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Tetrahydrocannabinol Inhibition of Macrophage Nitric Oxide Production*

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ABSTRACT. Δ^9-Tetrahydrocannabinol (THC) inhibited nitric oxide ($NO^{\cdot}$) production by mouse peritoneal macrophages activated by bacterial endotoxin lipopolysaccharide (LPS) and interferon-γ (IFN)-γ). Inhibition of $NO^{\cdot}$ production was noted at THC concentrations as low as 0.5 μg/mL, and was nearly total at 7 μg/mL. Inhibition was greatest if THC was added 1–4 hr before induction of nitric oxide synthase (NOS) by LPS and IFN-γ, and declined with time after addition of the inducing agents. This suggested that an early step such as NOS gene transcription or NOS synthesis, rather than NOS activity, was affected by THC. Steady-state levels of mRNA for NOS were not affected by THC. In contrast, protein synthesis was inhibited as indicated by immunoblotting. NOS activity was also decreased in the cytosol of cells pretreated with THC. Addition of excess cofactors did not restore activity. Inhibition of $NO^{\cdot}$ production was greater at low levels of IFN-γ, indicating the ability of the cytokine to overcome inhibition. The effectiveness of various THC analogues, in decreasing order of potency, was Δ^8-THC > Δ^9-THC > cannabidiol ≥ 11-OH-THC > cannabinol. The presumably inactive stereoisomer, (+)Δ^9-THC, and the endogenous ligand for cannabinoid receptors, anandamide, were weakly inhibitory. Inhibition may be mediated by a process that depends partly on stereoselective receptors and partly on a nonselective process. LPS, IFN-γ, hormone receptor agonists, and forskolin increased macrophage cyclic AMP levels. THC inhibited this increase, indicating functional cannabinoid receptors. Addition of 8-bromo-cyclic AMP increased $NO^{\cdot}$ 2-fold, and partially restored $NO^{\cdot}$ production that had been inhibited by THC. This occurred only under conditions of limited NOS induction, suggesting that the effect of THC on cyclic AMP was responsible for only a small portion of the inhibition of $NO^{\cdot}$ production. BIOCHEM PHARMACOL 52;5:743–751, 1996.

KEY WORDS. cyclic AMP; lipopolysaccharide; interferon-γ; cannabinoid; anandamide

THC‖ is the major psychoactive component of marijuana. It is being used to reduce nausea in cancer and AIDS patients undergoing chemotherapy [1] and is in limited trials for alleviating glaucoma and hypertension [2]. However, THC can be immunosuppressive [3], especially when combined with viral infections [4]. THC down-regulates the production of interferons [3, 5], the cytokines that constitute the chief defense against viruses. In addition, mice treated with THC are much more susceptible to infections with bacteria such as *Escherichia coli* [6] or *Legionella pneumophila* [7].

THC inhibits the activities of lymphocytes [8], natural killer cells [9], and macrophages [10, 11]. The capacity of mouse [12] and human [13] macrophages to spread on glass and to phagocytize yeast particles is inhibited significantly by THC. Treatment of mice with THC results in the reduced ability of subsequently harvested macrophages to kill amoebae, tumor cells, and virus-infected cells [10]. This is due, in part, to inhibition by THC of the production of TNF [14].

Another important product of activated macrophages that is cytotoxic for many parasites and certain cancer cells is $NO^{\cdot}$. $NO^{\cdot}$ and L-citrulline are formed from L-arginine by NOS, an enzyme that is potently induced in macrophages by a combination of IFN-γ and bacterial LPS [15, 16]. Macrophage NOS binds calmodulin tightly and requires NADPH, FAD, FMN, and H_4B.

Because of the increasing interest in the clinical uses of THC, often in immunologically compromised individuals,

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‖ *Abbreviations:* BMG, β_2-microglobulin; cAMP, adenosine 3′,5′-monophosphate; GCH, GTP cyclohydrolase I; H_4B, tetrahydrobiopterin; HB, homogenizing buffer; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NECA, 5′-(*N*-ethylcarboxamido)adenosine; $NO^{\cdot}$, nitric oxide; NOS, nitric oxide synthase; RT-PCR, reverse transcriptase-polymerase chain reaction; TG, thioglycollate; THC, Δ^9-tetrahydrocannabinol (unless another isomer or enantiomer is specified); and TNF, tumor necrosis factor-α.

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it is important to understand its effects on all aspects of the immune system. In this paper, we present evidence that THC inhibits induction of NO· generation by murine macrophages.

MATERIALS AND METHODS

Materials

All chemicals were from the Sigma Chemical Co., St. Louis, MO, unless stated otherwise. Δ^8-THC, cannabinol, and cannabidiol also were obtained from Sigma; (−)Δ^9-THC, (+)Δ^9-THC and 11-hydroxy-THC were received as ethanol solutions from the Research Technology Branch, National Institute of Drug Abuse; and anandamide was obtained from Biomol Research Laboratories Inc., Plymouth Meeting, PA. On the day of use, ethanol was evaporated from an aliquot of the stock solution with a stream of nitrogen gas, and THC was suspended in DMSO at a concentration of 20 mg/mL and then further diluted with the cell culture medium. Other cannabinoids were similarly dissolved in DMSO.

Macrophages

Female BALB/C mice were obtained from Jackson Laboratories, Bar Harbor, ME, and fed Purina mouse pellets and water *ad lib*. Peritoneal macrophages were harvested without elicitation (resident cells) or 4 days after thioglycollate administration (elicited cells) from mice aged 8–12 weeks (adult) or 2–3 weeks (young). Peritoneal resident cells were approximately 50% macrophages, whereas elicited cells were about 95% macrophages (esterase positivity); the harvested cells were used without further purification. Macrophages were incubated in 96-well flat-bottom plates (Costar) at 0.5×10^6 macrophages/mL in RPMI 1640 medium with 100 U/mL penicillin, 100 μg/mL streptomycin, 10^{-5} M 2-mercaptoethanol, and 5% fetal bovine serum. Cells were incubated with THC or analogues (final concentration, 0.5 to 7 μg/mL) for various times, followed by LPS (*E. coli* strain O26B6, 1 μg/mL) and recombinant murine IFN-γ (Genzyme, Cambridge, MA, 0.001 to 10 U/mL). Incubation was continued for 24 hr. Viability was assessed by the MTT assay [17].

Nitrite

Reactions were terminated by addition of an equal volume of the Griess reagent (naphthylethylene diamine · 2HCl, 0.05%; sulfanilamide, 0.5%; H_3PO_4, 2.5%) according to Green *et al.* [18], and nitrite, derived from NO·, was estimated spectrophotometrically at 540 nm in a Titertek Multi-Scan MC340 MKII semi-automatic plate reader (ICN). All incubation conditions were performed in replicates of 3–4. Results are expressed as micromolar nitrite in the 100 μL incubation volume or, to compare the effects of THC and other agents, simply as percent of control values. Statistical evaluations were performed by Student's unpaired, two-tailed *t*-test.

cAMP

Macrophages (0.5×10^6/mL) were incubated overnight in 48-well plates as described above. The medium was replaced with Hanks' balanced salt solution (prewarmed to 37°), the phosphodiesterase type IV inhibitor rolipram (50 μM) was added for 20 min, and then other reagents were added. After a further 20-min incubation, perchloric acid (0.2 M final concentration) was added, and the plates were frozen. cAMP was measured in supernatants after alumina column separation and acetylation, by the radioimmunoassay as described [19].

Gene Activation

RNA isolation from macrophages and performance of quantitative RT-PCR were performed as described previously [20]. Macrophages (3×10^6) were incubated with THC, IFN-γ, and LPS as above for times ranging from 1 to 12 hr. Total RNA was isolated by the single step method with TRI REAGENT (Molecular Research Center, Cincinnati, OH). Reverse transcription of total RNA was performed with avian myeloblastosis virus RT in a commercial reaction mixture (Reverse Transcription System, Promega, Madison, WI). The cDNAs were subjected to PCR with two primer sets, BMG-specific primers as endogenous standard and inducible macrophage NOS-specific or mouse GCH-specific primers, in the same tube. BMG and GCH primers were synthesized on a DNA synthesizer (PS250; Cruachem, Dulles, VA) with published sequences [21, 22]. Macrophage NOS primers were purchased from Clontech, Palo Alto, CA. The sequence of PCR amplification was 30 cycles of denaturation at 94° for 45 sec, annealing at 60° for 45 sec, and extension at 72° for 1.5 min. The PCR products were quantified by high-performance liquid chromatography with a TSK DEAE-NPR column (TosoHass, Montgomeryville, PA). Average elution times for NOS, GCH, and BMG were 8.36, 11.58, and 9.65 min, respectively. Peak absorbance (260 nm) was computer analyzed and normalized relative to the BMG amplicon, which was coamplified in each sample.

Immunoblotting

Macrophages (10×10^6) were incubated for 2 hr at 1×10^6 cells/mL with or without THC, and then with LPS and IFN-γ for 20 hr. Adhered cells were washed with warm PBS, chilled, scraped with a small volume of cold PBS, and centrifuged for 5 min at 1000 *g*. Cells were washed with cold PBS, and aliquots were removed for protein determination [23]. Cells were resuspended in 2% SDS-containing "electrophoresis sample buffer" (Transduction Laboratories, Lexington, KY), and aliquots representing 50 μg protein (about 50 μL) were electrophoresed on 7.5% poly-

acrylamide gels using a mini-gel apparatus (Bio-Rad Laboratories, Hercules, CA) according to the directions of the manufacturer. Gels were transferred to nitrocellulose membranes and incubated with anti-mouse macrophage NOS monoclonal antibody (Transduction Laboratories) according to instructions, washed, and developed with goat anti-mouse IgG-alkaline phosphatase conjugate (Bio-Rad).

NOS

Macrophages (20×10^6) were incubated, washed, chilled, scraped, and centrifuged as described above. Cells were resuspended in 1.3 mL HB: 50 mM HEPES · Na^+, pH 7.5, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μM leupeptin. Cells were sonicated with a Kontes Ultrasonic Cell Disruptor for 25 sec at a power of 6.5 and centrifuged at 44,000 *g* for 30 min at 4°. Supernatants (100 μL, approximately 50 μg protein) were incubated with the following reagents, all dissolved in HB: 0–50 μM H_4B (Bio-Mol), 20–400 μM NADPH, 5 μM FAD, and 10 μM (0.5 μCi) L-[^{14}]arginine (New England Nuclear, Boston, MA) in a total volume of 200 μl for 2 hr at 37°. Reactions were terminated with 0.75 mL "stop solution" (100 mM NaOAc, pH 5.5, 1 mM L-citrulline, 1 mM N^G-nitro-L-arginine, 2 mM EDTA) and passed through a 1-mL packed column of 200–400 mesh Dowex-50-Na^+ [24]. The eluate and 2×1 mL H_2O washes containing [^{14}C]-citrulline were collected, mixed with 19 mL scintillation fluid (Universol), and counted in a Beckman liquid scintillation counter. Blank values from samples lacking enzyme were subtracted.

RESULTS

THC Inhibition of NO· Production

Inhibition of NO· production by THC was concentration dependent (Fig. 1). The THC solvent DMSO (0.005 to 0.035%, equivalent to the concentration present with 1–7 μg/mL THC) had no effect on NO·. Nitrite produced after 24 hr of incubation of 50×10^4 macrophages in 0.1 mL containing 10 U/mL IFN-γ and 1 μg/mL LPS was greater for adult TG-elicited macrophages than for adult resident macrophages. There were no apparent differences in NO· produced by TG-elicited macrophages from adults compared with young mice. Resident macrophages from young mice were too few in number to compare with resident cells from adult mice. TG-elicited macrophages from young mice and adult resident macrophages appeared to be somewhat more sensitive to THC than elicited cells from adult mice, but the differences were not statistically significant. Inhibition was greater than 80% at 7 μg/mL THC. Based on both trypan blue uptake experiments and the MTT test [17], this level of THC was not toxic to macrophages during a 4-day incubation in 5% serum. Concentrations exceeding 7 μg/mL were associated with a small degree of cytotoxicity, so in subsequent experiments the maximum concentration of THC was 5 μg/mL. All further experiments were performed with adult TG-elicited macrophages.

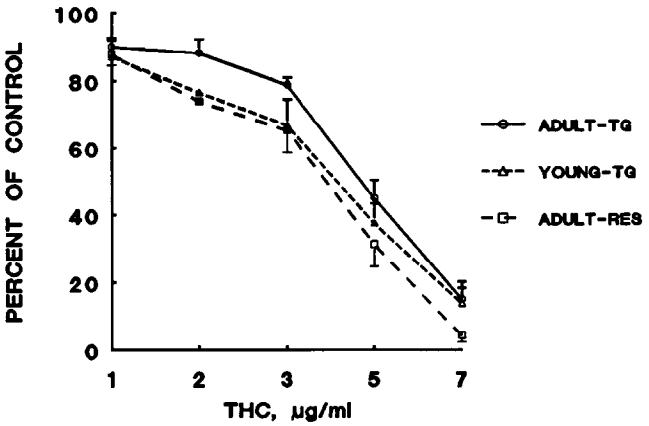

FIG. 1. THC inhibition of NO· production: concentration–response to THC. THC was added to mouse macrophages 2 hr before the NO· inducing agents (IFN-γ, 10 U/mL, +LPS, 1 μg/mL). Incubation was continued for another 24 hr, and nitrite was assayed as described in Materials and Methods. Key: (○) adult mouse TG-elicited macrophages, 6 experiments; (□) adult mouse resident macrophages, 4 experiments; and (△) young mouse TG-elicited macrophages, 4 experiments. Data are presented as percent of control nitrite values, which were 55.3 ± 4.0, 20.9 ± 2.8, and 55.6 ± 5.3 μM, respectively. In this and other figures, error bars represent SEM. All THC-treated cells produced significantly less NO· than controls ($P < 0.05$) except for the 1 μg/mL treatment of the young mouse cells ($P = 0.064$).

Timing of THC Treatment

Macrophage NOS mRNA is induced by LPS and IFN-γ; NO· appears at about 6 hr and the rate of its production remains almost constant for at least 48 hr [16]. Inhibition was greatest if THC was added 1–4 hr before the inducing agents (Fig. 2). Earlier times of THC addition such as 8–12 hr were less effective (not shown), and THC added 2 or more hr after the NOS induction period had diminishing effects. This suggested that THC directly or indirectly inhibits an early step such as NOS gene activation or enzyme synthesis rather than enzyme activity.

Apparent Reversal of Inhibition by IFN-γ

The ability of THC to inhibit production of NO· was increased greatly in conditions of limited NOS induction. IFN-γ concentrations ranging from 0.01 to 10 U/mL and a constant 1 μg LPS were added to macrophages 2 hr after incubating cells with THC, 5 μg/mL (Fig. 3). The effect of THC ranged from 37% inhibition at the highest concentration of IFN-γ to 92% at the lowest concentration (Fig. 4). THC concentration–responses are shown as percent of control values for IFN-γ concentrations of 0.01 to 10 U/mL. Significant inhibition (25%, $P < 0.05$) by 0.5 μg/mL THC was observed when the lowest concentration of IFN-γ was used. These results can be interpreted as a partial

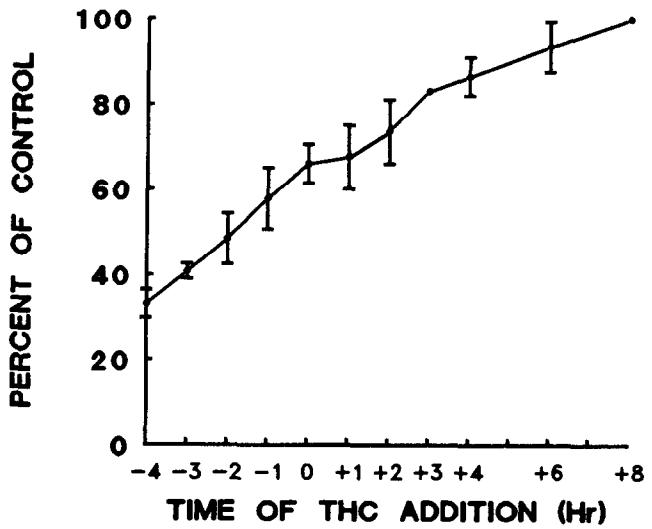

FIG. 2. THC inhibition of NO· production: Variation of time of THC addition. The results of 4 separate experiments are shown in which 5 μg/mL THC was added at various times before or after the inducing agents (LPS, 1 μg/mL, +IFN-γ, 10 U/mL, added at 0 hr). Data are presented as percent of control nitrite, which was 55.3 ± 4.7 μM. In two experiments (data not shown), 5 μg/mL THC was added at 8 and 12 hr before LPS and IFN-γ, causing a mean of 53 ± 10 and 29 ± 12% inhibition, respectively. In this and the following figures, experiments were performed with adult mouse TG-elicited macrophages.

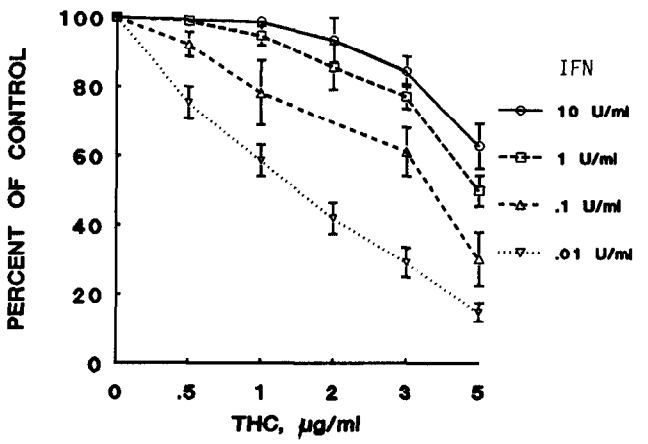

FIG. 4. Effects of variation of THC and IFN-γ concentrations on inhibition of NO· production. Macrophages were incubated with THC, 0.5 to 5 μg/mL for 2 hr, followed by LPS and IFN-γ as above. Results are shown as percent of control for 6–8 experiments. Control values (mean μM nitrite ± SEM) were 20.9 ± 1.7 μM (0.01 U/mL IFN-γ), 35.8 ± 1.6 μM (0.1 U/mL IFN-γ), 48.5 ± 2.0 μM (1.0 U/mL IFN-γ), and 54.9 ± 3.1 μM (10 U/mL IFN-γ). All values for cells treated with THC were significantly ($P < 0.05$) different from controls except those treated with 0.5 μg/mL THC and IFN-γ greater than 0.01 U/mL, or 1 μg/mL THC and IFN-γ greater than 0.1 U/mL.

reversal by IFN-γ of inhibition caused by THC. A similar effect was not observed when the concentration of LPS was varied (data not shown).

Levels of NOS and GCH mRNA

Because of the greater inhibition by THC when added before NOS induction, it was reasonable to expect an effect on induction of the mRNA for either NOS or GCH (the rate-limiting enzyme for H_4B synthesis). Using the quantitative RT-PCR/HPLC technique [20], we found that THC had no significant effect on steady-state levels of either of these mRNAs at 3, 6, or 12 hr of incubation with LPS and IFN-γ (Table 1).

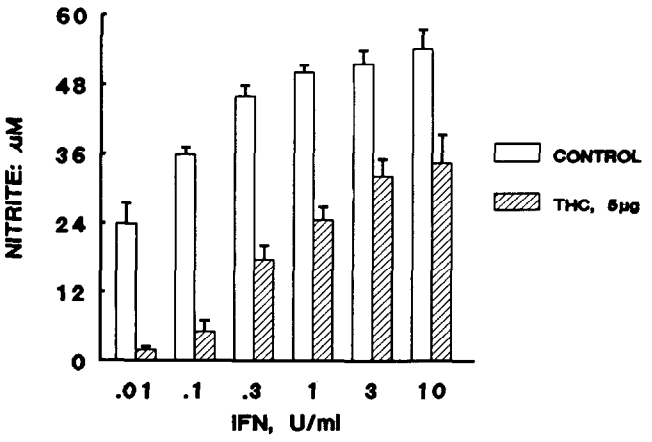

FIG. 3. Effects of variation of IFN-γ concentration on THC inhibition of NO· production. Macrophages were incubated with THC, 5 μg/mL, for 2 hr. LPS (1 μg/mL) and various concentrations of IFN-γ were then added for 24 hr. The results of 4 experiments are shown.

NOS

To determine whether THC pretreatment causes reduced NOS activity or simply affects NO· production in intact cells, NOS was measured in the cytosol of cells pretreated with THC (Table 2). Preliminary experiments established

TABLE 1. Effect of THC on NOS and GCH mRNA levels

		NOS mRNA levels		GCH mRNA levels	
Expt.	hr	Control	THC	Control	THC
1	1	<0.01	<0.01	1.43	1.42
	3	0.56	0.67	1.62	1.38
	6	1.49	1.29	1.93	1.60
	12	1.60	1.69	1.82	1.63
2	1	0.12	<0.01	ND*	ND
	3	1.29	1.03	1.81	2.07
	6	1.70	1.28	1.84	1.91
	12	1.59	1.74	1.70	2.01

Macrophages were treated with 5 μg/mL THC for 2 hr, and then with IFN-γ (1 U/mL) and LPS (1 μg/mL) for the times indicated. Total RNA was extracted and the levels of mRNAs were measured by quantitative RT-PCR as described in Materials and Methods. Levels of mRNA are presented as the ratios of target HPLC peak areas for reverse-transcribed mRNA of the enzymes NOS or GCH to that of the relatively constant BMG. Results of two separate experiments are shown.

* Not determined.

TABLE 2. Nitric oxide synthase

NADPH (μM)	H_4B (μM)	NOS activity Control (pmol/min/mg)	THC = 3 μg/mL (pmol/min/mg)	(% C)	THC = 5 μg/ml (pmol/min/mg)	(% C)
200	0	3.02 ± 0.17	2.60 ± 0.72	86	2.07 ± 0.34	69
200	10	6.22 ± 0.11	4.47 ± 0.93	72	3.21 ± 0.60	52
200	25	6.69 ± 0.30	4.39 ± 0.16	66	3.56 ± 0.30	53
200	50	6.57 ± 0.69	4.22 ± 0.19	64	3.41 ± 0.22	52
0	50	1.89 ± 0.06	1.82 ± 0.52	96	1.68 ± 0.34	89
20	50	4.92 ± 0.36	3.41 ± 0.05	69	2.69 ± 0.32	55
50	50	6.42 ± 0.82	4.61 ± 0.10	72	3.58 ± 0.11	56
100	50	6.60 ± 0.65	4.73 ± 0.30	72	3.36 ± 0.09	51
200	50	6.78 ± 0.48	4.86 ± 0.45	72	3.47 ± 0.19	51
500	50	7.64 ± 0.51	5.66 ± 0.80	74	4.41 ± 0.10	58
			Average:	74		59

Macrophages (25×10^6) were treated with 0, 3, or 5 μg THC/mL in 75 cm^2 plates (12.5 mL/plate) for 2 hr, and then with 1 U/mL IFN-γ and 1 μg/mL LPS for 20 hr. The medium was removed, and adherent cells were washed with warm PBS, scraped with a small volume of ice-cold PBS, and centrifuged. The cells were washed with cold PBS and resuspended in 1.25 mL of homogenizing buffer; NOS activity was measured as described in Materials and Methods. Results (mean ± SEM) are expressed as picomoles arginine converted to citrulline per minute per milligram of cytosolic protein for three experiments, and as percent of control values obtained with cells pretreatment with THC.

optimal levels of NADPH, FAD, and H_4B as approximately 200, 5, and 50 μM, respectively. NOS activity was inhibited about 26% by pretreatment of cells with 3 μg/mL THC and about 41% by 5 μg/mL THC. The extent of inhibition was not altered significantly by varying the concentrations of NADPH or H_4B (shown) or FAD (not shown). Neither dithiothreitol (1 mM) nor calmodulin/Ca^{2+} (5 μM/100 μM) reversed the inhibition. THC had no effect on NOS when added directly to macrophage cytosol.

Enzyme Transcription

Since THC pretreatment reduced NOS activity but did not affect NOS or GCH mRNA induction, immunoblotting experiments were performed to test whether THC affects protein synthesis (Fig. 5). There was no significant difference in total protein content in the various preparations. Using 0.1 U/mL IFN-γ, THC (5 μg/mL) diminished the density of the NOS band corresponding to about 130 kDa. With 1 U/mL IFN-γ, the 130 kDa bands were much darker, and the effect of THC was not as great.

Analogues of THC

The effects of (+)Δ^9-THC and its psychoactive enantiomer (−)Δ^9-THC were compared at concentrations of 0.5 to 5 μg/mL in Fig. 6. While (+)Δ^9-THC was nearly as efficacious as (−)Δ^9-THC at 5 μg/mL, (+)Δ^9-THC inhibited only 8% at 1 μg/mL compared with 30% for (−)Δ^9-THC. Several other cannabinoid analogues of Δ^9-THC were compared at 3 μg/mL in Fig. 7. Small differences in the inhibitory potency of Δ^9-THC, Δ^8-THC, 11-OH-THC, and cannabidiol were observed to depend somewhat on the concentration of IFN-γ. Cannabinol and the (+)Δ^9-THC enantiomer were considerably less inhibitory than the other agents. Anandamide, an endogenous derivative of arachidonic acid that mimics Δ^9-THC actions in brain [25], also inhibited NO· production but was much less potent than Δ^9-THC (Fig. 7).

cAMP

THC reduces basal as well as stimulated cAMP levels in lymphoid [26] and other [27] cells by a receptor-mediated activation of G_i. We found that THC also prevented stimulation of macrophage cAMP by several agonists (Table 3). Incubation overnight with IFN-γ and LPS increased the ability of macrophages to produce cAMP by about 10-fold.

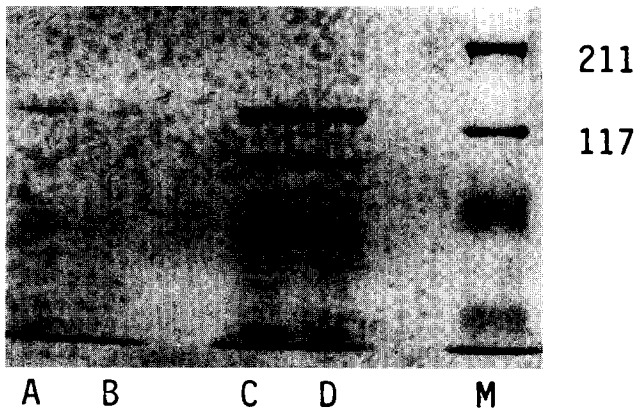

FIG. 5. NOS immunoblot. Macrophages were treated for 2 hr with or without THC, 5 μg/mL, followed by 1 μg/mL LPS and 0.1 or 1 U/mL IFN-γ for 20 hr. Lanes A and C: no THC; lanes A and B: IFN-γ = 0.1 U/mL; lanes C and D: IFN-γ = 1 U/mL; M = molecular weight markers. The NOS monomer band (130 kDa) appears between the 117 kDa β-galactosidase and the 211 kDa myosin markers. This experiment was repeated with similar results.

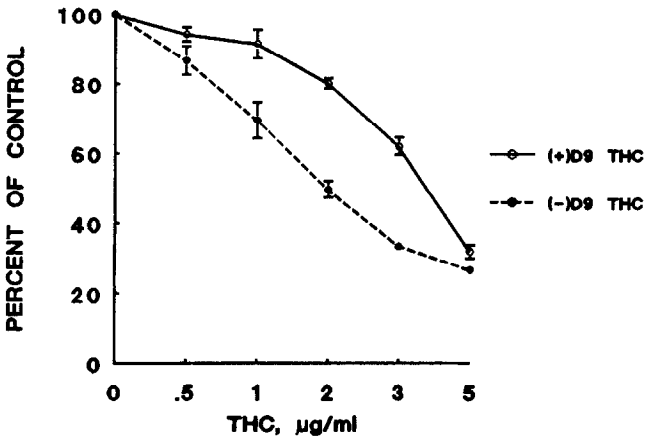

FIG. 6. Comparison of NO· inhibition by (+)Δ^9-THC and (-)Δ^9THC. Macrophages were incubated with 0.5 to 5 μg (-)Δ^9THC [denoted as (-)D9-THC] or the (+)-enantiomer [denoted as (+)D9-THC] for 2 hr, followed by 0.01 U/mL IFN-γ and 1 μg LPS for 24 hr. NO· was assayed as described in Materials and Methods. Control nitrite values were 22.5 μM. Results are means of two experiments and represent the mean ± range.

THC (5 μg/mL) pretreatment reduced this by 40%. The adenosine A2-receptor agonist NECA, the β-adrenergic receptor agonist isoproterenol, and the non-receptor agonist forskolin increased cAMP by 15-, 19-, and 5-fold, respectively. Overnight incubation with IFN-γ and LPS augmented cAMP another 2- to 5-fold. THC pretreatment inhibited the cAMP increases evoked by each of these agents to about the same extent, irrespective of whether the cells had been stimulated by IFN-γ and LPS.

In attempts to overcome THC inhibition of NO· production, 8-Br-cAMP was added. 8-Br-cAMP (1 mM) augmented NO· production by a factor ranging from 3.2 (when IFN-γ was 0.001 U/mL) to 1.4 (when IFN-γ was 10 U/mL). Although THC pretreatment decreased NO· production, the relative decrease was not as great in the presence of 8-Br-cAMP (Fig. 8). This relationship was statistically significant only at moderate NO· production levels, obtained with 0.01 U/mL IFN-γ or less, and only at THC concentrations of 1 μg/mL or less. The results suggest that the effect of THC to diminish NO· production by a cAMP-mediated mechanism applies only to these limiting conditions.

DISCUSSION

The present experiments clearly show that THC inhibits NO· production by mouse macrophages. The inhibition was concentration-dependent, and maximal if added 1–4 hr prior to the inducing agents LPS and IFN-γ. Inhibition was negligible if THC was added 6–8 hr after the inducing agents, a time when the rate of NO· production reaches a maximum and becomes constant [16, 24]. These data suggest that THC interferes with induction of the NOS gene, or a subsequent step in protein synthesis or processing, rather than with NOS activity *per se*. However, the measurement of mRNA levels indicated that THC had a minimal effect on the steady-state levels of NOS mRNA. Macrophage incubation times with inducing agents were varied from 1 to 12 hr in order to bracket the time of mRNA induction. THC also had little effect on mRNA of GCH, the rate-limiting enzyme for the synthesis of the cofactor H_4B. Furthermore, addition of excess H_4B did not restore NO· production by macrophages pretreated with THC.

Immunoblots with mAb to mouse macrophage inducible NOS indicated a large inhibition by 5 μg/mL THC of NOS synthesis if IFN-γ was 0.1 U/mL, and a smaller decrease in NOS if IFN-γ was 1 U/mL. These effects are qualitatively, but not quantitatively consistent with the NO· data, since 5 μg/mL THC inhibited NO· production by 86% at 0.1 U/mL IFN-γ and 50% at 1 U/mL IFN-γ (Fig. 3). However, THC inhibition of NOS synthesis is consistent with Cabral and Fischer-Stenger [28], who reported THC inhibition of induction of several macrophage proteins.

Burnette-Curley *et al.* [10] administered THC to mice and observed no change in the ability of subsequently isolated macrophages to attach to their targets (amoebae, parasites, or tumor cells). They also reported that macrophages of treated mice responded normally to activation by bacterial products with a sharp decline of ecto-5′-nucleotidase activity. They concluded that THC treatment, which severely reduced cytotoxic capabilities, affected a late, but not an early step of macrophage activation. If protein synthesis can be considered a late event compared with message induction, our results are consistent with their conclusion.

Inhibition of NO· production by THC was maximal at the lowest levels of IFN-γ, and was reversed, in part, by increasing the concentration of this cytokine. This suggests that THC may interfere specifically and reversibly with some signal evoked by IFN-γ. It also indicates that macrophages are not damaged by exposure to the concentrations of THC used here, in agreement with our microscopic observations and with data of others [10, 12, 13].

THC affects cells by both cannabinoid receptor-mediated and non-receptor pathways [3, 27]. A well-established receptor-mediated pathway involves the inhibition of adenylate cyclase, and this is mediated by the pertussis toxin-inhibitable GTP binding protein, G_i, in cells of the immune system [26] as well as other systems [29]. Kaminski *et al.* [30] identified stereospecific cannabinoid receptors on mouse spleen cells, and showed greater immunosuppression as well as binding of (-)-enantiomers compared to the (+)-enantiomers. They [31] showed that dibutyryl cAMP reverses the immunosuppression by THC. We found that IFN-γ and LPS caused large increases of macrophage cAMP synthesis, as did NECA, isoproterenol, and forskolin. THC significantly inhibited these cAMP increases. We also noted the ability of 8-Br-cAMP to augment NO· produc-

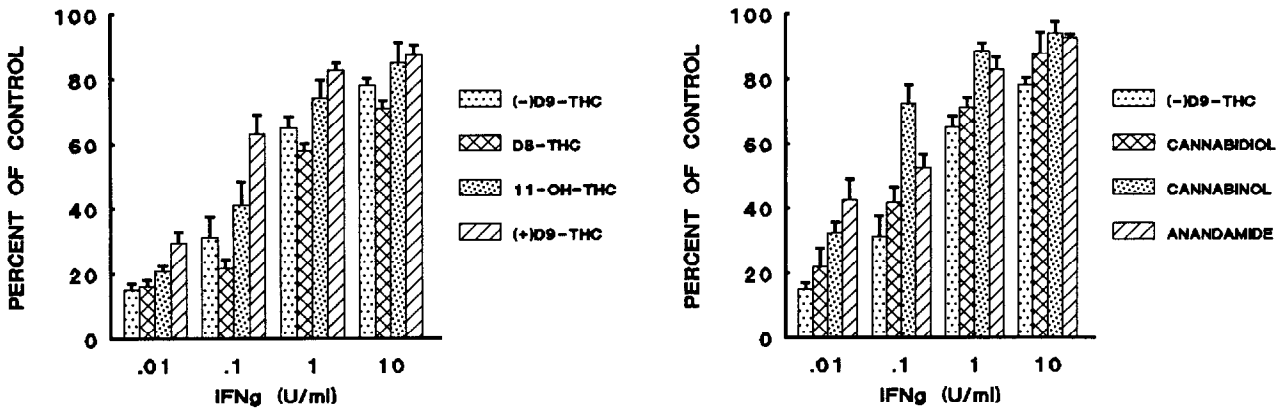

FIG. 7. Comparison of NO· inhibition by several cannabinoids and anandamide. Macrophages were incubated with 3 μg/mL THC and analogues for 2 hr, followed by 1 μg/mL LPS and various concentrations of IFN-γ for 24 hr. Results are means of two experiments and represent the mean ± range. Control values (mean μM nitrite ± difference from mean) were: 17.4 ± 2.2 μM (0.01 U/mL IFN-γ), 40.1 ± 3.1 μM (0.1 U/mL IFN-γ), 48.2 ± 3.1 μM (1.0 U/mL IFN-γ), and 54.8 ± 4.8 μM (10 U/mL IFN-γ).

tion, in agreement with several reports involving vascular smooth muscle cells [32], Kupffer cells [33], and macrophages [34]. However, 8-Br-cAMP was only marginally successful in reversing THC inhibition of NO· production. This indicates that the predominant effects of THC on NO· are not mediated by a receptor-linked reduction of cellular cAMP. Other candidates for a mediator of THC effects include arachidonic acid [35, 36] and hydroxyeicosatetraenoic acid (12-HETE) [37], which are increased markedly in other cells incubated with THC. However, we were unable to demonstrate NO· inhibitory effects of 0.1 to 10 μM arachidonic acid, 12(R)-HETE, or 12(S)-HETE in macrophages (data not shown).

To further probe the possibility of a receptor mechanism, several analogues of THC were used. The putatively inactive (+)Δ^9-THC enantiomer was much less inhibitory than the psychoactive (−)Δ^9-THC at low concentrations, supporting a role for stereospecific receptors. Since the purity of the compounds is uncertain, a portion of the activity of the (+)Δ^9-THC enantiomer could be attributed to contamination by the (−)-enantiomer. Anandamide, or arachidonic acid ethanolamide, is an endogenous mammalian compound that mimics the actions of THC in brain and competes with THC for binding to the brain CB1 receptor [25, 38]. Anandamide was a much less effective inhibitor than THC, possibly because of enzymatic degradation, although incubation with 1 mM phenylmethylsulfonyl fluoride, which inhibits anandamide hydrolysis in other systems, did not enhance its efficacy in macrophages (data not shown). Alternatively, anandamide may be less effective than Δ^9-THC because the peripheral THC receptor (termed CB2 or CX5) has a lower affinity for anandamide [38]. Munro *et al.* [39] characterized this peripheral receptor in the human promyelocytic cell line HL-60 and expressed it in COS cells. Relative binding affinities were 11-OH- > cannabinol > Δ^9-THC > anandamide > cannabidiol. By contrast, we observed relative inhibitory potencies of Δ^8-THC > Δ^9-THC > cannabidiol = Δ^{11}-THC > cannabinol. Differences in efficacies among these analogues were small, except for the relatively weak cannabinol. If the present results reflect affinities for the THC receptor, it is apparent that the mouse macrophage THC receptor is quite different from the promyelocytic HL-60 receptor.

IFN-γ and LPS, added separately, induced a small increase in mRNA for NOS, and the two agents acted synergistically to induce NOS in macrophages. Other cyto-

TABLE 3. Effect of THC on cAMP

	cAMP (fmol/10^6 cells)				Ratio +/− THC	
THC:	−	+	−	+		
IFN-γ:	−	−	+	+	−	+
Control	83 ± 13	84 ± 63	838 ± 63	501 ± 19	1.01	0.60
NECA	1275 ± 198	868 ± 100	4000 ± 452	3290 ± 148	0.69	0.82
Isoproterenol	1596 ± 10	1198 ± 45	2968 ± 196	2105 ± 248	0.75	0.71
Forskolin	414 ± 177	348 ± 28	2207 ± 11	1744 ± 46	0.81	0.80

Macrophages were treated with or without 5 μg/mL THC for 2 hr, and then with or without IFN-γ (1 U/mL) and LPS (1 μg/mL) for 20 hr. Cells were then washed and treated with NECA (100 μM), isoproterenol (10 μM), or forskolin (200 μM) for 20 min in the presence of 50 μM rolipram, and assays for cAMP were performed as described in Materials and Methods. Results are means ± SD for a single experiment with replicates of 3. Similar results were obtained in another experiment.

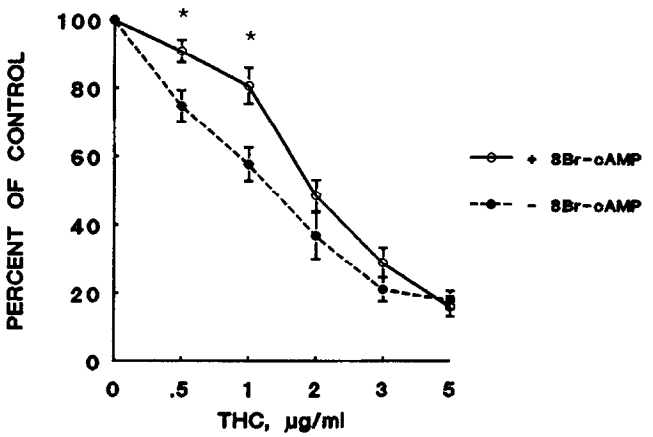

FIG. 8. Effect of 8-Br-cAMP on inhibition of NO· production by THC. Macrophages were incubated with or without THC for 2 hr. IFN-γ (0.01 U/mL), LPS (1 µg/mL), and 8-Br-cAMP (1 mM) were added and incubation was continued for another 24 hr. Data represent the mean percentages of control for 3 experiments ± SEM. Mean control values in the absence and presence of 8-Br-cAMP were 65 and 152 µM nitrite, respectively. Key: (*) $P < 0.05$.

kines including TNF, interleukin 1, and GM-CSF can induce NOS in hepatocytes, smooth muscle cells, and mesangial cells [40]. NO· is a free radical that is highly toxic to many malignant cells as well as bacterial and protozoal parasites. The levels of induced NO· are quite large, and account for much of the ability of macrophages to kill many parasites, bacteria, virus-infected cells, and tumor cells [16, 40]. Low levels of NO· may augment immune responses via stimulation of guanosine 3′,5′-cyclic monophosphate [41, 42]. However, high levels of NO· have undesirable effects, including the inhibition of lymphocyte responses [43] and autoimmune destruction of many cells such as pancreatic islets [44].

By inhibiting NO· production, THC would be expected to reduce host resistance to a variety of infections. MacMicking *et al.* [45] found greatly increased susceptibility to infections and cancer in mice lacking the gene for inducible NOS. Since cancer and AIDS are associated with high incidence of infections, the additional immunosuppressive effects of THC administration to these patients should be considered. THC promotes a relative reduction in CD4 lymphocytes [46] and would, therefore, reduce production of interleukin-2 and IFN-γ. Patients infected with retroviruses would thus be less able to produce IFN-γ, and consequently less able to enlist the NO· defense against infectious agents. The use of THC would be predicted to further compromise the ability of such patients to resist infection. Our preliminary results do suggest, however, that the concomitant administration of IFN-γ could, in part, overcome the inhibition caused by THC. In certain circumstances, THC could conceivably be beneficial. It could, for example, limit tissue destruction caused by NO· in autoimmune diseases and arthritis [47]. In the absence of clinical studies, however, no firm conclusions regarding the effect of THC on NO· production by human cells can be drawn from the data presented here.

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