were incubated with either AA, 2-AG, D₈-AA, or D₈-2-AG for 5 min. Incubations were stopped by adding 0.5 vol cold (4°C) stop solution containing 5 ng D₈-LTB₄ as internal standard. Samples were processed and analyzed of both LTB₄ and D₈-LTB₄, 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.

**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 × 10⁶ cells/ml) in HBSS containing 1.6 mM CaCl₂ 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 prior to the addition of either 2-AG or AA. All incubations were stopped by adding 0.5 vol cold (4°C) stop solution (MeOH/MeCN, 1/1, v/v) containing 12.5 ng 19-OH-PGB₂ and PGB₂ as internal standards and analyzed 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 CB2 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 × 10⁶ cells/ml in HBSS containing 1.6 mM CaCl₂) loaded with fura-2-AM were stimulated with either vehicle (DMSO), 30 nM LTB₄, 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 × 10⁶ cells/ml in HBSS containing 1.6 mM CaCl₂) were stimulated with 3 μM 2-AG, and 100 nM of either CP 55,940 (ALL), L-759,633 (CB₂), 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 CB₁ receptor antagonist AM 281 or the CB₂ 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 CB₁ and CB₂ receptors were cloned and deficient mice were engineered.  The finding that inflammation was enhanced in CB₂-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 CB₂ receptor agonists have been reported to stimulate proinflammatory functions of leucocytes 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 LTB₄, and the activation of BLT₁.

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 CB₁, CB₂, or GPR55.  The data presented in this work in which we were unable to observe the expression of CB₂ (Fig. 6) were generated using eosinophil-depleted neutrophils.  We favored this approach because eosinophils express the CB₂ receptor (26).  Given the strong mRNA expression of CB₂ receptor in human eosinophils, it is possible that they were involved in the previously observed CB₂ 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 (CB₁ and CB₂) 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 EP₃ (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 LTB₄.  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 fluorophosphatase-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...
AG inhibitors were 100 nM MAFP (MAGL), 100 nM pyrrophenone (cPLA2), and 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. Pre-warmed 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 (cPLA2), 30 nM L-739,010 (5-LO), 100 nM MK-0591 (FLAP), and 10 μM SC 57461A (LTA4H). Antagonists were 100 nM CP 105,696 (BLT1) and 100 nM MK-476 (CysLT1). The results are from one experiment representative of three.

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.

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 LTB4; and 3) activation of BLT1. The important role of LTB4 in the regulation of human neutrophils by 2-AG is evidenced by its ability to activate LTB4 receptors and induce neutrophil migration. Our findings suggest that 2-AG plays a key role in the regulation of human neutrophil functions, highlighting its importance in the context of inflammatory diseases.

FIGURE 7. Involvement of 2-AG hydrolysis and de novo LTB4 biosynthesis in the regulation of human neutrophil functions by 2-AG. A and B. Pre-warmed, 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 MAFP, 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. Pre-warmed 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 (cPLA2), 30 nM L-739,010 (5-LO), 100 nM MK-0591 (FLAP), and 10 μM SC 57461A (LTA4H). Antagonists were 100 nM CP 105,696 (BLT1) and 100 nM MK-476 (CysLT1). The results are from one experiment representative of three.

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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.

References

enzyme activities from an intracellular pool to plasma membrane upon cell activation. J. Biol. Chem. 266: 15638–15643.


