Effects of Cannabinoids on LPS-Stimulated Inflammatory Mediator Release From Macrophages: Involvement of Eicosanoids

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Abstract

Δ⁹-Tetrahydrocannabinol (Δ⁹-THC) is the major psychoactive component of marijuana and elicits pharmacological actions via cannabinoid receptors. Anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG) are endogenous ligands for cannabinoid receptors, which because of their structural similarities to arachidonic acid (AA), AEA, and 2-AG could serve as substrates for lipooxygenases and cyclooxygenases (COXs) that metabolize polyunsaturated fatty acids to potent bioactive molecules. In this study, we have compared the effects of Δ⁹-THC, AEA, 2-AG, and another cannabinoid agonist, indomethacin morpholinylamide (IMMA), on lipopolysaccharide (LPS)-induced NO, IL-6, and PGE₂ release from J774 macrophages. Δ⁹-THC, IMMA, and AEA diminish LPS-induced NO and IL-6 production in a concentration-dependent manner. 2-AG inhibits the production of IL-6 but slightly increases iNOS-dependent NO production. Δ⁹-THC and IMMA also inhibit LPS-induced PGE₂ production and COX-2 induction, while AEA and 2-AG have no effects. These discrepant results of 2-AG on iNOS and COX-2 induction might be due to its bioactive metabolites, AA and PGE₂, whose incubation cause the potentiation of both iNOS and COX-2 induction. On the contrary, the AEA metabolite, PGE₂-ethanolamide, influences neither the LPS-induced NO nor IL-6 production. Taken together, direct cannabinoid receptor activation leads to anti-inflammatory action via inhibition of macrophage function. The endogenous cannabinoid, 2-AG, also serves as a substrate for COX-catalyzing PGE₂ production, which in turn modulates the action of CB2. J. Cell. Biochem. 81:715–723, 2001. © 2001 Wiley-Liss, Inc.

Key words: Δ⁹-THC; anandamide; immune suppression; endotoxin; macrophage

Δ⁹-Tetrahydrocannabinol (Δ⁹-THC) is the major psychoactive component of marijuana and has been used to alleviate the nausea associated with cancer chemotherapy, pain, migraine, epilepsy, glaucoma, hypertension, and AIDS wasting [Razdan, 1986; Annas, 1997]. Two G-protein-coupled receptors with seven transmembrane domains have been identified as cannabinoid receptors and are referred to as CB1 and CB2 [Howlett, 1995; Adams and Martin, 1996; Felder and Glass, 1998]. CB1, initially designated the central cannabinoid receptor, is present in greatest abundance within the brain but is also present at low levels in peripheral tissues. CB2 is only present at the periphery and more particularly in cells of myeloid lineage. Both receptors are involved in mediating the diverse biological actions of cannabinoid compounds, including immune suppression via CB2 activation [Klein et al., 1998] and alterations in central nervous system function via CB1 activation [Herkenham et al., 1990; Abood and Martin, 1996]. To date, two types of arachidonic acid (AA)-containing molecules, anandamide and 2-arachidonoyl-glycerol (2-AG), have been reported to be endogenous ligands for the cannabinoid CB1 and CB2 receptors and to mediate the pleiotropic pharmacological effects as Δ⁹-THC [Felder and Glass, 1998].

Abbreviations used: AA, arachidonic acid; AEA, anandamide; 2-AG, 2-arachidonoyl-glycerol; COX-2, inducible COX; DMEM, Dulbecco's modified Eagle's medium; IL-6, interleukin-6; IMMA, indomethacin morpholinylamide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NO, nitric oxide; PCR, polymerase chain reaction; PGE₂, prostaglandin E₂; Δ⁹-THC, Δ⁹-tetrahydrocannabinol.

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Following reports indicating the wide distribution of AEA and 2AG, and their broad range of activity [Felder and Glass, 1998], the question arose as to whether both endogenous mediators exert their effects solely through the two cannabinoid receptors characterized so far. Indeed, cannabinoid receptor-independent effects of AEA have been suggested [Felder et al., 1993; Derocq et al., 1998]. Since AEA is a polyunsaturated fatty acyl amide with an unmodified arachidonate backbone, it is a substrate for the enzymes that metabolize AA and thereby plays a role as a precursor for other biologically active molecules. Recent in vitro studies have shown that AEA is a substrate for porcine leukocyte 12-lipoxygenase [Ueda et al., 1995], soybean 15-lipoxygenase [Hampson et al., 1995], rat brain 12-lipoxygenase [Hampson et al., 1995], and cyclooxygenase (COX) [Yu et al., 1997]. It was shown that in intact cells AEA was converted to 12(S)- and 15(S)-hydroxyarachidonyl ethanolamide by human polymorphonuclear leukocytes, and to 12(S)-hydroxyarachidonyl ethanolamide by human platelets [Edgemond et al., 1998]. Recently, several enzymes capable of hydrolyzing 2-AG to AA [Goparaju et al., 1998, 1999; Di Marzo et al., 1999], or to prostaglandin H2 glycerol ester [Kozak et al., 2000] were also demonstrated. Currently the physiological significance of AEA and 2-AG metabolism by COX and/or lipoxygenases is not known.

Immune suppression is one of the major actions of cannabinoids, which are able to inhibit lymphocyte proliferation [Pross et al., 1992; Schwarz et al., 1994], antibody production, natural killer cell activity [Specter et al., 1980], and macrophage activity [Tang et al., 1992; Burnette-Curley et al., 1993; Friedman et al., 1994]. Consistent with these inhibitory features, cannabinoids are known to diminish general resistance to bacterial or viral infection [Morahan et al., 1979; Klein et al., 1994; Newton et al., 1994], and to suppress host immune reactivity against tumor growth [Zhu et al., 2000]. Among immune responses, macrophages play an essential role in local host defense against invading microorganisms. In response to foreign invaders and/or chemical signals generated by the affected tissues, macrophages recognize, phagocytose, and destroy the foreign agent. During the activation process, such as by treatment with the bacterial endotoxin, lipopolysaccharide (LPS), macrophages are a major source releasing various inflammatory mediators which contribute to the local inflammatory response, including nitric oxide (NO), tumor necrosis factor-α, eicosanoid prostaglandin E2 (PGE2), and interleukin-6 (IL-6). Previous studies have provided evidence that the presence of CB2 receptors in macrophages [Jeon et al., 1996] underlies Δ9-THC inhibition of both iNOS induction [Coffey et al., 1996] and TNF-α production [Berdyshhev et al., 1997]. Studies by Jeon et al. [1996] indicated that the inhibition of cAMP signaling is responsible for the inhibition of iNOS transcription and NO production by Δ9-THC. So far, although it was demonstrated that AEA and 2AG are synthesized from macrophages [Pestonjamasp and Burstein, 1998; Varga et al., 1998; Di Marzo et al., 1999; Kozak et al., 2000], whether the two endogenous cannabinoids exhibit similar suppressive effects as that of Δ9-THC in macrophage activation is not yet established.

In this study we explore the pharmacological actions of cannabinoids in macrophage induction of NO, IL-6, and PGE2. The possible involvement of eicosanoid metabolites in the actions of AEA and 2AG is considered.

**MATERIALS AND METHODS**

**Cell Culture**

The mouse J774 macrophage cell line was obtained from American Type Culture Collection and grown in DMEM containing 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Cells cultured at 37 °C in a water-saturated atmosphere of 95% air and 5% CO2 were seeded into 24-well plates for nitrite, PGE2, and IL-6 assays and into 35-mm dishes for immunoblots.

**Nitrite, PGE2, and IL-6 Measurement**

For inflammatory mediator induction, fresh culture medium containing LPS and/or indicated agonists, at the concentrations indicated, was added to cells, then the mediators released into the medium were measured 24 h later. Nitrite, an indicator of NO synthesis, was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylene-diamide in 5% phosphoric acid) to 100-μl samples of culture medium. Then the optical density at 550 nm (OD550) was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the
OD$_{550}$ produced using standard solutions of sodium nitrite in culture medium. PGE$_2$ and IL-6 were measured using enzyme immunoassay kits from Cayman, following the manufacturer’s instruction.

Immunoblot Analysis

To quantify iNOS and COX-2 protein, cells were incubated for 24 h in the presence of various stimuli, after which they were washed twice in ice-cold phosphate-buffered saline, then solubilized in buffer containing 20 mM Tris-HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM p-methylsulphonyl fluoride, and 10 μg/ml leupeptin (pH 7.5). Samples of equal amounts of protein (100 μg) were subjected to SDS-PAGE on 7.5% (iNOS) or 9% (COX-2) polyacrylamide gels, then transferred onto a nitrocellulose membrane, which was then incubated in 150 mM NaCl, 20 mM Tris, and 0.02% Tween (pH 7.4) containing 5% milk. The iNOS and COX-2 bands were visualized by immunoblotting with specific antibodies. Immunoreactivity was detected by enhanced chemiluminescence, following the manufacturer’s instructions.

RNA Extraction and RT-PCR Analysis of IL-6 mRNA

Specific primers were synthesized to amplify murine macrophage IL-6 mRNA. The IL-6 primers used were: sense (277–294), 5'-TGC ACT TGC AGA AAA CAA TC-3' and antisense (607–624), 5'-TGG TCT TGG TCC TTA GCC-3'. β-Actin mRNA levels were used as internal controls. The β-actin primers used were: sense (613–632), 5'-GAC TAC CTC ATG AAG ATC CT-3' and antisense (1103–1122), 5'-CCA CAT CTG CTG GAA GGT GG-3'. After drug treatment, confluent cells were harvested. Total RNA was purified using the RNAzol reagent, and RT-PCR was carried out using an RNA PCR kit, according to the manufacturer’s instructions using 10 μg of total RNA as a template. Equal amounts (1 μg of cDNA) of each RT product were PCR-amplified with Taq polymerase in 30 cycles consisting of 40 sec at 95°C, 40 sec at 56°C, and 2 min at 72°C. The amplified cDNA was run on 1.2% agarose gels and visualized by ethidium bromide.

Materials

DMEM, fetal bovine serum, penicillin, streptomycin, Dulbecco’s PBS (phosphate buffered saline), and trypan blue dye were obtained from Gibco BRL (Grand Island, NY). Rabbit polyclonal antibodies specific for iNOS and COX-2 were respectively from Transduction Laboratories (Lexington, KY) and Santa Cruz Biotechnology (Santa Cruz, CA). Δ$^9$-THC, AEA, and 2-AG were obtained from RBI (Natick, MA). IMMA, PGE$_2$ and IL-6 assay kits were obtained from Cayman Chemicals (Ann Arbor, MI). The RT-PCR kit was purchased from Stratagene (La Jolla, CA). Oligonucleotides were synthesized on a PS 250 CRUACHEM DNA synthesizer, using the cyanoethyl phosphoroamidate method, and purified using gel filtration. Horse-radish peroxidase-coupled anti-rabbit antibody and the enhanced chemiluminescence (ECL) detection agent were purchased from Amer-sham Pharmacia Biotech (Arlington Heights, IL). All materials for SDS-polyacrylamide gel electrophoresis (PAGE) were obtained from Bio-Rad Laboratories (Hercules, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Statistical Evaluation

Values are expressed as the mean ± standard error of the mean (SEM) of at least three experiments. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a P value of less than 0.05 was considered statistically significant.

RESULTS

Effects of Cannabinoids on LPS-Induced Mediator Release

Murine J774 macrophages were used to examine the effects on inflammatory mediator release of several cannabinoids, including Δ$^9$-THC, AEA, 2-AG, and a CB2-preferring ligand, indomethacin morpholinylamide (IMMA) [Gallant et al., 1996]. In J774 macrophages, endotoxin LPS (1 μg/ml) stimulation leads to about 7- (from 6.1 ± 1 to 42 ± 5 μM), 6- (from 796 ± 280 to 4728 ± 991 pg/ml), and 7-fold (from 429 ± 104 to 3140 ± 500 pg/ml) increases in NO, IL-6, and PGE$_2$ release, respectively. Although these cannabinoids themselves cannot affect basal mediator release, co-incubation with LPS affects LPS responses in different ways. As shown in Figure 1A, 10 μM Δ$^9$-THC co-addition with LPS (1 μg/ml) concentration-dependently reduced NO, IL-6, and PGE$_2$ release (Table I). Since Δ$^9$-THC at 30 μM induced slight cytotoxi-
Involvement of COX-Dependent Metabolites in Cannabinoid Actions

Since AEA can be metabolized by COX into PGE2-ethanolamide [Yu et al., 1997] and 2-AG may generate AA [Di Marzo et al., 1999], it is crucial to explore the role that eicosanoids play in both cannabinoid effects. For this purpose, we first examined the effects of PGE2-ethanolamide, PGE2, and AA on LPS responses. We found that PGE2-ethanolamide at 30 μM could not alter the NO and IL-6 responses of LPS (Table I), while PGE2 elicited opposite changes in both responses of LPS. At the concentration range of 0.1 nM to 1 μM, PGE2 concentration-dependently increased NO formation, but attenuated IL-6 release, in response to 1 μg/ml LPS.

TABLE I. Effects of Cannabinoids and Related Metabolites on LPS-Induced Nitrite, IL-6 and PGE2 Release in J774 Macrophages

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of LPS-induced response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nitrite</td>
</tr>
<tr>
<td>THC (10 μM)</td>
<td>64 ± 2%*</td>
</tr>
<tr>
<td>AEA (30 μM)</td>
<td>61 ± 4%*</td>
</tr>
<tr>
<td>2-AG (30 μM)</td>
<td>117 ± 2%*</td>
</tr>
<tr>
<td>IMMA (30 μM)</td>
<td>64 ± 3%*</td>
</tr>
<tr>
<td>PGE2-ethanolamide (30 μM)</td>
<td>92 ± 5%</td>
</tr>
<tr>
<td>AA (30 μM)</td>
<td>172 ± 17%*</td>
</tr>
<tr>
<td>PGE2 (1 μM)</td>
<td>134 ± 5%*</td>
</tr>
</tbody>
</table>

ND, not determined.
Each drug at indicated concentration was added with 10 μg/ml LPS for 24 h. The data represent the mean ± SEM from 3–5 independent experiments.
*P < 0.05 as compared to the response of LPS alone.

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Fig. 1. Effects of Δ9-THC, AEA, and 2-AG on LPS-induced nitrite, IL-6, and PGE2 formation in J774 macrophages. Cells were treated with 1 μg/ml LPS alone or with Δ9-THC (1–10 μM) (A), AEA (3–30 μM) (B), 2-AG (3–30 μM) (C), or IMMA (D) for 24 h, then the amounts of nitrite, IL-6, and PGE2 released into the medium were determined. LPS alone at 1 μg/ml increased nitrite, IL-6, and PGE2 release from the basal level of 6 ± 2 μM, 693 ± 190 pg/ml, and 402 ± 118 pg/ml to 49 ± 5 μM, 5278 ± 813 pg/ml and 3275 ± 412 pg/ml, respectively. The data represent the mean ± SEM of 3–7 experiments. *P < 0.05 indicates significant inhibition or potentiation of the LPS response.
Effects on NO, PGE₂, and IL-6 Release by Cannabinoids

(Fig. 2, Table I). Consistent with the effects of PGE₂, AA at 30 μM increased NO release, but decreased IL-6 formation stimulated by LPS (Table I).

In considering that the actions of AA might be mediated by either a COX- or lipoxygenase-dependent process, we examined this issue from pharmacological approaches. As shown in Figure 3, pretreatment with either a non-selective COX inhibitor (3 μM indomethacin) or a COX-1 inhibitor (10 μM valeryl salicylate) both abrogated the effects of AA. Alternatively, the enhanced NO release and reduced IL-6 formation following AA co-incubation was mediated by generation of COX-dependent metabolites. On the contrary, when the 5-lipoxygenase pathway was blocked by the 5-lipoxygenase-activating protein inhibitor, MK886 (10 μM), or the 12-lipoxygenase pathway was blocked by baicalein (10 μM), NO potentiation and IL-6 inhibition caused by AA was unaffected (Fig. 3). All these data suggest that a COX metabolite, possibly PGE₂, mediates the immunoregulatory action of AA in macrophages.

Effects of Cannabinoids on iNOS, COX-2, and IL-6 Expression

To confirm the inhibitory effects of cannabinoids on mediator release, we examined their effects on the expression of the inducible proteins, NOS, and COX-2, as well as on inducible IL-6 gene transcription. Immunoblot analysis revealed that Δ⁹-THC, AEA, and IMMA could inhibit LPS-mediated iNOS induction to 25% ± 8% (n = 3), 63% ± 4% (n = 3), and 43% ± 8% (n = 3) of the control, respectively, while 2-AG slightly enhanced iNOS immunoreactivity to 120% ± 4% (n = 3) of the control (Fig. 4). With respect to COX-2, Δ⁹-THC and IMMA could inhibit LPS-mediated COX-2 induction to 54% ± 6% (n = 3) and 79% ± 4% (n = 3) of the control, respectively, whereas AEA (93% ± 6% (n = 3) of the control) and 2-AG (110% ± 8% (n = 3) of the control) showed no significant changes (Fig. 4). RT-PCR analysis indicated that transcription of IL-6 mRNA could be induced after LPS stimulation for 4 h. Upon co-incubation with Δ⁹-THC, AEA, or 2-AG, this stimulated gene transcription was reduced, indicating that cannabinoids antagonize LPS-elicited IL-6 production through reducing IL-6 gene transcription.

To further characterize whether the non-significant inhibition of COX-2 induction by AEA and 2-AG results from the opposite effects caused by COX-dependent metabolites, we examined the effects of PGE₂, PGE₂-ethanolamide, and AA on COX-2 induction. As shown in
Figure 5A, PGE₂, but not PGE₂-ethanolamide, incubation for 12 h increased COX-2 expression in a concentration-dependent manner. Likely, AA at 10 μM also induced COX-2, and this effect was abolished by pretreatment with the specific COX-1 inhibitor, valeryl salicylate, but not by MK886, baicalein, or NDGA (a non-selective lipoxygenase inhibitor).

**DISCUSSION**

Many previous reports have shown that cannabinoids possess immunosuppressive effects. Cannabinoids diminish general resistance to bacterial or viral infection [Morahan et al., 1979; Klein et al., 1994; Newton et al., 1994], lymphocyte proliferation, antibody production, natural killer cell activity, and macrophage activity [Friedman et al., 1994]. At the molecular level, such modulation is expressed by the inhibition of the production of cytokines such as interferon [Blanchard et al., 1986], TNF-α [Zheng and Specter, 1996], and IL-2 [Nakano et al., 1992]. In addition, other studies indicated that Δ⁹-THC could inhibit LPS-induced nitrite production in mouse peritoneal macrophages [Coffey et al., 1996]. In human blood monocytes, Δ⁹-THC at low concentrations inhibits TNF-α, IL-6, and IL-8 production, while potentiating these mediators at high concentrations [Berdyshev et al., 1997]. Not only was anti-inflammation action seen for Δ⁹-THC, but the endogenous cannabinoid, AEA, was also shown to regulate nitrite release through activation of cNOS and by affecting iNOS expression in endothelial cells [Stefano et al., 1998], and by inhibiting IL-4, IL-6, IL-8, TNF-α, and IFN-γ production in human blood monocytes [Berdyshev et al., 1997].

Based on all the immunoregulatory actions of cannabinoids previously reported, we herein further delineated and compared the actions of four cannabinoid agonists on macrophage release of NO, PGE₂, and IL-6 in response to LPS. We chose murine J774 macrophages as the target cell system, as the murine macrophage...
cell line has been shown to express CB2 [Jeon et al., 1996]. All three mediators are known to be induced through transcriptional signaling pathways and to play crucial roles in immunomodulation and inflammation responses upon exposure to endotoxin and many inflammatory insults [Grimble, 1990, Graeve et al., 1993]. Our results showed that Δ⁹-THC, mimicking its inhibitory actions on the release of other cytokines as previously observed, is capable of reducing LPS-elicited iNOS, COX-2 protein induction, and IL-6 gene transcription, which thereby lead to the attenuation of NO, PGE₂, and IL-6 production. Interestingly, we found that although these concentration-dependent inhibitory actions of Δ⁹-THC are mimicked by the synthetic cannabinoid agonist, IMMA, they cannot be completely mimicked by the two endogenous cannabinoids, AEA and 2-AG. With respect to IL-6 production, all cannabinoids examined, without exception, concentration-dependently produced inhibitory action. Regarding iNOS-related NO production, Δ⁹-THC, AEA, and IMMA caused inhibition, while a slight enhancement was observed for 2-AG. In contrast to the inhibition of COX-2 induction by Δ⁹-THC, AEA, and 2-AG did not elicit a similar effect.

The distinct effects of AEA and 2-AG as compared to those of Δ⁹-THC and IMMA might depend on their structural similarity to AA, which provides their susceptibilities for eicosanoid enzyme metabolism and produces pharmacological actions through eicosanoid receptor interaction. Indeed, accumulating studies have identified some bioactive metabolites of AEA and 2-AG in different cell types. AEA can be metabolized to many products, such as PGE₂-ethanolamide, 12(S)-HAEA, and 15(S)-HAEA, through many enzymes [Yu et al., 1997; Edgemon et al., 1998]. 2-AG also can rapidly be metabolized to AA [Goparaju et al., 1998, 1999; Di Marzo et al., 1999], which is further converted to PGE₂ through COX, or to HPETE(s) and leukotrienes by lipoxygenase [Di Marzo et al., 1999]. In this study, pharmacological evidence supports the notion that AA and its downstream COX, but not lipoxygenase, metabolites participate in the actions of 2-AG. First, the iNOS-dependent NO potentiation induced by 2-AG was mimicked by exogenous AA and PGE₂. This study supports our previous report demonstrating that PGE₂ is able to potentiate LPS-elicited macrophage iNOS induction through a PKA-dependent mechanism [Lin et al., 1999]. Second, the lack of inhibition by 2-AG on COX-2 induction, as contrasted with the ability of Δ⁹-THC and IMMA to do so, might be explained by the enhancement ability of AA and PGE₂ on COX-2 induction, which counteracts the inhibitory outcome ascribed from CB2 activation. Third, iNOS potentiation and IL-6 inhibition elicited by exogenous AA co-incubation were abolished by the presence of the COX-1 inhibitor, valeryl salicylate, instead of the lipoxygenase inhibitors, MK886, baicalein, and NDGA. Our present data do not seem to support the possible involvement of the metabolite, PGE₂-ethanolamide, in AEA action, because exogenous PGE₂-ethanolamide did not affect NO or IL-6 production by LPS. Nonetheless, at present, we still cannot explain the ineffectiveness of AEA on LPS-induced iNOS induction and NO production. We can not rule out whether other unidentified mechanisms or metabolites underlie the cumulative effects of AEA.

In summary, cannabinoids mediate the inhibition of LPS-induced NO, PGE₂, and IL-6 release in J774 macrophages through CB2 activation. In addition to acting as a CB2 activator, endogenous cannabinoid 2-AG itself serves as the AA precursor, whose metabolism by COX and generation of PGE₂ participates in the positive modulation of iNOS and COX-2 induction.

REFERENCES


