

# Mediation of Cannabidiol Anti-inflammation in the Retina by Equilibrative Nucleoside Transporter and A<sub>2A</sub> Adenosine Receptor

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**PURPOSE.** Cannabidiol (CBD), a nonpsychotropic, nontoxic compound has been shown to block diabetes- and endotoxin-induced retinal damage. However, the protective mechanism of this anti-inflammatory cannabinoid is not completely understood. The goal of this study is to determine the role of adenosine signaling in retinal inflammation and its potential modulation by CBD.

**METHODS.** The adenosine receptor (AR) subtypes expressed in rat retinal microglial cells were assessed by quantitative real-time RT-PCR. AR function was determined via in vitro and in vivo inflammatory models. Microglial cells or rats were treated with or without lipopolysaccharide (LPS) in the presence or absence of adenosine, adenosine receptor agonists/antagonists, or CBD. Adenosine uptake and tumor necrosis factor (TNF)- $\alpha$  release in cells or in retinas were determined.

**RESULTS.** The results showed that A<sub>2A</sub>ARs are abundantly expressed in rat retinal microglial cells. When the cells or rats were treated with LPS, activation of the A<sub>2A</sub>AR was the most efficient in mediating AR agonist- or CBD-induced TNF- $\alpha$  inhibition. CBD inhibited adenosine uptake via equilibrative nucleoside transporter 1 and synergistically enhanced adenosine's TNF- $\alpha$  suppression after treatment with LPS.

**CONCLUSIONS.** These results suggest that the activated A<sub>2A</sub>AR in the retinal microglial cells plays a major anti-inflammatory role in the retina and that CBD's anti-inflammatory effects are linked to the inhibition of adenosine uptake. (*Invest Ophthalmol Vis Sci.* 2008;49:5526–5531) DOI:10.1167/iops.08-2196

Retinal neuroinflammation is the common pathway in infectious and autoimmune retinitis, glaucoma, age-related macular degeneration, ischemic retinopathy in diabetic retinopathy and retinal vein occlusions.<sup>1–6</sup> Cytokine expression is induced via lipid mediators such as platelet activating factor

and leukotrienes, oxygen-derived free radicals, complement components, and debris from dead or dying cells.<sup>7</sup> Proinflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1, and IL-6, are generated in tissues under infectious and ischemic insult, which serves to promote wound healing and clearance of necrotic tissue. However, excessive or chronic inflammation, especially in the context of neural tissue, leads to propagation of tissue injury and is the target for therapy in a variety of retinal diseases.<sup>4</sup>

Activated retinal microglia and macrophages are implicit in the pathogenesis of sight-threatening disease but are difficult to study in vivo due to their migratory behavior and morphologic transformation.<sup>8</sup> Cultured retinal microglia activated by lipopolysaccharide (LPS) have been used to study microglial behavior as an in vitro model of neuroinflammation.<sup>9</sup> Studies have shown that after neuroinflammation through brain injury, activated microglia release proinflammatory cytokines, such as TNF- $\alpha$ , that instigate a cascade of inflammation and neurodegeneration.<sup>10</sup> We hypothesize that activated microglia in the diseased retina play a role similar to that of activated microglia in the brain.

Adenosine, a purine nucleoside, regulates a variety of physiological functions by stimulating specific extracellular receptors.<sup>11</sup> Under adverse conditions such as inflammation, adenosine production by damaged neurons is increased and helps to protect tissue against excessive damage.<sup>12</sup> Adenosine delivers potent suppressive effects on virtually all cells of the immune system by interacting with four subtypes of adenosine receptors (ARs): A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>.<sup>13</sup> The G-protein-coupled A<sub>2A</sub>AR is most widely recognized as attenuating inflammation via a cAMP-mediated pathway, and its activation leads to inhibition of T-cell expansion and differentiation, downregulation of neutrophil superoxide production and degranulation, and inhibition of proinflammatory cytokine expression, including TNF- $\alpha$ .<sup>12</sup>

However, adenosine usually disappears very rapidly under physiological conditions, in part, due to rapid uptake into adjacent cells and subsequent intracellular metabolism,<sup>14</sup> preventing it from interacting with immune cell adenosine receptors and preventing sustained tissue protection. One way of retarding the disappearance of adenosine is by introducing nucleoside transporter inhibitors.<sup>15</sup>

It has recently been shown that nanomolar concentrations of cannabidiol (CBD), a nonpsychotropic and nontoxic cannabinoid, inhibit uptake of adenosine by nucleoside transporters in both murine microglia and RAW264.7 macrophages. Moreover, in vivo treatment with a low dose of CBD was reported to decrease serum TNF- $\alpha$  levels in LPS-treated mice. This effect of CBD was reversed by treatment with the A<sub>2A</sub>AR antagonist ZM 241385 and was completely absent in A<sub>2A</sub>AR<sup>-/-</sup> mice.<sup>16</sup> These studies demonstrated that CBD enhances adenosine signaling by inhibiting its extracellular removal, revealing a non-cannabinoid receptor-mediated mechanism by which CBD decreases inflammation. Our previous work has shown that CBD inhibits glutamate-, LPS-, and diabetes-induced retinal inflam-

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mation (El-Remessy AB, et al., manuscript submitted).<sup>5,17</sup> However, whether CBD inhibits these inflammatory diseases by blocking adenosine uptake has not been studied to our knowledge. The goal of this study was to determine whether adenosine mitigates the release of TNF- $\alpha$  in activated retinal microglial cells and to determine whether CBD curtails inflammation via enhancement of adenosine concentrations.

## METHODS

### Animal Treatment

All animal studies were performed in accordance with the National Institutes for Health Guide for the Use and Care of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats (200–225 g) were pretreated IP for 1 hour with CBD [Cayman Chemical, Ann Arbor, MI] or vehicle, dimethylsulfoxide (DMSO) (a solution of CBD or DMSO/Cremophor [BASF Pharma, Florham Park, NJ]/saline at a ratio of 1:1:18). After 30 minutes, the rats were given 8-cyclopentyl-1,3-dipropylxanthine (CPX; Sigma-Aldrich, St. Louis, MO), 4-[2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM 241385; Tocris Bioscience, Ellisville, MO), or vehicle IP (CPX or ZM or control DMSO, Cremophor, and saline at a ratio of 1:1:8). After drug pretreatment, the rats were anesthetized with isoflurane and given a single LPS (*Salmonella enterica*; Sigma-Aldrich) injection at 0.35 mg/kg. After 24 hours, the rats were killed by decapitation after anesthesia with isoflurane. Retinas and vitreous were collected for assay of total protein (DC protein assay; Bio-Rad, Hercules, CA) and for TNF- $\alpha$  levels by enzyme-linked immunosorbent assay (ELISA).

### Primary Rat Retinal Microglia Cultures

Microglial cells were isolated from retinas of newborn (within 24 hours) rats as described previously, with minor modifications (El-Remessy AB, et al., manuscript submitted).<sup>9</sup> Briefly, retinas were collected and washed twice with ice-cold PBS and digested with 0.125% trypsin at 37°C for 3 minutes. Trypsin was inactivated by adding culture medium (DMEM/F12 (1:1) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin). The tissues were triturated with a plastic pipette and washed twice. The cells were filtered through a nylon mesh (mesh opening, 100  $\mu$ m; Nitex Sheets; Sefar America Inc., Kansas City, MO), collected by centrifugation, resuspended in culture medium, and plated onto 100-cm<sup>2</sup> cell culture flasks at a density of  $2 \times 10^5$  cells/cm<sup>2</sup>. All cultures were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub> and fed on the third day, then once every 4 days. After 2 weeks, microglial cells were harvested in culture medium by shaking the flasks at 100 rpm for 1.5 hours. The cell suspension was centrifuged and the detached cells replated in culture medium at designated densities for various experiments. The purity of the microglial cultures was ~98%, as determined by immunocytochemical staining analysis for OX42, a macrophage/microglial marker. The morphology of microglia in culture was carefully examined by phase-contrast and fluorescence microscopy (El-Remessy AB, et al., manuscript submitted).

### Quantitative Real-Time RT-PCR

Total RNA was isolated from rat retinal microglial cells (RNAqueous 4PCR; Ambion, Austin, TX). Subsequently, 1  $\mu$ g of total RNA was reverse transcribed by using a mixture of random and poly-T primers according to the manufacturer's protocol (Invitrogen). Primers were designed for the rat A<sub>1</sub> (FWD, 5'-TCATTGCCCTTGGTCTCTG-3'; REV, 5'-GTGGAAGTAGGCTGTGG-3'), A<sub>2A</sub> (FWD 5'-TGCTTCCATCATGTCCTTGC-3'; REV, 5'-GCCCTTCGCTTCATACC-3'), A<sub>2B</sub> (FWD, 5'-TTGGCATTTGGATTGACTC-3'; REV, 5'-TATGAGCAGTGGAGGAAG-3'), and A<sub>3AR</sub> (FWD, 5'-TGCTTCCATCATGTCCTTGC-3'; REV, 5'-TGCTTCCATCATGTCCTTGC-3') on computer (Beacon Design software; Bio-Rad). PCR amplification (in SYBR Green Supermix) was performed using a

thermocycler (iCycler iQ; Bio-Rad) for 40 cycles of 25 seconds at 95°C followed by 45 seconds at an optimized annealing temperature for each AR. The cycle threshold, determined as the initial increase in fluorescence above background, was ascertained for each sample. Melting curves were calculated on completion of the cycles to ensure that nonspecific products were absent. For quantification of AR transcripts, a standard curve plotting cycle threshold versus copy number was constructed for each receptor subtype by analyzing 10-fold serial dilutions of plasmids containing the full-length rat AR clones.<sup>18</sup> AR transcript levels were expressed as copies per 50 ng of total RNA.

### Cell Treatment

Microglial cells collected from culture flasks were seeded at a density of  $1 \times 10^5$  cells/well in 96-well plates. One day after seeding, the culture wells were washed (Cellgro Complete; Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with 1  $\mu$ M CBD or vehicle dimethylsulfoxide (DMSO) for 30 minutes at 37°C, followed by adenosine (Sigma-Aldrich), AR agonists (all from Sigma-Aldrich), or AR antagonists (all from Sigma-Aldrich except ZM 241385) at the indicated concentrations for 30 minutes at 37°C, finally treated with 30 ng/mL LPS (*Escherichia coli* OB4:1111; Sigma-Aldrich). After the indicated time course, culture media were collected and assayed for TNF- $\alpha$  by ELISA. Subsequently, the cells were lysed with 0.4 N NaOH and assayed for total protein by protein assay (DC; Bio-Rad).

### Adenosine Uptake Assays

Assays were performed at 37°C in Earle's normal balanced salt solution (EBSS). Cells seeded at a density of  $5 \times 10^5$  cells/well in 24-well plates were washed once in EBSS and preincubated for 30 minutes at 37°C with drug or DMSO vehicle. Uptake began after addition of 0.5  $\mu$ Ci [<sup>3</sup>H]adenosine (37 MBq; GE Healthcare, Chalfont St. Giles, UK). Nonspecific uptake was defined as uptake in the presence of 1 mM adenosine, which was added 1 minute before [<sup>3</sup>H]adenosine. After 1 minute, buffer was rapidly aspirated, and cells were washed once with ice-cold PBS. The cells were solubilized in 0.2 M NaOH/1% SDS, and radioactivity was determined.

### Enzyme-Linked Immunosorbent Assay (ELISA) for TNF- $\alpha$

TNF- $\alpha$  levels in the supernatants of culture media were estimated with ELISA kits (R&D Systems, Minneapolis, MN) per the manufacturer's instructions. Briefly, diluted samples were added to a microplate pre-coated with a monoclonal antibody specific for rat TNF- $\alpha$ . After washing to remove unbound substances, an enzyme-linked polyclonal antibody specific for TNF- $\alpha$  was added. After a wash to remove unbound antibody-enzyme reagent, a substrate solution was added. The colored product yielded by the enzyme reaction was measured at 450 nm. The minimum detectable level for TNF- $\alpha$  with this assay is 5 pg/mL. The sample levels were calculated from a standard curve. Subsequently, the cells were lysed with 0.4 N NaOH and assayed for total protein by the protein assay (DC; Bio-Rad). TNF- $\alpha$  release was expressed, after adjustment for protein, as a percentage of the maximum TNF- $\alpha$  released from vehicle-treated cells. Retinal microglial cells were treated with LPS, and culture media were collected at 5, 15, 20, and 30 minutes and 1, 2, 4, 6, 12, and 24 hours after treatment and assayed for TNF- $\alpha$ . Levels of TNF- $\alpha$  began to increase significantly after 4 hours of LPS treatment, peaking at hour 6, then slowly leveling off within a 24-hour period. The levels of TNF- $\alpha$  in control culture medium without LPS did not change throughout the experimental period (El-Remessy AB, et al., manuscript submitted).

Retinal and vitreous lysates from rat eyes were used for TNF- $\alpha$  ELISA, according to the manufacturer's instructions (R&D Systems), with modifications. Pooled retinas and vitreous from each animal were placed in 150  $\mu$ L of lysis buffer (20 mM imidazole HCl, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 1% Triton X-100, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, and 1 mM EDTA [pH 6.8])<sup>19</sup> supplemented with a protease inhibitor

cocktail (Sigma-Aldrich) followed by homogenization (Mini-Bead Beater; Biospec Products, Inc., Bartlesville, OK) with treated Ottawa sand.<sup>5</sup> The lysate was cleared of debris by centrifugation at 10,000 *g* for 15 minutes (4°C), and 50  $\mu$ L of the supernatant was used for ELISA directly without further dilution. The sample TNF- $\alpha$  levels were calculated from a standard curve, corrected for protein concentration, and expressed as a percentage of maximum TNF- $\alpha$  released from vehicle- and LPS-treated animals.

### Data Analysis

The results are expressed as the mean  $\pm$  SEM. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by Fisher's PLSD post hoc test when indicated. Significance was defined as  $P < 0.05$ .

## RESULTS

### AR Expression in Retinal Microglial Cells

We first quantified mRNA expression of the four AR subtypes by real-time RT-PCR. The absolute copy numbers of the AR transcripts were calculated based on standard curves generated with AR cDNA clones.<sup>18</sup> As illustrated in Figure 1, we detected abundant mRNA expression of A<sub>2A</sub>AR (940  $\pm$  95 copies/50 ng of RNA) mRNA in rat retinal microglial cells. mRNA expression of A<sub>1</sub>AR, A<sub>2B</sub>AR, or A<sub>3</sub>AR mRNA were barely detectable above background levels.

### A<sub>2A</sub>AR Regulation of TNF- $\alpha$ in Retinal Microglial Cells

To identify the AR subtype(s) involved in inhibiting TNF- $\alpha$  release in the retinal microglia in response to LPS stimulation, we examined the effect of the nonselective AR agonist adenosine-5'-*N*-ethylcarboxamide (NECA) in the presence of AR subtype-selective antagonists. The concentrations of each antagonist chosen for this study were based on the affinity and selectivity for the recombinant mouse AR subtypes determined by radioligand binding studies.<sup>18</sup> As shown in Figure 2A, cells pretreated with vehicle showed a 10-fold increase in LPS-induced TNF- $\alpha$  release compared with vehicle-treated control cells. Treatment with NECA at a concentration of 1  $\mu$ M potently inhibited LPS-stimulated TNF- $\alpha$  release (40%). When the cells were pretreated with the A<sub>1</sub>AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (CPX; 100 nM), the A<sub>2B</sub>AR antagonist 8-[4-(((4-cyanophenyl) arbamoylmethyl) oxy)phenyl]-1,3-

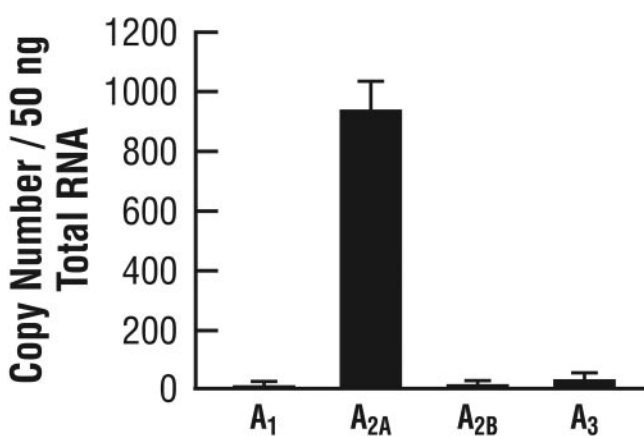


FIGURE 1. Expression of AR subtypes in retinal microglial cells. Expression levels of mRNA of the four AR subtypes in rat retinal microglia, as quantified by real-time RT-PCR ( $n = 4-5$ ).

di(*n*-propyl) xanthine hydrate (MRS 1754; 1  $\mu$ M), or the A<sub>3</sub>AR antagonist 3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine-carboxylate (MRS 1523; 10  $\mu$ M), the inhibitory effect of NECA on TNF- $\alpha$  release was not affected. However, this effect was successfully blocked by 4-[2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3- $\alpha$ ][1,3,5]triazin-5-ylamino]ethyl]phenol (ZM 241385) at concentrations (100 and 500 nM) capable of blocking A<sub>2A</sub>ARs. These results suggest that NECA inhibited LPS-induced TNF- $\alpha$  release from rat retinal microglia via the A<sub>2A</sub>AR.

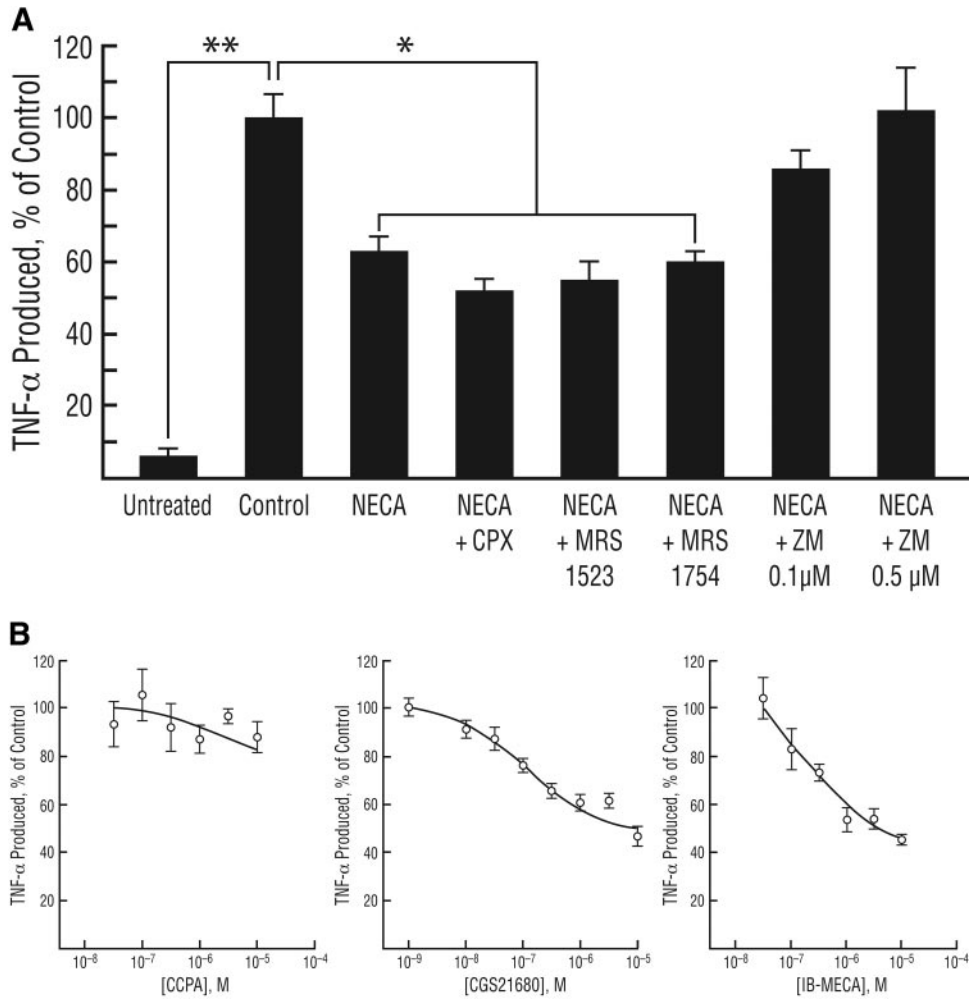
To confirm the role of the A<sub>2A</sub>AR in regulating TNF- $\alpha$  release, we compared concentration-response curves generated with the A<sub>1</sub>AR agonist 2-chloro-*N*<sup>6</sup>-cyclopentyladenosine (CCPA), the A<sub>2A</sub>AR agonist 2-*p*-[2-carboxyethyl]phenethyl-amino-5'-*N*-ethylcarboxamidoadenosine (CGS 21680), and the A<sub>3</sub>AR agonist *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA). As revealed in Figure 2B, treatment with CGS 21680 concentration-dependently inhibited LPS-stimulated TNF- $\alpha$  release. In contrast, IB-MECA and CCPA inhibited TNF- $\alpha$  release, but their potencies were low, reflecting their low affinity for the A<sub>2A</sub>AR, although, it was noticed that the potencies of IB-MECA and CGS 21680 were very similar. This similarity was probably because the A<sub>3</sub>AR-selective IB-MECA can inhibit TNF- $\alpha$  release via the A<sub>2A</sub> and/or A<sub>2B</sub>AR in A<sub>2A</sub>AR-dominant systems.<sup>18</sup> These results suggest a TNF- $\alpha$  release regulatory effect mediated by the A<sub>2A</sub>AR.

### CBD Effects on Adenosine Uptake in Retinal Microglial Cells

CBD has the ability to enhance adenosine signaling through competitive inhibition of adenosine uptake by equilibrative nucleoside transporter (ENT) 1 in murine microglia and macrophages.<sup>16</sup> To determine whether ENT1, which is *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR) sensitive, is expressed in retinal microglial cells, we demonstrated NBMPR sensitivity in these cells. [<sup>3</sup>H]adenosine uptake assays were performed in cells in the presence of 0.1  $\mu$ M NBMPR. To specifically examine inward [<sup>3</sup>H]adenosine transport, we recorded uptake during the first minute after addition of [<sup>3</sup>H]adenosine. As shown in Figure 3, [<sup>3</sup>H]adenosine uptake was partially inhibited by NBMPR, suggesting the presence of ENT1 in these cells. To determine whether CBD inhibits adenosine uptake in retinal microglial cells via ENT1, we determined the effect of CBD on [<sup>3</sup>H]adenosine transport in the presence of 0, 0.02, 0.1, and 0.5  $\mu$ M NBMPR. NBMPR alone dose-dependently inhibited [<sup>3</sup>H]adenosine transport. CBD at 0.5  $\mu$ M also inhibited [<sup>3</sup>H]adenosine uptake. NBMPR at all levels did not enhance this inhibition, suggesting CBD is competing with NBMPR for ENT1 (Fig. 3). To determine whether this inhibitory effect is dependent on known cannabinoid receptors, HU 210, a synthetic cannabinoid agonist that has an affinity for CB<sub>1</sub> and CB<sub>2</sub> receptors in the picomolar range<sup>20</sup> was tested for its ability to decrease [<sup>3</sup>H]adenosine uptake. HU 210 at 1  $\mu$ M did not inhibit [<sup>3</sup>H]adenosine uptake (Fig. 3). These data suggest an adenosine uptake inhibitory effect of CBD that is independent of known cannabinoid receptors.

### Adenosine Mediation of CBD's Effects on TNF- $\alpha$ in Retinal Microglial Cells

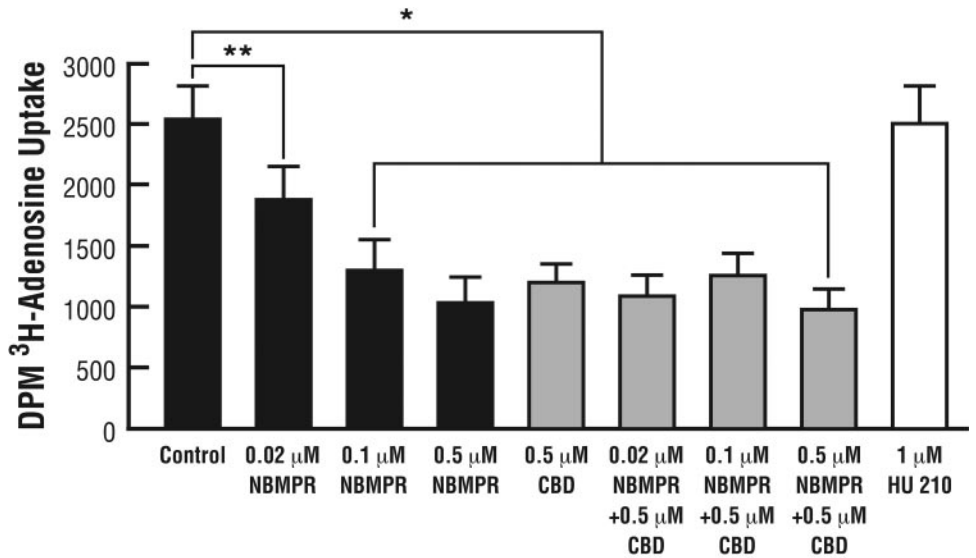
We tested the hypothesis that the TNF- $\alpha$ -inhibiting effect of CBD is mediated through enhancement of adenosine, by comparing concentration-response curves generated with CBD or adenosine alone and with the two reagents combined. Cells that were pretreated with adenosine alone from 1.5 to 100  $\mu$ M followed by LPS showed a gradual decrease in TNF- $\alpha$  from 100% to 50% (Fig. 4). Cells that were pretreated with CBD alone from 0.03 to 1  $\mu$ M followed by LPS showed a more



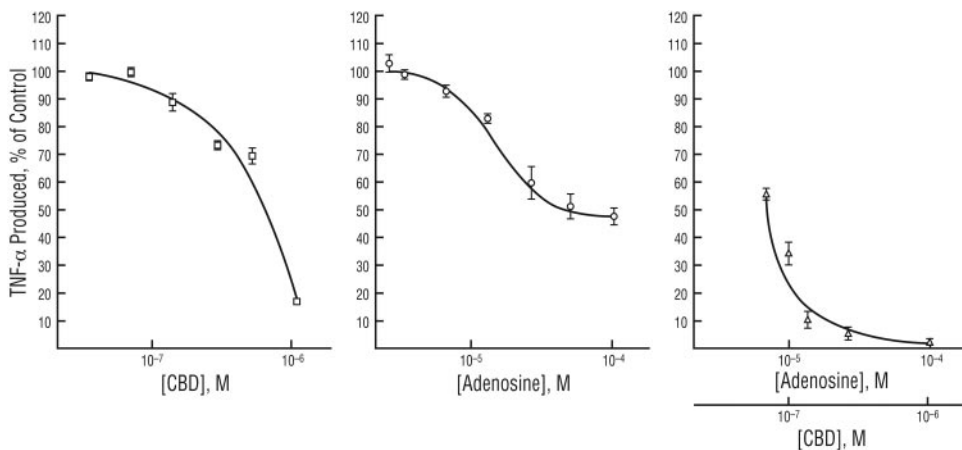
**FIGURE 2.** Functional identification of AR subtypes in retinal microglial cells. TNF-α production was determined in LPS-treated (30 ng/mL, 12 hours) retinal microglial cells. (A) In the presence of nonselective AR agonist NECA (1 μM) and subtype-selective AR antagonists for A<sub>1</sub>AR (CPX, 100 nM), A<sub>2A</sub>AR (ZM 241385, 100 and 500 nM), A<sub>2B</sub>AR (MRS 1754, 1 μM), and A<sub>3</sub>AR (MRS 1523, 10 μM). TNF-α levels were determined by ELISA (n = 4–8). \*\*P < 0.005, \*P < 0.05, compared with the vehicle control (one-way ANOVA followed by the post hoc Fisher PLSD). (B) In the presence of various concentrations of subtype-selective AR agonists: A<sub>1</sub>AR, CCPA; A<sub>2A</sub>AR, CGS 21680; and A<sub>3</sub>AR, IB-MECA.

drastic decrease in TNF-α from 100% to 20%. The drastic TNF-α-reducing effect by CBD at or near 1 μM may be due to CBD's antioxidant properties that enable it to scavenge oxygen radicals.<sup>21</sup> Of interest, cells that were cotreated with adenosine from 6 to 100 μM and CBD from 0.06 to 1 μM showed a decrease in TNF-α that was much more drastic than the effect

of either agent alone. This effect is particularly obvious at low concentrations of adenosine and CBD where the TNF-α-inhibiting effect was not observed for either reagent alone (Fig. 4). Taken together, these results suggest that by inhibiting adenosine uptake, CBD synergistically enhances adenosine's TNF-α suppression on LPS treatment.



**FIGURE 3.** CBD or NBMPR, but not HU 210, inhibited [<sup>3</sup>H]adenosine uptake in retinal microglial cells. Cells were pretreated with NBMPR (0, 0.02, 0.1, and 0.5 μM), CBD (0.5 μM), NBMPR combined with CBD, or HU 210 (1 μM) for 30 minutes at 37°C, and uptake of 0.5 μCi [<sup>3</sup>H]adenosine over a period of 1 minute was assayed. Nonspecific uptake, determined in the presence of 1 mM adenosine, was subtracted from each data point (n ≥ 5). \*P < 0.005, \*\*P < 0.05, compared with the vehicle control (one-way ANOVA followed by the post hoc Fisher PLSD).



**FIGURE 4.** CBD enhanced adenosine's ability to inhibit TNF- $\alpha$ . Retinal microglial cells were pretreated with CBD (0.03–1  $\mu$ M) or vehicle for 0.5 hour. The cells were then treated with vehicle or adenosine (1.5–100  $\mu$ M) for another 0.5 hour. Last, the cells were treated with LPS (30 ng/mL). Twelve hours after LPS treatment, the culture media were collected. TNF- $\alpha$  levels were determined by ELISA ( $n = 4-8$ ).

### Adenosine Mediation of CBD's Effects on TNF- $\alpha$ in the Retina

Adenosine uptake inhibitors decrease TNF- $\alpha$  in LPS-treated mice by increasing the amount of endogenous adenosine available to bind the  $A_{2A}$ AR.<sup>15</sup> Because CBD inhibits adenosine uptake, we hypothesized that the  $A_{2A}$ AR mediates TNF- $\alpha$  inhibition by CBD (El-Remessy AB, et al., manuscript submitted).<sup>5</sup> Rats were pretreated with CBD (1 mg/kg, IP) and with the  $A_1$ AR antagonist CPX (3 mg/kg, IP) or the  $A_{2A}$ AR antagonist ZM 241385 (10 mg/kg, IP) before LPS treatment (0.35 mg/kg, footpad). CBD-pretreated rats showed significantly decreased retinal TNF- $\alpha$  levels, compared with vehicle-pretreated rats (Fig. 5). Although this decrease remained unchanged on co-treatment with CPX, pretreatment with ZM 241385 reversed the effects of CBD on TNF- $\alpha$  (Fig. 5). Neither antagonist alone significantly altered the effect of LPS to increase TNF- $\alpha$  levels

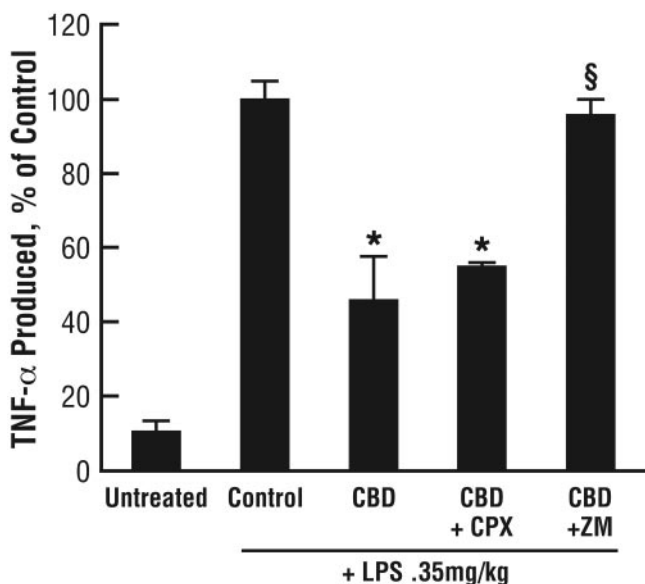
(data not shown). These results correlate with the in vitro data obtained with microglial cells, showing that CBD enhances the ability of adenosine to inhibit LPS-induced TNF- $\alpha$  release via the  $A_{2A}$ AR.

### DISCUSSION

Inflammation-mediated neurodegeneration is of utmost clinical relevance. Inflammation in neural tissues involves production of reactive oxygen species that stimulate cellular release of proinflammatory cytokines. We studied the mechanisms for the activation of LPS-treated animals and retinal microglial cells as a model to simulate neuroinflammation because, in uveitis or diabetes, retinal microglia activated by reactive oxygen species release proinflammatory cytokines causing neurodegeneration and vascular permeability.<sup>8</sup> Adenosine has been shown to mitigate the proinflammatory cytokine release response in central neural tissue. A more complete understanding of adenosine receptor function in the retina should help develop novel therapeutic approaches to treat retinal disorders that are associated with inflammation.

CBD has been shown to block NMDA-, LPS-, or diabetes-induced retinal damage (El-Remessy AB, et al., manuscript submitted),<sup>5,17</sup> but the mechanism of protection is not completely understood. It has recently been demonstrated in macrophage/microglial cell lines and in LPS-treated mice that CBD may function by enhancing adenosine signaling.<sup>16</sup> In the present study, we demonstrated for the first time that CBD functions by a similar mechanism in the retina. In this study, we observed that the  $A_{2A}$ AR is highly expressed in retinal microglial cells and that activation of this receptor inhibits TNF- $\alpha$  production in response to LPS. CBD inhibited adenosine uptake in retinal microglial cells and synergistically enhanced adenosine's effect in suppressing LPS-induced TNF- $\alpha$  release. CBD has been shown to competitively inhibit adenosine uptake by ENT1, which is NBMPR sensitive.<sup>16</sup> We determined that adenosine transport in retinal microglia is partially NBMPR sensitive and that the combined effect of adenosine uptake inhibition by NBMPR and CBD is not additive. This finding suggests that CBD competitively inhibits adenosine transport via the ENT1 transporter in retinal microglial cells as well. In vivo, this inhibition increased the availability of endogenous adenosine to produce anti-inflammatory activity, because the effects of CBD on TNF- $\alpha$  production induced by systemic administration of LPS were blocked by an  $A_{2A}$  receptor antagonist. This mechanism unveils a likely explanation for the anti-inflammatory activity of CBD identified in other rat models (El-Remessy AB, et al., manuscript submitted).<sup>5</sup>

CBD may mitigate inflammation through other pathways as well as enhancing adenosine signaling. For example, CBD is



**FIGURE 5.** CBD decreases TNF- $\alpha$  via activation of  $A_{2A}$ AR in the retina. Male SD rats were pretreated before LPS injection with a single dose of CBD (1 mg/kg, IP) or vehicle. Thirty minutes later, the rats were given vehicle, CPX (3 mg/kg, IP), or ZM 241385 (ZM, 10 mg/kg, IP). The rats were treated with LPS (0.35 mg/kg, footpad) 1 hour after CBD injection. Twenty-four hours after LPS treatment, the rats were killed, and the retina and vitreous were collected. TNF- $\alpha$  levels were determined by ELISA ( $n = 6$ ). \* $P < 0.05$  compared with vehicle control; § $P > 0.05$  compared with control and  $P < 0.05$  compared with CBD alone (one-way ANOVA followed by the post hoc test (Fisher's PLSD)).

known to scavenge reactive oxygen species.<sup>21</sup> Therefore, the anti-inflammatory effect of CBD, especially at 0.5 to 1  $\mu\text{M}$  or higher concentrations, may be due to its ability to block oxidative stress that causes microglial activation and inflammation.

Adenosine binds to four different subtypes of G-protein-coupled receptors ( $A_1$ ,  $A_{2A}$ ,  $A_{2B}$ , and  $A_3$  receptors) that are variably expressed in immune cells. Previous studies have shown that AR subtypes except the  $A_1$ AR are expressed in primary microglia and microglia cell lines.<sup>22,23</sup> In this study, the real-time RT-PCR and pharmacologic experiments with AR agonists as well as AR antagonists have shown that  $A_{2A}$ AR is the most likely candidate for mediating the adenosine effect on TNF- $\alpha$  suppression in retinal microglial cells.  $A_{2A}$ AR mediates the suppressive effects of adenosine in macrophages as well as microglial cells.<sup>18</sup> LPS treatment induces macrophage infiltration in the uveitic retina (El-Remessy AB, et al., manuscript submitted).<sup>24</sup> Therefore, the effects of CBD on TNF- $\alpha$  production in the eye may be due to enhanced  $A_{2A}$ AR signaling in infiltrated macrophages in the retina. This mechanism highlights the role of macrophages or activated retinal microglia in retinal inflammation.

Drugs that enhance extracellular adenosine signaling have been of clinical interest in treatment of inflammation after myocardial or cerebral ischemia.<sup>25,26</sup> CBD as an anti-inflammatory drug is an attractive alternative to smoking marijuana because of its lack of psychoactive effects.<sup>27</sup> CBD is known to be nontoxic in humans,<sup>28</sup> which has previously been a problem for other nucleoside inhibitor drugs.<sup>29,30</sup> Another potential problem with chronic consumption of adenosine transporter inhibitor is the development of tolerance due to cell tolerance of adenosine through receptor desensitization.<sup>31</sup> It is important to determine the long-term effects of CBD use on adenosine receptors.

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