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Anandamide inhibits IL-12p40 production by acting on the promoter repressor element GA-12: possible involvement of the COX-2 metabolite prostamide E_2

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The eCB [endoCB (cannabinoid)] system is being considered as a novel therapeutic target for immune disorders. Cytokines of the IL-12 (interleukin-12) family have essential functions in cell-mediated immunity. In the present study, we have addressed the mechanisms of action of the eCB AEA (anandamide) on the regulation of IL-12p40 in activated microglia/macrophages. We demonstrated that AEA can inhibit the expression of p35, p19 and p40 subunits, which form the biologically-active cytokines IL-12 and IL-23 in microglia stimulated with LPS (lipopolysaccharide)/IFNγ (interferon γ). Additionally, we have provided evidence that AEA reduces the transcriptional activity of the IL-12p40 gene in LPS- and IFNγ-co-activated cells, and this is independent of CB or vanilloid receptor activation. Site-directed mutageneis of the different elements of the p40 promoter showed that AEA regulates IL-12p40 expression by acting on the repressor site GA-12 (GATA sequence in IL-12 promoter). Prostamide E_2 (prostaglandin E_2 ethanolamide), a product considered to be a putative metabolite of AEA by COX-2 (cyclo-oxygenase 2) oxygenation, was also able to inhibit the activity of the IL-12p40 promoter by acting at the repressor site. The effects of AEA and prostamide E_2 on p40 transcription were partially reversed by an antagonist of EP_2 (prostanoid receptor-type 2), suggesting the possibility that prostamide E_2 may contribute to the effects of AEA on IL-12p40 gene regulation. Accordingly, the inhibition of COX-2 by NS-398 partially reversed the inhibitory effects of AEA on IL-12 p40. Overall, our findings provide new mechanistic insights into the activities of AEA in immune-related disorders, which may be relevant for the clinical management of such diseases.

Key words: anandamide (AEA), endocannabinoid, interleukin-12 (IL-12) p40 subunit, microglia, multiple sclerosis (MS), prostamide E_2.

INTRODUCTION

eCBs [endoCBs (cannabinoids)] are lipid signalling mediators that play an important role in immunomodulation, and have also been shown to be useful in the treatment of chronic inflammatory diseases [1,2]. Microglial cells constitute a population of 'facultative' macrophages adapted to the neural environment which play a critical role in CNS (central nervous system) immune surveillance and in the initiation and maintenance of intracerebral immune responses. Neurological disorders, such as MS (multiple sclerosis), involve activation and even dysregulation of microglial cells that, in addition to their phagocytic function, participate in the regulation of non-specific inflammation as well as in adaptive immune responses [3]. Both exogenous and endogenous CBs have been shown to be efficient in attenuating disease symptomatology and inflammatory reactions in chronic relapsing-remitting EAE (experimental autoimmune encephalomyelitis) and TMEV (Theiler's murine encephalomyelitis virus), which are experimental models of MS [4–9].

IL-12 (interleukin-12) is a heterodimeric cytokine formed from two subunits, p35 and p40, which regulates innate immunity and determines the type and duration of adaptive immunity [10]. IL-12 is produced mainly by monocytes, macrophages and dendritic cells, as well as brain microglia [11]. Besides forming the IL-12p70 heterodimer, the p35 and p40 subunits may dimerize with alternative partners to form distinct bimolecular complexes. For example, the p19 subunit, which lacks biological activity, combines with the p40 subunit to form IL-23, which regulates the clonal expansion of T-cells [12]. There is evidence suggesting that dysregulation of the IL-12 gene may contribute to the initiation and perpetuation of various autoimmune and chronic inflammatory diseases [13]. Despite the described reduction in IL-12 in *Legionella pneumoniae* infection after administration of the main psychoactive compound of *Cannabis sativa*, Δ^9-tetrahydrocannabinol, the mechanisms underlying this response are still unclear [14].

The eCB family contains several structurally-related lipids which may be synthesized and released by immune cells to act locally in an autocrine or paracrine manner [15]. CBs mainly act through two distinct G-protein-coupled receptors, CB_1 and CB_2. However, these CB molecules have been demonstrated to exert effects independently of CB_1 and CB_2 receptor activation. These effects have been proposed to be mediated through several unidentified receptors, or to occur in a membrane receptor-independent fashion [1]. Within the CNS, neurons, microglial cells and astrocytes are able to synthesize the eCBs AEA (anandamide) and 2-arachidonoylglycerol [16–18]. During neuroinflammation, eCBs are released mainly by microglial

Abbreviations used: AEA, anandamide; CB, cannabinoid; C/EBP, CCAAT/enhancer-binding protein; CNS, central nervous system; COX-2, cyclo-oxygenase 2; DMEM, Dulbecco's modified Eagle's medium; EAE, experimental autoimmune encephalomyelitis; eCB, endoCB; EP_2 etc., prostanoid receptor-type 2 etc.; ETS, E twenty-six; FAAH, fatty acid amidohydrolase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFNγ, interferon γ; IL-12 etc., interleukin-12 etc.; GA-12, GATA sequence in IL-12 promoter; LPS, lipopolysaccharide; MS, multiple sclerosis; NF-κB, nuclear factor κB; prostamide E_2, prostaglandin E_2 ethanolamide; RT–PCR, reverse transcription–PCR; SR1, SR141716A; SR2, SR144528; TMEV, Theiler's murine encephalomyelitis virus; TRPV, transient receptor potential vanilloid.

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cells and exert neuroprotective actions. In particular, AEA has been shown to protect neurons from inflammatory damage [19]. However, despite the therapeutic relevance of eCBs, the mechanisms of their actions on the immune system remain unknown, and studies on the role of AEA on microglial function are scarce. As a typical eCB, AEA is inactivated by a reuptake system and is metabolized mainly by FAAH (fatty acid amidohydrolase) [20]. In addition, AEA may also be oxygenated by COX-2 (cyclo-oxygenase 2) to form prostamide E_2 (prostaglandin E_2 ethanolamide) [21].

In the present study, we investigated the role of AEA in the regulation of IL-12 and related cytokines in activated macrophages and microglial cells. First, we assessed whether AEA modified the level of mRNA expression of the p19, p35 and p40 subunits of IL-12 and IL-23, as well as expression of IL12p40 protein in LPS (lipopolysaccharide)/IFNγ-activated microglial cells. We further analysed the regulation of IL-12p40 promoter activity by AEA using RAW 264.7 and BV-2 murine microglial cells as cellular models. We identified the response element for AEA action on the IL-12 p40 promoter. The involvement of CB and vanilloid receptors in the activity of AEA was also explored, as well as the putative involvement of the COX-2 pathway in modulation of the effects of AEA.

EXPERIMENTAL

Materials

Culture medium and FBS (fetal bovine serum) were from Invitrogen. AEA and LPS were from Sigma. Murine recombinant IFNγ (interferon γ) was from PeproTech EC (London, U.K.). The CB antagonists SR1 (SR141716A) [N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazol-carboxamide] and SR2 (SR144528) {N-(1S)-endo-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-5-(4-chloro-3methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide} were supplied by Sanofi Recherche (Montpellier, France). Prostamide E_2 and AH6809 (6-isopropoxy-9-oxoxanthene-2-carboxilic acid) were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). AH23848B was kindly supplied by GlaxoSmithKline (GSK). Capsazepine {N-[2-(4-chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2*H*-2-benzazepine-2-carbothionamide} was obtained from Alexis Biochemicals (Lausen, Switzerland). NS-398 was purchased from Calbiochem. All other reagents were obtained from standard suppliers.

Animals

Balb/c mice from our in-house colony (Cajal Institute, Madrid, Spain) were used. Animals were housed in cages with filter tops in a laminar flow hood and maintained on food and water *ad lib.* in a 12 h dark/light cycle. Handling of animals was performed in compliance with the Guidelines of Animal Care set by the European Union (86/609/EEC).

Cell line cultures

The murine macrophage cell line RAW 264.7 was purchased from the Center of Biological Sciences [CIB (Centro de Investigaciones Biológicas)–CSIC (Consejo Superior de Investigaciones Científicas), Madrid, Spain]. Cells were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 2 mM L-glutamine, 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin, and passaged every 5 days for a maximum of 30 passages. Cells were grown in a humidified environment containing 5 % CO_2 and at a constant temperature of 37 °C. BV-2 murine microglial cells were purchased from the ICLC (Interlab Cell Line Collection; Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy). These cells exhibit morphological and functional properties comparable with primary microglia.

Microglial cultures

Primary mixed glial cultures were prepared as described previously [22]. Briefly, forebrains of newborn Balb/c mice were dissociated mechanically, filtered through a 150 μm nylon mesh, resuspended in DMEM containing 10 % (v/v) heat-inactivated FBS, 10 % (v/v) horse serum and 1 % (w/v) penicillin/streptomycin and plated on poly-L-lysine-coated 75 cm^2 flasks (Falcon; Le Pont de Claix, France). After 15 days in culture, the flasks were shaken at 230 rev./min at 37 °C for 3 h to remove loosely adherent microglia. The supernatant was plated on multiwell culture plates for 2 h. After this, the medium was changed to remove non-adherent cells. Cells were grown in a humidified environment containing 5 % CO_2 and held at a constant temperature of 37 °C. The purity of microglial cultures was assessed by examining cell morphology under phase-contrast microscopy and was confirmed by immunostaining with anti-CD11b antibody (Serotec).

Cell treatment

Prior to each experiment, DMEM containing serum was removed and replaced with fresh serum-free DMEM. Microglia or cell lines were stimulated with both LPS (50 ng/ml) and IFNγ (100 units/ml) concurrently. The concentrations of the various treatments used in the experiments were: AEA (1, 5 and 10 μM), SR1 (1 μM), SR2 (1 μM), capsazepine (10 μM), prostamide E_2 (10 μM unless stated otherwise), NS-398 (10 μM), AH6809 (10 μM) and AH23848B (30 μM).

Plasmids

Plasmid constructs for the luciferase reporter vector were generated as described previously for the p40/pGL2B [23] and for the p40/pXP1 [24] constructs. Mutant p40/pXP1 plasmids were generated by site-directed mutagenesis as described by Becker et al. [24]. Mutant primer sequences are as follows (mutated bases are underlined): ETS (E twenty-six): 5′-CCCAAAAGTCATTAA-CTCTTAGTTC-3′; NF-κB (nuclear factor κB): 5′-GAACTT-CTTGAAATTAGCCCAGAAGG-3′; GA-12 (GATA sequence in IL-12 promoter) primer 1: 5′-CTCGTTTTTCTACACA-CAC-3′; GA-12 primer 3: 5′-CTCGTTTTGATACATCCAC-3′; and C/EBP (CCAAT/enhancer-binding protein): 5′-TGTTT-TCAATGTTCTAACAAGTCAGT-3′.

Transfections and reporter gene analysis

The p40/pGL2B or the p40/pXP1 firefly reporter vector (200 ng/cm^2) along with a *Renilla* luciferase expression vector (Promega) were transiently transfected into RAW 264.7 cells using Lipofectamine™ (Invitrogen) according to the manufacturer's instructions. After 18 h incubation, the medium was removed and replaced with fresh serum-free DMEM and, after a further 2 h incubation, cells were stimulated as described in each case. Stimulation was allowed to proceed for 18 h before cells were harvested, washed with 0.1 M PBS (pH 7.4) and lysed in cell lysis buffer (Promega). Luciferase activity (both firefly and *Renilla* luciferase activity) was evaluated using the Dual-Luciferase® reporter assay system (Promega), which allows

simultaneous expression and measurement of two individual reporter enzymes within a single system. Values were normalized to the *Renilla* luciferase activity (Promega). Thus the experimental reporter (firefly luciferase) was correlated with the effect of specific experimental conditions, whereas the activity of the co-transfected control (*Renilla* luciferase) reporter provided an internal control. Normalizing the activity of the experimental reporter to the activity of the internal control minimized experimental variability due to differences in cell toxicity, transfection efficiency and proliferation. Luciferase activity (firefly and *Renilla*) was mesaured as light emission over a period of 10 s with a standard luminometer (Sirius, Berthold Technologies, Bad Wildbad, Germany).

Decoy oligonucleotides assay

Decoy double-stranded oligonucleotides were transfected into RAW 264.7 cells in an attempt to interfere with the binding of LPS- and IFNγ-activated transcription factors to their *cis*-acting elements within the GA-12 sequence. The sequences of the oligonucleotides used are as follows: decoy GA-12 wild-type, 5′-CTCGTTTTGATACACACACAGAGA-3′; and decoy GA-12 mutant 1, 5′-CTCGTTTTTCTACACACACAGAGA-3′. These oligonucleotides were co-transfected into the RAW 264.7 cells together with the reporter constructs. After 18 h incubation, cell lysates were prepared and assayed for dual-luciferase activity as described above.

Semi-quantitative RT–PCR (reverse transcription–PCR)

Total RNA from microglial primary cultures was prepared using RNAeasy extraction columns (Qiagen). Samples (1 μg) of total mRNA were transcribed into cDNA using the Reverse Transcription system kit (Promega) according to the manufacturer's instructions. cDNA libraries were amplified by PCR with oligonucleotides for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), IL-12p35 (p35) subunit, IL-12/23p40 (p40) subunit and IL-23p19 (p19) subunit (PCR products of 800, 300, 250 and 190 bp respectively). The GAPDH primers sequences were: sense 5′-CATTGACCTCAACTACATGGT-3′ and antisense 5′-CATTGACCTCAACTACATGGT-3′. The p35 oligonucleotides were: sense 5′-ATGACCCTGTGCCTTGGTAG-3′ and antisense 5′-CAGATAGCCCATCACCCTGT-3′. The p40 oligonucleotides were: sense 5′-AGGTGCGTTCCTCGTAGAGA-3′ and antisense 5′-AAAGCCAACCAAGCAGAAGA-3′. The p19 oligonucleotides were: sense 5′-GACTCAGCCAACTCCTCCAG-3′ and antisense 5′-GGCACTAAGGGCTCAGTCAG-3′. The conditions for amplification of GAPDH were 94 °C for 1 min (denaturation), 60 °C for 1 min (primer annealing) and 72 °C for 1 min (elongation); for 35 cycles. The amplification conditions for p35 and p40 were 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min; for 40 cycles. The amplification conditions for p19 were 94 °C for 1 min, 55 °C for 1 min and 72 °C elongation for 1 min; for 35 cycles. The PCR products were analysed by separation on 1.5 % (w/v) agarose gels. The specificity of the PCR products was confirmed by direct sequencing, which demonstrated 100 % homology with the original sequences.

IL-12p40 ELISA

Levels of IL-12p40 in microglial culture supernatants were quantified using specific ELISA kits purchased from Biosource International (Camarillo, CA, U.S.A.), according to the manufacturer's instructions. The assay detected >2 pg/ml and the intra- and inter-assay coefficients of variations were between 3.3 and 4.5 % and 5.6 and 6.7 % respectively.

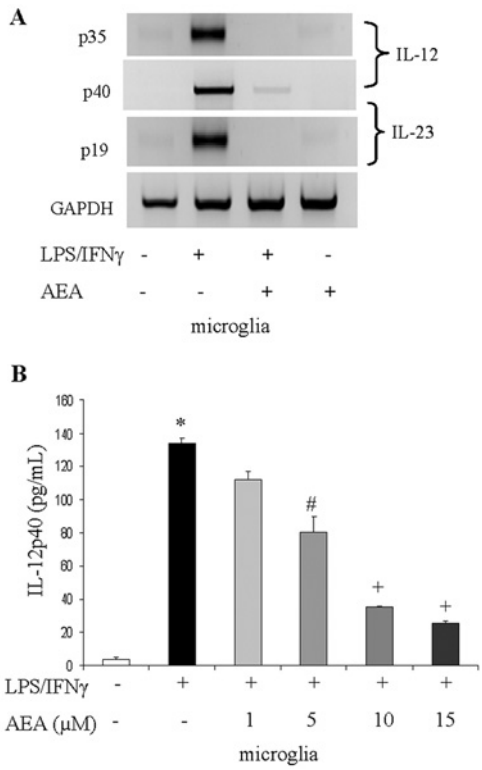

Figure 1 AEA represses the expression of IL-12p35, IL-12p40 and IL-23p19 subunits in activated microglia

(**A**) Microglial cells stimulated for 18 h with both LPS and INFγ concurrently (LPS/IFNγ) showed an induction in the mRNA expression of p35, p40 and p19 subunits, as determined by semi-quantitative RT–PCR. This induction was down-regulated by co-treatment with AEA (10 μM). (**B**) Levels of IL-12p40 protein were determined by ELISA in the supernatant of microglial cultures after 18 h stimulation with both LPS and INFγ. The augmented levels of the p40 subunit in the supernatants from LPS and IFNγ-co-stimulated (LPS/IFNγ) microglia were diminished after co-treatment with 0, 1, 5, 10 or 15 μM AEA in a dose–response fashion. Results are means $\pm$ S.E.M. ($n = 3$), with each experiment performed in triplicate. *, $P < 0.05$ compared with control; #, $P < 0.05$ compared with LPS and IFNγ; +, $P < 0.01$ compared with LPS and IFNγ.

Statistical analysis

Results are means $\pm$ S.E.M. of at least three experiments performed with different cell preparations. Analysis of variance (ANOVA) followed by the Tukey test for multiple comparison was used to determine statistical significance (95 %; $P < 0.05$).

RESULTS

AEA negatively regulates the induction of both IL-12 (p40/p35) and IL-23 (p40/p19) in LPS- and IFNγ-activated microglia

We first studied the microglial responses to AEA treatment when it was co-administered with both LPS and IFNγ (50 ng/ml and 100 units/ml respectively). The stimulation of murine microglial cells with both LPS and IFNγ up-regulated the levels of mRNA for IL-12 (p40/p35) and IL-23 (p40/p19) (Figure 1A), as shown by semi-quantitative RT–PCR. To assess the effects of AEA on the expression of these subunits, we treated microglial cells with LPS and IFNγ concurrently, and with AEA. Experiments with different AEA doses (1, 5, 10 and 15 μM) on IL-12p40 protein

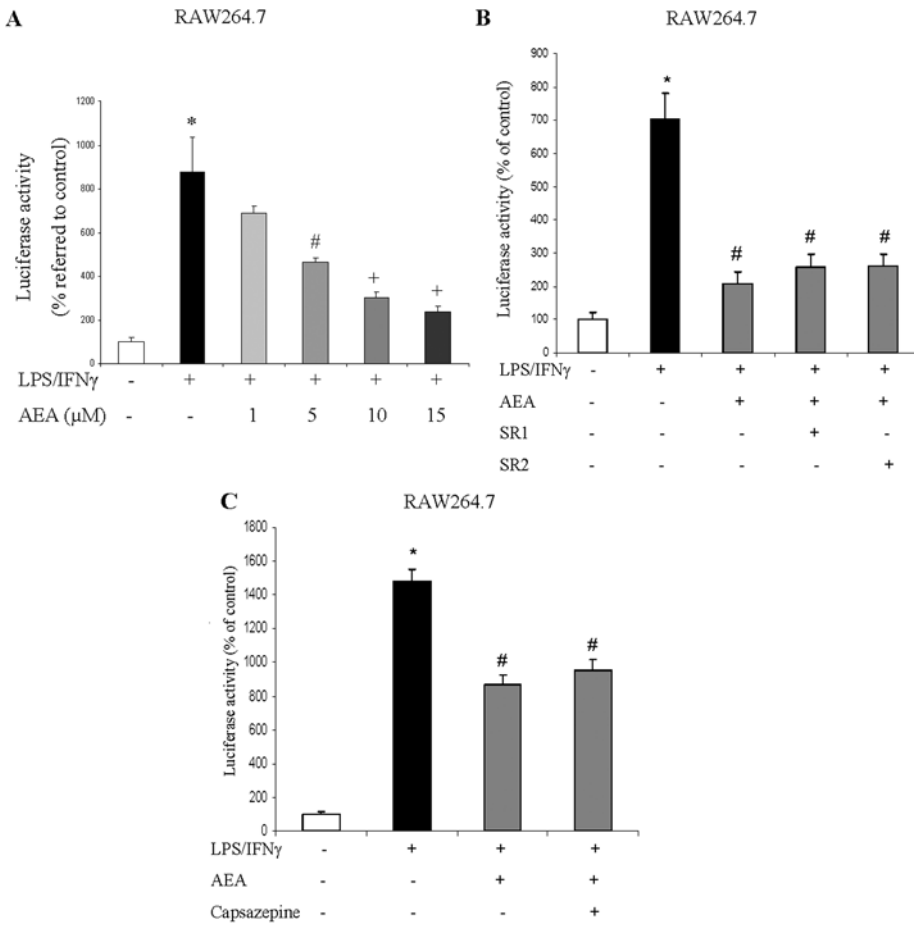

Figure 2 AEA reduces IL-12p40 promoter activity independently of CB or vanilloid receptors

(**A**) RAW 264.7 cells were transiently transfected with a wild-type p40/pGL2B promoter gene construct. Cells were left untreated or were stimulated for 18 h with both LPS and IFNγ concurrently (LPS/IFNγ) in the presence or absence of different doses of AEA (0, 1, 5, 10 or 15 μM) prior to luciferase measurement. Luciferase activity was normalized to Renilla luciferase activity. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). *, $P < 0.02$ compared with control; #, $P < 0.05$ compared with LPS and IFNγ; +, $P < 0.01$ compared with LPS and IFNγ. (**B**) RAW 264.7 cells were transiently trasfected with a wild-type p40/pGL2B promoter gene construct. Transfected cells were pre-treated with the CB antagonists SR1 or SR2 (1 μM) for 1 h, and subsequently treated with both LPS and IFNγ concurrently (LPS/IFNγ) and with AEA (10 μM). Luciferase activity was measured 18 h after the last treatment. Neither SR1 nor SR2 were capable of reversing the effects of AEA on p40 promoter activity. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). *, $P < 0.005$ compared with control; #, $P < 0.002$ compared with LPS and IFNγ. (**C**) RAW 264.7 cells were transiently transfected with 400 ng/cm^2 of the wild-type p40/pGL2B promoter gene construct. Transfected cells were pre-treated with the TRPV$_1$ antagonist capsazepine (10 μM) for 1 h and then treated for 18 h with both LPS and IFNγ concurrently (LPS/IFNγ) and with AEA (10 μM). Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). *, $P < 0.001$ compared with control; #, $P < 0.005$ compared with LPS and IFNγ.

expression (Figure 1B) led us to select the dose of 10 μM AEA for subsequent experiments. None of the doses of AEA (1–15 μM) exhibited cell toxicity effects, as detected by the lactate dehydrogenase assay (results not shown). In all cases, AEA (10 μM) was able to reverse the induction of p40, p35 and p19 subunits caused by LPS and IFNγ treatment (Figure 1A).

AEA suppresses IL-12p40 promoter activity independently of activation of CB_1, CB_2 or $TRPV_1$ (transient receptor potential vanilloid$_1$) receptors

Since p40 is the common subunit in both biologically active IL-12 and IL-23, we decided to focus our study on the effects of AEA on this particular subunit. To delineate the mechanisms underlying the inhibitory effects of AEA on the production of IL-12p40, its effects on LPS and IFNγ activation of the p40 promoter were explored. The RAW 264.7 murine macrophage cell line has been extensively used as a cellular model to study the regulation of p40 promoter activity [23–25]. RAW 264.7 cells also express CB_1 and CB_2 receptors, making them suitable for studying the regulation of IL-12p40 expression by eCBs [26]. Therefore we used RAW 264.7 cells as the main cellular model for reporter gene experiments in the present study. Cells were transiently transfected with a luciferase reporter gene construct driven by the wild-type IL-12p40 promoter [23]. Stimulation with both LPS and IFNγ caused a strong induction of IL-12p40 transcriptional activity, which was significantly reversed by co-treatment with AEA at different doses, as shown in Figure 2(A). AEA, when applied alone, did not induce any change in p40 promoter activity. We also confirmed the inhibitory effects of AEA on IL-12 p40 transcription in the BV-2 microglial cell line (results not shown). The involvement of the CB receptors CB_1 and CB_2 in mediating the inhibitory effects of AEA was explored by assessing the ability of the CB_1 and CB_2 receptor antagonists, SR1 and SR2 respectively, to regulate the inhibitory effect of AEA. As shown in Figure 2(B), the presence of SR1 or SR2 had no influence on the inhibitory effect of AEA on IL-12 p40 transcription. This suggests a lack of involvement of the CB_1 or CB_2 receptors in mediating the effects of AEA. Because several studies have demonstrated that AEA also behaves as a full

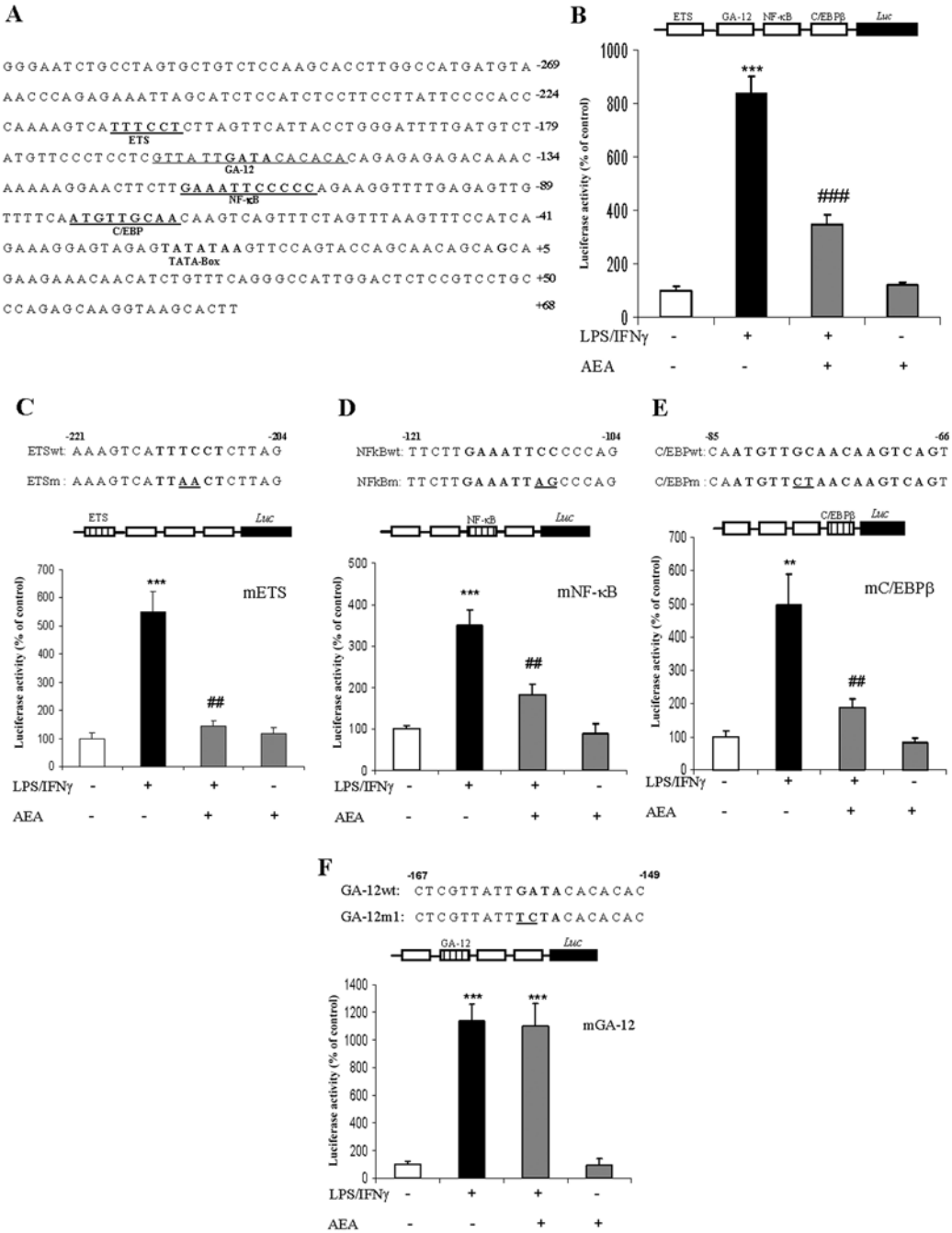

Figure 3 AEA reduces the activity of the p40 promoter through activation of the repressor site GA-12

(**A**) Summary of consensus binding sites on the −300 to +68 promoter region of the IL-12p40 gene. (**B**) RAW 264.7 cells were transiently transfected with a reporter gene construct containing the wild-type p40 promoter. Cells were stimulated for 18 h with both LPS and IFNγ together (LPS/IFNγ) and with AEA (10 μM) prior to luciferase activity measurement. Non-treated transfected cells were used as a control. Results are means ± S.E.M. of luciferase activity ($n = 12$). ***, $P < 0.001$ compared with control; ###, $P < 0.001$ compared with LPS and IFNγ. (**C**–**E**) RAW 264.7 cells were transiently transfected with mutant p40/pXP1 reporter gene constructs carrying 2 bp mutations at the indicated (underlined) promoter sites [ETS (mETS) (**C**), NF-κB (mNF-κB) (**D**) or C/EBPβ (mC/EBPβ) (**E**)], as indicated in each panel. In all three cases, induction of p40 promoter activity was lower when compared with the wild-type promoter (ETSwt, NFκBwt and C/EBPwt respectively). The mutation was unable to interfere with the actions of AEA (10 μM) in reducing the induction caused by stimulus with both LPS and IFNγ (LPS/IFNγ). Results are means ± S.E.M. of luciferase activity. ***, $P < 0.001$ compared with control; ##, $P < 0.005$ compared with LPS and IFNγ (**C**); ***, $P < 0.001$ compared with control; ##, $P < 0.001$ compared with LPS and IFNγ (**D**); **, $P < 0.002$ compared with control; ##, $P < 0.01$ compared with LPS and IFNγ (**E**). (**F**) RAW 264.7 cells were transiently transfected with 2 bp mutated (underlined) GA-12 region (mGA-12) p40/pXP1 construct. The mutation of the repressor element interfered with the actions of 10 μM AEA. Results are means ± S.E.M. of luciferase activity. ***, $P < 0.001$ compared with control (GA-12wt). In all cases $n = 12$.

agonist and a modulator of the vanilloid receptor, namely $TRPV_1$ [27], we investigated whether the effects of AEA could be the result of AEA modulating $TRPV_1$ receptors. RAW 264.7 cells transfected with the p40 promoter construct were treated with the $TRPV_1$ antagonist capsazepine at the effective dose (10 μM) [27] prior to treatment with LPS, IFNγ and AEA. As shown in Figure 1(C), pre-treatment with capsazepine was unable to prevent the effects of AEA on p40 promoter activity.

The GATA repressor promoter site is critical for the inhibitory actions of AEA on IL-12p40 gene expression

Studies on the regulation of IL-12p40 promoter activity indicate that the IL-12p40 promoter contains several binding sites for transcription factors that contribute to gene regulation in LPS-stimulated macrophages. The three control elements that have been most extensively characterized bind NF-κB, C/EBPβ and ETS transcription factor family members. In addition, a control repressor element, termed GA-12, has been characterized [24]. To investigate the molecular mechanisms controlling the transcription of IL-12p40 under AEA exposure, we performed a series of experiments to study the response elements contained in the IL-12p40 sequence (Figures 3A and 3B). RAW 264.7 cells were transfected with various constructs of the IL-12p40 promoter containing 2 bp mutations in a luciferase reporter vector. These mutant promoter constructs have been described to induce loss of promoter activity in other models [24]. Site-directed mutagenesis of the C/EBPβ, NF-κB and ETS sites of the IL-12p40 promoter were unable to reverse the inhibitory effects of AEA on reporter vector activity (Figures 3C–3E). Interestingly, when RAW 264.7 cells were transiently transfected with constructs carrying a mutation on the GA-12 motif, AEA was unable to reverse LPS- and IFNγ-induced activation of the p40 promoter (Figure 3F). These results strongly suggest that the main mechanism of action of AEA is activation of the GA-12 repressor site on the IL-12p40 promoter. To confirm the critical role of the GATA repressor element in inhibition by AEA, we performed decoy oligonucleotide assays. A decoy double-stranded oligonucleotide (with the sequence corresponding to the GATA sequence) was generated and transfected into RAW 264.7 cells in an attempt to interfere with binding of transcription factors modulated by AEA to their *cis*-acting elements within the IL-12p40 promoter sequence (Figure 4). The use of this decoy oligonucleotide completely blocked the effects of AEA on IL-12p40 promoter activity (Figure 4). To confirm further that this was a specific effect of the decoy sequence, we used a GATA sequence decoy oligonucleotide containing a 2 bp mutation as a control (Figure 4). The mutated decoy oligonucleotide was unable to reverse the effects of AEA on the down-regulation of IL-12p40 promoter activity. These results strongly suggest that AEA reverses LPS- and IFNγ-induced IL-12p40 expression mainly by acting on the GA-12 repressor site.

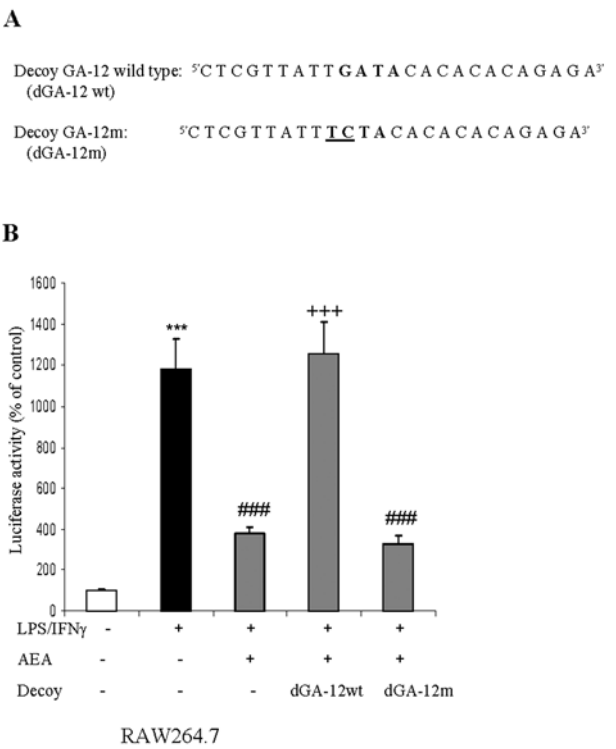

Figure 4 Decoy GA-12 wild-type oligonucleotide is able to reverse the effects of AEA on the activation of the repressor site at the p40 promoter

(**A**) Sequences of the GA-12 wild-type (dGA-12wt) and mutated (dGA-12m) decoy double-stranded oligonucleotides used in this study. The residues which are mutated are underlined. (**B**) RAW 264.7 cells were transiently transfected with wild-type p40/pXP1 reporter gene construct with or without double-stranded decoy oligonucleotides corresponding to wild-type GA-12 (dGA-12wt) or 2 bp mutated GA-12 (dGA-12m) region. Co-transfection of GA-12 wild-type oligonucleotide with p40/pXP1 completely blocked the induction-reducing effects of 10 μM AEA on p40 promoter activity, which was induced by both LPS and IFNγ co-stimulation (LPS/IFNγ), whereas co-transfection of dGA-12m oligonucleotide with p40/pXP1 had no effect. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). ***, $P < 0.001$ compared with control; ###, $P < 0.001$ compared with LPS and IFNγ; +++, $P < 0.001$ compared with LPS and IFNγ + AEA.

Interactions of AEA and the COX-2 pathway: inhibition of IL-12 p40 promoter activity by prostamide E_2 involves the activation of EP_2 (prostanoid receptor-type 2)

Overall, our results show that the inhibitory effect of AEA on the regulation of IL-12p40 gene transcription is mediated by a mechanism which is independent of activation of the CB_1 or CB_2 receptors. Previous evidence [21,28] indicates that COX-2 is capable of oxygenating eCB to generate new types of prostanoids, and thus COX-2 may recognize AEA and catalyse its conversion into prostaglandin endoperoxidase ethanolamides, such as prostamide E_2 (Figure 5A). The above findings prompted us to investigate the effects of prostamide E_2 in the regulation of IL-12 p40. First, we studied the effects of prostamide E_2 on the induction of p40, p35 and p19 subunits caused by both LPS and IFNγ in primary microglial cells. As shown in Figure 5(B), prostamide E_2 (10 μM) markedly reversed the LPS- and IFNγ-induced up-regulation of p40 mRNA expression, as observed by semi-quantitative RT–PCR. Interestingly, induction of the p35 and p19 genes was also reduced by prostamide E_2 (Figure 5B). In the following experiments, we then addressed whether prostamide E_2 could regulate IL-12p40 promoter activity. To analyse this, luciferase reporter gene assays were performed. As shown in Figure 5(C), p40 promoter activity induced by LPS and IFNγ was decreased in a dose-dependent manner by prostamide E_2 (0.1, 1 and 10 μM). We also showed that prostamide E_2 acts on the GA-12 repressor site on the IL-12p40 promoter. Thus when cells were transiently transfected with constructs carrying a mutated GA-12 motif, prostamide E_2 was unable to reverse LPS- and IFNγ-induced activation of the p40 promoter (Figure 5D). It is well known that cells of the myeloid lineage express different types of prostanoid receptors. Therefore to investigate whether AEA-induced inhibitory effects on IL-12p40 promoter activity may involve the generation of prostamide E_2, we used a pharmacological approach by treating cells with AH6809, an antagonist of EP_2, as EP_2 is also known to be activated by prostamide E_2 [28]. AEA and prostamide E_2 were added alone or in the presence of AH6809, together with both LPS and IFNγ in RAW 264.7 cells. As shown in Figure 5(E), the presence of AH6809 partially reversed the inhibitory effect of both AEA and prostamide E_2 on the transcriptional activity of IL-12p40. Interestingly, the effect of AEA was reversed in the same manner when EP_2 was antagonized, strongly suggesting that a proportion of the effects of AEA are mediated by EP_2 activation. EP_4 does

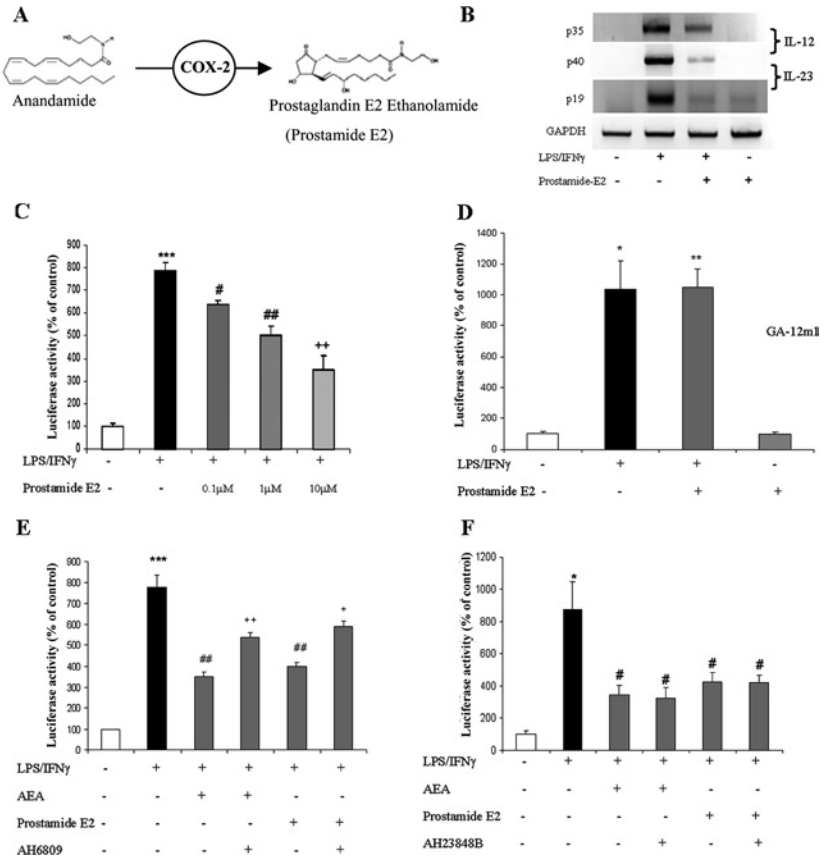

Figure 5 Prostamide E_2 reduces IL-12p40 promoter activity by activating the repressor site GA-12: involvement of EP_2

(**A**) Scheme showing the metabolic pathway by which AEA is converted into prostamide E_2. (**B**) Microglial cells which were stimulated for 18 h with both LPS and IFNγ (LPS/IFNγ) showed an increase in the expression of p35, p40 and p19 subunits at the mRNA level. When co-treated with prostamide E_2 (10 μM), this induction was reduced. (**C**) RAW 264.7 cells were transiently transfected with p40/pGL2B reporter gene contruct. Cells were left unstimulated or treated with both LPS and INFγ concurrently (LPS/IFNγ) in the presence or absence of different doses of prostamide E_2 (0.1, 1 or 10 μM). Prostamide E_2 reduced p40 promoter activity in a dose-dependent fashion. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). ***, $P < 0.001$ compared with control; #, $P < 0.02$ compared with LPS and IFNγ; ##, $P < 0.01$ compared with LPS and IFNγ; ++, $P < 0.005$ compared with LPS and IFNγ. (**D**) RAW 264.7 cells were transiently transfected with wild-type p40/pXP1 reporter gene construct and transfected with or without double-stranded decoy oligonucleotides corresponding to the GA-12 wild-type or 2 bp mutated (GA-12m1) sequence. Co-transfection of dGA-12wt with p40/pXP1 completely blocked the reducing effects of 10 μM AEA on the induction of p40 promoter activity [induced by LPS and IFNγ co-treatment (LPS/IFNγ)], whereas co-transfection of GA-12m1 with p40/pXP1 had no effect on AEA downregulation of p40 promoter activity. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). **, $P < 0.001$ compared with control; *, $P < 0.001$ compared with control. (**E**) RAW 264.7 cells were transiently transfected with wild-type p40/pXP1 reporter gene construct. Transfected cells were left unstimulated or treated with both LPS and INFγ concurrently (LPS/IFNγ) in the presence or absence of AEA (10 μM) or prostamide E_2 (10 μM). Pre-treatment with the EP_2 antagonist AH6809 (10 μM) partially reversed the effects of AEA and prostamide E_2 on p40 promoter activity. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). ***, $P < 0.001$ compared with control; ##, $P < 0.003$ compared with LPS and IFNγ; ++, $P < 0.01$ compared with LPS and IFNγ + AEA; +, $P < 0.01$ compared with LPS and IFNγ+prostamide E_2. (**F**) RAW 264.7 cells were transiently transfected with wild-type p40/pXP1 reporter gene construct. Transfected cells were left unstimulated or treated with both LPS and INFγ concurrently (LPS/IFNγ) in the presence or absence of AEA (10 μM) or prostamide E_2 (10 μM). Pre-treatment with the EP_4 antagonist AH23848B (30 μM) did not modify the effects of AEA and prostamide E_2 on p40 promoter activity. Results are means $\pm$ S.E.M. of luciferase activity ($n = 12$). *, $P < 0.02$ compared with control; #, $P < 0.05$ compared with LPS and IFNγ.

not seem to be involved, since treatment with the EP_4 antagonist AH23848B did not modify the inhibitory effects of AEA or prostamide E_2 on IL-12p40 gene transcription (Figure 5F). These results suggest that AEA could exert part of its effects on the regulation of IL-12p40 gene transcription by the generation of prostamide E_2.

AEA and prostamide E_2 down-regulate the induction of IL-12p40 protein by LPS- and IFNγ-activated microglia

Next, we attempted to delineate AEA and prostamide E_2 actions at the IL-12p40 protein level. AEA (10 μM) inhibited LPS- and IFNγ-mediated up-regulation of p40 protein in primary microglial cell cultures, as evaluated by ELISAs on tissue-culture medium supernatant (Figure 6A). To explore whether AEA-induced inhibition of IL-12p40 protein synthesis may involve prostanoids, we co-treated microglial cells with both LPS and IFNγ, and AEA or prostamide E_2 in the presence or absence of the EP_2 antagonist AH6809. When we antagonized EP_2 on AEA-treated microglia, a partial reversion of AEA effects on the production of IL-12p40 protein was oberved in cells stimulated by LPS and IFNγ, as similarly seen following the addition of prostamide E_2 (Figure 6A). Again, treatment with the EP_4 antagonist AH23848B (Figure 6B) was unable to modify AEA or prostamide E_2 effects on IL-12p40 protein expression.

Inhibition of the COX-2 pathway reverses the effects of AEA on both p40 promoter activity and IL-12p40 expression

Since both AEA and prostamide E_2 modulate IL-12p40 at both the protein and promoter levels, and AEA can be oxygenated by COX-2 to generate prostamides, we postulated that inhibition

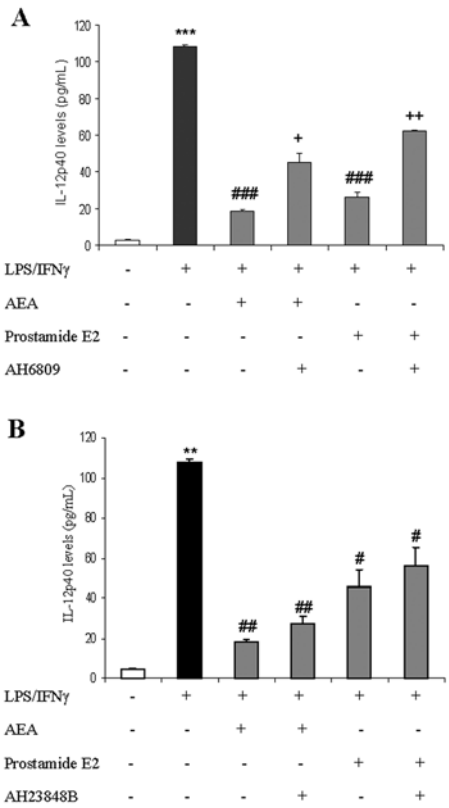

Figure 6 Prostamide E_2 negatively modulates p40 protein levels in activated microglial cells

(**A**) Microglial cells were co-treated overnight with both LPS and IFNγ (LPS/IFNγ) and with AEA (10 μM), prostamide E_2 (10 μM) and/or EP_2 antagonist AH6809 (10 μM). Antagonism of EP_2 partially blocked the effects of both AEA and prostamide E_2 on p40 protein expression. Supernatants were collected and p40 protein was measured by ELISA. Results are means $\pm$ S.E.M. ($n = 3$), with each experiment performed in triplicate. ***, $P < 0.001$ compared with control; ###, $P < 0.001$ compared with LPS and IFNγ; +, $P < 0.05$ compared with LPS and IFNγ + AEA; ++, $P < 0.005$ compared with LPS and IFNγ+prostamide E_2. (**B**) When microglial cells were pre-treated with the EP_4 antagonist AH23848B (30 μM), there was no modification of the effects of 10 μM AEA and 10 μM prostamide E_2 on p40 protein expression as measured by ELISA. Results are means $\pm$ S.E.M. ($n = 3$), with each experiment performed in triplicate. **, $P < 0.002$ compared with control; #, $P < 0.05$ compared with LPS and IFNγ; ##, $P < 0.005$ compared with LPS and IFNγ.

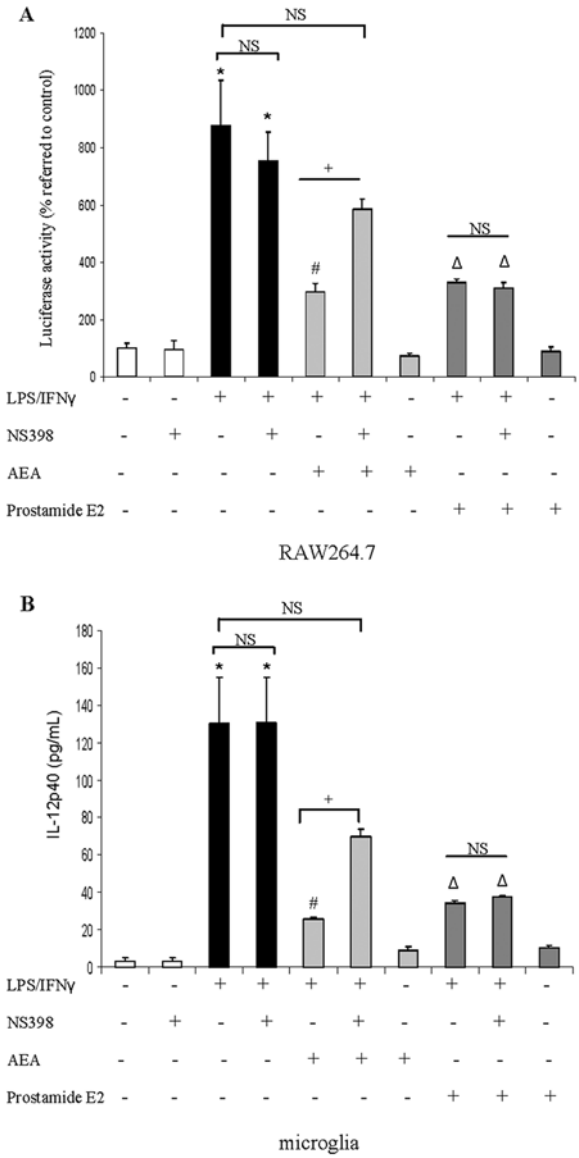

Figure 7 Pharmacological inhibition of COX-2 reverses the effect of AEA both at protein and promoter levels

(**A**) RAW 264.7 cells were transiently transfected with a reporter gene construct containing the wild-type p40 promoter. Cells were stimulated for 18 h with both LPS and IFN γ concurrently (LPS/IFNγ), and with AEA (10 μM) or prostamide E_2 (10 μM), in the presence or absence of the specific COX-2 inhibitor NS-398 (NS398; 10 μM), prior to luciferase activity measurement. Results are means $\pm$ S.E.M. of luciferase activity ($n = 9$). *, $P < 0.05$ compared with control; #, $P < 0.01$ compared with LPS and IFNγ; +, $P < 0.05$ compared with LPS and IFNγ + AEA; Δ, $P < 0.01$ compared with LPS and IFNγ. (**B**) IL-12p40 protein expression of microglia treated with NS-398 (NS398; 10 μM). COX-2 inhibition significantly reduced the magnitude of AEA(10 μM) inhibition on IL-12p40 protein expression (as measured by ELISA), whereas there was no effect on prostamide E_2 action, supporting the involvement of this enzyme in the effects of AEA. Results are means $\pm$ S.E.M. ($n = 3$), with each experiment performed in triplicate. *, $P < 0.05$ compared with control; #, $P < 0.01$ compared with LPS and IFNγ; +, $P < 0.05$ compared with LPS and IFNγ + AEA; Δ, $P < 0.01$ compared with LPS and IFNγ.

of COX-2 could result in a reversion of AEA effects on the IL-12p40 promoter. We thus treated RAW 264.7 cells with the COX-2-specific inhibitor NS-398. In support of the contribution of COX-2 to AEA actions, the treatment of activated RAW 264.7 cells with 10 μM NS-398 significantly reversed the inhibition of p40 promoter activity by AEA (Figure 7A). Interestingly, NS-398 had no effect by itself and did not modify the p40 promoter activity when co-treated with both LPS and IFNγ. In addition, the presence of 10 μM NS-398 was unable to reverse the effects of prostamide E_2, a downstream metabolite in the COX-2 pathway (Figure 7A). We further confirmed these results at the protein level in primary microglial cells, obtaining similar results to those of p40 promoter activity (Figure 7B). Again, COX-2 inhibition with 10 μM NS-398 reversed the effects of AEA on IL-12p40 protein expression and had no effect by itself, in the presence of LPS and IFNγ and with prostamide E_2 (Figure 7B). Overall, these results highlight the contribution of COX-2 to AEA actions.

DISCUSSION

CB compounds were previously demonstrated to reduce the development of CREAE (chronic relapsing-remitting experimental autoimmune encephalomyelitis) and TMEV models of MS [4–6]. Chronically-activated microglia and astrocytes produce pro-inflammatory molecules that may contribute to the loss of

oligodendrocytes, which are compromised in MS. Previously, our group and others have reported that CB agonists inhibit the production of pro-inflammatory molecules by CNS glial cells [7, 29–31]. In the present study, we addressed the question of whether AEA might be involved in the regulation of IL-12 and related cytokines on microglial cells, which are capable of producing eCBs during CNS inflammation [18,19]. This is of interest because of the role these cytokines play in the induction and maintenance phases of autoimmune inflammatory demyelination [32,33]

Our results show that activated microglial cells express the p19, p35 and p40 subunits, therefore making the formation of IL-12 and IL-23 possible, and then raising the possibility that microglia may contribute to Th1-skewing within the CNS. In addition, for the first time, we demonstrated that AEA is a potent inhibitor of mRNA expression of p19, p35 and p40 subunits and of IL-12p40 protein release by activated microglia. IL-12 (p35/p40 subunits) and IL-23 (p19/p40 subunits) are known to play a crucial role in the development of EAE [34]. Thus our results support the involvement of the eCB system in the modulation of neuroinflammatory processes, in line with its ability to limit the immune response in animal models of MS.

The mechanisms by which AEA inhibit the production of IL-12 family cytokines in activated microglia are unknown. Because the p40 subunit is shared by both IL-12 and IL-23, we concentrated our studies from a mechanistic point of view on its regulation. In activated cells, C/EBP, NF-κB and ETS are some of the enhancer sites in the p40 promoter that may up-regulate the expression of this subunit [24]. In contrast, in resting cells, there is a constitutively activated repressor site, called GA-12. In an approach aimed at understanding p40 promoter regulation by AEA, we performed site-directed mutagenesis experiments in regions of the IL-12p40 promoter. We observed that mutations within the core region of the GA-12 site abolished the ability of this mutated construct to respond to AEA, strongly suggesting that the mechanism of action of AEA is related to the activation of this repressor site of the IL-12p40 promoter. Furthermore, decoy oligonucleotide experiments also support the idea that AEA down-regulates IL-12p40 expression by acting on GA-12. Our data demonstrate for the first time that AEA-mediated down-regulation of p40 promoter activity suggests a mechanism in which the GA-12 repressor site is critical.

Studies on the regulation of the IL-12p40 promoter indicated that AEA suppression of IL-12p40 promoter activity was independent of both CB_1 and CB_2 receptor activation. Although functional interactions between the eCB and vanilloid systems have been postulated [27], our results also indicate a lack of involvement of the vanilloid system in AEA-induced suppression of IL-12p40. At this point, it is interesting to note that prostamide E_2, an active metabolite of AEA through COX-2 activity [35], was able to down-regulate the transcriptional activity of IL-12p40 in a dose-dependent manner. Although little is known about the mechanisms of prostamide E_2-induced biological actions, it is capable of activating the prostanoid receptor EP_2 [28]. Supporting this, prostamide E_2-induced reversion of IL-12p40 gene transcription by LPS and IFNγ was attenuated by pretreatment with the prostanoid EP_2 antagonist. It is important to note that the EP_2 antagonist AH6809 also caused a partial but significant attenuation of AEA-mediated inhibition of IL-12p40 transcriptional activity and protein secretion. Moreover, AH6809 was as efficient in reversing the effects of AEA as was prostamide E_2, which would suggest that these compounds share signalling mechanisms. Therefore the production of prostamide E_2 through metabolism of AEA by COX-2 could mediate a proportion of the effects of AEA on IL-12 p40 regulation. Pretreatment with the EP_4 prostanoid receptor antagonist was ineffective, indicating the specificity of the effects of EP_2s. However, we cannot exclude the involvement of other mechanisms for AEA action in IL-12 p40 regulation, including the existence of unknown CB receptors for AEA [36], or the participation of lipid rafts. The up-regulation of COX-2 may be important in terms of favouring the oxygenation of AEA and generation of prostamide E_2, which would contribute to the inhibition of IL-12p40. If COX-2 plays a role in the metabolism of AEA and generates prostamide E_2 which, in turn, contributes to the inhibition of IL-12p40, then inhibition of COX-2 should produce a positive influence on IL-12p40 production. The suppressive effects of AEA on IL-12p40 levels were observed to be attenuated after treatment with the specific COX-2 inhibitor NS-398, but there was no inhibition of the effects of prostamide E_2. Therefore we suggest that during inflammation, over-expression of COX-2 may drive AEA metabolism towards a derived prostanoid, prostamide E_2, which may contribute to the negative regulation of IL-12p40 production. Supporting this, the results by Rockwell and Kaminski [37] show that AEA caused a concentration-dependent inhibition of IL-2 by murine splenocytes through the generation of a COX-2 metabolite of AEA, independently of CB_1/CB_2 activation.

In the present study, AEA concentrations were used at the low micromolar range (1–15 μM), in agreement with doses reported in other studies [37–39]. Even though AEA levels in rat and human plasma have been detected in the low nanomolar range, the local concentrations of AEA under pathological conditions could be higher. In addition, AEA has usually been measured in normal healthy tissue and plasma samples, whereas these levels differ substantially in inflamed or damaged tissues [19].

The eCB system is highly activated during CNS inflammation [19]. Microglial cells are capable of producing eCBs during CNS inflammation [18,19], and the different components of eCBs are regulated in brain cells from MS patients [40]. Activation of the eCB system may represent a mechanism for controlling excessive inflammation. The inhibition of IL-12p40 may help to control and limit the local immune response in order to prevent over-activation of cellular immunity. Our results also suggest that excessive COX-2 resulting from inflammation has a significant impact on eCB-derived prostanoid signalling in immune reactivity.

Here, we present evidence that the repressor element GA-12 is critical for AEA-mediated down-regulation of IL-12p40 promoter activity. Although the physiological relevance of these findings needs to be established, *in vivo* production of prostamide E_2 has been described as occuring after AEA administration, especially in the case of FAAH knockout mice [41]. The identification of the mechanisms underlying the limiting effects of eCBs on the production of pro-inflammatory cytokines such as IL-12p40 may provide insights relevant for consideration of the eCB system as a novel and promising target for therapeutic treatment of autoimmune or chronic inflammatory diseases. Our observations in microglial cells suggest that it may be of interest to study whether eCBs can regulate IL-12 and IL-23 and then coordinate Th1/Th2 responses on CNS autoimmune diseases, such as MS. Further studies are warranted to answer this question.

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