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RESEARCH PAPER

Cannabinoid CB_2 receptor attenuates morphine-induced inflammatory responses in activated microglial cells

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BACKGROUND AND PURPOSE

Among several pharmacological properties, analgesia is the most common feature shared by either opioid or cannabinoid systems. Cannabinoids and opioids are distinct drug classes that have been historically used separately or in combination to treat different pain states. In the present study, we characterized the signal transduction pathways mediated by cannabinoid CB_2 and μ-opioid receptors in quiescent and LPS-stimulated murine microglial cells.

EXPERIMENTAL APPROACH

We examined the effects of μ-opioid and CB_2 receptor stimulation on phosphorylation of MAPKs and Akt and on IL-1β, TNF-α, IL-6 and NO production in primary mouse microglial cells.

KEY RESULTS

Morphine enhanced release of the proinflammatory cytokines, IL-1β, TNF-α, IL-6, and of NO via μ-opioid receptor in activated microglial cells. In contrast, CB_2 receptor stimulation attenuated morphine-induced microglial proinflammatory mediator increases, interfering with morphine action by acting on the Akt-ERK1/2 signalling pathway.

CONCLUSIONS AND IMPLICATIONS

Because glial activation opposes opioid analgesia and enhances opioid tolerance and dependence, we suggest that CB_2 receptors, by inhibiting microglial activity, may be potential targets to increase clinical efficacy of opioids.

Abbreviations

AM 251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM 630, 6-iodopravadoline; CHO-hCB_2, CHO cells transfected with human CB_2 receptor; CTAP, D-Phe-*cyc*[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2; DAMGO, Tyr-DAla-Gly-[*N*MePhe]-NH$(CH_2)_2$; DPDPE, *cyc*[DPen^2, DPen^5]enkephalin; FBS, fetal bovine serum; JWH-015, (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone; PE, phycoerythrin; siRNA, small interfering RNA; THC, Δ^9tetrahydrocannabinol; U69593, 5α,7α,8β-(-)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxasipro(4,5)dec-8-yl)benzeneacetamide

Introduction

Opioids produce their pharmacological effects by acting mainly through three types of receptors, namely μ, δ and κ (Satoh and Minami, 1995; receptor nomenclature follows Alexander *et al.* (2011). Opioids are the drugs of choice for treating severe pain, despite the development of tolerance, dependence and hyperalgesia (da Fonseca Pacheco *et al.*, 2008). The cellular and molecular mechanisms involved in this phenomenon are complex and may involve receptor desensitization and endocytosis, intracellular signalling hyperactivity, secondary activation of excitatory amino acid receptors, and subsequent intracellular cascades, as well as glial activation and the release of proinflammatory mediators (Mayer *et al.*, 1999; Watkins *et al.*, 2005). Glial activation by opioids is an important phenomenon to understand, as it opposes opioid analgesia and enhances opioid tolerance, dependence and other negative side-effects, such as respiratory depression (Watkins *et al.*, 2009). In particular, there is good evidence that glia and glia-derived proinflammatory mediators, such as IL-1β, TNF-α and IL-6, could be involved in tolerance to the anti-nociceptive properties of morphine. Glial cells are also considered to be crucial sources of nitric oxide (NO), responsible for morphine tolerance (Mayer *et al.*, 1999; Chen and Sommer, 2009). Repeated morphine treatment can activate glia and hence up-regulate these various mediators (Raghavendra *et al.*, 2002; 2004; Johnston *et al.*, 2004; Watkins *et al.*, 2005) through the MAPK, PKC and PI3K/Akt pathways, key players in the intracellular signalling cascade leading to the development of morphine tolerance (Mayer *et al.*, 1999; Watkins *et al.*, 2001; Raghavendra *et al.*, 2002; 2004; Galeotti *et al.*, 2006; Cunha *et al.*, 2010).

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 receptors have been identified by molecular cloning – CB_1 and CB_2 receptors. The CB_1 receptors are expressed by the neurons and regulate the release of neurotransmitters, while CB_2 receptors are expressed by the microglia, regulating their motility and immunomodulator production (Atwood and Mackie, 2010; Pertwee *et al.*, 2010). In the nervous system, activation of CB_1 and CB_2 receptors is induced by the endocannabinoids, arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoylglycerol, produced by the neurons and glia. However, ample evidence suggests that additional receptors may contribute to the behavioural, vascular and immunological actions of Δ^9-tetrahydrocannabinol (THC), the major psychoactive constituent of marijuana, and endocannabinoids (Begg *et al.*, 2005). In particular, it has been established that GPR55 is an additional novel cannabinoid receptor (Lauckner *et al.*, 2008).

Microglia, a specialized population of macrophages found in the CNS, are quiescent in normal brain. However, after CNS injury or after interaction of LPS 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 proinflammatory and neurotoxic factors released by activated microglia (Jung *et al.*, 2010).

CB receptor agonists produce pain relief in a variety of animal models (Richardson, 2000). Cannabinoids act on glia and neurons to inhibit the release of proinflammatory molecules, including IL-1β, TNF-α and NO (Molina-Holgado *et al.*, 1997; 2002; Shohami *et al.*, 1997; Puffenbarger *et al.*, 2000; Cabral *et al.*, 2001), and enhance the release of the anti-inflammatory cytokines IL-4, IL-10 (Klein *et al.*, 2000) and IL-1 receptor antagonist (Molina-Holgado *et al.*, 2003). It is known that the anti-inflammatory properties of CB receptor agonists are expressed through the activation of CB_2 receptors (Klein and Newton, 2007; Romero-Sandoval *et al.*, 2009; Correa *et al.*, 2010; Hsieh *et al.*, 2011). In particular, the 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; Merighi *et al.*, 2012). Furthermore, such activation protects against human microglial neurotoxicity by enhancing IL-10 production (Klegeris *et al.*, 2003; Correa *et al.*, 2005; 2010; Eljaschewitsch *et al.*, 2006). The activation of CB_2 receptors also reduces the release of proinflammatory factors in animal models of peritoneal hypoxia-ischemia and Huntington's disease (Benito *et al.*, 2008). In the periphery, both CB_1 and CB_2 receptors participate in pain control (Malan *et al.*, 2001).

Interestingly, receptors for opioids and cannabinoids are coupled to similar intracellular signalling mechanisms, leading to a decrease in cAMP production through the activation of G_i proteins (Satoh and Minami, 1995; Pertwee *et al.*, 2010). Therefore, following the discovery that opioids and cannabinoids produce not only similar biochemical effects but also similar pharmacological effects, the interaction between these two classes of drugs has been extensively studied (Manzaneres *et al.*, 1999). Cannabinoids can enhance the antinociceptive properties of opioids (Cichewicz, 2004; Wilson *et al.*, 2008; Parolaro *et al.*, 2010) and adolescent exposure to chronic THC blocks opiate dependence in maternally deprived rats (Morel *et al.*, 2009). However, the molecular signalling underlying the participation of cannabinoids in the side effects induced by opioids is still unknown. Nevertheless, it is possible that cannabinoids may interfere with the tolerance and dependence effects induced by opioids because the administration of low-dose combinations of cannabinoids and opioids seems to be an alternative regimen that reduces the need to escalate opioid dose, while increasing opioid potency.

Therefore, the aim of the present study was to explore whether and how CB_2 receptor stimulation affected opioid actions on activated microglia, with a view to improving pain control by increasing the clinical efficacy of opioids.

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. The results of all studies involving animals are

reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). One-day-old Balb/c mice (50 in total) were obtained from Charles River (Calco, Italy).

Cell line, reagents and antibodies

CHO cells transfected with the human recombinant CB_2 receptor cDNA (CHO-hCB_2) were purchased from PerkinElmer (Milan, Italy). Tissue culture media and growth supplements were obtained 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-ERK1/2 pAb were provided by Promega (Milan, Italy). Phosphorylated (Thr^{180}/Tyr^{182}) and total p38, phosphorylated (Thr^{183}/Tyr^{185}) and total JNK1/2 antibodies were from Cell Signalling Technology (Celbio, Milan, Italy). JWH-015 (1-propyl-2-methyl-3-(1-naphthoyl)indole) (soluble in ethanol), SH5 (inhibitor of Akt), blocking peptide for pAb to CB_2 receptor (ALX-153-027) and anti-CB_2 receptor rabbit polyclonal antibody (ALX-210-198) were from Enzo Life Sciences (Vinci-Biochem, Florence, Italy). The immunogen for the CB_2 receptor antibody was a synthetic peptide corresponding to aa 20–33 of the human CB_2 receptor N-terminal. Anti-MOR-1 (H80) (sc-15310), small interfering RNA (siRNA) for the CB_2 receptor (sc-39913) and MOR-1 siRNA (sc-35958) 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 (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).

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, Carros, France) and decapitation, forebrains from newborn Balb/c mice were excised, meninges removed and tissue dissociated mechanically. Cells were re-suspended in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin, then plated on poly-D-lysine-coated (5 $\mu g \cdot mL^{-1}$) 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 microglia) or Ham's (CHOh-CB_2 cells) medium containing 10% fetal calf serum, penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$), and L-glutamine (2 mM) at 37°C in 5% CO_2/95% air. Geneticin (G418, 0.4 $mg \cdot mL^{-1}$) was added to CHO-hCB_2 cells. Cells were split two or three times weekly at a ratio between 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, or isotype-matched irrelevant IgG-PE (Beckman Coulter, Fullerton, CA, USA) as negative control. Cells were washed with PBS and characterized for CD11b and glial fibrillary acidic protein (GFAP) expression by flow cytometry with PE-labelled anti-CD11b MoAb (BD Pharmingen) 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 microglial cell exposure to cannabinoids, opioids 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 $\mu g \cdot mL^{-1}$ LPS (from *Escherichia coli*, serotype 055:B5) (soluble in cell culture medium) before commencing incubation with CB and opioid receptor ligands. Unless otherwise stated, the concentration of morphine (Salars, Como, Italy), naloxone, Tyr-DAla-Gly-[*N*MePhe]-$NH(CH_2)_2$ (DAMGO), *cyc*[$DPen^2$, $DPen^5$]enkephalin (DPDE), 5α,7α, 8β-(-)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro(4,5)dec-8-yl)benzeneacetamide (U69593), D-Phe-*cyc*[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2 (CTAP), JWH-015 (Enzo Life Sciences, Vinci-Biochem, Vinci, Florence, Italy), AM 630 and AM 251 was 100 nM, which is the ligand concentration able to occupy 99% of the receptors at equilibrium. Microglia were then maintained in DMEM containing cannabinoids, opioids or their vehicle, and harvested after treatment at the indicated times.

Nitrite assay for primary microglial cells

NO synthase 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, Stockholm, Sweden). Values were obtained by comparison with reference concentrations of sodium nitrite.

ELISA

The levels of IL-1β, TNF-α and IL-6 protein secreted by the cells in the medium were determined by ELISA kits (R&D Systems). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of drugs. The medium was collected, and IL-1β, TNF-α and IL-6 protein concentrations were measured by ELISA according to the manufacturer's instructions. The results were normal-

ized to the number of cells per plate. The data are presented as mean ± SE from four independent experiments performed in triplicate.

Western blotting for primary 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 JNK1/2 (1:1000 dilution), phosphorylated or total Akt (1:1000 dilution), for CB_2 and μ-opioid receptors. Specific reactions were revealed with enhanced chemiluminescence Western blotting detection reagent (Amersham Corp., Arlington Heights, IL, USA). 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 was normalized to the results obtained with control cell cultures. The ratio of phospho-protein to total protein was reported in a densitometric analysis.

Treatment of primary microglial cells with siRNA

Microglial cells were plated in six-well plates and grown to 50–70% confluence before transfection. Transfection of $siRNA_{CB2}$ or $siRNA_{\mu}$ 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 μ-opioid receptor protein. A randomly chosen non-specific siRNA was used under identical conditions as control (Merighi *et al.*, 2005; 2007).

Statistical analysis

All data are reported as mean ± SEM of independent experiments and are indicated in the figure legends. Each experiment was performed by using the microglial cells derived from one single mouse, and was performed in triplicate. The experiments were repeated at least four times as indicated from *n* values that represent the number of mice used.

Data sets were examined by ANOVA for comparisons between multiple groups and Dunnett's test for comparing a control group to all other groups (when necessary). A P value < 0.05 was considered statistically significant.

Results

CB_2 and μ-opioid receptor expression in primary mouse microglial cells

The 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

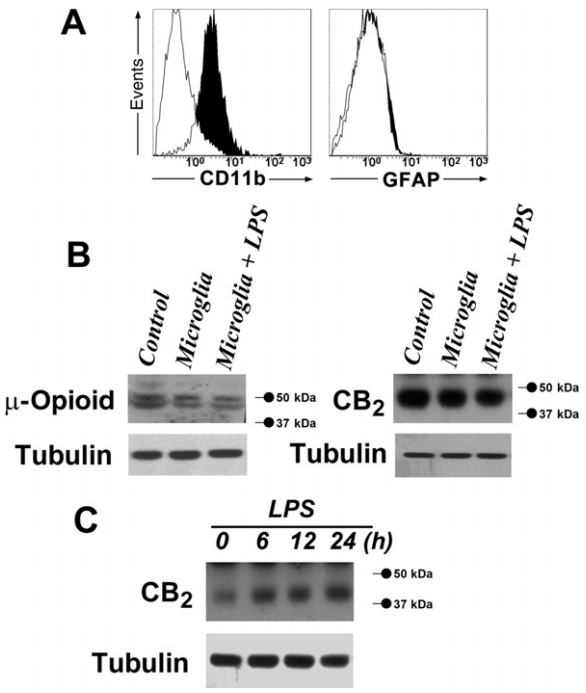

Figure 1

Detection of CB_2 and μ-opioid receptors in primary microglial cells. (A), Cell surface expression of CD11b and intracellular expression of GFAP by flow cytometry analysis. Primary microglial cells were treated with specific monoclonal antibodies (black histograms) or with isotype-matched irrelevant monoclonal antibodies (empty histograms, controls). (B), CB_2 and μ-opioid receptor detection by Western blot assay in quiescent and LPS-activated (1 μg·mL^{-1} for 30 min) primary microglial cells. Tubulin shows equal loading of protein. The expression of CB_2 receptors in CHO-hCB_2 cells and of μ-opioid receptors in mouse brain extracts, used as positive controls (Control), is shown. (C), CB_2 receptor detection by Western blot assay in quiescent and LPS-activated (1 μg·mL^{-1} for 6, 12 and 24 h) primary microglial cells. Tubulin shows equal loading of protein.

for CD11b, as compared to the isotype control, thereby indicating high expression levels of the microglial cell marker CD11b (Figure 1A).

The expression of CB_2 receptors in CHO-hCB_2 cells (used as positive control), in quiescent and LPS-activated primary microglial cells is shown in Figure 1B. The molecular weight of the protein detected in these cells was 50 kDa, comparable with the calculated molecular weight of CB_2 receptors. To ascertain the specificity of the CB_2 receptor antibody used in Western blots, antigen preabsorption experiments were carried out with the corresponding blocking peptide. Co-incubation with the immunizing peptide completely prevented the signal (data not shown). CB_2 receptor protein expression was not modified by 30-min treatment with 1 μg·mL^{-1} LPS (Figure 1B). Similarly, the expression of μ-opioid receptors in mouse brain extracts (used as positive control) in quiescent and LPS-activated primary microglial cells is shown in Figure 1B. Therefore, CB_2 and μ-opioid receptors were expressed in primary mouse microglial cells. To evaluate whether LPS induced changes in CB_2 receptor expression, we assayed CB_2 receptors over 24 h of LPS treat-

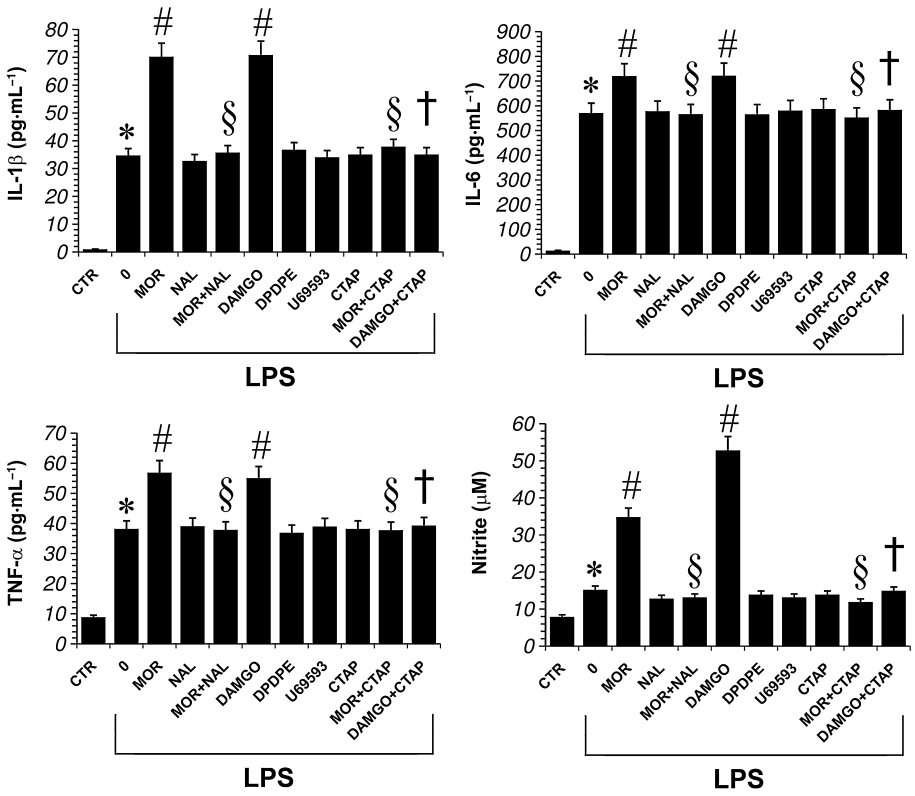

Figure 2

Effect of opioid receptor ligands on LPS-induced production of proinflammatory cytokines and nitrite in primary microglial cells. Microglial cells were treated with either LPS alone, LPS + morphine 100 nM (MOR), LPS + naloxone, a non-selective opioid receptor antagonist (Nal; 100 nM,), LPS + DAMGO, a selective μ-opioid receptor agonist (100 nM), LPS + DPDPE, a selective δ-opioid receptor agonist (100 nM), LPS + U69593, a selective κ-opioid receptor agonist (100 nM) or LPS + CTAP, a selective μ-opioid receptor antagonist (100 nM) for 24 h. The antagonists were added 30 min before morphine. Data shown are mean ± SEM values of four separate experiments performed in triplicate ($n = 4$). $^{*}P < 0.01$ significantly different from control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ significantly different from LPS conditions (0); $^{\S}P < 0.01$ significantly different from LPS + MOR; $^{\dagger}P < 0.01$ significantly different from LPS + DAMGO; analysis was by ANOVA followed by Dunnett's test.

ment. In agreement with published data (Carlisle *et al.*, 2002), LPS 1 μg·mL^{-1} produced a time-dependent increase in CB_2 receptor expression over the period from 6 to 24 h (Figure 1C).

Effect of opioid receptor ligands on cytokine and nitrite production in primary microglial cells

To investigate the effect of opioid receptor ligands on microglial activation, the ability of morphine to induce inflammatory mediator production, such as IL-1β, TNF-α, IL-6 and nitrite, was examined in LPS-stimulated microglial cells. The TLR-4 agonist LPS was used to induce IL-1β, TNF-α, IL-6 and NO (as nitrite) release from primary microglial cells. Preliminary studies in our laboratory demonstrated that LPS induced release of these mediators from primary microglial cells in a concentration-dependent manner, with maximum release occurring at a concentration of 1 μg·mL^{-1} (data not shown). As shown in Figure 2, non-stimulated microglial cells showed a very low level of IL-1β, TNF-α, IL-6 and nitrite but LPS triggered a robust increase in the release of these mediators into the culture media. Then, we went on to investigate how the activation of opioid receptors interferes with the signalling pathways modulated by LPS by maintaining primary microglial cells in LPS-supplemented (1 μg·mL^{-1}) DMEM in combination with the opioid receptor agonist morphine (100 nM) for 24 h. As shown in Figure 2, LPS-induced cytokine and nitrite release was significantly increased in the presence of morphine in primary microglial cells. The stimulatory response of morphine on cytokine and nitrite production was reversed in the presence of the broad-range opioid receptor-antagonist naloxone (Figure 2). Furthermore, DAMGO (100 nM), a μ-opioid receptor-selective agonist, mimicked the effects of morphine on microglial cytokine and nitrite induction (Figure 2). Conversely, DPDPE and U-69593, δ- and k-receptor-selective agonists, respectively, had no effect on LPS-induced microglial cytokine and nitrite production (Figure 2). Finally, pretreatment of microglial cells with CTAP (100 nM), a selective antagonist of μ-opioid receptors, before treatment with morphine (100 nM) or DAMGO (100 nM), abolished the effects of opioid receptor agonists on LPS-induced cytokine and nitrite production by microglia (Figure 2).

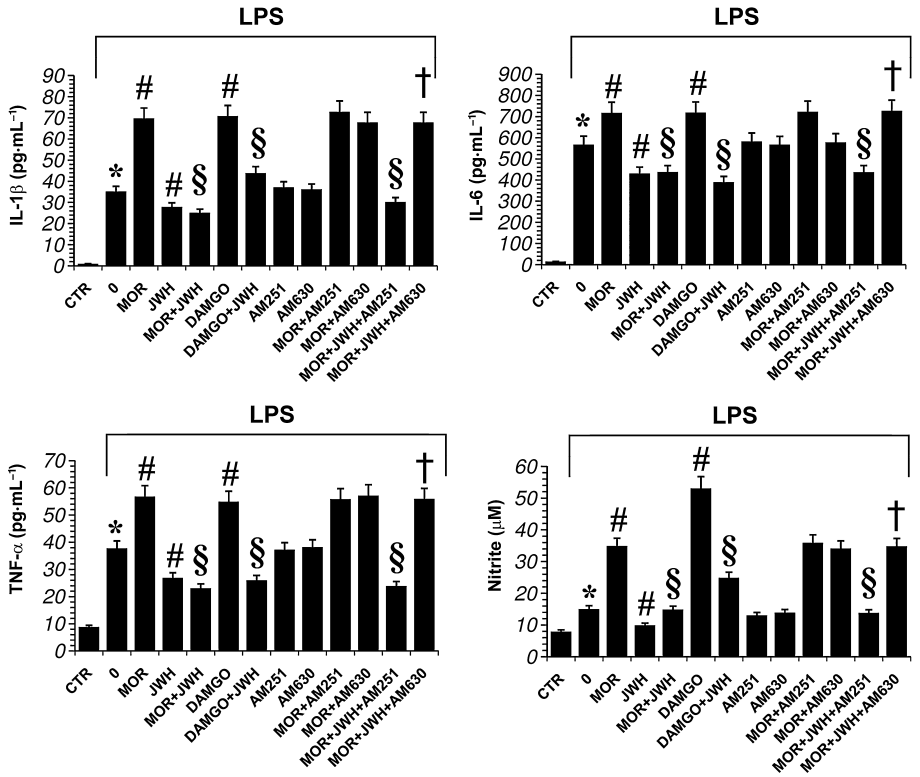

Figure 3

Effect of opioid and CB receptor ligands on LPS-induced production of proinflammatory cytokine and nitrite in primary microglial cells. Microglial cells were treated with either LPS alone, LPS + morphine (MOR; 100 nM), LPS + JWH-015 a CB_2 receptor agonist (JWH; 100 nM)), LPS + DAMGO (100 nM), LPS + AM 251 a selective CB_1 receptor antagonist (100 nM) or LPS + AM 630 a selective CB_2 receptor antagonist (100 nM) for 24 h. The antagonists were added 30 min before morphine. Data shown are mean ± SE values of four separate experiments performed in triplicate ($n = 4$). *$P < 0.01$ significantly different from control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ significantly different from LPS conditions (0); $^{\S}P < 0.01$ significantly different from LPS + MOR; $^{\dagger}P < 0.01$ significantly different from LPS + MOR + JWH; analysis was by ANOVA followed by Dunnett's test.

Influence of CB_2 receptors on μ-opioid receptor-induced cytokine and nitrite production in primary microglial cells

The effect of CB_2 receptor stimulation on μ-opioid receptor-induced cytokine and nitrite production in primary microglial cells was studied using JWH-015, a CB receptor agonist known to bind more readily to CB_2 than CB_1 receptors (Merighi *et al.*, 2010). By this means we tested whether CB_2 receptors can modulate cytokine and nitrite production in activated microglial cells. As shown in Figure 3, JWH-015 (100 nM) significantly decreased LPS-induced IL-1β, TNF-α, IL-6 and nitrite levels. Furthermore, co-administration of LPS (1 μg·mL^{-1}), morphine (100 nM) and JWH-015 (100 nM) significantly attenuated the morphine-induced increases in cytokine and nitrite production (Figure 3). The anti-inflammatory response of JWH-015 in LPS-activated microglial cells treated with morphine was reversed in the presence of the CB_2 receptor antagonist AM 630 (100 nM) (Figure 3). In contrast, the CB_1 antagonist AM 251 (100 nM) did not affect the ability of JWH-015 to down-regulate the increase in IL-1β, TNF-α, IL-6 and nitrite induced by morphine in LPS-activated microglial cells (Figure 3).

Signalling induced by morphine on quiescent and activated microglial cells

We went on to investigate whether the stimulatory effect of morphine on cytokine and nitrite release induced by LPS is mediated via the MAPK and Akt pathways. We tested whether morphine could induce ERK1/2, p38, JNK1/2 and Akt phosphorylation in primary murine microglial cells, treated for 30 min at 37°C, in a concentration-dependent manner. As shown in Figure 4A, morphine (1–1000 nM) increased p-ERK1/2 and pAkt expression levels but did not modulate pp38 and pJNK1/2. In particular, morphine (100 nM) induced a rapid and sustained (up to 60 min) stimulation of ERK1/2 and pAkt (Figure 4B). Furthermore, we have investigated how the activation of microglial cells interferes with the signalling pathways modulated by morphine. Thus, microglial cells were maintained in DMEM containing LPS (1 μg·mL^{-1}), and the ability of morphine (100 nM) to modulate ERK1/2 and Akt phosphorylation was evaluated at 15, 30 and 60 min. As shown in Figure 4B, LPS stimulation of microglial cells resulted in a rapid (15 min) increase in ERK1/2 and Akt phosphorylation, which was maximal at 30 min and declined towards basal levels within 60 min. In the presence

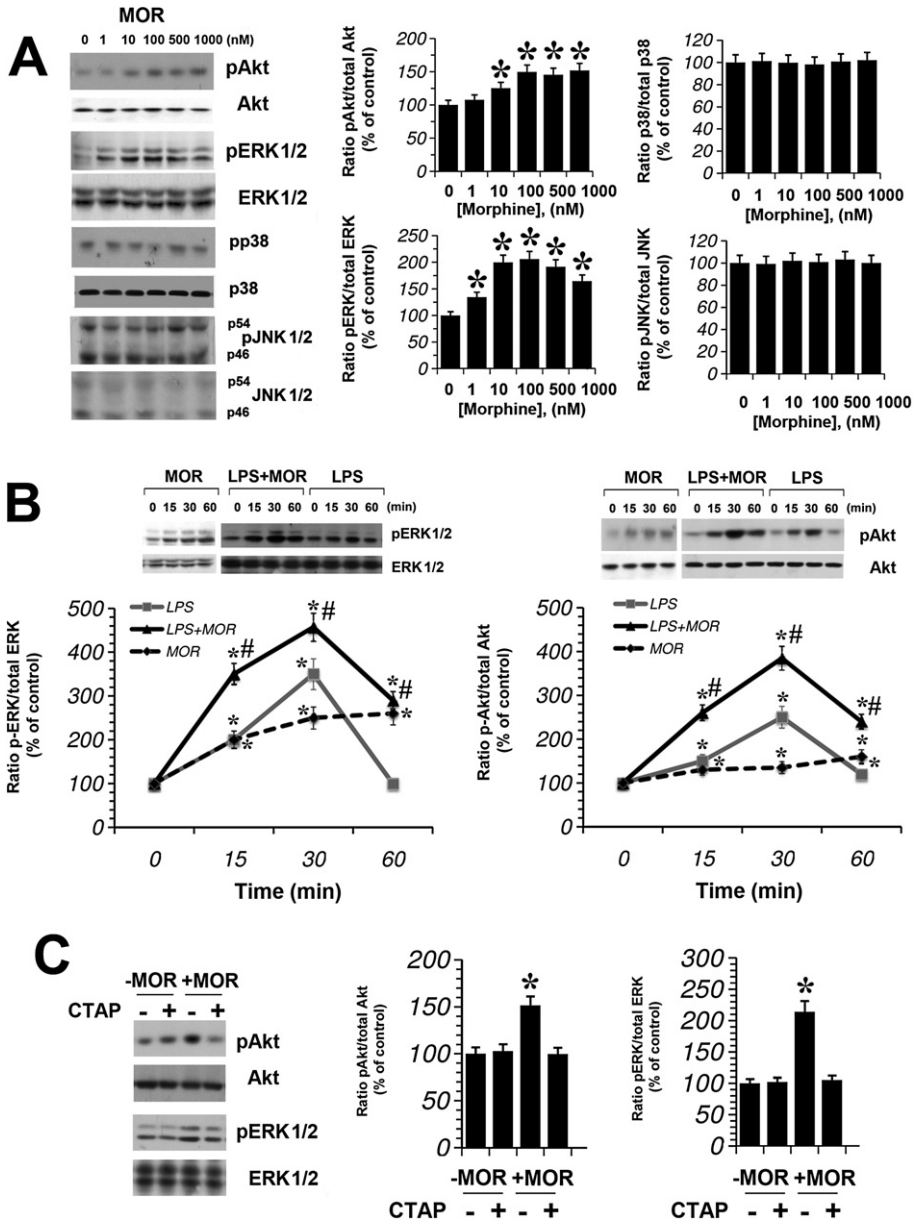

Figure 4

Morphine-enhanced Akt and ERK1/2 phosphorylation in a μ-opioid receptor-dependent manner. (A), Western blot analysis of Akt, ERK1/2, p38, and JNK1/2 phosphorylation in primary microglial cells incubated for 30 min with morphine (MOR) (1–1000 nM). The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). The ratio of phospho-protein to total protein is used. The mean values of four independent experiments (one of which is shown) were normalized to the result obtained with morphine-untreated cell cultures (0). Densitometric analysis is shown. The unstimulated control (0, cells in the absence of morphine) was set to 100%. Data shown are mean ± SE values of four separate experiments performed in triplicate ($n = 4$). *$P < 0.05$ significantly different from unstimulated control; analysis was by ANOVA followed by Dunnett's test. (B), Primary microglial cells were treated with morphine (100 nM) for 0, 15, 30 and 60 min, subjected to Western blot analysis and probed with anti-pERK1/2, anti-pAkt, then Akt and ERK1/2 antibody. The ratio of phospho-protein to total protein is used. Data shown are mean ± SE values of four separate experiments performed in triplicate ($n = 4$). *$P < 0.01$ significantly different from control conditions (0); #$P < 0.01$ significantly different from LPS conditions; analysis was by ANOVA followed by Dunnett's test. (C), Image of Western blot membrane probed with anti-pERK1/2, anti-pAkt, then Akt and ERK1/2 antibody of primary microglial cells pre-treated for 30 min with 0 or 100 nM of the μ-opioid receptor antagonist CTAP, then treated for 30 min with 0 or 100 nM morphine. The ratio of phospho-protein to total protein is used. Data shown are mean ± SEM values of four separate experiments performed in triplicate ($n = 4$). *$P < 0.01$ significantly different from untreated cells; analysis was by ANOVA followed by Dunnett's test.

of morphine (100 nM), ERK1/2 and Akt phosphorylation were significantly higher than LPS alone. To evaluate whether the morphine-induced changes in ERK1/2 and Akt phosphorylation levels were μ-opioid receptor-dependent, we used the selective antagonist of μ-opioid receptors, CTAP. Primary microglial cells were pretreated with CTAP, then exposed to morphine for 30 min. Morphine-induced p-ERK1/2 and p-Akt increased levels were reduced with CTAP pretreatment (Figure 4C), indicating that the effect of morphine was mediated via the μ-opioid receptor subtype.

Signalling induced by morphine and JWH-015 on LPS-activated microglial cells

Primary microglial cells were treated with either LPS alone, LPS + JWH-015 (100 nM) or LPS + morphine (100 nM) for 30 min. As shown in Figure 5, LPS significantly increased all kinases measured. Furthermore, while the CB_2 receptor agonist JWH-015 failed to modulate the activity of LPS on p38 and JNK1/2, it significantly decreased ERK1/2 and Akt phosphorylation levels in LPS-activated cells (Figure 5). Similarly, the phosphorylation of ERK1/2 and Akt were significantly increased by morphine (100 nM) added LPS-activated cells, compared with treatment with LPS alone. In contrast, morphine did not modulate LPS-induced phosphorylation of p38 and JNK (Figure 5). Finally, when microglial cells were treated with LPS (1 $\mu g \cdot mL^{-1}$) plus morphine (100 nM) in combination with JWH-015 (100 nM), we found that JWH-015 decreased ERK1/2 and Akt activation induced by LPS plus morphine, without affecting pp38 and pJNK1/2 levels (Figure 5).

The effect of morphine and JWH-015 on ERK1/2 and Akt activation was reversed in the presence of the antagonists naloxone and AM 630 respectively (Figure 6). In contrast, the CB_1 receptor antagonist AM 251 (100 nM) did not reverse the ability of JWH-015 to down-regulate the increase in p-ERK1/2 and p-Akt induced by morphine (Figure 6).

Akt and MEK-1/2 inhibition decreases LPS-induced inflammatory effects in microglial cells

To elucidate the mechanisms involved in the effects of morphine and JWH-015 on cutokine and nitrite production by activated microglial cells, we investigated the effects of inhibiting ERK1/2 or Akt signalling pathways,.with the MEK1/MEK2 inhibitor U0126 (1 μM) or the Akt inhibitor SH-5 (1 μM). To confirm the activity of these inhibitors, primary microglial cells were treated with U0126 (1 μM) and with SH-5 (1 μM) for 30 min and ERK-1/2 or Akt activation were measured. We observed that 30 min incubations of microglial cells with either U0126 or SH-5 resulted in significant decreases in active ERK-1/2 or Akt respectively, when compared with control cells incubated for 30 min with DMSO (U0126 = 32 ± 5% of control; n = 3; SH-5 = 28 ± 3% of control; n = 3). As shown in Figure 7, U0126 significantly reduced the production of TNF-α, IL-6 and nitrite induced by LPS-stimulated microglial cells. Similarly, the inhibition of the Akt pathway, by SH-5 (1 μM), resulted in a significant reduction of TNF-α, IL-6 and nitrite levels (Figure 7). However, while LPS-induced expression of IL-1β was significantly reduced by addition of SH-5, IL-1β expression was not

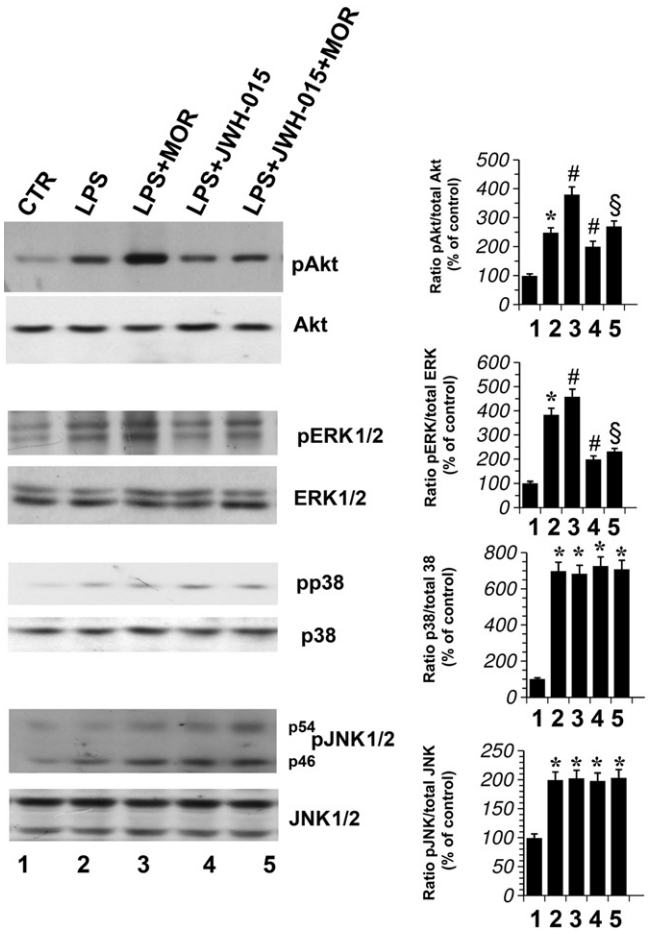

Figure 5

Effect of CB_2 receptor stimulation in morphine-treated activated primary microglial cells. JWH-015 and morphine (MOR) effect on Akt, ERK1/2, p38 and JNK1/2 phosphorylation in primary microglial cells treated with LPS. Microglial cells were incubated with DMSO vehicle (CTR), with MOR (100 nM), or with JWH-015 (100 nM) alone and in combination in the presence of LPS 1 $\mu g \cdot mL^{-1}$ for 30 min. The mean values of four independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS (CTR). Data shown are mean ± SEM values of four separate experiments performed in triplicate (n = 4). CTR was set to 100%. The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). Densitometric analysis of kinase activation is shown. The ratio of phospho-protein to total protein is used. $^{*}P < 0.05$ significantly different from CTR; $^{\#}P < 0.05$ significantly different from cells treated with LPS; $^{\S}P < 0.05$ significantly different from cells treated with LPS + morphine; analysis was by ANOVA followed by Dunnett's test.

affected by U0126 in LPS-treated microglia (Figure 7). These results suggest that ERK and Akt could mediate the enhancing effects of LPS on TNF-α, IL-6 and nitrite induction. In contrast, while the Akt pathway was involved in LPS-induced IL-1β production, the ERK pathway was not implicated.

CB_2 and μ-opioid receptor gene silencing in microglial cells

To confirm the apparent role of CB_2 receptors and to investigate the involvement of μ-opioid receptors, we reduced CB_2

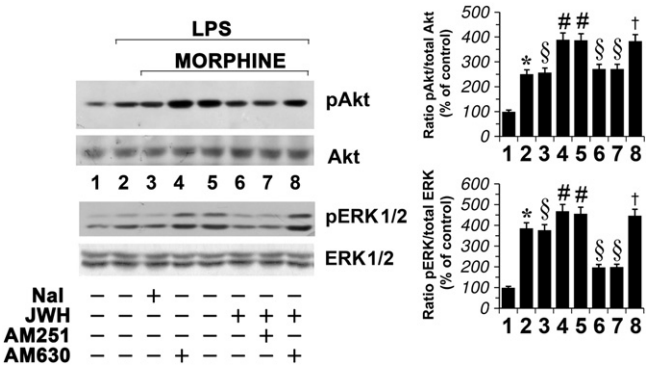

Figure 6

Effect of CB and opioid receptor blockade in morphine-treated activated primary microglial cells. Primary microglial cells were incubated with either DMSO vehicle (lane 1), morphine (MOR; 100 nM), naloxone a non-selective opioid receptor antagonist (Nal; 100 nM,), JWH-015 100 nM, AM 251 a selective CB_1 receptor antagonist (100 nM) or AM 630 a selective CB_2 receptor antagonist (100 nM) in the presence of LPS (1 $\mu g \cdot mL^{-1}$) for 30 min. The mean values of four independent experiments (one of which is shown) were normalized to the result obtained in cells in the absence of LPS (lane 1). Data shown are mean ± SEM values of four separate experiments performed in triplicate ($n = 4$). The unstimulated control (lane 1) was set to 100%. The immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). Densitometric analysis of kinase activation is shown. The ratio of phospho-protein to total protein is used. $^{*}P < 0.05$ significantly different from untreated cells (lane 1); $^{\#}P < 0.05$ significantly different from cells treated with LPS (lane 2); $^{\S}P < 0.05$ significantly different from cells treated with LPS + morphine (lane 5); $^{\dagger}P < 0.05$ significantly different from LPS + morphine + JWH-015 (lane 6); analysis was by ANOVA followed by Dunnett's test.

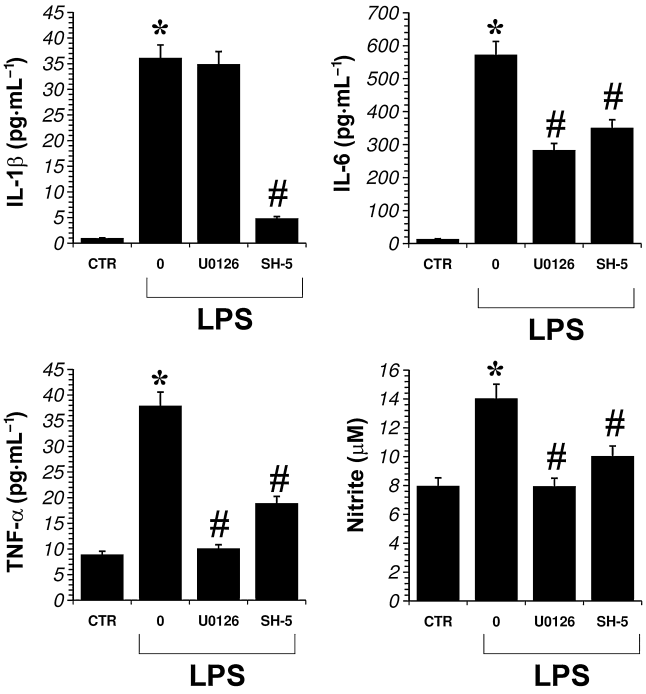

Figure 7

Effect of kinase inhibitors on LPS-induced production of proinflammatory cytokines and nitrite in primary microglial cells. Microglial cells were treated with LPS with either the MEK-1/2 inhibitor U0126 (1 μM) or the Akt inhibitor SH-5 (1 μM) for 24 h. The inhibitors were added 30 min before LPS. Data shown are mean ± SEM values of four separate experiments performed in triplicate ($n = 4$); $^{*}P < 0.01$ significantly different from control conditions (CTR); $^{\#}P < 0.01$ significantly different from LPS conditions; analysis was by ANOVA followed by Dunnett's test.

and μ-opioid receptor expression in primary microglial cells by siRNA transfection, in order to cause transient knockdown of the CB_2 and μ-opioid receptor genes. Primary microglial cells were transfected with non-specific random control ribonucleotides (siRNA scramble, $siRNA_{ctr}$) or with small interfering RNAs that target CB_2 or μ-opioid receptor mRNAs ($siRNA_{CB2}$ or $siRNA_{\mu}$, respectively) for degradation.

As shown in Figure 8, CB_2 and μ-opioid receptor protein expression were strongly reduced after 48 and 72 h of treatment with $siRNA_{CB2}$ and $siRNA_{\mu}$ respectively. Therefore, 48 h after $siRNA_{CB2}$ or $siRNA_{\mu}$ transfection, primary microglial cells were treated with either LPS (1 $\mu g \cdot mL^{-1}$), JWH-015 (100 nM) or morphine (100 nM), alone and in combination, for 30 min, after which ERK1/2- and Akt-phosphorylated protein levels were measured. This revealed that inhibition of CB_2 receptor expression was sufficient to block the JWH-015-induced inhibition of ERK1/2 and Akt phosphorylation levels, increased by morphine in LPS-treated microglia (Figure 9). Furthermore, inhibition of the expression of μ-opioid receptors blocked morphine-induced increases in pERK1/2 and pAkt in microglia (Figure 9). These results clearly show the connection between CB_2 receptor stimulation, morphine, ERKs and Akt signalling in activated primary microglial cells.

We also measured cytokine and nitrite levels in microglial cells in which CB_2 or μ-opioid receptors were down-regulated. We found that, in microglial cells with μ-opioid receptors down-regulated, morphine did not significantly increase IL-1β, TNF-α, IL-6 and nitrite protein levels when compared to LPS. Similarly, the CB_2 agonist JWH-015 did not significantly reduce cytokine and nitrite levels in microglial cells with down-regulated CB_2 receptors, compared with those after LPS alone (Figure 10).

Discussion and conclusions

The presence of opioid receptors on glia and the ability of morphine to prime microglia for enhanced production of proinflammatory cytokines supports a possible direct interaction of morphine with glial cells (Chao *et al.*, 1994). As microglial differentiation and immune function is regulated by activation of CB_2 receptors (Stella, 2010), we set out to characterize the signalling pathways modulated by both μ-opioid and CB_2 receptors expressed in microglial cells. CB_2 and μ-opioid receptor ligands (agonist and antagonist) and CB_2- and μ-opioid receptor-knockout microglial cells were used to determine the role of the CB and opioid system in primary microglial cells. We have described the mechanisms by which CB_2 and opioid receptors modulate the MAPK signal response to LPS, an agent widely used experimentally to create inflammation in the brain (Lehnardt *et al.*, 2002).

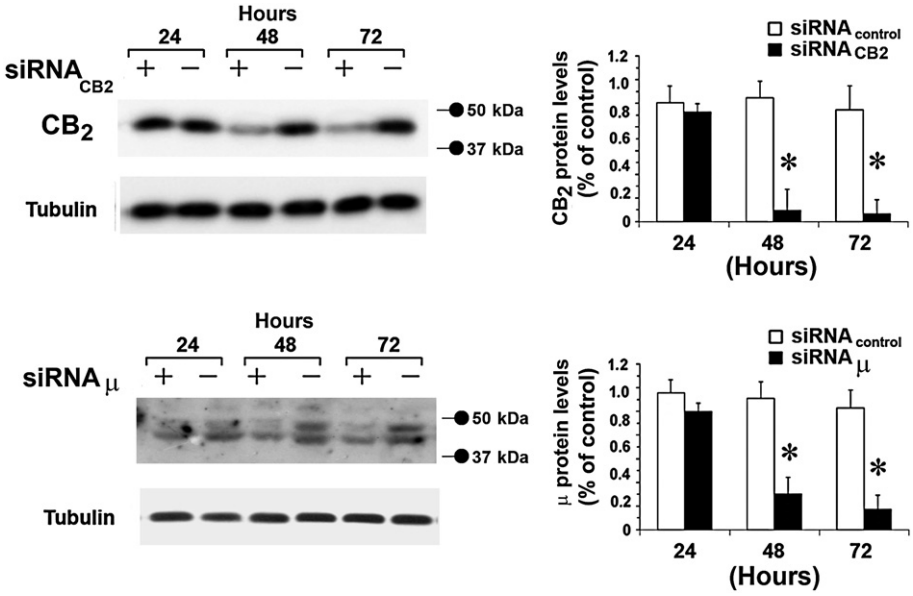

Figure 8

CB_2 and μ-opioid receptor expression silencing. (A), Primary microglial cells were treated with either scrambled (-) ($siRNA_{control}$), with $siRNA_{CB2}$ (+) or with $siRNA_{\mu}$ and cultured for 24, 48 and 72 h. Tubulin shows equal loading of protein. Densitometric quantification of CB_2 and μ-opioid receptor by Western blot is shown; the immunoblot signals were quantified using a VersaDoc Imaging System (Bio-Rad). Plots are mean ± SEM values of four separate experiments performed in triplicate ($n = 4$); *$P < 0.01$ significantly different from control (scrambled siRNA transfected cells); analysis was by ANOVA followed by Dunnett's test.

In this study, we demonstrated that (1) morphine enhanced the release of NO and the proinflammatory cytokines, IL-1β, IL-6, TNF-α, from activated microglial cells; (2) CB_2 receptor stimulation attenuated morphine-induced microglial proinflammatory mediator increases; (3) morphine-induced microglial proinflammatory mediator increases were μ-opioid receptor dependent; and (4) CB_2 receptor stimulation interfered with morphine action by acting on Akt-ERK1/2 signalling. Together, these results suggest that CB_2 receptors are critical to the activity of microglia, opposing the morphine-induced release of NO and cytokines from microglial cells. Therefore, we suggest a novel interaction between μ-opioid- and CB_2-receptor systems in microglia, which is likely to be mediated via the Akt/ERK1/2 pathways.

The influence of CB_2 receptor stimulation on μ-opioid receptor-induced cytokine and nitrite production in primary microglial cells was studied using JWH-015, a CB receptor agonist known to bind more readily to CB_2 than CB_1 receptors (Merighi *et al.*, 2010). However, JWH-015 also stimulates GPR55 (Lauckner *et al.*, 2008). Similarly, different studies have demonstrated that the CB_1 receptor antagonist AM251 induces GPR55 activity (Lauckner *et al.*, 2008; Henstridge *et al.*, 2010; Anavi-Goffer *et al.*, 2012). Therefore, even if the activity of CB receptor ligands at GPR55 is influenced by the assay used to assess receptor-mediated downstream signalling (Henstridge *et al.*, 2010), a role for GPR55 activation needs to be considered in further pharmacological studies of cannabinoid actions. Furthermore, a non-selective CB agonist enhanced morphine antinociception via the CB_1 receptor, pointing to the involvement of CB_1 receptors in cannabinoid antinociception produced by CB receptor ligands (Wilson *et al.*, 2008).

The first report linking glia to morphine tolerance demonstrated that chronic systemic morphine increased glia activation in the spinal cord (Song and Zhao, 2001). Other authors have also shown that chronic morphine administration activated astroglia and microglia (Raghavendra *et al.*, 2002; Cui *et al.*, 2006). Activated microglial cells in the spinal cord may release proinflammatory cytokines and other substances thought to facilitate pain transmission (Watkins *et al.*, 2001; 2003). Therefore, pharmacological attenuation of glial activation represents a novel approach for controlling neuropathic pain (Watkins *et al.*, 2005). Neuropathic hyperalgesia could lead to lowered morphine efficacy and quicker development of morphine tolerance (Mayer *et al.*, 1999), and some authors have suggested that uncontrolled activation of microglial cells after nerve injury can lead to altered activities of opioid systems or opioid-specific signalling (Watkins *et al.*, 2005; 2007). It is already known that microglia release neuroexcitatory substances in response to morphine, thereby opposing its effects (Watkins *et al.*, 2001; 2005; 2007). This raises an older hypothesis that suppression of glial activation and the resulting blockade of proinflammatory cytokine synthesis can improve morphine efficacy (Song and Zhao, 2001; Raghavendra *et al.*, 2002; Watkins *et al.*, 2007). The mechanism underlying the involvement of glial cells in morphine tolerance is unclear. It is possible that morphine can act directly on glial cells, triggering alterations in their morphology and functions (Raghavendra *et al.*, 2002; 2004). Additionally, glial cells are also considered to be crucial sources of NO, cytokines and cyclooxygenase products that influence synaptic transmission in the CNS. Inhibition of these factors may delay morphine tolerance (Powell *et al.*, 1999).

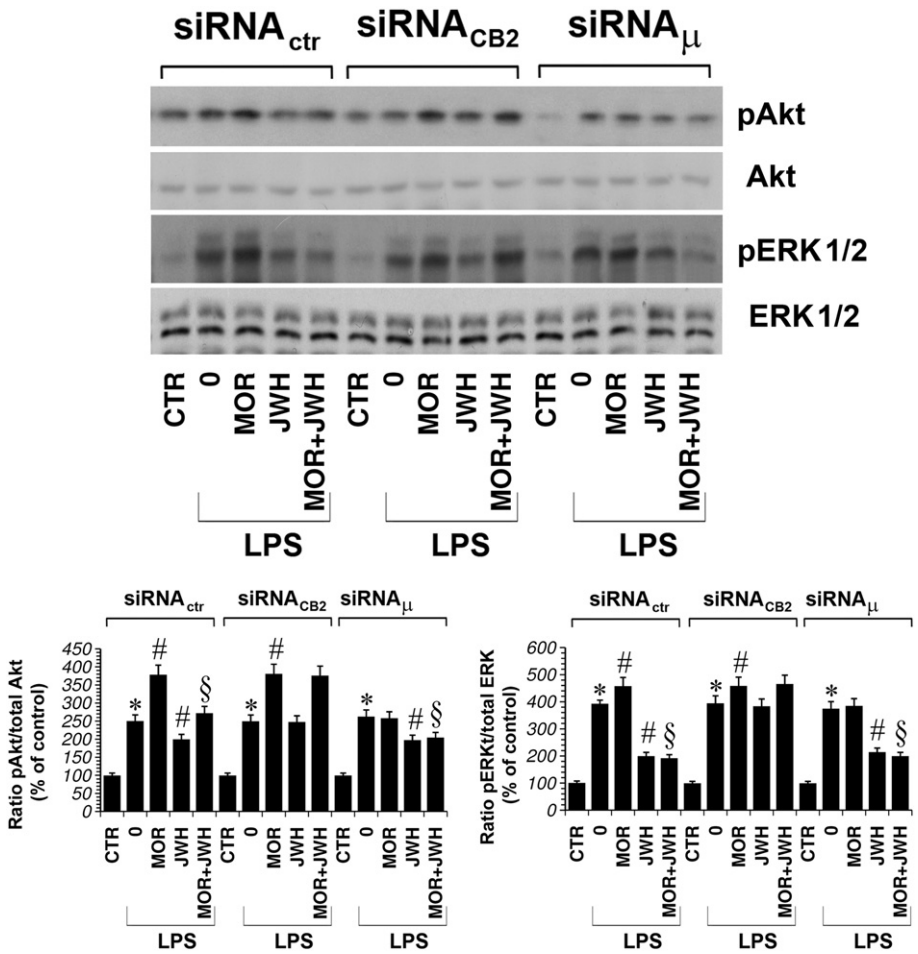

Figure 9

Primary microglial cells were treated with either $siRNA_{ctr}$, $siRNA_{CB2}$ or $siRNA_{\mu}$ for 48 h and cultured with LPS (1 $\mu g \cdot mL^{-1}$) alone or plus morphine (MOR; 100 nM), JWH-015 (JWH; 100 nM) alone and in combination for 30 min. Densitometric analysis of phosphorylated isoform is shown. 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 four 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 (CTR); $^{\#}P < 0.05$ significantly different from cells treated with LPS; $^{\S}P < 0.05$ significantly different from LPS + MOR; analysis was by ANOVA followed by Dunnett's test.

Many current studies aim to find substances inhibiting the biosynthesis of proinflammatory cytokines. Propentofylline, minocycline and ibudilast inhibit cytokines and decrease astroglia and microglia activation, thereby suppressing the development of neuropathic pain (Romero-Sandoval *et al.*, 2008). The beneficial effects of minocycline are associated with a reduction of inducible NO synthase and cyclooxygenase-2 expression and a decrease in cytokine and prostaglandin release in microglia (Yrjanheikki *et al.*, 1998; 1999). Further studies have shown that minocycline reduced microglial activation by inhibiting p38 MAPK in microglia and, in this way, delayed morphine tolerance (Romero-Sandoval *et al.*, 2008). Ibudilast may counteract opioid tolerance by blocking the activation of glial cells in the spinal cord in rodents (Romero-Sandoval *et al.*, 2008). In the present work, we found that in activated microglial cells, morphine increased Akt and ERK kinase phosphorylation. ERK1/2 kinases are known to regulate the production of proinflammatory mediators from glial cells (Watkins *et al.*, 2001). Furthermore, p38 and ERK kinases have been implicated in the development of morphine-induced hyperalgesia and antinociceptive tolerance (Cui *et al.*, 2006; Wang *et al.*, 2009). Therefore, it is of interest that CB_2 receptor stimulation was able to downregulate Akt and ERK1/2 activation induced by morphine in activated microglial cells. Our data indicate that the CB_2 receptor did not mediate its effects through p38 and JNK1/2 kinases. However, β-caryophyllene, a CB_2 receptor selective agonist, modulates JNK1/2 in LPS-stimulated monocytes (Gertsch *et al.*, 2008). As microglia are considered as the resident macrophage-like cells in the brain, we suggest that this contrasting behaviour may be due to the different experimental conditions, for example, time of ligand incubation (30 min vs. 3 h for microglia and monocytes respectively) or LPS concentration (1 $\mu g \cdot mL^{-1}$ vs. 0.313 $\mu g \cdot mL^{-1}$ for microglia and monocytes respectively). As for ERK1/2 signalling, 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

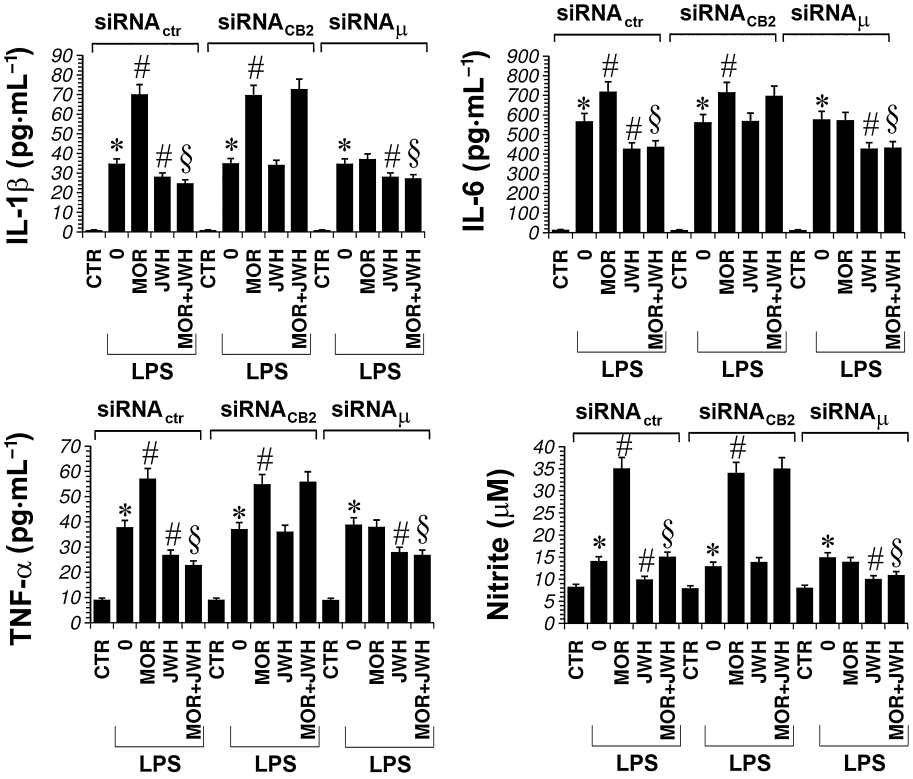

Figure 10

Primary microglial cells were treated with either $siRNA_{ctr}$, $siRNA_{CB2}$ or with $siRNA_{\mu}$ for 48 h and cultured with LPS (1 μg·mL^{-1}) alone or plus morphine (MOR; 100 nM) or JWH-015 (JWH; 100 nM) alone and in combination for 24 h. The unstimulated control (CTR) was set to 100%. Data shown are mean ± SEM values of four separate experiments performed in triplicate ($n = 4$); *$P < 0.05$ significantly different from CTR; $^{\#}P < 0.05$ significantly different from LPS; $^{\S}P < 0.05$ significantly different from LPS + MOR; analysis was by ANOVA followed by Dunnett's test.

et al., 2008; Correa *et al.*, 2010). Similarly, a more recent study has demonstrated that CB_2 receptor stimulation in microglial cells induced an anti-inflammatory phenotype and reduced migration via MKP-induced ERK dephosphorylation (Romero-Sandoval *et al.*, 2009). Agonist at CB receptors inhibited the production of proinflammatory molecules, which were 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 influence the production of the potent inflammatory mediator NO, released from quiescent, and, to a greater extent, from activated microglia (Stella, 2010). In this work, we have described the activation of the ERK1/2 and Akt pathways by LPS leading to an increment in TNF-α, IL-6 and nitrite production. In contrast, while Akt is engaged as the signalling pathway generating IL-1β in LPS-activated microglia, the ERK pathway was not implicated. The involvement of the ERK-MAPK pathway in IL-1β production by LPS is controversial. Some reports show that the ERK cascade is important for LPS-stimulated production of IL-1β in macrophage cell lines and monocytes (Scherle *et al.*, 1998; Caivano and Cohen, 2000). However, it has also been reported that the ERK pathway is not essential for IL-1β production in BV-2 microglia (Watters *et al.*, 2002). Consistent with findings on BV-2 microglia cell lines, our study indicates that the regulation of LPS-stimulated IL-1β production is ERK-independent also in primary microglia.

According to previous studies showing that CB_2 receptor mRNA and protein are modulated *in vitro* differentially in relation to cell activation state (Carlisle *et al.*, 2002; Cabral *et al.*, 2008), we have demonstrated that LPS increases CB_2 receptor expression level in primary microglial cells. It is important to mention that CB_2 receptors, identified in the healthy brain, mainly in glial elements, and, to a lesser extent, in certain subpopulations of neurons, are dramatically up-regulated in response to damaging stimuli, which supports the idea that the cannabinoid system behaves as an endogenous neuroprotective system. This CB_2 receptor up-regulation has been found in many neurodegenerative disorders, which supports the beneficial effects found for CB_2 receptor agonists in these pathologies (Fernández-Ruiz *et al.*, 2011). Now, we have characterized, for the first time, the events occurring in LPS-activated microglia via CB_2 receptor stimulation, which reduces not only ERK1/2- but also Akt-phosphorylation increases induced by LPS. Therefore, CB_2 receptors expressed in microglia may participate in regulating neuroinflammation and provide neuroprotection by tempering morphine-induced cytokine and NO synthesis through ERK1/2 and Akt signalling in activated microglia. Interestingly, in microglia, we showed that the effects of morphine were mediated by the μ-opioid receptor subtype. This accords with previous observations describing the involvement of endocannabinoids in the peripheral antinociception induced by the μ-opioid receptor agonist mor-

phine. In contrast, the release of endocannabinoids appears not to be involved in the peripheral antinociceptive effect induced by κ- and δ-opioid receptor agonists (da Fonseca Pacheco *et al.*, 2008). At the same time, it would be possible that certain effects of CB_2 receptor agonists in different models for inflammation and possibly their analgesic effects previously reported, actually reflect their interaction with endogenous opioids. In particular, it has been demonstrated that morphine is present in human gliomas (Olsen *et al.*, 2005) and that it increases the proliferation of human glioblastoma cells (Lazarczyk *et al.*, 2010). Because morphine is used to alleviate pain associated with cancers, this study suggests that a combination of CB_2 receptor agonists to prevent morphine-induced proliferation may have clinically important implications.

In conclusion, the novel finding of this study is the existence of a receptor–receptor interaction when the receptors are co-expressed in the same cells leading to the interaction of their intracellular pathways. In particular, CB_2 receptor stimulation counteracts the ability of morphine to upregulate Akt and ERK1/2 activation induced by LPS, thus reducing NO and proinflammatory cytokine release, a process which is ERK- and Akt-dependent. The studies presented here are the first to assess the signalling mechanisms through which CB_2 receptor stimulation modulates morphine effects on microglia and MAPK activation. The ability to modulate microglia and MAPK is very interesting because their activation in the central and peripheral nervous system contributes to morphine tolerance and dependence (Mayer *et al.*, 1999; Watkins *et al.*, 2001; Raghavendra *et al.*, 2002; 2004; Galeotti *et al.*, 2006; Cunha *et al.*, 2010). Our results indicate a regulatory role for CB_2 receptors in preventing excessive microglial cell response to injury in activated microglia. Based on the findings obtained in the present study, we will advance our research to reinforce the idea that the cannabinoid system exerts an important control on the tolerance and dependence effects induced by opioids. In particular, it will be interesting to evaluate the impact of low-dose combinations of cannabinoids and opioids to effectively treat acute and chronic pain, especially pain that may be resistant to opioids alone. It is well known that the use of cannabinoids, like that of the opioids, has liability for abuse potential. The use of marijuana as a therapeutic pain management tool has generated a great deal of publicity and controversy. However, it should be noted that CB_2 receptor agonists, in comparison with CB_1 agonists, lack the undesirable CNS side effects, like sedation and psychotomimetic effects (Fernández-Ruiz *et al.*, 2011). Therefore, the development of selective CB_2 receptor agonists might open new avenues of therapeutic intervention to reduce the release of proinflammatory mediators especially during morphine therapy. Accordingly, CB_2 receptors may be potential targets for reducing morphine tolerance and dependence.

Acknowledgements

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Conflict of interest

On behalf of all the authors, I declare that we have no conflicts of interest.

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