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The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia

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Increased production of proinflammatory cytokines has a prominent role in tolerance to opioids. The objectives of this study were to examine whether μ-opioid receptor affects proinflammatory signalling through the activation of NF-kB in microglia. The novelty of the described research is that a low dose of morphine, exerting its effects via the μ-opioid receptor, increases the DNA-binding activity of NF-kB via PKCε, while a high dose of morphine triggers a nonopiate receptor response mediated by TLR4 and, interestingly, PKCε signalling. The identification of morphine as a crucial upstream regulator of PKCε-NF-κB signalling in microglia argues for a central role of these pathways in neuroinflammation development and progression. Therefore, the morphine-PKCε-NF-κB pathway may provide novel targets to induce neuroprotective mechanisms, thereby reducing tolerance to opioids.

Keywords: Akt; mitogen-activated protein kinases; neuroinflammation; nuclear factor-kB; opioid receptor; PKCε

Opioids are useful analgesic drugs for the management of pain. They induce important signalling transduction mechanisms through three G protein-coupled receptors named μ-, δ- and κ-opioid receptors. The well-known effects of morphine are mediated specifically by the μ-opioid receptor subtype, which is able to bind not only morphine but also the opioid drugs used in therapy [1]. Unfortunately, the long treatment with opiates results in a significant decrease in their pharmacological effects, thereby leading to a lack of analgesic efficacy. As a consequence, the extended application of these drugs in therapeutic protocols has to be prevented. It has not been well explained how opiates induce tolerance but proinflammatory cytokines produced by microglia seem to play an important pathogenic role. Indeed, activated microglia can promote a neuroinflammatory response through the release of inflammatory mediators, including interleukin 1β (IL-1β), tumour necrosis factor α (TNF-α), prostaglandin E_2 (PGE_2) and nitric oxide (NO) [2–4]. Furthermore, microglia has the ability to distribute proinflammatory cytokines in the spinal cord, thus promoting pain states and morphine tolerance [5–8]. Therefore, a primary area of interest in this research field is drug screening and lead optimization programmes that characterize selective and potent inhibitors of proinflammatory cytokine release to manage pain and analgesic drug tolerance [9,10].

Opiates can activate the novel PKCε isoform which in turn induces a specific signalling inside the cells

Abbreviations
ANOVA, analysis of variance; DAPI, 4′,6′-diamino-2-phenyl-indole; DMEM/F12, Dulbecco's modified Eagle medium; ERK, extracellular signal-regulated kinase; IKK, IkB kinase; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-kB, nuclear factor-kB; NO, nitric oxide; PE, phycoerythrin; PGE2, prostaglandin E_2; PKC, protein kinase C; PS, phosphatidyl serine; siRNA, small interfering RNA; TLR, toll-like receptor; TNF, tumour necrosis factor.

mediating opioid tolerance and dependence. Not least, PKCε has a central role in different pain states, for instance, acute versus chronic pain [11]. The signalling induced by morphine involves the PKCε pathway through Akt and ERK phosphorylation, receptor desensitization and proinflammatory cytokine secretion [12,13]. The antinociceptive tolerance is reduced by PKC inhibitors [14] according to the increased responses to morphine observed in mice that lack PKCε isozyme ($PKC\varepsilon^{-/-}$) [15]. These animals have macrophages inadequate to produce TNF-α, IL-1β, PGE_2 and NO, and to activate mitogen-activated protein kinase (MAPK) and nuclear factor-kB (NF-*k*B) [16,17]. In particular, $PKC\varepsilon^{-/-}$ murine macrophages were found to display an impairment in LPS-induced IkB-α degradation and consecutive NF-kB activation [16]. Furthermore, it has been reported that NF-kB activity in different cell types is either increased [18,19] or decreased [20–22] in response to opioids.

We designed the present study to address the molecular mechanisms linking morphine to the increase in inflammatory mediators in LPS-activated microglial cells. In particular, the aim of this work was to define whether morphine, through its μ-opioid receptors, regulates NF-kB via the PKCε-Akt-MAPK pathway in activated microglia.

Materials and methods

Animals

One-day-old Balb/c mice were obtained from Charles River (Calco, Italy). Animal care procedures conformed to the guidelines issued by the European Council (86/609/EEC) were approved by the local Animal Care and Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [23].

Reagents and antibodies

Tissue culture media and growth supplements were from Euroclone (Milan, Italy). Morphine was from Salars (Como, Italy). U0126 was from Promega (Milan, Italy). The NF-κB pathway sampler kit containing IKKα, IKKβ, NF-κB p65/RelA and IκBα and anti-iNOS were from Cell Signalling Technology (Euroclone, Milan, Italy). SH5 was from Enzo Life Sciences (Vinci-Biochem, Vinci, Florence, Italy). Small interfering RNA (siRNA) PKCε and siRNA MOR-1 were from Santa Cruz Biotechnology (DBA, Milan, Italy). RNAiFect™ Transfection Kit was from Qiagen (Milan, Italy). BAY 11-7082 was from Calbiochem (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 [24]. 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 Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10% FBS, penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) at 37 °C in 5% CO_2/95% air.

Flow cytometry of primary microglial cells

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 GFAP expression by flow cytometry with PE-labelled anti-CD11b MoAb (BD Pharmingen) and FITC-labelled anti-GFAP MoAb (BD Pharmingen). In particular, GFAP immunophenotyping was performed in permeabilized cells, using IntraPrep™ fixing/permeabilization reagent (Beckman Coulter) [25]. Analysis was performed on an Epics XL flow cytometer (Beckman Coulter) using EXPO ADC software (Beckman Coulter).

Primary microglial cell exposure to opioids and LPS treatment

Microglial cells were treated with 1 $\mu g \cdot mL^{-1}$ LPS (from *Escherichia coli,* serotype 055:B5) before incubation with opioid ligands. Unless otherwise stated, the concentration of morphine, naloxone, DAMGO, DPDE, U69593 and CTAP was 100 nM, which is the ligand concentration able to occupy 99% of the receptors at equilibrium.

PKCε activity

PKCε activity has been modulated by εV1-2 (CEAVSLKPT) peptide conjugated by a cysteine disulphide bound to $TAT_{47–57}$ (CYGRKKRRQRRR) [26], generously donated by Prof. P. Mirandola, University of Parma, Italy. Briefly, εV1-2 is a specific PKCε inhibitor designed from C2 region of PKCε protein that acts as a competitor of binding between PKCε and its anchoring protein εRACK. Microglial cells were treated with 1 μM of εV1-2.

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) according to a modified Griess method [27]. Values were obtained by comparison with the reference concentrations of sodium nitrite.

Enzyme-linked immunosorbent assay

The levels of IL-1β, TNF-α and IL-6 protein secreted by the cells in the medium were determined by ELISA kits according to the manufacturer's instructions (R&D Systems) [28]. The data were presented as mean ± SE from four independent experiments performed in triplicate.

NF-kB activation assay

Nuclear extracts from microglial cells were obtained using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The NF-kB activation was evaluated by detecting phosphorylated p65 proteins in nuclear extracts using the TransAM NF-kB kit (Active Motif).

Western blotting

Western blot assay was performed as previously described [27]. Aliquots of protein sample were analysed using antibodies specific for the key proteins in the NF-κB pathway (IKKα, IKKβ, NF-κB p65/RelA and IκBα). The cytosolic and nuclear fractions of cells were also prepared for western blot analysis of p65. Specific reactions were revealed with Enhanced Chemiluminescence western blotting detection reagent (Amersham Corp., Arlington Heights, IL, USA). The membranes were then stripped and reprobed 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.

Treatment of primary microglial cells with siRNA

Transfection of $siRNA_{PKC\varepsilon}$ or $siRNA_{\mu}$ was performed at a concentration of 100 nM using RNAiFect™ Transfection Kit (Qiagen). Total proteins were isolated at 24, 48 and 72 h for western blot analysis of PKCε and μ-opioid receptor protein. A randomly chosen nonspecific siRNA was used under identical conditions as control [27].

Statistical analysis

All data are reported as mean ± SE of independent experiments and are indicated in the figure legends. Each experiment was performed 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 analysis of variance (ANOVA) for comparisons between multiple groups and Dunnett's test for comparing a control group to all other groups (when necessary). A P value of less than 0.05 was considered statistically significant.

Results

Morphine alters the LPS-induced activation of NF-kB

We determined the expression of the myeloid cell surface antigen CD11b in primary microglial cells through flow cytometry analysis. Cells were treated with specific MoAbs or isotype-matched irrelevant MoAbs. We found that microglia were negative for the astrocyte-specific protein GFAP while showed significant positive staining for CD11b, as compared to the isotype control. These results indicate high expression levels of the marker CD11b in the isolated microglial cells (data not shown).

We examined the activation of the NF-κB signalling pathway by detecting phosphorylated p65 proteins in nuclear extracts using the TransAM NF-kB kit in microglia. Our results show that the treatment with morphine alone (0.1 and 10 μM), for 15 min, did not change NF-κB activity. On the contrary, NF-κB activity was increased in microglia stimulated with LPS 1 $\mu g \cdot mL^{-1}$ for 15 min. Furthermore, morphine 0.1 and 10 μM for 15 min significantly increased NF-κB activity in LPS-activated microglial cells (Fig. 1A). To evaluate the potential role of μ-opioid receptors, we reduced their expression in primary microglial cells by siRNA transfection. Primary microglial cells were transfected with nonspecific random control ribonucleotides (siRNA scramble, $siRNA_{ctr}$) or with small interfering RNAs that target μ-opioid receptor mRNA ($siRNA_{\mu}$) for degradation. Forty-eight hours after $siRNA_{\mu}$ transfection, primary microglial cells were treated with either LPS 1 $\mu g \cdot mL^{-1}$, or LPS + morphine 0.1 and 10 μM, for 15 min, after which nuclear fractions of cells were prepared for NF-kB activity assay. We found that the inhibition of μ-opioid receptor expression blocked the increases of NF-kB activity in LPS-activated microglia induced by morphine 0.1 μM, while only

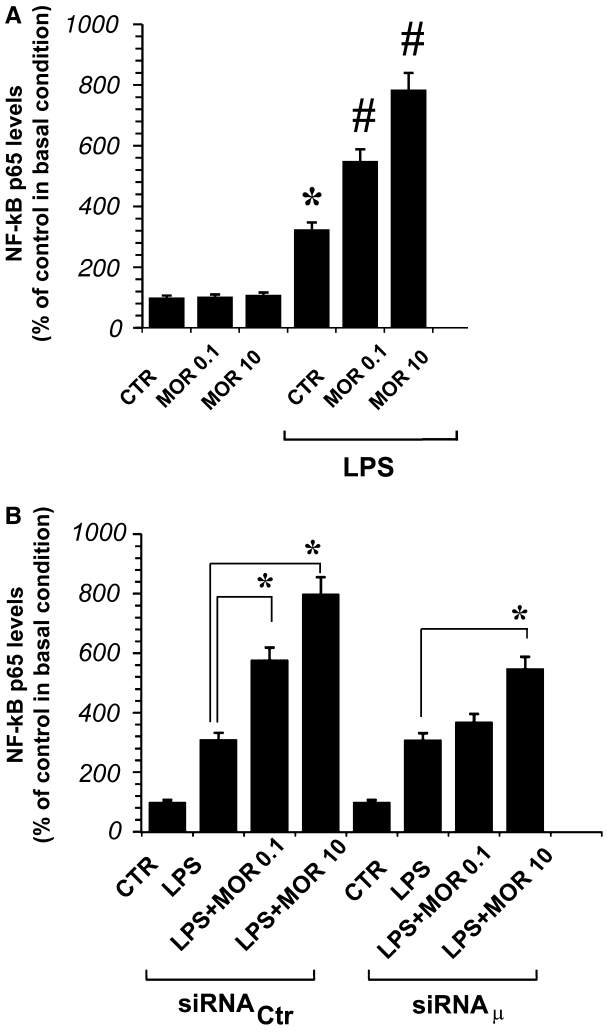

Fig. 1. Effect of morphine on NF-kB activation which was evaluated by detecting activated p65 proteins in nuclear extracts. (A) Microglial cells were treated with either morphine 0.1 μM (MOR 0.1), morphine 10 μM (MOR 10), LPS 1 $\mu g \cdot mL^{-1}$, LPS + morphine 0.1 μM, or LPS + morphine 10 μM for 15 min. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions (CTR-LPS); analysis was by ANOVA followed by Dunnett's test. (B) Primary microglial cells were treated with either $siRNA_{ctr}$ or $siRNA_{\mu}$ for 48 h and cultured without or with LPS 1 $\mu g \cdot mL^{-1}$ alone or plus morphine 0.1 μM (LPS + MOR 0.1), or morphine 10 μM (LPS + MOR 10), for 15 min. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus LPS conditions; analysis was by ANOVA followed by Dunnett's test.

reduced those induced by morphine 10 μM (Fig. 1B). These results indicate that the low dose effect of morphine is via the μ-opioid receptor while a high dose of morphine would engage a nonopiate receptor response.

Effect of opioid receptor ligands on LPS-induced activation of NF-kB

To confirm that the low dose effect of morphine 100 nM is via the μ-opioid receptor, we analysed the effect of opioid receptor ligands on LPS-induced activation of NF-kB. Pretreatment of cells with the broad-range opioid receptor antagonist naloxone 100 nM, for 30 min, abolished the induction of NF-kB induced by morphine 100 nM at 15 min in LPS-activated microglial cells (Fig. 2A). Furthermore, DAMGO (100 nM), a μ-opioid receptor selective agonist, mimicked morphine effects on microglial NF-kB activation (Fig. 2A). Conversely, DPDPE (100 nM) and U-69593 (100 nM), δ- and k-receptor selective agonists respectively, had no effect on LPS-induced NF-kB activation (Fig. 2A). Finally, pretreatment of microglial cells with CTAP 100 nM, a μ-opioid receptor selective antagonist, before the treatment with DAMGO 100 nM, abolished μ-opioid receptor agonist's effect on LPS-induced NF-kB activation (Fig. 2A). These results demonstrate that morphine 100 nM activates NF-kB in mouse-activated microglial cells via the μ-opioid receptor.

To investigate the signalling engaged by the high dose of morphine (10 μM), we analysed the effect of εV1-2 (1 μM), a selective PKCε inhibitor, and of CLI-095 (1 μM), a toll-like receptor (TLR) 4 inhibitor, on LPS-elicited NF-kB activity. Microglial cells were pretreated with εV1-2 (1 μM) or CLI-095 (1 μM) for 2 h and then treated with LPS 1 $\mu g \cdot mL^{-1}$ alone or LPS + morphine 10 μM. Subsequently, whole-cell lysates were prepared and analysed for NF-kB activity. PKCε and TLR4 inhibitors reduced LPS-induced activation of NF-kB. Similarly, morphine 10 μM did not increase NF-kB activity in activated microglial cells pretreated with εV1-2 and CLI-095, indicating that the increased DNA-binding activity induced by morphine 10 μM was dependent on the activation of PKCε and TLR4 (Fig. 2B).

Morphine alters the LPS-induced activation of NF-kB through PKCε-Akt-ERK1/2 signalling

Microglial cells were pretreated with U0126 and SH-5, inhibitors of ERK1/2 and Akt, respectively, for 30 min before LPS 1 $\mu g \cdot mL^{-1}$ plus morphine 100 nM stimulation for 15 min. Figure 3A shows that morphine 100 nM did not significantly increase NF-kB activity in activated microglial cells in the presence of SH-5 and U0126. These results indicate that the activation of Akt and ERK1/2 by morphine 100 nM in reactive microglia occurs upstream NF-kB involvement.

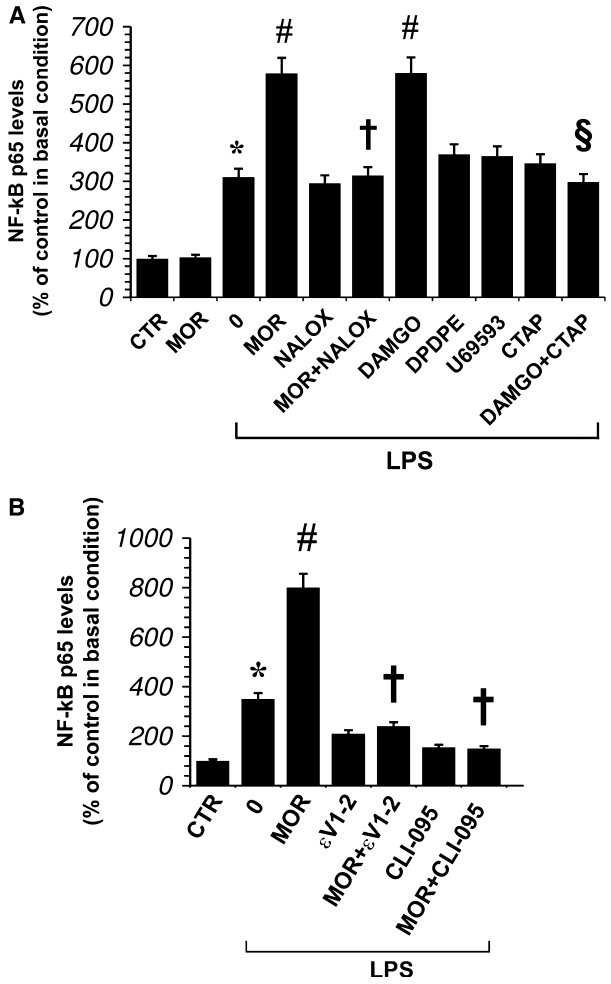

Fig. 2. Effect of opioid receptor ligands and of signalling inhibitors on NF-kB activation which was evaluated by detecting activated p65 proteins in nuclear extracts. (A) Microglial cells were treated with either LPS 1 $\mu g \cdot mL^{-1}$ alone, LPS + morphine 100 nM (MOR), LPS + Naloxone 100 nM (NALOX), LPS + DAMGO 100 nM, LPS + DPDPE 100 nM, LPS + U69593 100 nM, or LPS + CTAP 100 nM for 15 min. The antagonists were added 30 min before morphine or DAMGO. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions (0); $^{\dagger}P < 0.01$ versus LPS + MOR; $^{\S}P < 0.01$ versus LPS + DAMGO; analysis was by ANOVA followed by Dunnett's test. (B) Microglial cells were pretreated with εV1-2 (1 μM), or CLI-095 (1 μM) before LPS and morphine 10 μM stimulation for 15 min. NF-kB activation, which was evaluated by detecting activated p65 proteins in nuclear extracts, is shown. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$) and normalized to the result obtained in untreated cell cultures (CTR) set to 100%. $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions; $^{\dagger}P < 0.01$ versus LPS + MOR; analysis was by ANOVA followed by Dunnett's test.

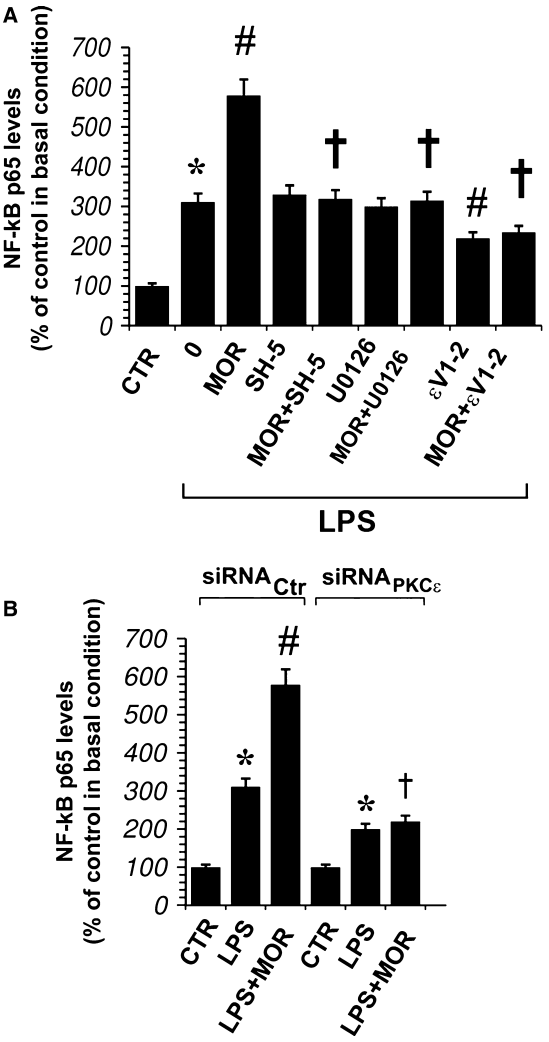

Fig. 3. PKCε is involved in mediating morphine-induced Akt-ERK1/2-NF-kB activation in primary microglial cells. (A) Microglial cells were pretreated with εV1-2 (1 μM), U0126 (1 μM) or SH-5 (1 μM) before LPS and morphine 100 nM stimulation for 15 min. NF-kB activation, which was evaluated by detecting activated p65 proteins in nuclear extracts, is shown. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$) and normalized to the result obtained in untreated cell cultures (CTR) set to 100%. $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions; $^{\dagger}P < 0.01$ versus LPS + MOR; analysis was by ANOVA followed by Dunnett's test. (B) Primary microglial cells were treated with either siRNA$_{ctr}$ or siRNA$_{PKCε}$ for 24 h and cultured with LPS 1 $\mu g \cdot mL^{-1}$ alone or plus morphine 100 nM (MOR) for 15 min. NF-kB activation, which was evaluated by detecting activated p65 proteins in nuclear extracts, is shown. 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%. $^{*}P < 0.05$ with respect to the unstimulated control (CTR); $^{\#}P < 0.05$ with respect to the cells treated with LPS; $^{\dagger}P < 0.01$ versus LPS + MOR of cells treated with siRNA$_{ctr}$; analysis was by ANOVA followed by Dunnett's test.

To confirm the role of PKCε in the signalling under investigation, we examined the effect of εV1-2 (1 μM), a selective PKCε inhibitor, and of $siRNA_{PKC\varepsilon}$ on μ-opioid receptor-elicited NF-kB activity.

Microglial cells were pretreated with εV1-2 (1 μM) for 2 h and then treated with LPS 1 $\mu g \cdot mL^{-1}$ alone or LPS + morphine 100 nM. Subsequently, whole-cell lysates were prepared and analysed for NF-kB activity. PKCε inhibitor reduced LPS-induced activation of NF-kB. Similarly, morphine 100 nM did not increase NF-kB activity in activated microglial cells pretreated with εV1-2, indicating that the increased DNA-binding activity induced by morphine 100 nM was dependent on the activation of PKCε.

We reduced PKCε expression in primary microglial cells by siRNA transfection. Primary microglial cells were transfected with nonspecific random control ribonucleotides (siRNA scramble, $siRNA_{ctr}$) or with small interfering RNAs that target PKCε mRNA ($siRNA_{PKC\varepsilon}$) for degradation. Twenty-four hours after $siRNA_{PKC\varepsilon}$ transfection, primary microglial cells were treated with either LPS 1 $\mu g \cdot mL^{-1}$ alone or LPS + morphine 100 nM for 15 min, after which NF-kB activity was measured. The results revealed that the inhibition of PKCε expression is sufficient to block NF-kB activity increased by morphine 100 nM in LPS-treated microglia (Fig. 3B).

μ-opioid receptor activation results in the activation and nuclear translocation of p65 NF-kB protein

To determine whether the increased DNA-binding activity of NF-kB was due to nuclear translocation of NF-kB p65 subunit, we analysed phosphorylation levels and nuclear translocation of p65 in microglial cells by western blotting (Fig. 4). In particular, microglial cells were treated with either LPS 1 $\mu g \cdot mL^{-1}$ alone or LPS + morphine 100 nM for 0, 15, 30, 60 or 120 min. The cytosolic and nuclear fractions of cells were subsequently prepared for western blot analysis of p65. The presence of p65 was determined in unstimulated microglial cells. We found that LPS induces translocation of p65 to the nucleus in a time-dependent manner (Fig. 4A–B). In the presence of morphine 100 nM, p65 translocation induced by LPS was significantly higher than LPS alone, with a maximal activation at 15 min (Fig. 4A–B). A decrease in p65 translocation induced by LPS + morphine was observed at 30 min (Fig. 4A–B). Furthermore, to confirm that the translocation of p65 leads to p65 activation, we have shown the presence of phosphorylated p65 at Ser536 on nuclear fractions. As shown in Fig. 4C–D, there was a significant increase in the nuclear expression of p65 phosphorylation induced by

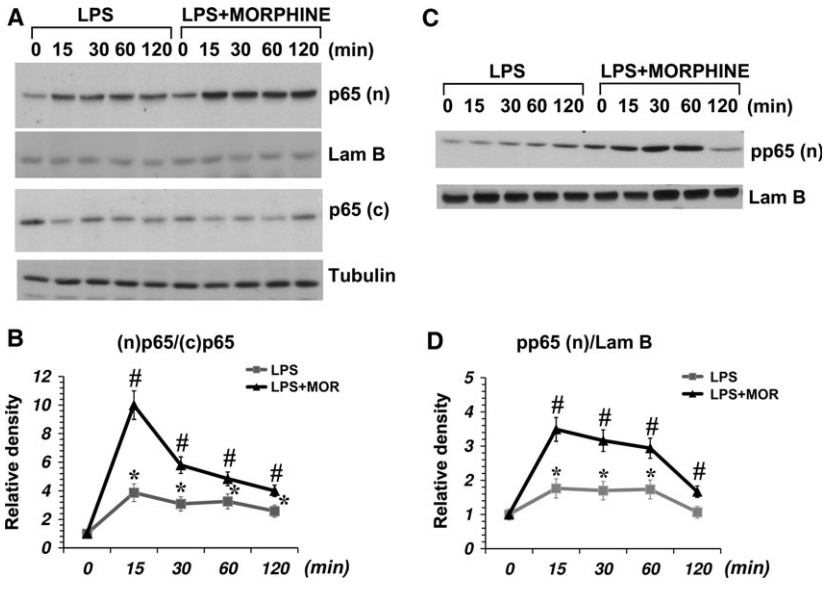

Fig. 4. Expression and morphine-induced phosphorylation and nuclear translocation of p65 isoform in LPS-activated primary microglia. Microglial cells were treated with either LPS (1 $\mu g \cdot mL^{-1}$) alone or LPS + morphine (100 nM) for 0–120 min. (A) p65 expression in nuclear (n) and cytosplasmatic (c) fractions of cells. (B) Densitometry of nuclear versus cytosplasmatic p65. (C) Phospho-p65 expression in nuclear fractions of cells is shown. (D) Densitometry of phospho-p65 expression relative to Lam B. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, 0); $^{\#}P < 0.01$ versus LPS conditions; analysis was by ANOVA followed by Dunnett's test.

LPS + morphine (for 15 min), significantly higher than LPS alone. These data indicate that the activation of μ-opioid receptor induces nuclear translocation of the p65 subunit of NF-kB, which could result in enhanced DNA-binding activity of NF-kB.

Effect of μ-opioid receptor activation on the LPS-induced degradation of IkBα and activation of IKKα-β, IkBα and p65 in microglial cells

In its inactive state, NF-kB binds with its inhibitor protein, IkBα, in the cytoplasm. However, after cellular stimulation, IkBα is phosphorylated and undergoes polyubiquitination and proteasomal degradation, which frees NF-kB and allows it to be translocated to the nucleus [29]. As shown in Fig. 5, IkBα became phosphorylated and increased upon the treatment with LPS 1 $\mu g \cdot mL^{-1}$ for 15 min. Morphine and DAMGO 100 nM significantly increased IkBα phosphorylation in LPS-activated microglial cells. The increases observed with morphine and DAMGO were abrogated by the antagonists naloxone and CTAP 100 nM respectively. Accordingly, LPS-activated cells contain ~ 70% of IkBα protein levels as compared with the control level (nonactivated samples) and morphine and DAMGO 100 nM increased the LPS-induced degradation of IkBα in microglial cells. The response of morphine and DAMGO on IkBα degradation was reversed in the presence of the antagonists naloxone and CTAP 100 nM respectively (Fig. 5). As IKKα and ß are upstream kinases of IkBα in the NF-kB signal pathway, we examined the effects of μ-opioid receptor activation on LPS-induced IKKα and IKKß activation. It was found that morphine and DAMGO markedly increased LPS-induced IKKα/ß phosphorylation, whereas they did not affect the total amounts of IKKα

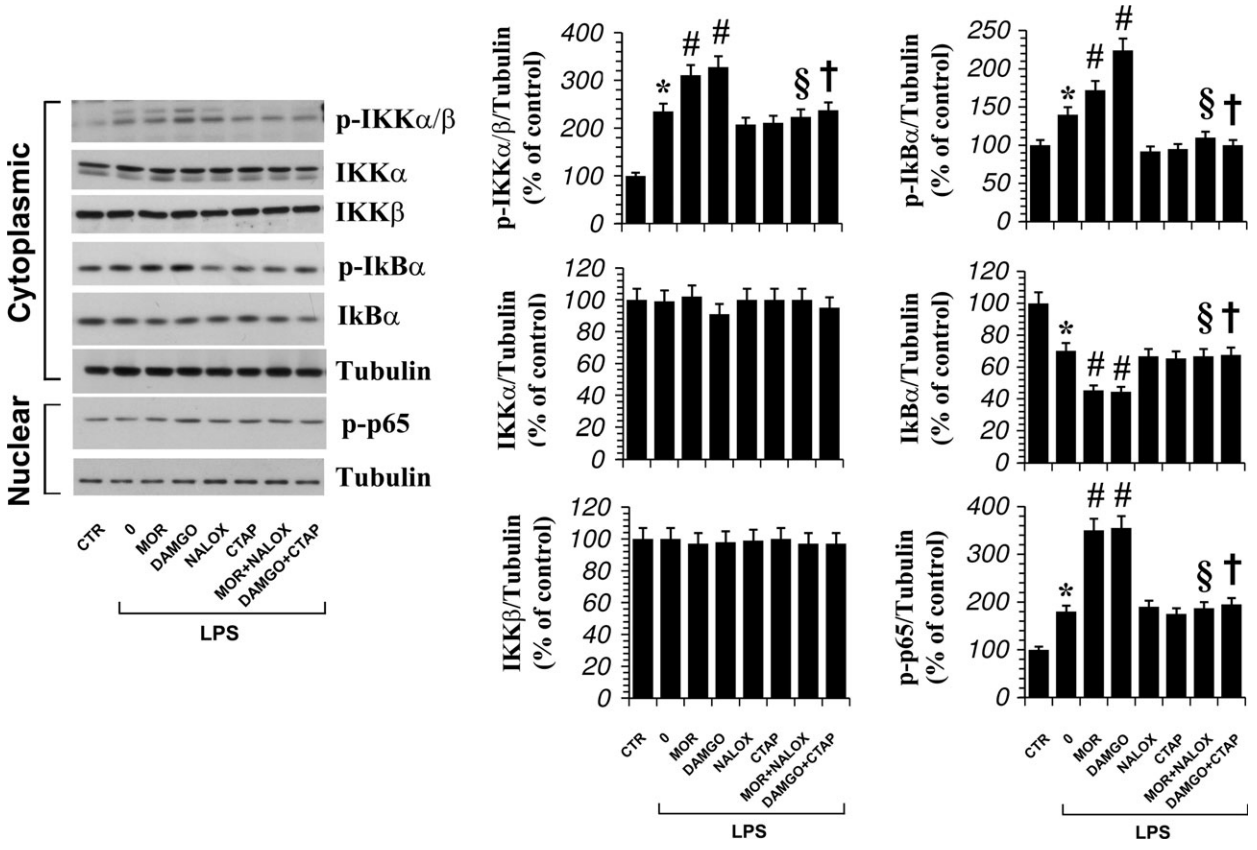

Fig. 5. Modulation of the total level of IkBα, IKKα, IKKβ and of IKKα/β, IkBα and p65 phosphorylation by opioid receptor ligands in microglia. The cells were treated with either LPS alone, LPS + morphine 100 nM (MOR), LPS + DAMGO 100 nM, LPS + Naloxone 100 nM (NALOX), or LPS + CTAP 100 nM for 15 min. The antagonists were added 30 min before morphine or DAMGO. At the end of the incubation, cytoplasmic or nuclear extracts were collected and western blot was performed to measure p-IKKα/β, IKKα, IKKβ, p-IkBα, IkBα and p-p65 respectively. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions (0); $^{\S}P < 0.01$ versus LPS + MOR; $^{\dagger}P < 0.01$ versus LPS + DAMGO; analysis was by ANOVA followed by Dunnett's test.

and IKKß. The increases of IKKα/ß phosphorylation induced by morphine and DAMGO in LPS-activated microglial cells were abrogated by the antagonists naloxone and CTAP 100 nM respectively. Finally, we observed that also the increases of p65 phosphorylation induced by morphine and DAMGO in LPS-activated microglial cells were abrogated by the antagonists naloxone and CTAP 100 nM respectively.

PKCε activation results in nuclear translocation of p65 NF-kB protein

To verify whether the effect of LPS + MOR on the nuclear translocation of NF-kB is dependent on PKCε, we examined the effect of $siRNA_{PKCε}$ on μ-opioid receptor-elicited NF-kB activation. Microglial cells with PKCε expression strongly reduced after 24 h of $siRNA_{PKCε}$ treatment were treated with either LPS 1 $μg·mL^{-1}$, LPS + morphine 100 nM or LPS + DAMGO for 15 min, then protein extracts were prepared for NF-kB analysis. A reduction in the phosphorylation of IKKα/β was detected in LPS- and LPS + morphine- or LPS + DAMGO-treated microglia with PKCε downregulated, when compared with that of controls. Furthermore, pretreatment with $siRNA_{PKCε}$ showed an impaired increase in IkBα phosphorylation in response to LPS (Fig. 6), demonstrating that LPS increases IkBα activation through PKCε. Accordingly, in the presence of $siRNA_{PKCε}$, both morphine and DAMGO 100 nM attenuated degradation of IkBα, when compared with IkBα level in untransfected cells treated with morphine and DAMGO. In agreement with these results, while LPS activation for 15 min resulted in profound phosphorylation of the p65 NF-kB subunit, this activation was impaired following the pretreatment of microglial cells with

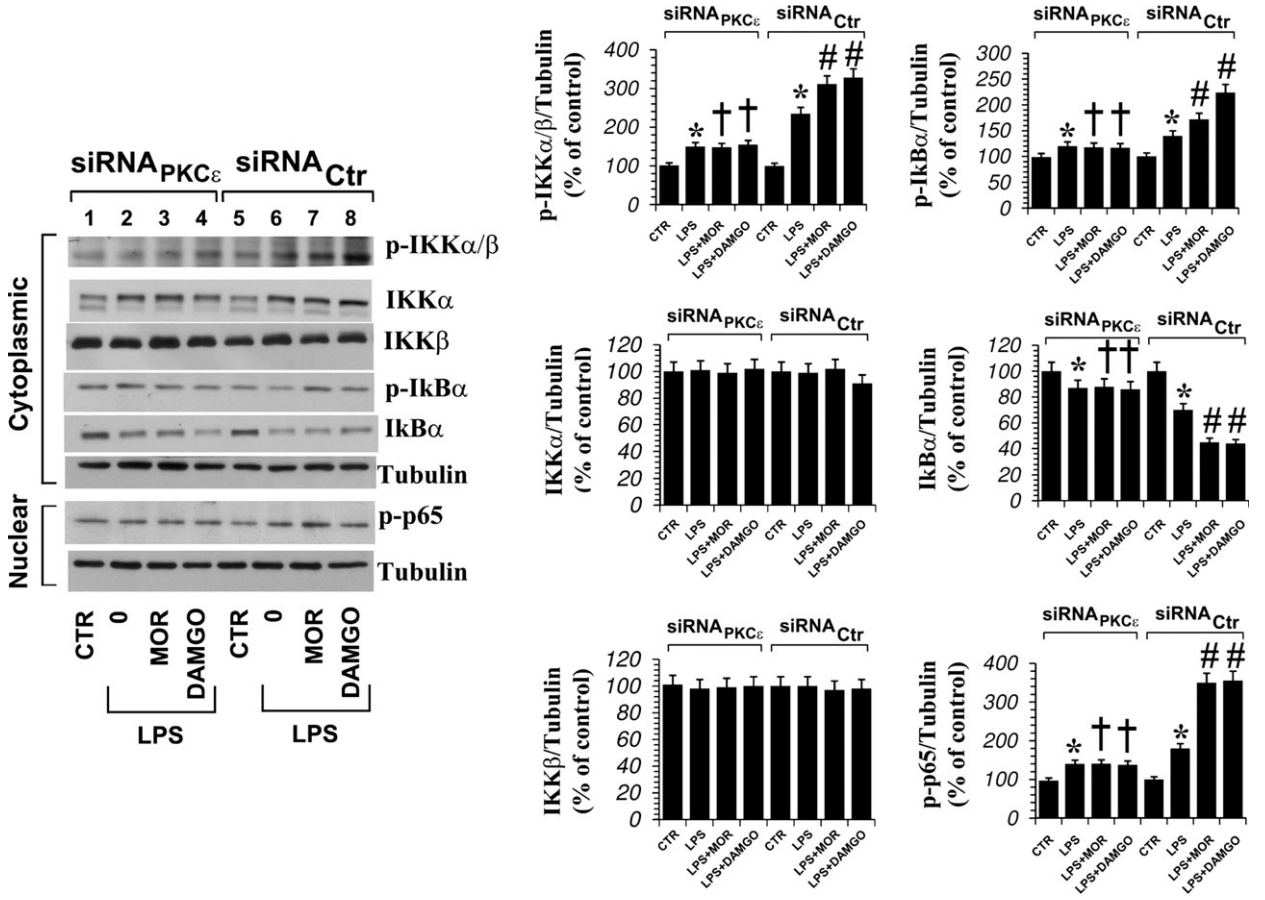

Fig. 6. Modulation of the total level of IkBα, IKKα, IKKβ and of IKKα/β, IkBα and p65 phosphorylation by opioid receptor ligands in microglial cells treated with either $siRNA_{ctr}$ or $siRNA_{PKCε}$ for 24 h and cultured with LPS 1 $μg·mL^{-1}$ alone, plus morphine 100 nM (MOR) or plus DAMGO for 15 min. At the end of the incubation, cytoplasmic or nuclear extracts were collected and western blot was performed to measure p-IKKα/β, IKKα, IKKβ, p-IkBα, IkBα and p-p65 respectively. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions (0); $^{\dagger}P < 0.01$ versus LPS + MOR of cells treated with $siRNA_{ctr}$; analysis was by ANOVA followed by Dunnett's test.

$siRNA_{PKC\varepsilon}$, indicative that downregulation of PKCε results in impaired NF-κB signalling.

μ-opioid receptor gene silencing and translocation of p65 NF-kB protein

Microglial cells with μ-opioid receptor expression reduced after 48 h of treatment with $siRNA_{\mu}$ were treated with either LPS 1 $\mu g \cdot mL^{-1}$, LPS + morphine 100 nM or LPS + DAMGO for 15 min, after which protein extracts were prepared for NF-kB analysis. We found that the inhibition of μ-opioid receptor expression blocks morphine and DAMGO increases in the translocation of NF-kB p65 protein in microglia. Indeed, in the presence of $siRNA_{\mu}$, both morphine and DAMGO 100 nM attenuated degradation of IkBα, when compared with IkBα level in untransfected cells treated with morphine and DAMGO. Accordingly, pretreatment with $siRNA_{\mu}$ showed an impaired increase in IkBα phosphorylation in response to LPS + morphine or LPS + DAMGO, demonstrating that morphine and DAMGO increased IkBα degradation through μ-opioid receptor. Furthermore, a reduction in the phosphorylation of IKKα/β was detected in LPS-treated microglia cells with μ-opioid receptor downregulated, when compared with that of controls. These results show the connection between μ-opioid receptor stimulation, morphine, and NF-kB signalling in activated primary microglial cells (Fig. 7).

Activation of NF-kB by morphine in reactive microglia occurs downstream Akt-ERK1/2 kinase

To identify whether Akt and ERK1/2 signalling pathways are necessary for μ-opioid receptor-PKCε-induced activation of NF-kB, we selectively blocked

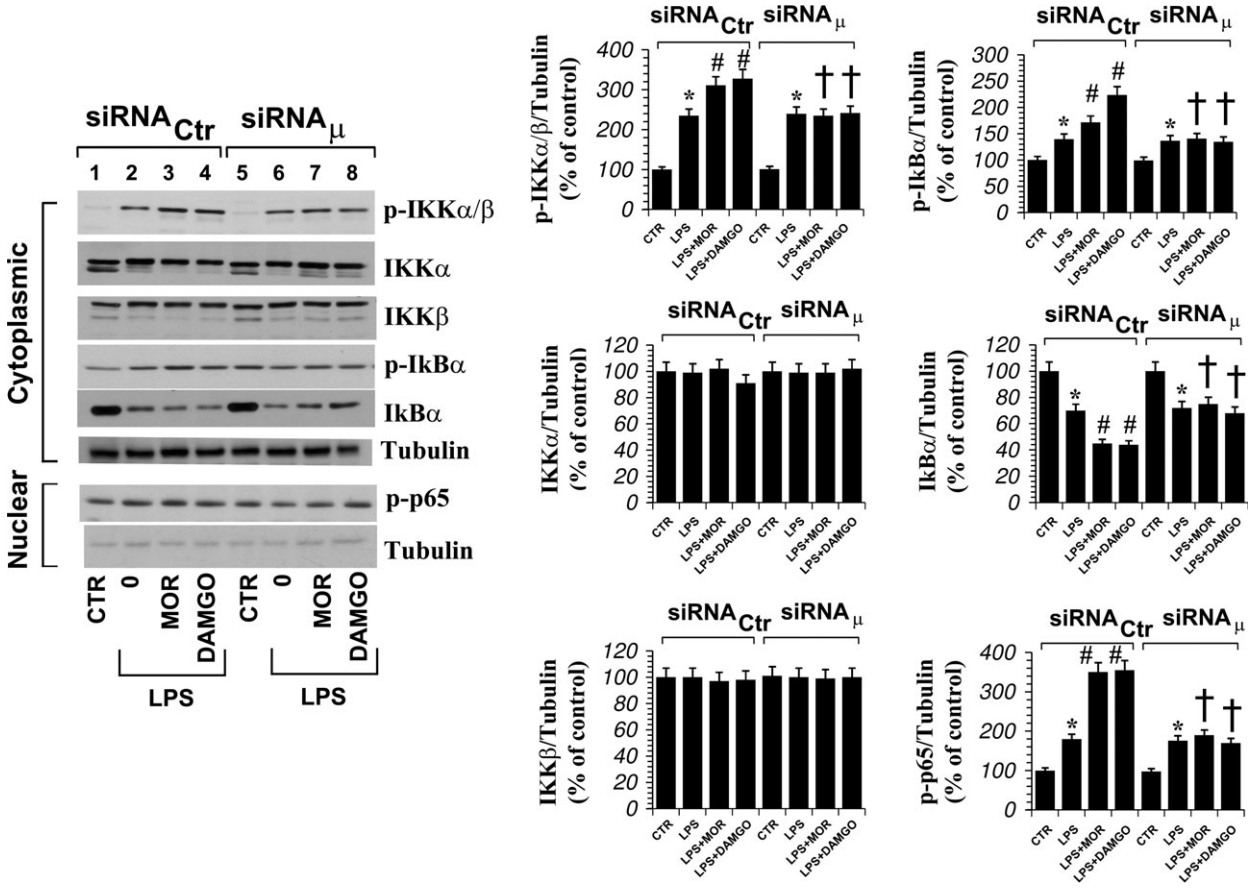

Fig. 7. Modulation of the total level of IkBα, IKKα, IKKβ and of IKKα/β, IkBα and p65 phosphorylation by opioid receptor ligands in microglial cells treated with either $siRNA_{ctr}$ or $siRNA_{\mu}$ for 48 h and cultured with LPS 1 $\mu g \cdot mL^{-1}$ alone, plus morphine 100 nM (MOR) or plus DAMGO for 15 min. At the end of the incubation, cytoplasmic or nuclear extracts were collected and western blot was performed to measure p-IKKα/β, IKKα, IKKβ, p-IkBα, IkBα and p-p65 respectively. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions (0); $^{\dagger}P < 0.01$ versus LPS + MOR of cells treated with $siRNA_{ctr}$; analysis was by ANOVA followed by Dunnett's test.

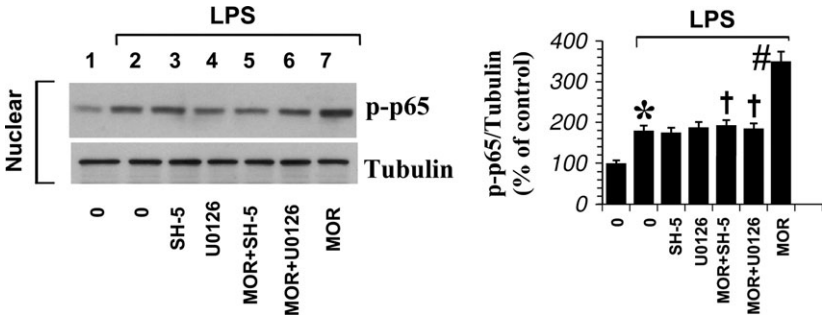

Fig. 8. Modulation of p65 phosphorylation by SH-5 (1 μM) or U0126 (1 μM) alone or in combination with morphine (100 nM) before LPS and morphine stimulation for 15 min. The inhibitors were added 30 min before morphine. At the end of the incubation, nuclear extracts were collected and western blot was performed to measure p-p65. The mean values of three independent experiments (one of which is shown) were normalized to the result obtained in the cells in the absence of LPS. The unstimulated control was set to 100%. $^{*}P < 0.05$ with respect to the unstimulated control (0); $^{\#}P < 0.05$ with respect to the cells treated with LPS; $^{\dagger}P < 0.01$ versus LPS + MOR; analysis was by ANOVA followed by Dunnett's test.

Akt and ERK1/2 pathways with SH-5 and U0126, inhibitors of Akt and ERK1/2, respectively, for 30 min before LPS plus morphine 100 nM stimulation for 15 min. Figure 8 shows that the inhibition of ERK1/2 and Akt by U0126 (1 μM) and SH-5 (1 μM), respectively, abolished LPS + morphine-induced phosphorylation of NF-kB p65 protein in activated microglial cells. These results indicate that morphine utilizes both the Akt and ERK1/2 signalling pathways to activate NF-kB in microglial activated cells.

Activation of NF-kB is required for the induction of chemokine and NO production by μ-opioid receptor

We measured cytokine and nitrite levels in microglial cells pretreated with the NF-kB inhibitors BAY 11-7082 and MG132. BAY 11-7082 was used as it blocks the NF-kB signalling pathway by inhibiting the phosphorylation of IkB, while MG132 is a proteasome inhibitor that inhibits the degradation of phosphorylated IkB. These inhibitors allowed us to explore the role of NF-kB in the regulation of cytokine expression and nitrite levels. Primary microglial cells were maintained in LPS in combination with morphine 100 nM for 24 h. We show that LPS-induced IL-1β, TNF-α, IL-6 and nitrite release was significantly increased in the presence of morphine in primary microglial cells. However, the stimulatory response of morphine on cytokine and nitrite production was reversed in the presence of MG132 or BAY 11-7082 1 μM (Fig. 9A).

Finally, microglial cells were pretreated with BAY 11-7082 and MG132 for 30 min before LPS plus morphine 100 nM stimulation for 4 h. Subsequently, whole-cell lysates were prepared for western blot analysis of iNOS. The results demonstrate that BAY 11-7082 and MG132 significantly blocked the ability of morphine to increase iNOS production in the presence of LPS (Fig. 9B), indicating that the activation of NF-kB occurs upstream iNOS induction induced by LPS and morphine in reactive microglia.

Discussion

Microglial cells express the TLR4 which increases inflammation [30]. Previous work has shown that morphine may activate glia through different ways from classical opioid receptors by an interaction with TLR4-MD2 [31–34]. In particular, opioid receptor antagonists reversed this activation in a nonstereospecific fashion [35–37]. These data indicated that morphine and other opioids may signal as direct TLR4 ligands leading to neuroinflammation [38]. Accordingly, here we have found that the high dose of morphine is not mediated by the μ-opioid receptor, as indicated in the literature data showing that morphine mediates neuroinflammatory effects not via μ-opioid receptors, but in a manner parallel to endotoxin, through the activation of TLR4 signalling [34,38]. Similarly, we have found that the activation of NF-kB induced by the high dose of morphine is mediated through TLR4 and, interestingly, via PKCε signalling.

However, in the present study, based on the KO data and through the use of a μ-opioid specific agonist, we have demonstrated that the μ-opioid receptor is playing a role in microglial cells. While morphine by itself had no effect, we have found that the low dose of morphine stimulates μ-opioid receptors in activated microglial cells, thereby inducing the transcription factor NF-kB and that this pathway serves as downstream signalling target of the ε-isoform of PKC. The activation of Akt and ERK1/2 kinases is a critical

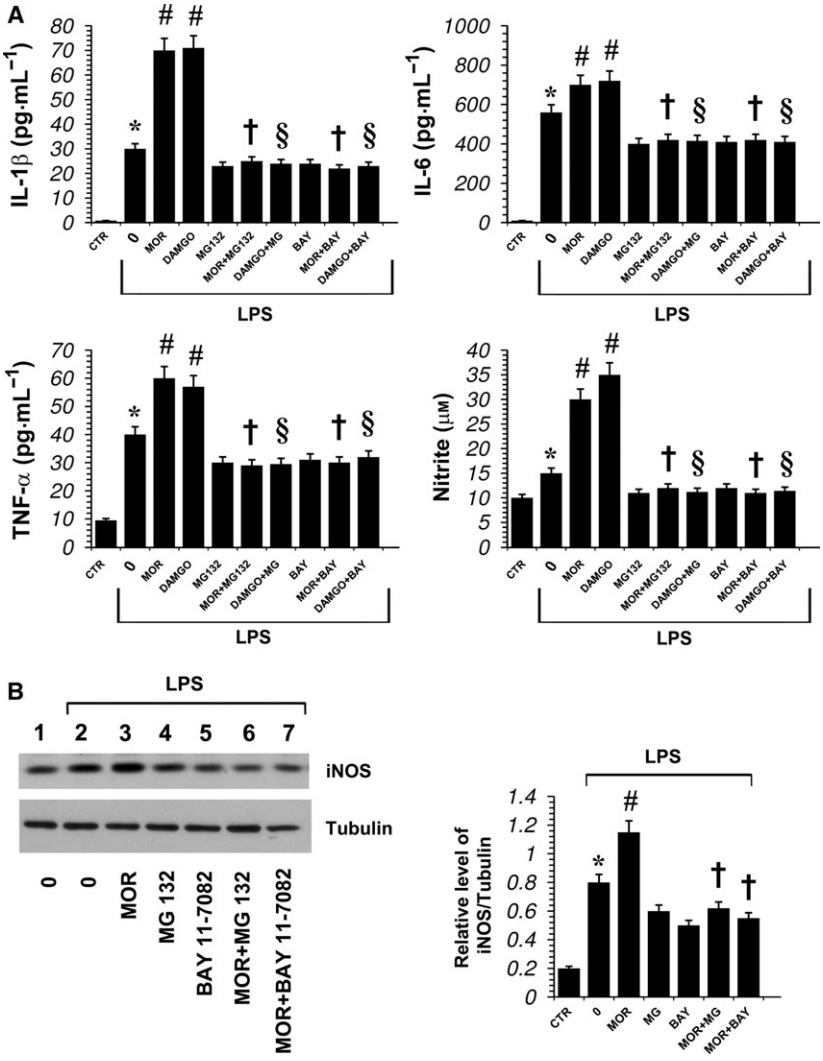

Fig. 9. Primary microglial cells were treated with either LPS 1 $\mu g \cdot mL^{-1}$ alone or plus morphine 100 nM (MOR), DAMGO (100 nM), MG 132 1 μM, BAY 11-7082 1 μM, alone or in combination, for 24 h (IL-1β, IL-6, TNF-α, Nitrite, panel A) or 4 h (iNOS, panel B). Densitometric analysis of iNOS is reported. Plots are mean ± SE values of four separate experiments (one of which is shown) performed in triplicate ($n = 4$). $^{*}P < 0.01$ versus control conditions (absence of drugs, CTR); $^{\#}P < 0.01$ versus LPS conditions; $^{\dagger}P < 0.01$ versus LPS + MOR; $^{\S}P < 0.01$ versus LPS + DAMGO; analysis was by ANOVA followed by Dunnett's test.

intermediate signalling step during μ-opioid receptor-PKCε-induced activation of NF-kB and both of these pathways are necessary to produce the activation of the transcription factor. Furthermore, we have demonstrated that μ-opioid receptor determined NF-kB-regulated proinflammatory IL-1ß, TNF-α, IL-6 cytokines along with iNOS and NO production increase.

The involvement of PKC and opioids in regulating NF-kB has been reported [39,40]. In particular, in previous studies performed in rat cortical neurons [41], in the human neuroblastoma cells [18] and in murine macrophages [42], morphine treatment differentially modulates LPS-inducible gene expression through the regulation of NF-kB. The effects appear diverse and often cell type-dependent, even if NF-kB is considered as a critical component in opioid function and receptor gene expression [43]. However, the implication of individual PKC isozymes in the control of this pathway remained to be elucidated. In human leucocytes, opioids increased chemokine expression through NF-kB activity induced by PKCζ [44]. Here, we found that μ-opioid receptors, by the recruitment of Akt and ERK1/2 pathways, increase IkBα phosphorylation, thus resulting in the stabilization of p65 subunit in the cytoplasm. As a consequence, NF-kB translocates into the nucleus then driving an enhanced inflammatory response via PKCε signalling. In particular, this study provides evidence that, in microglial cells, PKCε depletion or inhibition diminishes LPS-induced IκBα phosphorylation and degradation, NF-κB nuclear translocation, and transactivation potential. Therefore, PKCε is required for the increased NF-κB activation observed in morphine-stimulated microglial cells.

To our knowledge, this is the first report describing that μ-opioid receptors through PKCε-Akt-ERK1/2 signalling govern the activity of NF-kB in microglia,

further underscoring the role of opioid system in microglia activation.

The establishment of NF-kB role in morphine signalling may provide a means to treat opioid development of tolerance and dependence. However, even if ideally the inhibition of NF-kB would be a worthwhile therapeutic strategy, the blockade of important physiological functions, such as innate immunity and cell survival, mediated by NF-kB proteins may account for the difficulty in the validation of a NF-kB inhibitor as a drug. Therefore, it is possible to target the pathway in some conditions but problems may arise.

Consequently, the identification of PKCε as a crucial upstream regulator of NF-κB signalling in microglia argues for a fundamental role of this kinase in the control of pathways involved in neuroinflammation development and progression. It is important to highlight that there has been significant interest in the development of PKCε inhibitors as anti-inflammatory and anticancer agents [45–47]. Certainly, the *in vivo* observations that PKC inhibitors can reduce morphine antinociceptive tolerance [14] provide evidence that the selective inhibition of those PKCs involved might provide useful adjuncts to chronic therapy with opioid drugs. Furthermore, selective PKCε inhibitors can be effective candidates for the sensitization of microglia to therapeutic agents, as shown previously for NF-κB inhibitors. Inhibition of a kinase required for so many important biological functions may be expected to result in substantial side effects. However, several inhibitors of PKC have proven to be well tolerated in clinical trials [48–50]. We may hope for a similar advance with regard to PKCε selective inhibition. Thus, targeting the PKCε-NF-κB pathway may provide novel means to induce neuroprotective mechanisms, thereby reducing tolerance to opioids.

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Author contributions

SM supervised the study and wrote the manuscript; PAB made manuscript revisions; SG designed the experiments; SB and DF performed the experiments; KV analysed the data.

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