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RESEARCH PAPER

Cannabinoid CB_2 receptors modulate ERK-1/2 kinase signalling and NO release in microglial cells stimulated with bacterial lipopolysaccharide

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BACKGROUND AND PURPOSE

Cannabinoid (CB) receptor agonists have potential utility as anti-inflammatory drugs in chronic immune inflammatory diseases. In the present study, we characterized the signal transduction pathways affected by CB_2 receptors in quiescent and lipopolysaccharide (LPS)-stimulated murine microglia.

EXPERIMENTAL APPROACH

We examined the effects of the synthetic CB_2 receptor ligand, JWH-015, on phosphorylation of MAPKs and NO production.

KEY RESULTS

Stimulation of CB_2 receptors by JWH-015 activated JNK-1/2 and ERK-1/2 in quiescent murine microglial cells. Furthermore, CB_2 receptor activation increased p-ERK-1/2 at 15 min in LPS-stimulated microglia. Surprisingly, this was reduced after 30 min in the presence of both LPS and JWH-015. The NOS inhibitor L-NAME blocked the ability of JWH-015 to down-regulate the LPS-induced p-ERK increase, indicating that activation of CB_2 receptors reduced effects of LPS on ERK-1/2 phosphorylation through NO. JWH-015 increased LPS-induced NO release at 30 min, while at 4 h CB_2 receptor stimulation had an inhibitory effect. All the effects of JWH-015 were significantly blocked by the CB_2 receptor antagonist AM 630 and, as the inhibition of CB_2 receptor expression by siRNA abolished the effects of JWH-015, were shown to be mediated specifically by activation of CB_2 receptors.

CONCLUSIONS AND IMPLICATIONS

Our results demonstrate that CB_2 receptor stimulation activated the MAPK pathway, but the presence of a second stimulus blocked MAPK signal transduction, inhibiting pro-inflammatory LPS-induced production of NO. Therefore, CB_2 receptor agonists may promote anti-inflammatory therapeutic responses in activated microglia.

Abbreviations

2-AG, 2-arachidonoylglycerol; AEA, anandamide; AM 251, *N*-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM 630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone(6-iodopravadoline); CB, cannabinoid; CHO-hCB_2, CHO cells transfected with human CB_2 cannabinoid receptor; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; iNOS, inducibleNOS; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; L-NAME, L-N^G-nitroarginine methyl ester (hydrochloride); PE, phycoerythrin; PTX, *Pertussis* toxin; siRNA, small interfering RNA; SNP, sodium nitroprusside; TLR4, Toll-like receptor 4

Introduction

In the nervous system, neurotransmission and neuroinflammation are mediated by the endocannabinoid signalling system (Fernández-Ruiz, 2009; Marrs *et al.*, 2010). To date, two cannabinoid (CB) receptors have been identified by molecular cloning, namely, CB_1 and CB_2. CB_1 receptors are expressed by the neurones and regulate the release of neurotransmitters, while CB_2 receptors are expressed by the microglia, regulating their motility and immunomodulator production (Howlett *et al.*, 2002; Pertwee *et al.*, 2010; receptor nomenclature follows Alexander *et al.*, 2011).

Both CB_1 and CB_2 receptors are $G_{i/o}$-protein-coupled and are able to signal through the three main components of the MAPK pathway, namely, ERK, JNK and p38 (Howlett *et al.*, 2002). However, activation of CB receptors is heavily influenced by cell type specificity, and their signalling is remarkably complex (Bosier *et al.*, 2010); indeed, they are able to exploit a wide variety of pathways to regulate the activities of MAPKs. In fact, CB receptor coupling to MAPKs is extremely dependent on the cellular context and a wide range of activation and inhibition has been observed dependent on cell type, cell differentiation status and co-modulators of MAPK cascades (Howlett, 2005). In the nervous system, activation of CB_1 and CB_2 receptors is induced by the endocannabinoids, arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), produced by the neurones and glia.

During CNS inflammation, both CB_1 and CB_2 receptors mediate the induction of MAPK phosphatase-1 (MKP-1) by the endocannabinoid AEA. Rapid AEA-induced MKP-1 expression switches off MAPK signal transduction in microglial cells and thereby protects neurones from inflammatory damage (Eljaschewitsch *et al.*, 2006). Furthermore, it has recently been reported that both the pharmacological stimulation of CB_2 receptors and the manipulation of endocannabinoid tone by inhibiting AEA hydrolysis in microglia, could modify the cytokine milieu, thereby contributing to the accumulation of anti-inflammatory microglia at lesion sites in multiple sclerosis (Correa *et al.*, 2010; 2011). Moreover, another recent study has demonstrated that administration of 2-AG ameliorates both acute and chronic experimental autoimmune encephalomyelitis, a widely used model of multiple sclerosis (Lourbopoulos *et al.*, 2011).

Microglia, a specialized macrophage population found in the CNS, are quiescent in the normal brain. However, after CNS injury or upon LPS interaction with Toll-like receptor (TLR)-4 during bacterial infection (González-Scarano and Baltuch, 1999), these cells can be activated by cytokines, produced by infiltrating immune effector cells. Thus, LPS stimulation of the microglia is a useful model for the study of mechanisms underlying neuronal injury by various pro-inflammatory and neurotoxic factors released by activated microglia (Jung *et al.*, 2010). Microglial activation is associated with increased phagocytosis and the release of oxygen radicals, proteases, pro-inflammatory cytokines and NO. Cumulative evidence shows that, while the NO free radical may play an important role in host defence against certain intracellular pathogens, it may also contribute to neuropathology (Hanisch and Kettenmann, 2007). Hence, agents that suppress microglial cell activation may be beneficial in the treatment of neurodegenerative diseases.

Activation of CB_2 receptors has anti-inflammatory effects (Klein and Newton, 2007; Romero-Sandoval *et al.*, 2009; Correa *et al.*, 2010; Hsieh *et al.*, 2011). In particular, activation of CB_2 receptors expressed in brain microglia during neuroinflammation (Benito *et al.*, 2008; Atwood and Mackie, 2010) reduced NO production and TNF-α in primary microglia (Ehrhart *et al.*, 2005). Furthermore, it protected against human microglial neurotoxicity by enhancing IL-10 production (Klegeris *et al.*, 2003; Correa *et al.*, 2005; 2010; Eljaschewitsch *et al.*, 2006).

However, the specific intracellular mechanism of action by which CB_2 receptor activation alters the microglial phenotype still remains to be elucidated. Hence, building on previous studies of the role of CB_2 receptors in microglial cells, the rationale behind this study was to confirm and further explore the role of this receptor subtype in immune modulation within the CNS. In particular, we set out to characterize CB_2 receptor cell signalling, to verify whether the CB_2 receptor is coupled to the activation of MAPKs and to determine its role in NO production. In this way, we aimed to provide a comprehensive description of the signal transduction mechanisms coupled to CB_2 receptor activation.

To this end, we examined the effect of stimulating CB_2 receptors in quiescent and LPS-activated murine microglial cell line N9 and primary murine microglia. We found that CB_2 receptors exerted different effects on ERK activation in microglial cells, depending on the duration of cannabinoid challenge and the state of cell activation. We observed reversal of ERK phosphorylation in the LPS-treated microglial cells following 30 min of CB_2 receptor activation, a negative feedback loop dependent on NO. This phenomenon was shown to diminish the response of microglial cells to LPS, by reducing NO synthesis and thereby regulating neuroinflammation. Hence, our findings suggest that CB_2 receptor agonists may promote therapeutic anti-inflammatory responses in activated microglia.

Methods

Animals

All animal care and experimental procedures conformed to the guidelines issued by the European Council (86/609/EEC) and were approved by the local Animal Care and Ethics Committee. One-day-old Balb/c mice were obtained from Charles River (Calco, Italy).

Cell lines

The N9 murine microglial cell line (kindly donated by Dr P Ricciardi-Castagnoli, University of Milan, Milan, Italy) was generated by infecting primary mouse microglial cultures with the J2 retrovirus carrying v-raf/v-myc oncogenes (Corradin *et al.*, 1993). CHO cells transfected with the human recombinant CB_2 receptor cDNA (CHO-hCB_2) were obtained from PerkinElmer (Milan, Italy).

Primary microglial cell cultures

Primary glial cultures were prepared as described in a previous study (Molina-Holgado *et al.*, 2002). Briefly, after anaesthesia

(Zoletil 100, 30 mg·kg^{-1}, Virbac Laboratories, France) and decapitation, forebrains from newborn Balb/c mice were excised, meninges were removed and tissue was dissociated mechanically. Cells were re-suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin, then plated on poly-D-lysine-coated (5 μg·mL^{-1}) 75 cm^2 flasks (Falcon; Celbio, Milan, Italy). After 15 days, the flasks were shaken vigorously to remove loosely adherent microglia. The supernatant was plated on multi-well culture plates for 2 h, and the medium was changed to remove non-adherent cells. Cells were grown in a humidified environment containing 5% CO_2 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 flow cytometry with Mac-1 anti-CD11b antibody (BD Pharmingen, Milan, Italy).

Cell cultures

Cells were maintained in DMEM (primary and N9) or Ham's (CHOh-CB_2) medium containing 10% fetal calf serum, penicillin (100 U·mL^{-1}), streptomycin (100 μg·mL^{-1}) and L-glutamine (2 mM) at 37°C in 5% CO_2/95% air. Geneticin (G418, 0.4 mg·mL^{-1}) was added to CHO-hCB_2 cells. Cells were split two or three times weekly at a ratio of 1:5 and 1:10.

Flow cytometry of primary microglial cells

Aliquots of 0.5 × 10^6 cells were incubated for 40 min at 4°C with either specific phycoerythrin (PE)-labelled antibodies (Abs) or isotype-matched irrelevant IgG-PE (Beckman Coulter, Fullerton, CA) as negative control. Cells were washed with PBS and characterized for expression of CD11b and the glial fibrillary acidic protein (GFAP) by flow cytometry with PE-labelled anti-CD11b monoclonal antibody (MoAb) (BD Pharmingen, Milan, Italy) and the fluorescein isothiocyanate (FITC)-labelled anti-GFAP MoAb (BD Pharmingen). In particular, GFAP immunophenotyping was performed in permeabilized cells, using IntraPrep™ fixing/permeabilization reagent (Beckman Coulter) (Gobbi *et al.*, 2003). Analysis was performed on an Epics XL flow cytometer (Beckman Coulter) using Expo ADC software (Beckman Coulter).

Primary and N9 microglial cell exposure to cannabinoids and LPS treatment

LPS, a cell wall component of Gram-negative bacteria, is a potent activator of glia. Hence, microglial cells were treated with 1 μg·mL^{-1} LPS (from *Escherichia coli*, serotype 055:B5, dissolved in cell culture medium) before commencing incubation with cannabinoid ligands. Microglia were then maintained in DMEM containing cannabinoids or their vehicle and harvested after treatment at the indicated times.

Nitrite assay for primary microglial cells

NOS activity was assessed indirectly by measuring nitrite (NO_2^-) accumulation in the cell culture media using a colorimetric kit (Calbiochem, Milan, Italy). At the end of the treatment period, the nitrite concentration in the conditioned media was determined according to a modified Griess method (Green *et al.*, 1982). Briefly, the NADH-dependent enzyme nitrate reductase was used to convert the nitrate to nitrite prior to quantification of the absorbance, measured at 540 nm by a spectrophotometric microplate reader (Fluoroskan Ascent, Labsystems, Sweden). Sodium nitrite was used as the standard compound.

Western blotting in primary and N9 microglial cells

Western blot assay was performed as previously described (Merighi *et al.*, 2009). Aliquots of total protein sample (50 μg) were analysed using antibodies specific for phosphorylated or total p44/p42 MAPK (1:5000 dilution), phosphorylated or total p38 (1:1000 dilution), phosphorylated or total JNK 1/2 (1:1000 dilution) and for anti-CB_2 receptor and anti-TLR4 sera. Specific reactions were revealed with Enhanced Chemiluminescence Western blotting detection reagent (Amersham Corp., Arlington Heights, IL). The membranes were then stripped and re-probed with tubulin (1:250) to ensure equal protein loading.

Densitometry analysis

The intensity of each immunoblot assay band was quantified using a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Mean densitometric data from independent experiments were normalized to the results obtained with control cell cultures. The ratio of phospho-protein to total protein was reported in a densitometric analysis. In particular, MAPK phosphorylation was expressed as ratios between phosphorylated and total MAPK intensity (pMAPK/MAPK).

siRNA transfection of N9 microglial cells

Microglial cells were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of siRNA$_{CB2}$ or siRNA$_{TLR4}$ was performed at a concentration of 100 nM using RNAiFect™ Transfection Kit (Qiagen). Cells were cultured in complete media, and total proteins were isolated at 24, 48 and 72 h for Western blot analysis of CB_2 and TLR4 receptor protein. A randomly chosen non-specific siRNA was used under identical conditions as control (Merighi *et al.*, 2005; 2007).

Immunocytochemistry in cultured N9 microglial cells

Immunostaining was performed directly on cells seeded on glass coverslips. ERK-1 and ERK-2 were detected by anti-ACTIVE MAPK pAb, an affinity-purified rabbit serum that specifically recognizes the dual-phosphorylated active forms of MAP (also known as p44/ERK-1 and p42/ERK-2) enzymes. Microglial cells were seeded on glass coverslips in 24-well plates overnight at 37°C in a humidified atmosphere containing 5% CO_2. Microglial cells were treated with 1 μg·mL^{-1} LPS alone or in combination with JWH-015. After 15 or 30 min, the medium was removed, and cells were washed twice with PBS, mixed in 10% paraformaldehyde for 30 min, permeabilized in a PBS solution containing 0.1% Triton X-100 and incubated for 30 min with PBS-plus 5% goat serum and 0.5% BSA. Cells were then stained (for 24 h at 4°C in a humidified chamber) with a 1:200 dilution of anti-ACTIVE MAPK pAb in 0.5% of goat serum and 0.5% BSA PBS solution. Excess antibody was washed away with PBS, and rabbit antibodies were detected with FITC-labelled goat anti-rabbit IgG. Coverslips

were stained with 4′,6′-diamino-2-phenyl-indole (DAPI), mounted in DABCO glycerol-PBS and observed with a Nikon fluorescent microscope.

Data analysis

All values in the figures and text are expressed as mean ± SE of *n* observation ($n \geq 3$). For the primary cell work, *n* is equal to the number of mice from which the cells were derived. Data sets were examined by ANOVA and Dunnett's test (when necessary). A *P*-value of less than 0.05 was considered statistically significant.

Materials

Tissue culture media and growth supplements were acquired from Cambrex (Bergamo, Italy). U0126 (MEK-1 and MEK-2 inhibitor, soluble in DMSO), human anti-ACTIVE®MAPK (phosphorylated Thr^{183}/Tyr^{185}) and human anti-ERK 1/2 pAb were purchased from Promega (Milan, Italy). Phosphorylated (Thr^{180}/Tyr^{182}) and total p38, phosphorylated (Thr^{183}/Tyr^{185}) and total JNK-1/2, anti-iNOS and phosphorylated Ser^{338} Raf antibodies were from Cell Signaling Technology (Celbio, Milan, Italy). JWH-015 (1-propyl-2-methyl-3-(1-naphthoyl)indole) (soluble in ethanol) and anti-CB_2 receptor rabbit polyclonal antibody (ALX-210–198) were provided by Enzo Life Sciences (Vinci-Biochem, Vinci, Florence, Italy).

The immunogen for the CB_2 receptor antibody was a synthetic peptide corresponding to residues 20–33 of the human CB_2 receptor N-terminal. Anti-TLR4 (sc-30002), small interfering RNA (siRNA) for TLR4 (sc-40261) and siRNA for the CB_2 receptor (sc-39913) were from Santa Cruz Biotechnology (DBA, Milan, Italy). AM 251, *N*-(piperidin-1-yl)-1-(2, 4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide and AM 630 (AM 630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl) methanone(6-iodopravadoline) (both soluble in DMSO) were from Tocris Bioscience (Bristol, UK).

RNAiFect™ Transfection Kit was from Qiagen (Milan, Italy). Unless otherwise stated, all other chemicals were purchased from Sigma (Milan, Italy). L-NAME (L-N^G-nitroarginine methyl ester (hydrochloride)) was dissolved in DMSO, and *Pertussis* toxin (PTX) and sodium nitroprusside (SNP) were dissolved in distilled water.

Results

CB_2 receptor expression in primary and N9 mouse microglial cells

Expression of the myeloid cell surface antigen CD11b was analysed in primary microglial cells by flow cytometry. Cells were treated with specific MoAbs or isotype-matched irrelevant MoAbs. Microglia were negative for the astrocyte-specific protein GFAP but showed significant positive staining for CD11b, as compared with the isotype control, thereby indicating high expression levels of the microglial cell marker CD11b (Figure 1A).

The expression of CB_2 cannabinoid receptor in CHO-hCB_2 (used as positive control) in quiescent and LPS-activated primary and N9 mouse microglial cells is illustrated in Figure 1. The molecular weight of the protein detected in these cells was 50 kDa, comparable with the calculated molecular weight of CB_2 receptors. Expression of CB_2 receptor protein was not modified by 30 min treatment with LPS (1 μg·mL^{-1}; Figure 1B).

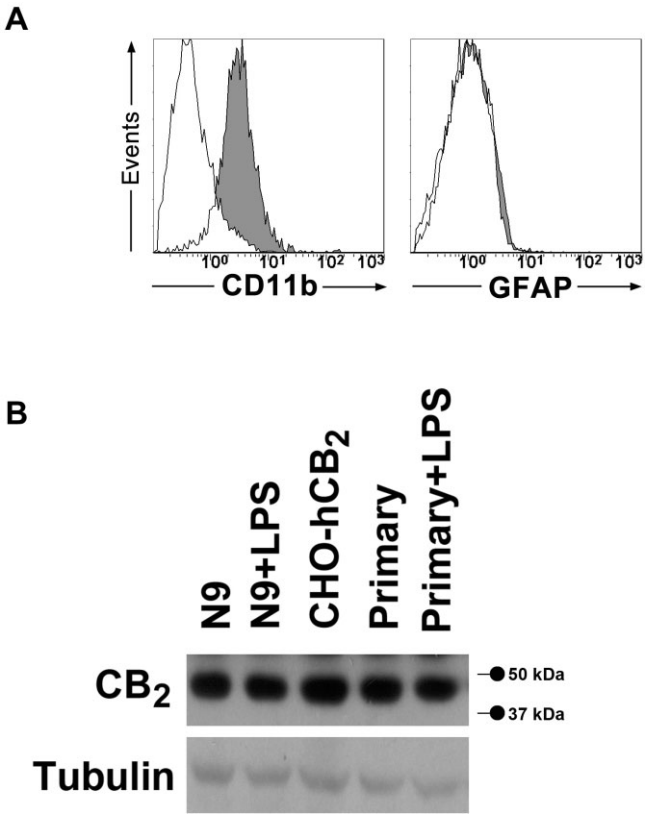


Figure 1

Detection of CB_2 cannabinoid receptors. (A) Cell surface expression of CD11b and intracellular expression of GFAP by flow cytometry analysis. Primary microglial cells were treated with specific MoAbs (grey histograms) or with isotype-matched irrelevant MoAbs (empty histograms, controls). (B) CB_2 receptor detection by Western blot assay in CHO-hCB_2 cells, in quiescent and LPS-activated (1 μg·mL^{-1} for 30 min) primary and N9 microglial cells. Tubulin shows equal loading of protein.

To evaluate the previously reported effect of LPS-induced up-regulation of CB_2 receptors, we assayed CB_2 receptors over 4 h of LPS treatment. In agreement with published data (Mukhopadhyay *et al.*, 2006), no significant increase in CB_2 receptor immunoreactivity was observed by Western blot in 4 h after LPS treatment (data not shown).

Effects of activating CB_2 receptors on MAPKs in quiescent N9 microglial cells

The effects of CB_2 cannabinoid receptor stimulation were studied using JWH-015, a cannabinoid receptor agonist known to bind more readily to CB_2 than CB_1 receptors (Merighi *et al.*, 2010), to test whether CB_2 receptors could induce ERK-1/2, p38 and JNK-1/2 phosphorylation in murine N9 microglial cells. As shown in Figure 2A, JWH-015 (10 nM) induced a rapid and sustained (up to 60 min) stimulation of ERK-1/2 and JNK-1/2. Conversely, p38 phosphorylation levels were not affected.

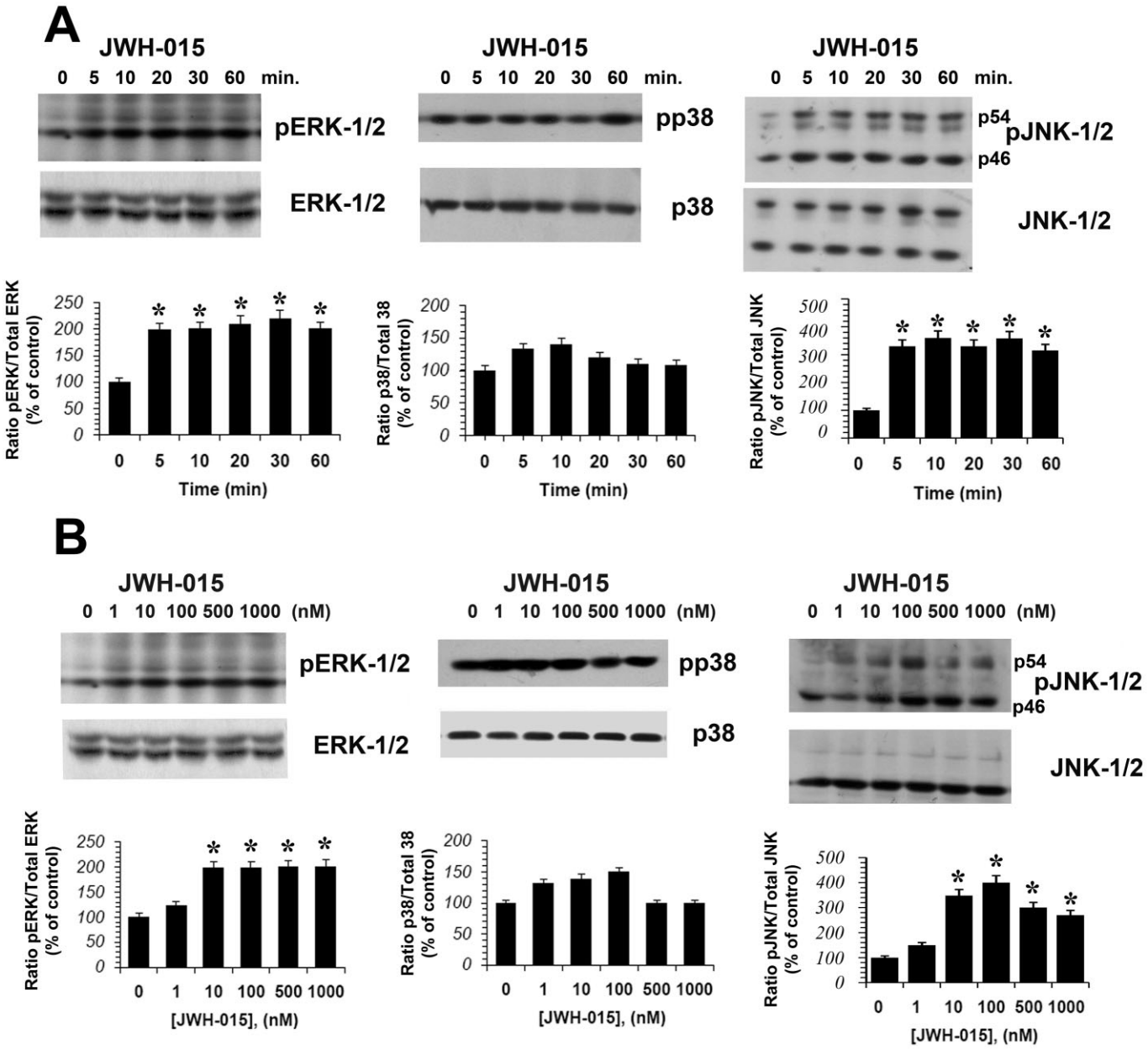


Figure 2

Effects of stimulating CB_2 cannabinoid receptors in N9 cells. (A) N9 cells were incubated with 10 nM JWH-015 for the indicated time and ERK-1/2, p38 and JNK-1/2 phosphorylation levels were analysed by Western blot. (B) Western blot analysis of ERK-1/2, p38 and JNK-1/2 phosphorylation in N9 cells incubated for 30 min with JWH-015 (1–1000 nM). The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). MAPK phosphorylation was expressed as ratios between phosphorylated and total MAPK intensity (pMAPK/MAPK). The mean values of three independent experiments (one of which is shown) were normalized to the result obtained with JWH-015-untreated cell cultures. Densitometric analysis is reported. The unstimulated control (0, cells in the absence of JWH-015) was set to 100%. *$P < 0.05$, significantly different from unstimulated control; ANOVA followed by Dunnett's test.

We then set out to evaluate the concentration-dependent effects of JWH-015 on N9 cells treated for 10 min. As shown in Figure 2B, JWH-015 (10–1000 nM) increased p-ERK-1/2 and p-JNK-1/2 expression levels but did not modulate p-p38. Furthermore, addition of the antagonist AM 630 (0.1–1 μM) blocked the JWH-015-induced (10–1000 nM) increase in ERK-1/2 and JNK-1/2 phosphorylation (Figure 3A,B). The CB_1 receptor antagonist AM 251 (1 μM), on the other hand, had no effect on the increase in ERK-1/2 and JNK-1/2 phosphorylation levels induced by JWH-015 (1 μM; Figure 3B).

To further investigate the possible involvement of the G_i/G_o-proteins in the signal transduction pathway responsible for ERK-1/2 and JNK-1/2 modulation, N9 cells were treated with PTX (50 ng·mL^{-1}). Blockade of G_i/G_o-protein dis-

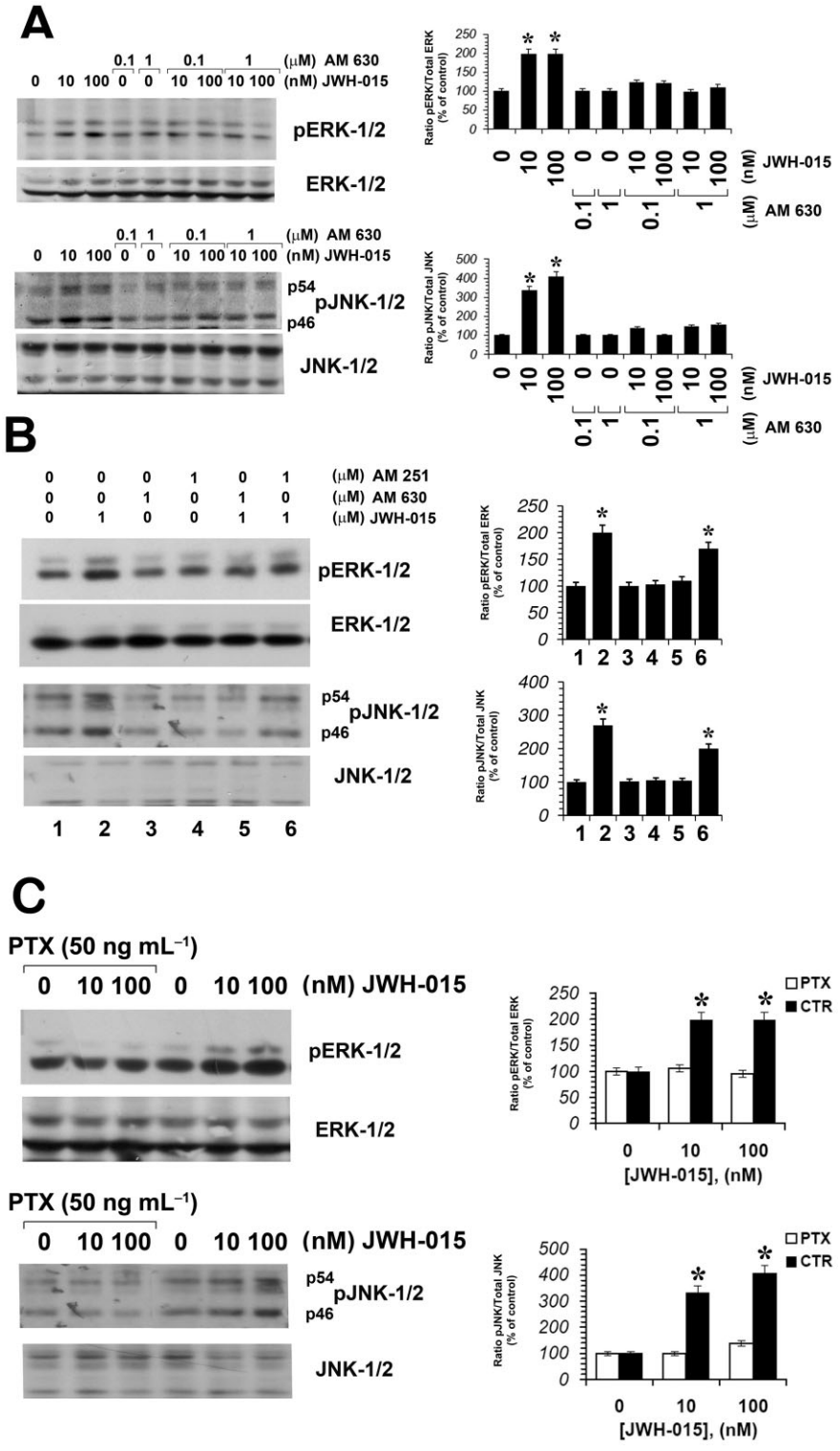


Figure 3

The CB_2 cannabinoid receptor and the G_i/G_o-protein pathway in N9 cells. (A) Western blot analysis of p-ERK-1/2 and p-JNK-1/2 expression levels. N9 cells were incubated with JWH-015 (10 and 100 nM) and AM 630 (0.1 and 1 μM) alone or in combination. (B) Western blot analysis of p-ERK-1/2 and p-JNK-1/2 expression levels. N9 cells were incubated with JWH-015 (1 μM), AM 630 91 μM) and AM 251 (1 μM), alone or in combination. (C) N9 cells were incubated with or without PTX (50 ng·mL^{-1} for 14 h) and then with or without JWH-015 (10 and 100 nM) for additional 10 min. The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). MAPK phosphorylation was expressed as ratios between phosphorylated and total MAPK intensity (pMAPK/MAPK). Densitometric analysis is reported. The unstimulated control (0, cells in the absence of JWH-015) was set to 100%. *$P < 0.05$, significantly different from unstimulated control; ANOVA followed by Dunnett's test.

sociation with PTX abolished the JWH-015-induced activation of ERK-1/2 and JNK-1/2 in N9 cells (Figure 3C).

Effects of activating CB_2 receptors on MAPKs in activated N9 and primary microglial cells

We went on to investigate how the activation of microglial cells interferes with the signalling pathways modulated by CB_2 receptors by maintaining microglial N9 cells in LPS-supplemented (1 μg·mL^{-1}) DMEM; the ability of JWH-015 (10 and 100 nM) to modulate MAPKs was evaluated after 15 and 30 min. As shown in Figure 4A, LPS significantly increased all kinases under investigation. In contrast, the CB_2 receptor agonist JWH-015 failed to modulate the activity of LPS on p38 and JNK-1/2 at any time, but p-ERK-1/2 levels in LPS-treated cells were increased at 15 min. Conversely, JWH-015 significantly decreased ERK-1/2 phosphorylation levels in LPS-activated cells after 30 min of treatment (Figure 4A).

These results were confirmed in primary microglial cells, which yielded similar results to those observed in N9 cells. In particular, as shown in Figure 4B, JWH-015 (100 nM) significantly decreased ERK-1/2 phosphorylation levels in LPS-activated primary cells (after 30 min of treatment).

Figure 4C shows that, in LPS-treated primary microglial cells, the CB_2 antagonist AM 630 (1 μM) abolished the effect of JWH-015 on ERK-1/2 phosphorylation, thereby confirming CB_2 receptor involvement. In contrast, the CB_1 antagonist AM 251 (1 μM) did not reverse the ability of JWH-015 to down-regulate the LPS-induced increase in p-ERK-1/2 (Figure 4C).

We then studied the time-course of the effects of the CB_2 receptor agonist on LPS signalling. As shown in Figure 4D, LPS stimulation of microglial cells resulted in a rapid (after 15 min) increase in ERK-1/2 phosphorylation, which was maximal at 30 min and declined towards basal levels within 60 min. In the presence of the CB_2 agonist JWH-015 (100 nM), on the other hand, ERK-1/2 phosphorylation was significantly higher at 15 min than with LPS alone. In contrast, ERK-1/2 phosphorylation levels were significantly reduced by LPS+JWH-015 at the 30 min incubation time point, as compared with LPS alone. Activation of ERK-1/2 returned to baseline levels within 120 min.

Finally, we studied the intracellular distribution of activated ERK-1 and ERK-2 MAPKs in N9 cells during LPS (1 μg·mL^{-1}) and JWH-015 (100 nM) treatment, alone and in combination, at 15 and 30 min. As seen in Figure 5, faint fluorescence caused by anti-ERK-1 and anti-ERK-2-activated isoforms was detected in the cytoplasm of cells treated with drug vehicle, contrasting with the strong fluorescence present in the nuclei of LPS- and JWH-015-treated cells after 15 and 30 min of treatment. Activated ERKs were also detected in the nuclei of N9 cells after 15 min of treatment with LPS in combination with JWH-015, but not after 30 min of combined treatment.

CB_2 receptors modulate Raf-1 and MEK-1/2 kinases in quiescent N9 microglial cells

We investigated the levels of p-ERK upstream effectors, namely, p-Raf-1 and p-MEK-1/2, after CB_2 receptor stimulation in N9 cells. A significant increase in p-Raf expression levels was noted after 5 min of treatment with JWH-015 in the range 1–1000 nM (Figure 6A). The increase in Raf phosphorylation was marked until 30 min but had disappeared entirely by 60 min. Interestingly, the kinetics of Raf phosphorylation were comparable with those of ERK-1/2 phosphorylation. Furthermore, the JWH-015-induced increase of Raf activity was blocked by the addition of the selective CB_2 antagonist AM 630 (0.1–1 μM) (Figure 6A).

An increase in MEK-1/2 phosphorylation in N9 cells was promptly (5 min) induced by JWH-015 (1–500 nM; Figure 6B), an effect that was prevented by the CB_2 receptor antagonist AM 630 (0.1–1 μM) (Figure 6B).

p-Raf and p-MEK-1/2 levels were then evaluated in N9 cells treated with 1 μg·mL^{-1} LPS for 5 and 20 min. LPS increased p-Raf and p-MEK-1/2 levels at 5 and 20 min of treatment, JWH-015 had no effect on LPS activity at 5 min (Figure 6C), while CB_2 receptor activation inhibited LPS stimulation of Raf and MEK-1/2 after 20 min of treatment (Figure 6D). Moreover, the antagonist AM 630 (1 μM) blocked the ability of JWH-015 to decrease the LPS-modulated p-Raf and p-MEK-1/2 expression levels at 20 min (Figure 6D). Figure 6E shows that blockade of G_i/G_o-protein dissociation by PTX (50 ng·mL^{-1}) abolished the ability of JWH-015 (10 and 100 nM) to impair LPS-induced Raf, MEK-1/2 and ERK-1/2 phosphorylation.

CB_2 receptors modulate ERK-1/2 kinases by a NO-dependent pathway in activated primary microglial cells

To define the signalling responsible for ERK-1/2 activation, downstream of CB_2 receptors, in microglia, quiescent and activated primary microglial cells were treated for 30 min with JWH-015 (100 nM) in the presence of the NOS inhibitor L-NAME (100 μM). Figure 7A shows that L-NAME failed to alter the increased p-ERK-1/2 levels provoked by JWH-015, indicating that activation of the CB_2 receptor increased ERK activation in quiescent microglia cells in a NO-independent manner. In contrast, L-NAME blocked the ability of JWH-015 to down-regulate LPS-induced ERK activation in activated microglial cells, suggesting that CB_2 receptor stimulation reduced the effects of LPS on ERK-1/2 phosphorylation via a NO-mediated mechanism (Figure 7B).

To investigate the potential role of NO in LPS-mediated up-regulation of p-ERK-1/2, primary microglial cells were treated with either LPS alone, LPS + JWH-015 (100 nM) or LPS + the NO donor SNP (2.5 mM) for 30 min. As shown in Figure 7C, LPS significantly increased ERK-1/2 phosphorylation levels in primary microglial cells, an effect that was attenuated by JWH-015 and also by SNP, thereby confirming that an excess of intracellular NO is sufficient to impair p-ERK expression levels in primary microglial cells activated.by LPS.

In order to analyse iNOS protein expression, primary microglial cells were then stimulated with LPS, with or without JWH-015, and Western blotting was performed. As shown in Figure 7D, iNOS protein was induced after 4 h of stimulation with LPS (1 μg·mL^{-1}). This induction was reduced by co-treatment with either JWH-015 or U0126 (0.5–1 μM).

Temporal conditions and concentrations of LPS required for optimal production of NO in primary microglial cells were then defined. Nitrite levels were measured at 30 min and 4 h in culture supernatants of microglial cells maintained in DMEM supplemented with LPS (1 μg·mL^{-1}; Figure 7E,F).

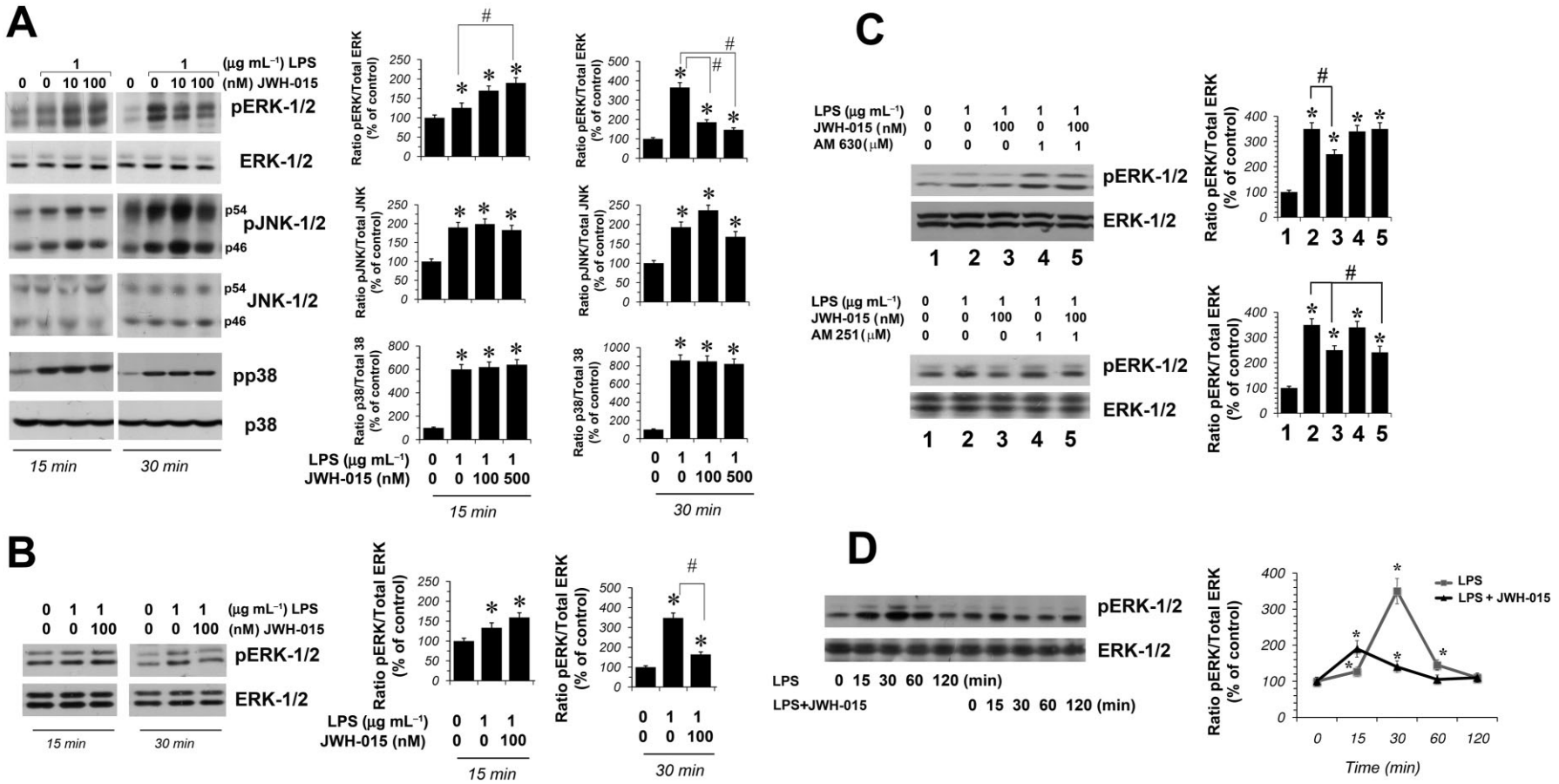


Figure 4

Effect of CB_2 cannabinoid receptor stimulation in activated microglia cells. (A) JWH-015 effect on ERK-1/2, JNK-1/2 and p38 phosphorylation in N9 cells treated with LPS. N9 cells were incubated with DMSO vehicle (lane 0) or with JWH-015 (10 and 100 nM) in the presence of LPS (1 μg·mL^{-1}) for 15 and 30 min. (B) JWH-015 effect on ERK-1/2 phosphorylation in primary microglia cells treated with LPS. Microglial cells were incubated with DMSO vehicle (lane 0) or with JWH-015 (100 nM) in the presence of LPS (1 μg·mL^{-1} for 15 and 30 min). (C) Primary microglial cells were incubated with DMSO vehicle (lane 1, control), LPS (1 μg·mL^{-1}; lane 2), LPS + JWH-015 (100 nM), LPS + AM 630 (1 μM; upper panel)/AM 251 (1 μM; lower panel) (lanes 3 and 4, respectively), and with LPS+JWH-015 + AM 630/AM 251 (lane 5) for 30 min. (D) Time-dependent pERK-1/2/MAPK phosphorylation induced by LPS (1 μg·mL^{-1}) and LPS + JWH-015 (100 nM) after 0, 15, 30, 60 and 120 min. The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS. Plots are mean ± SE values ($n = 3$). The unstimulated control (0) was set to 100%. The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). Densitometric analysis of MAPK activation is shown as the ratio of phospho-protein to total protein. *$P < 0.05$, significantly different from untreated cells; #$P < 0.05$, significantly different from cells treated with LPS alone; ANOVA followed by Dunnett's test.

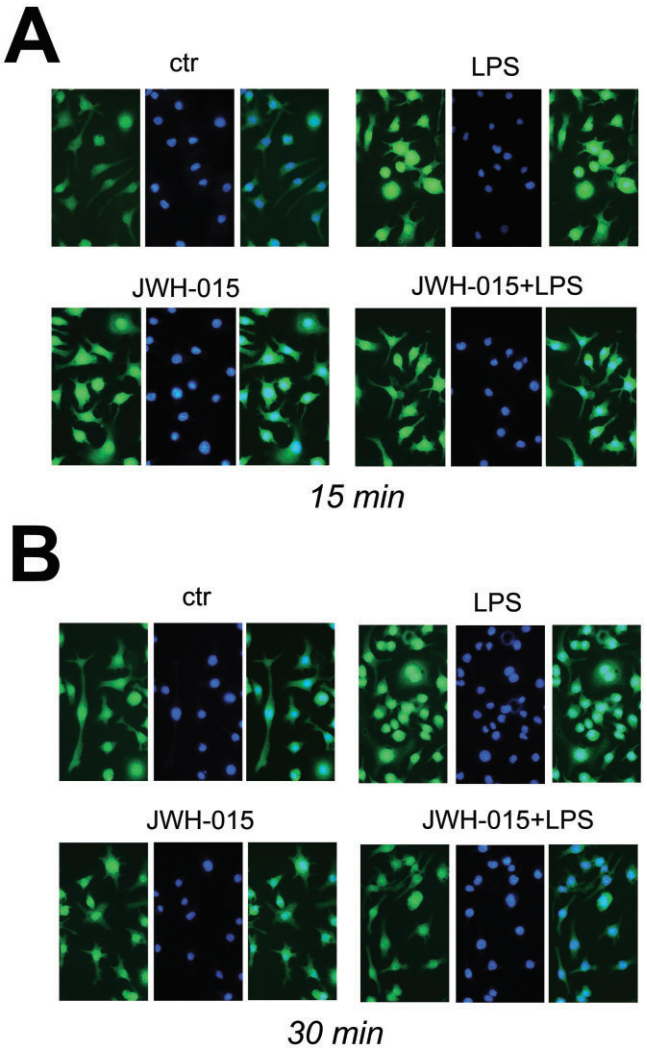


Figure 5

Intracellular localization of pERK-1/2. N9 cells (untreated cells, ctr) were treated with LPS (1 μg·mL^{-1}) alone or in combination with JWH-015 (100 nM) for 15 or 30 min. Cells were then stained with antibody to ERK-1/2 phosphorylated isoforms and detected by goat FITC anti-rabbit IgG. The fluorescent dye DAPI (blue panel) was used to stain the nuclei of all microglial cells. Overlap of DAPI and FITC is shown (right panels). Original magnification, ×40.

Primary microglial cultures were incubated with JWH-015 (0.5–1 μM) before and after LPS activation. Different effects of CB_2 receptor stimulation on nitrite levels were seen in quiescent versus LPS-activated microglial cells. In the former, JWH-015 increased LPS-induced nitrite levels at 30 min of treatment, when this increase was particularly significant (Figure 7E). In contrast, at 4 h, nitrite levels induced by LPS were decreased by CB_2 receptor stimulation (Figure 7F).

Subsequently, the effect on nitrite release of pre-treatment of microglial cells with the CB_2 antagonist AM 630 administered before exposure to JWH-015 was examined. 1 μM AM 630 alone had no effect on microglial cell release of nitrite but did reverse the activity of JWH-015 (Figure 7E,F).

The effect on nitrite release of pre-treatment of microglial cells with U0126 (1 μM), a specific inhibitor of MEK-1/2, the upstream regulator of ERK-1/2 phosphorylation, before exposure to LPS and JWH-015 was investigated; U0126 administered alone had no effect on nitrite release by microglial cells. Furthermore, in cells pre-treated with U0126, LPS for 30 min induced no significant increase in nitrite levels, as compared with cells treated with LPS alone (Figure 7E), indicating that LPS increased nitrite in an ERK-1/2-dependent manner. In contrast, the potentiating effect of JWH-015 on LPS seen at 30 min was not abolished by U0126. There, unlike LPS, CB_2 receptor activation must increase NO production in microglial cells by ERK-independent signalling. These data are in agreement with those reported in Figure 7B, which apparently shows p-ERK downstream of NO in CB_2-mediated signalling. At 4 h, JWH-015 reduced LPS-mediated NO accumulation; this activity was mimicked by U0126, which suggests that, at 4 h, the effects of CB_2 receptor stimulation in LPS-stimulated cells are consequent to the down-regulation of ERK-1/2 activation (Figure 7F).

Similar results were obtained with the MEK-1 inhibitor PD98059 in LPS- and JWH015-treated cell cultures (30 min and 4 h, data not shown).

CB_2 and TLR4 receptor gene silencing in N9 cells

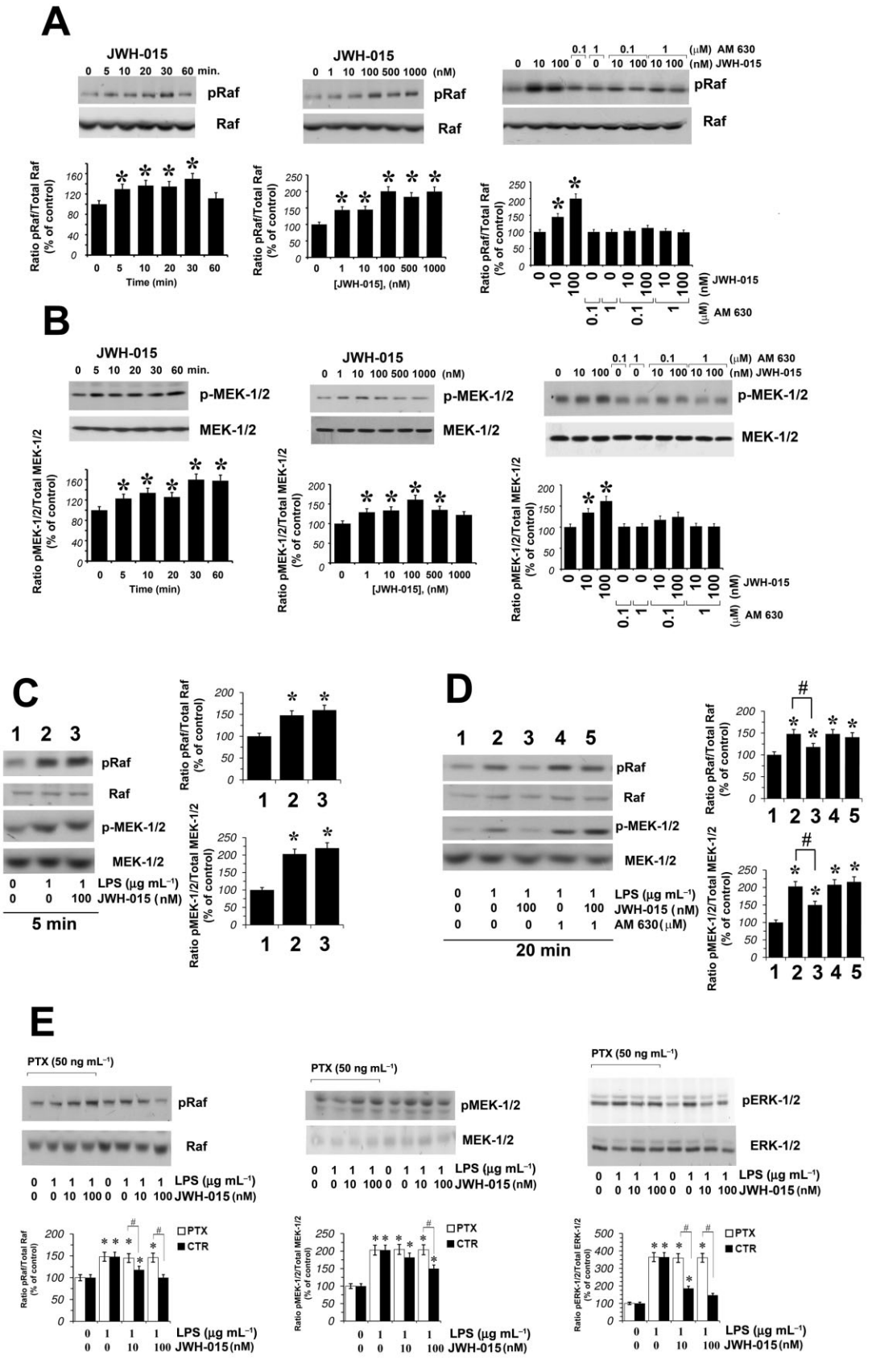
To confirm the apparent role of CB_2 receptors and to investigate the involvement of TLR4, we reduced CB_2 and TLR4 receptor expression in N9 cells by siRNA transfection, in order to cause transient knockdown of the CB_2 and TLR4 receptor genes. N9 cells were transfected with non-specific random control ribonucleotides (siRNA scrambled, $siRNA_{ctr}$) or with small interfering RNAs that targeted CB_2 or TLR4 receptor mRNAs ($siRNA_{CB2}$ or $siRNA_{TLR4}$, respectively) for degradation.

As seen in Figure 8A, CB_2 receptor protein expression was strongly reduced after 48 and 72 h of treatment with $siRNA_{CB2}$. Therefore, 48 h after $siRNA_{CB2}$ transfection, N9 cells were exposed to LPS (1 μg·mL^{-1}) and JWH-015 (100 nM) for 30 min, after which ERK-phosphorylated protein levels were measured. This revealed that inhibition of CB_2 receptor expression was sufficient to block the JWH-015-induced inhibition of ERK-1/2 phosphorylation levels increased by LPS (Figure 8B). These results clearly show the connection between CB_2 receptor stimulation and ERK signalling in activated microglial cells.

Likewise, Figure 8C shows that TLR4 receptor expression was reduced after 48 and 72 h of treatment with $siRNA_{TLR4}$. Therefore, 72 h post-$siRNA_{TLR4}$ transfection, N9 cells were exposed to LPS (1 μg·mL^{-1}) and JWH-015 (100 nM) for 30 min. Inhibition of TLR4 receptor expression blocked LPS-induced ERK-1/2 activation (Figure 8D). In contrast, JWH-015 significantly increased ERK-1/2 phosphorylation levels even in the absence of TLR4, indicating that this agonist acted in a TLR4-independent manner.

Discussion and conclusions

As microglial differentiation and immune function is regulated by activation of CB_2 receptors (Stella, 2010), we set out


A
JWH-015
0 5 10 20 30 60 min.
pRaf
Raf
JWH-015
0 1 10 100 500 1000 (nM)
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0.1 1 0.1 1 (μM) AM 630
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Raf
Ratio pRaf/Total Raf (% of control)
Time (min)
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B
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MEK-1/2
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p-MEK-1/2
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0 0 10 100 0 0 10 100 JWH-015 (nM)
Ratio pRaf/Total Raf (% of control)
Ratio pMEK-1/2/Total MEK-1/2 (% of control)
Ratio pERK-1/2/Total ERK-1/2 (% of control)
PTX
CTR
0 1 1 1 LPS (μg mL⁻¹)
0 0 10 100 JWH-015 (nM)

Figure 6

CB_2 cannabinoid receptors modulate Raf and MEK-1/2 phosphorylation in quiescent (A–B) and activated (C–E) N9 cells. Cells were incubated with 10 nM JWH-015 for the indicated time (from 0 to 60 min) or for 30 min with different concentrations of JWH-015 (1 – 1000 nM). Raf (A) and MEK-1/2 (B) kinase phosphorylation levels were quantified by Western blot analysis. Furthermore, N9 cells were incubated with JWH-015 (10 and 100 nM). AM 630 (0.1 and 1 μM) was added alone or in the presence of JWH-015 (10 and 100 nM) before determination of Raf (A) and MEK-1/2 (B) phosphorylation. N9 cells were treated with LPS (1 $\mu g \cdot mL^{-1}$) for 5 (C) or 20 (D) min. The effect of the antagonist AM 630 (1 μM) is shown. (E) N9 cells were incubated with or without PTX (50 $ng \cdot mL^{-1}$ for 14 h) and then with or without LPS (1 $\mu g \cdot mL^{-1}$) alone or in combination with JWH-015 (10 and 100 nM) for additional 30 min. The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of JWH-015. Densitometric analysis is shown as the ratio of phospho-protein to total protein. The unstimulated control (0, cells in the absence of JWH-015) was set to 100%. *$P < 0.05$, significantly different from unstimulated control; analysis was by ANOVA followed by Dunnett's test.

to characterize the signalling pathways modulated by these receptors expressed in microglial cells. CB_2 receptor ligands (agonist and antagonist) and CB_2-receptor-knockout microglial cells were thus used to determine the role of the cannabinoid system in *in vitro* models, using N9 and primary microglial cells. By this means we described the mechanism by which CB_2 cannabinoid receptors modulated the MAPK signalling in response to LPS, an agent widely used experimentally to create inflammation in the brain (Lehnardt *et al.*, 2002).

Multiple signalling molecules are activated in response to LPS in microglia (Aloisi, 2001). In particular, while microglial cells display a quiescent phenotype in the normal brain, they become highly activated upon insult, altering their phagocytic and antigen presentation functions, as well as the production of cytokines and NO release (Lehnardt *et al.*, 2002; Hanisch and Kettenmann, 2007). Pathways associated with the induction of pro-inflammatory mediators of microglia include reactive oxygen species, PI3K/Akt, MAPKs and NF-κB (Aloisi, 2001; Hanisch and Kettenmann, 2007).

The discrepancies between physiological microglia and microglial cell cultures, including primary cultured microglia, have been much discussed (Horvath *et al.*, 2008), and N9, being an artificially immortalized cell line, may not entirely reflect the physiological functions of microglia. In an attempt to clarify this situation, we compared mouse primary microglial and N9 cells in experiments assessing the phosphorylation of ERKs and production and release of NO. As N9 cells responded in a similar fashion to primary microglia, the cell line did, in our system, provide an adequate model of primary microglia.

Moreover, in this study, we showed that CB_2 cannabinoid receptors had different effects on ERK activation in microglial cells, depending on the duration of cannabinoid exposure and the state of cell activation. In particular, CB_2 receptor stimulation increased ERK-1/2 phosphorylation levels in quiescent and LPS-treated cells for 15 min, while, surprisingly, in the presence of both stimuli (LPS and JWH-015), phosphorylation of ERK-1/2 was reduced following 30 min of treatment. The signalling revealed in the presence of $siRNA_{CB2}$ confirmed the role of CB_2 receptors in microglial cells. Thus, we demonstrated that CB_2 receptor stimulation provoked a state of activation in microglial cells by rapid MAPK-phosphorylation but prevented over-activation in the presence of a second stimulus.

It has been previously observed that CB_2 receptor stimulation leads to ERK-mediated cellular activation and anti-inflammatory effects in monocytes/macrophages and microglia (Gertsch *et al.*, 2008; Correa *et al.*, 2010). Furthermore, the endocannabinoid AEA has been shown to protect neurones from inflammatory damage during CNS inflammation by rapid $CB_{1/2}$-receptor-mediated induction of MKP-1 in microglial cells (Eljaschewitsch *et al.*, 2006). Hence, rapid AEA-induced MKP-1 expression switches off MAPK signal transduction in activated microglial cells. Similarly, a more recent study has demonstrated that CB_2 receptor stimulation in microglial cells induces an anti-inflammatory phenotype and reduces migration via MKP-induced ERK dephosphorylation (Romero-Sandoval *et al.*, 2009). Interestingly, here we show that the signalling observed in ERK-1/2 kinase is reflected in Raf and MEK-1/2, thereby indicating that the CB_2 receptor is coupled to the activation of the ERK-1/2 kinase cascade through a classical upstream mechanism involving G_i/G_o-protein βγ–subunits, Raf and MEK-1/2.

It has been reported that CB receptor agonists inhibit the production of pro-inflammatory molecules, which can be induced by LPS, in CNS glial cells (Molina-Holgado *et al.*, 2002; Fachinetti *et al.*, 2003; Ortega-Gutiérrez *et al.*, 2005; Sheng *et al.*, 2005; Correa *et al.*, 2008; 2009). In particular, CB_2 receptors affect the production of the potent inflammatory mediator NO, released from quiescent and, to a greater extent, from activated microglia (Stella, 2010). Here we characterized, for the first time, the events occurring when quiescent microglia are exposed to LPS and CB_2 receptor agonist for 30 min, showing that CB_2 receptor stimulation increased their response to LPS without involving the TLR-4 pathway. Interestingly, at 15 and 30 min, the increase in ERK activation and nitrite levels in response to LPS plus CB_2 receptor stimulation was found to be additive rather than synergistic. However, at 30 min, CB_2 receptor stimulation was shown to reduce LPS-induced ERK-1/2 increase in LPS activated microglia through modulation of NO production. These data suggest that CB_2 receptor stimulation alone activates the MAPK pathway but switches off MAPK signal transduction by rapid induction of NO, in the presence of a second microglial stimulus. Therefore, CB_2 receptors expressed in microglia may participate in regulating neuroinflammation and provide neuro-protection by tempering LPS-induced NO synthesis.

In agreement with previous reports that all three MAPK pathways are involved in iNOS gene regulation in response to inflammatory stimuli in astrocytes and microglia (Bhat *et al.*, 1998; Bodles and Barger, 2005; Wang *et al.*, 2006), we showed that CB_2 receptor stimulation specifically reduced iNOS activation via inhibition of ERK-1/2 phosphorylation. This indicates that inhibition of MAPK pathways, and hence reduction in iNOS expression and NO production, is an important

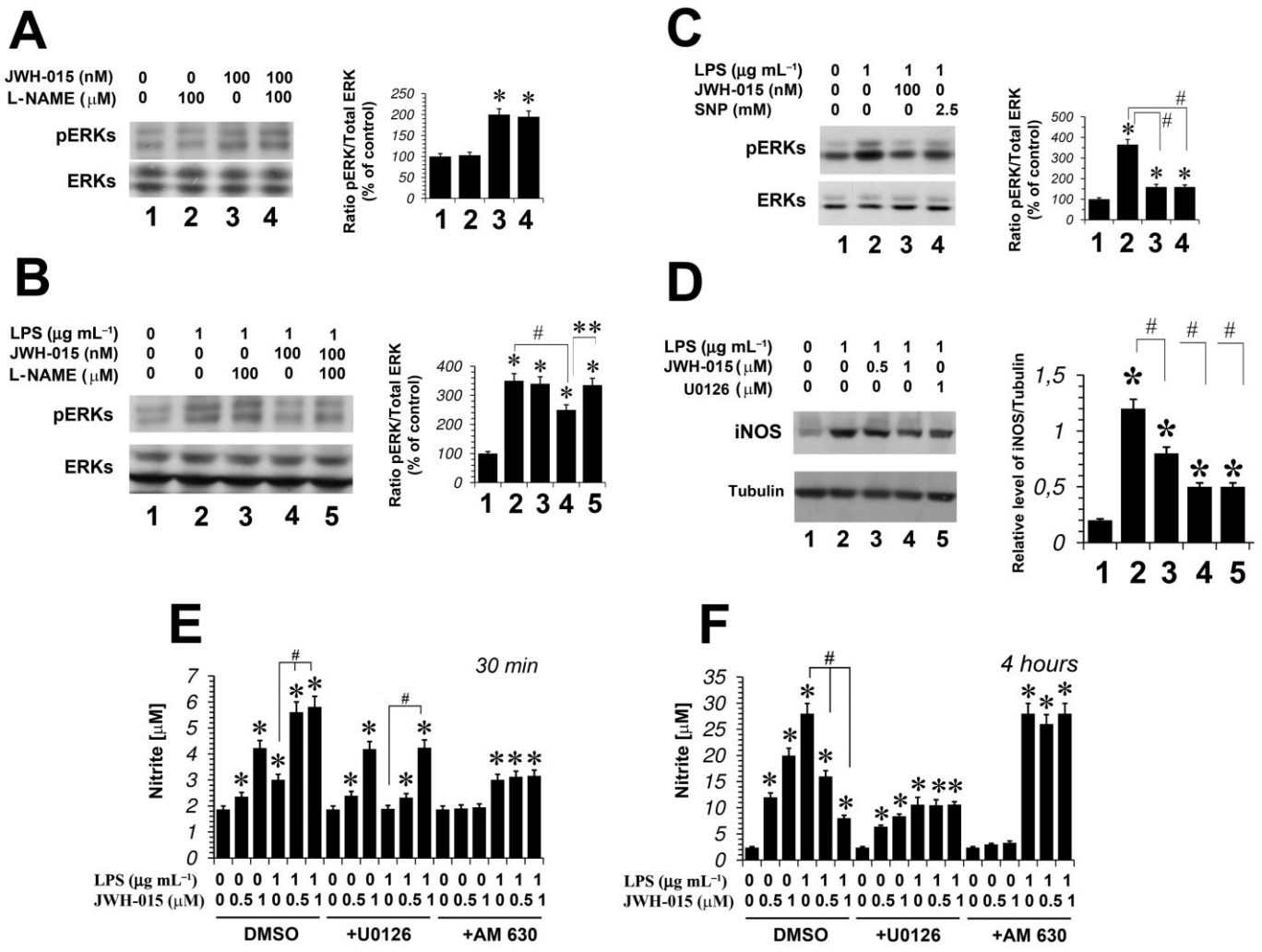

Figure 7

The CB_2 cannabinoid receptor and the NO pathway in primary microglial cells. (A) Primary microglial cells were incubated with DMSO vehicle (lane 1, control), L-NAME (100 μM; lanes 2, 4) and with JWH-015 (100 nM; lanes 3–4). (B) Primary microglial cells were incubated with DMSO vehicle (lane 1, control), LPS (1 μg·mL^{-1} ; lane 2) and with JWH-015 (100 nM; lanes 4–5) or L-NAME (100 μM lanes 3, 5) for 30 min. (C) Primary microglial cells were incubated for 30 min with DMSO vehicle (lane 1) or LPS (1 μg·mL^{-1}; lanes 2–4), JWH-015 (100 nM; lane 3) or SNP (2.5 mM; lane 4). The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS. Densitometric analysis is reported as the ratio of phospho-protein to total protein. The unstimulated control was set to 100%. *$P < 0.05$ with respect to unstimulated control; $^{\#}P < 0.05$, significantly different from cells treated with LPS alone; **$P < 0.05$, significantly different from LPS + JWH-015; ANOVA followed by Dunnett's test. (D) Primary microglial cells were treated with LPS (1 μg·mL^{-1}) for 4 h without and with JWH-015 (0.5 and 1 μM) to assess the effect on iNOS levels. These cells were harvested for Western analysis of iNOS expression. The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS. The unstimulated control was set to 100%. *$P < 0.05$, significantly different from unstimulated control; $^{\#}P < 0.05$, significantly different from cells treated with LPS alone; ANOVA followed by Dunnett's test. (E–F) Effect of LPS and CB_2 receptor stimulation on nitrite levels in microglial cells. Primary microglial cells were treated with JWH-015(0.5 and 1 μM), with or without LPS (1 μg·mL^{-1}) and culture supernatants were assayed for nitrite after 30 min (E) and 4 h (F) of treatment. The effects of U0126 (1 μM) and of AM 630 (1 μM) are shown. *$P < 0.01$, significantly different from untreated cells; $^{\#}P < 0.05$, significantly different from LPS-treated cells. *$P < 0.01$, significantly different from untreated cells; $^{\#}P < 0.05$, significantly different from LPS-treated cells; ANOVA followed by Dunnett's test.

cellular signalling cascade in glial cell activation, suggesting a functional inhibitory role for the cannabinoid system in NO production. This accords with previous observations describing cannabinoid responses in activated microglia and in macrophage-like cells (Stefano *et al.*, 1996; Ross *et al.*, 2000; Walter *et al.*, 2003; Ehrhart *et al.*, 2005; Cabral *et al.*, 2008; Kreitzer and Stella, 2009; Pietr *et al.*, 2009; Oh *et al.*, 2010). Indeed, endogenous or synthetic cannabinoids are known to inhibit LPS-induced inducible NOS expression (mRNA and protein) through CB_1 receptors in astrocyte cultures. In addition, Δ^9-tetrahydrocannabinol has been reported to produce a marked inhibition of NO release in the macrophage cell line RAW 264.7 by inhibiting NF-κB/Rel activation and increasing iNOS transcription (Jeon *et al.*, 1996). Moreover, the CB_1

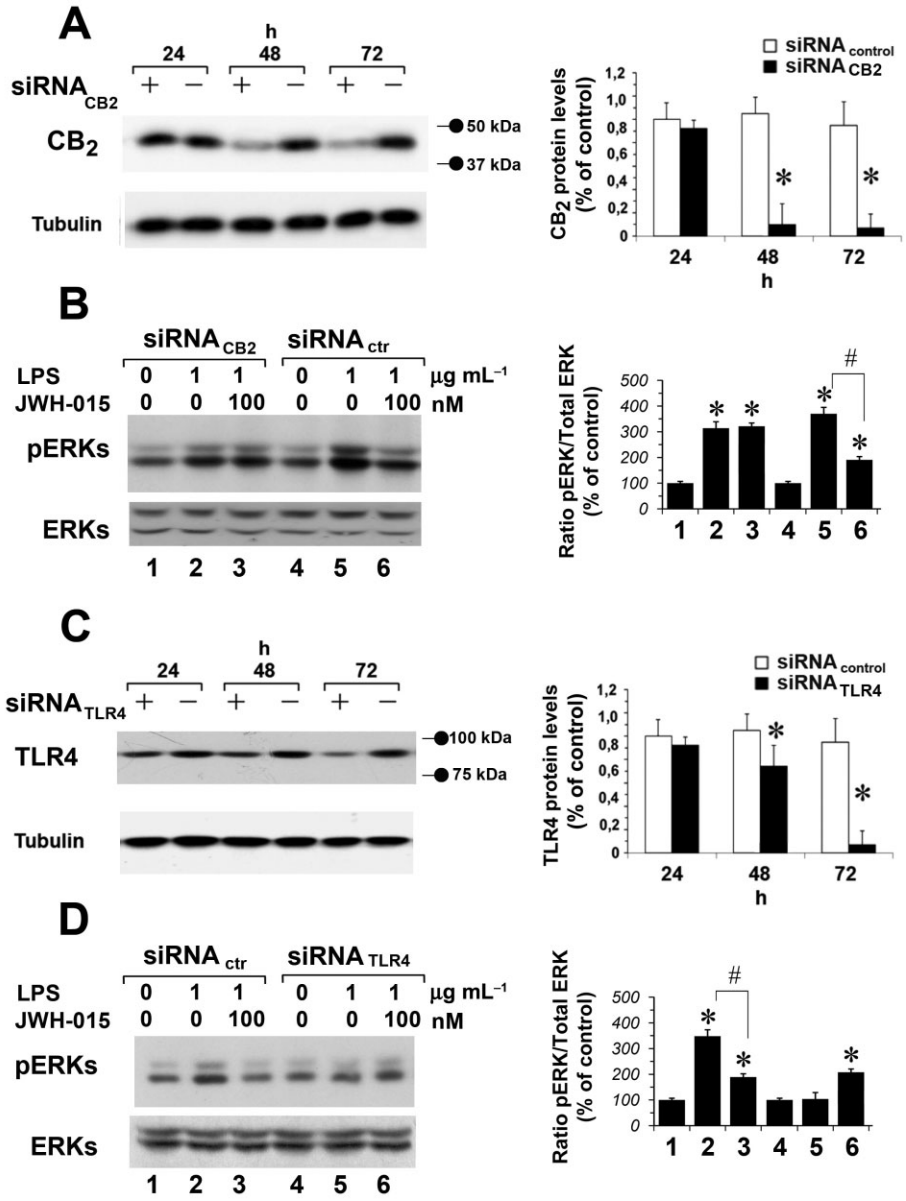

Figure 8

CB_2 and TLR4 receptor expression silencing. (A) N9 microglial cells were treated with scrambled (–) (control) or with $siRNA_{CB2}$ (+) and cultured for 24, 48 and 72 h. Tubulin shows equal loading of protein. Densitometric quantification of CB_2 receptors by Western blot; plots are mean ± SE values ($n = 3$); *$P < 0.01$, significantly different from the control (scrambled transfected cells). (B) N9 microglial cells were treated with $siRNA_{CB2}$ or without (control) $siRNA_{CB2}$ for 48 h and cultured with LPS (1 µg·mL^{-1}) alone or in combination with JWH-015 (100 nM) for 30 min. Densitometric analysis of phosphorylated isoform is shown as the ratio of phospho-protein to total protein The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS. The unstimulated control was set to 100%. *$P < 0.05$, significantly different from unstimulated control; $^{\#}P < 0.05$, significantly different from cells treated with LPS alone; ANOVA followed by Dunnett's test. (C) N9 microglial cells were treated with scrambled (–) (control) or with $siRNA_{TLR4}$ (+) and cultured for 24, 48 and 72 h. Tubulin shows equal loading of protein. Densitometric quantification of TLR4 receptor by Western blot; plots are mean ± SE values ($n = 3$); *$P < 0.01$ compared with the control (scramble transfected cells). (D) N9 microglial cells were treated with $siRNA_{TLR4}$ or without (control) $siRNA_{TLR4}$ for 72 h and cultured with LPS (1 µg·mL^{-1}) alone or in combination with JWH-015 (100 nM) for 30 min. Densitometric analysis of phosphorylated isoform is reported as the ratio of phospho-protein to total protein is used. The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS. The unstimulated control was set to 100%. *$P < 0.05$, significantly different from unstimulated control; $^{\#}P < 0.05$, significantly different from cells treated with LPS alone; ANOVA followed by Dunnett's test.

receptor has been implicated in the regulation of neuronal NOS in cerebellar granule neurons (Hillard *et al.*, 1999). In cultured rat microglial cells, on the other hand, inhibition of LPS/IFN-γ-induced NO release by the agonist CP-55940 has been shown to act via a CB_1 receptor-mediated pathway (Waksman *et al.*, 1999). However, in macrophages, the inhibition of LPS-mediated NO release by WIN-55212 occurs after activation of CB_2 receptors (Ross *et al.*, 2000).

In addition, it has been recently reported that pharmacological stimulation of CB_2 receptors and manipulation of endocannabinoid tone, via inhibition of AEA hydrolysis in microglia, could result in modification of the cytokine milieu, contributing to the accumulation of anti-inflammatory microglia at lesion sites, in this case in multiple sclerosis (Correa *et al.*, 2010). In particular, it has been demonstrated that AEA inhibits the production of biologically active IL-12p70 and IL-23. These cytokines play a crucial role in the pathogenesis of multiple sclerosis by activating human (and murine) microglial cultures, acting through the ERK-1/2 and JNK pathways to down-regulate IL-12p70 and IL-23 and up-regulate IL-10, effects that are partially mediated by CB_2 receptor activation (Correa *et al.*, 2009; 2010).

Furthermore, it is well known that NO has an influence on critical signalling proteins; indeed, exposure of cells to NO and NOS inhibitors has revealed their complex effects on the production of cytokines. Precursors of IL-1β and IL-18 are not cleaved in the presence of NO, which also inhibits the expression of IL-8 and MCP-1. In contrast, NO increased the release of TNFα and IL-6 acting simultaneously as a pro-inflammatory and anti-inflammatory agent (Murphy, 2000). Therefore, the modulation of NO mediated by CB_2 receptors may affect important target proteins in the CNS.

In conclusion, our results indicate a regulatory role for the CB_2 receptor in preventing excessive microglial cell response to injury in activated microglial cells. Hence, the development of selective CB_2 receptor agonists may open new avenues of therapeutic intervention, with the aim of reducing the release of pro-inflammatory mediators, especially in chronic neuropathologies. This is particularly exciting because selective CB_2 receptor agonists are devoid of the psychoactive side effects, characteristic of CB_1 receptor activation in the CNS (Fernández-Ruiz *et al.*, 2007).

Acknowledgements

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Conflicts of interest

None.

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