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Morphine Withdrawal Inhibits IL-12 Induction in a Macrophage Cell Line through a Mechanism That Involves cAMP

Jennifer Kelschenbach, ‡ Jana Ninkovic, ‡ Jinghua Wang,* Anitha Krishnan,* Richard Charboneau,‡ Roderick A. Barke,*‡ and Sabita Roy2*†‡

There are very few studies that examine the effects that morphine withdrawal has on immune functioning, and of these even fewer describe the mechanisms by which withdrawal brings about these changes. Our previous work demonstrated that morphine withdrawal contributed to Th cell differentiation by biasing cells toward the Th2 lineage. A major finding from these studies was that IL-12 was decreased following withdrawal, and it was concluded that this decrease may be a mechanism by which morphine withdrawal is mediating Th2 polarization. Therefore, it was the aim of the current studies to develop an in vitro model to examine the process of morphine withdrawal and to understand the signaling mechanisms that withdrawal may use to effect IL-12 production through the use of this model. It was demonstrated and concluded that morphine withdrawal may be effecting IL-12 production by increasing cAMP levels, which activates protein kinase A. Protein kinase A activation then prevents the phosphorylation and subsequent degradation of IκB, which in turn prevents translocation of the NF-κB p65 subunit to the nucleus to transactivate the IL-12 p40 gene, ultimately resulting in decreased IL-12 production following LPS stimulation. The Journal of Immunology, 2008, 180: 3670–3679.

Although chronic morphine use and abuse have been documented to result in severe immune consequence (1–3) with increased prevalence of opportunistic infections (4–6), very few studies have been dedicated to examining the effects of opiate withdrawal on immune functioning. Initial studies by Tomei and Renaud (7) demonstrated that opioid withdrawal in an in vitro model significantly decreases FcyR-mediated macrophage phagocytosis and the decrease correlated with altered intracellular cAMP levels (8). More recent studies from Eisenstein and colleagues (9, 10) show that both abrupt and precipitated morphine withdrawal results in significant immunosuppression and the observed dysfunction was a result of impaired macrophage function, as evidenced by the findings that macrophages obtained from withdrawn spleens displayed reduced expression of the costimulatory molecule B7.2 and had depressed cytokine production. Additional studies from this group (11, 12) further demonstrated that morphine-withdrawn mice when administered a sublethal dose of LPS exhibited 100% lethality that was accompanied by a decrease in IL-12 production. Similarly, we have also recently shown that morphine withdrawal results in a significant decrease in LPS-induced IL-12 synthesis in an in vivo morphine-withdrawal model (13).

IL-12 is a pivotal cytokine involved in cell-mediated immunity and impairment in IL-12 synthesis results in increased susceptibility to intracellular bacterial pathogens (14–18). Thus, the observation that morphine withdrawal results in reduction in IL-2 synthesis is clinically significant and may account for the increased susceptibility to opportunistic infection in the drug abuse population.

IL-12 is a heterodimeric cytokine (referred to as IL-12p70) and is comprised of two disulfide-linked subunits known as p35 and p40, which corresponds to their approximate molecular mass (19, 20). Although p35 is produced by most hematopoetic cells (21, 22), IL-12p40 is tightly regulated and the active form of IL-12 is produced only by APCs (19, 20). Most studies indicate that levels of IL-12p40 are a better indicator of IL-12 production, and as a result, the IL-12p40 gene has been studied for transcriptional regulation of IL-12 (21). The production of IL-12p40 in both macrophages and dendritic cells is transcriptionally regulated by key transcription factors which include AP-1, PU.1, C/EBP, and NF-κB (22–27). Recent studies (21) show that NF-κB activation is essential for LPS-induced IL-12 production and mutation of the IL-12 promoter at the NF-κB site significantly reduced IL-12 transcription (21).

The production of IL-12p40 in both macrophages and dendritic cells is transcriptionally regulated by key transcription factors which include AP-1, PU.1, C/EBP, and NF-κB (22–27). Recent studies (21) show that NF-κB activation is essential for LPS-induced IL-12 production and mutation of the IL-12 promoter at the NF-κB site significantly reduced IL-12 transcription (21).

The goal of the current studies was to initially establish an in vitro model of withdrawal and using this model examine the mechanism by which morphine withdrawal modulates IL-12 production.

Materials and Methods

Reagents

LPS (from Escherichia coli O127:B7) was obtained from Sigma-Aldrich and used as an immune stimulus. Protein kinase inhibitors (H-89, SB203580, Rp cAMP, and PD98059) were obtained from Calbiochem/EMD Biosciences and were used to assess cell signaling components involved in morphine-withdrawal mediated IL-12 production.
Animals
Six- to 8-wk-old B6129SF2 male mice were used in the experiments described within. Animals were housed four animals per cage under controlled conditions of temperature and lighting (12-h light/dark) and given free access to standard food and tap water. All animals were allowed to acclimate to their environment for at least 7 days before any experimental manipulations. Sacrifice was performed by carbon dioxide asphyxiation and spleen tissue was harvested aseptically. Discomfort, distress, and injury to the animals were minimized. The Institutional Animal Care and Use Committee at the University of Minnesota have approved all protocols in use, and all procedures are in agreement with the guidelines set forth by the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Purification of macrophages from splenocytes
Spleens were removed aseptically and suspensions were prepared by forcing the tissue through a tissue sieve with a sterile syringe plunger. Splenocytes were allowed to adhere in serum-free medium for 1 h at 37°C. Non-adherent cells were washed and adherent cells were resuspended in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% newborn calf serum and 1% penicillin-streptomycin (Sigma-Aldrich). The composition of the adherent populations was evaluated using FACS analysis. Flow cytometric analysis revealed that adherent cells were primarily macrophages, with almost a 5-fold enhancement of F4/80 staining compared with the total splenocyte population. CD3+ T lymphocytes were not detected in the adherent population. The adherent cells were plated at a concentration of 1 × 10^6 cells/ml in triplicate onto 6-well culture plates. Cells were subjected to the in vitro withdrawal paradigm described below and stimulated with LPS (Sigma-Aldrich) at 10 μg/ml LPS (Sigma-Aldrich; incubated either 1–2 g/ml LPS (Sigma-Aldrich) at 5 μg/ml (a concentration determined to have significant stimulatory effect when measuring subsequent protein levels) and incubated overnight at 37°C in 5% CO2.

Cell culture
The mouse alveolar macrophage cell line CRL-2019 (American Type Culture Collection) was also used to examine cell signaling properties and IL-12p40 promoter activity following morphine withdrawal. Cells were maintained in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS and 1% penicillin-streptomycin (Sigma-Aldrich). Cells were plated at a concentration of 1–2 × 10^6 cells/ml in triplicate onto 6-well culture plates. Cells were subjected to the in vitro withdrawal paradigm described below and stimulated with LPS (Sigma-Aldrich) at 5 μg/ml (a concentration determined to have significant stimulatory effect when measuring subsequent protein levels) and incubated overnight at 37°C in 5% CO2.

In vitro withdrawal paradigm
To replicate conditions tested in vivo (13), either primary cells or the cell line was plated as described above. Following plating, cells were treated with 100 nM morphine sulfate (National Institute on Drug Abuse, Research Triangle Park, NC) once per day for 3 consecutive days. Parallel cultures were treated with vehicle and designated vehicle control groups. Following the third day of morphine treatment, cells were vigorously washed three to five times with PBS (Invitrogen Life Technologies) to simulate withdrawal. Parallel cultures were washed in the presence of morphine and incubated with morphine and designated as the chronic morphine group. Cells were then stimulated with 5 μg/ml LPS (Sigma-Aldrich; incubated either overnight or for various time points depending on the type of experiment at 37°C in 5% CO2.

ELISA
ELISAs were performed as described previously by our laboratory (13). Briefly, quantikine ELISA kits were obtained (R&D Systems) and assays run according to the manufacturer’s directions. Sample supernatant was assayed in triplicate per experimental condition. Following the incubation period, plates were washed and 100 μl of detection Ab conjugated to HRP was added to the wells. Finally, 100 μl of substrate solution was added and absorbance was read at 450 nm using a standard plate reader (Packard SpectraCount Microplate Photometer). OD measurements for the standards were used to generate a standard curve, and the concentration of the particular cytokine in each of the samples was extrapolated from this standard curve. Concentrations are presented as pg/ml.

RT-PCR
RT-PCR experiments were used to assess mRNA levels of cytokines, and were performed as described previously by our laboratory (13). Briefly, total RNA was extracted from splenocytes using an RNasy Mini kit (Qiagen), a system which uses spin columns to extract and purify RNA from cells. Before RT-PCR, RNA samples were treated with deoxyribonuclease 1 (DNase I; Invitrogen Life Technologies) according to the manufacturer’s instructions. To ensure quality of RNA samples, 260/280 UV absorbance readings were performed and the 260/280 ratios were in range for pure RNA. Total RNA (1 μg) was then reverse transcribed to synthesize first-strand cDNA (45°C, 45 min) using random hexamers (2.5 μM). Moloney murine leukemia virus reverse transcriptase (2.5 U), and 1 mM each of dATP, dCTP, dGTP, and dTTP for a final reaction volume of 40 μl (Applied Biosystems). Following first-strand synthesis, the reaction mixture was heated at 95°C for 5 min to inactivate reverse transcriptase. Amplification steps were performed using upstream and downstream primers specific for mouse IL-12p40 (Clontech Laboratories) and β2-microglobulin (Clontech Laboratories). The primer sequences are as follows: IL-12p40, sense 5’-ACT CAT ATC TTC TGC TCC AC-3’, antisense 5’-CTG GTT TGA TGA TGT CCT G3’; and β2-microglobulin, sense 5’-ATG GCT CGC TCG TGT ACC CTA-T3’, antisense 5’-TCA TGA TGG TAT ATC CAT GTC TCG-3’. PCR buffer containing 2 mM MgCl2, 0.1 μM primer, and 2.5 U of AmpliTaq DNA polymerase was prepared and −5 μl of the first-strand cDNA reaction mixture was added for a final volume of 50 μl. PCR conditions consisted of 35 cycles of 94°C for 45 s (denaturation), 60°C for 45 s (annealing), and 72°C for 45 s (extension), followed by a final extension at 72°C for 15 min. Internal controls included both a negative control (containing only PCR mix) and a control lacking reverse transcriptase used at the point of cDNA synthesis. PCR products were separated using 1.5% agarose gels and visualized by ethidium bromide staining.

Real-time RT-PCR
Real-time RT-PCR experiments were used to assess mRNA levels of IL-12p40. Total cellular RNA was isolated using the RNeasy Mini kit per the manufacturer’s instructions (Qiagen). Total RNA was DNase I treated per the manufacturer’s instructions (Invitrogen Life Technologies) and quantitated using the A260:A280 ratio. Reverse transcription and real-time PCR was performed on Applied Biosystems Prism 7500 using SYBR Green PCR Master Mix (Applied Biosystems). The established primers for IL-12p40 were determined using a 2-microglobulin, sense 5’-GTC TGC 3’; and β2-microglobulin, sense 5’-ATG GCT CGC TCG TGT ACC CTA-T3’, antisense 5’-TCA TGA TGG TAT ATC CAT GTC TCG-3’. PCR buffer containing 2 mM MgCl2, 0.1 μM primer, and 2.5 U of AmpliTaq DNA polymerase was prepared and −5 μl of the first-strand cDNA reaction mixture was added for a final volume of 50 μl. PCR conditions consisted of 35 cycles of 94°C for 45 s (denaturation), 60°C for 45 s (annealing), and 72°C for 45 s (extension), followed by a final extension at 72°C for 15 min. Internal controls included both a negative control (containing only PCR mix) and a control lacking reverse transcriptase used at the point of cDNA synthesis. PCR products were separated using 1.5% agarose gels and visualized by ethidium bromide staining.

EMSA
Transcription factor interactions with DNA response elements were assessed using EMSAs as described previously by our laboratory (12). Briefly, nuclear extracts were prepared as described below. NF-κB consensus oligonucleotides were purchased (Santa Cruz Biotechnology) and end-labeled with 32P according to the manufacturer’s instructions (Promega). Approximately 10 μg of nuclear extracts was incubated with 0.5 ng of labeled probe in binding buffer. DNA-protein complexes were resolved using nondenaturing acrylamide gels. Gels were then dried and visualized by either autoradiography or phosphorimaging techniques.

cAMP immunoassay
Cells were exposed to withdrawal as described above. At 5, 10, and 30 min following withdrawal, cells were washed in PBS and resuspended at a density of 1 × 10^7 cells/ml and lysed in cell lysis buffer. The supernatant from lysed cells was analyzed using cAMP assay kits (R&D Systems) according to the manufacturer’s instructions.

Western blotting
Protein levels of various NF-κB subunits and IκBα were determined using Western blot techniques. First, nuclear protein extracts were prepared using the CellLytic Nuclear Extract Kit (Sigma-Aldrich). Briefly, cells were resuspended in lysis buffer containing DTT and protease inhibitors. Cells were then homogenized and centrifuged to separate cytoplasmic and nuclear proteins. Proteins were then separated by SDS-PAGE using 10% gels and the gels were then transferred to nitrocellulose membranes using a semidry transfer procedure. Membranes were blocked using Superblock and probed with a 1/1000 dilution of rabbit monoclonal primary Ab (Cell Signaling Technology). Membranes were washed and probed with a 1/5000 dilution of a goat × rabbit secondary Ab conjugated to HRP (Sigma-Aldrich). Membrane blots were then
exposed to ECL detection reagents (SuperSignal West Pico Chemiluminescent Substrate; Pierce) and visualized using x-ray films.

**Transient transfection**

The human IL-12p40 promoter-luciferase reporter plasmid was provided by Dr. A. Kumar (University of Ottawa, Ottawa, Ontario, Canada) and the construction of this plasmid has been previously described (21). CRL-2019 cells were transfected with plasmids using the Effectene reagent (Qiagen) according to the manufacturer’s instructions. Briefly, 10 μg of IL-12p40 promoter-firefly luciferase reporter plasmid and 0.5 μg of pRL-TK-reporter internal control plasmid (Promega) were incubated for 10 min with Effectene reagent in standard RPMI 1640 medium to allow formation of liposome complexes. Complexes were added directly to each well of a 6-well plate and cells were maintained at 37°C in 5% CO₂ culture conditions. Transfections were performed on day 3 of these 5-day experiments. Following the treatment paradigm, cells were lysed and luciferase activity was measured using a Dual-Luciferase Reporter Assay system (Promega) and a Turner Biosystems TD 20/20 luminometer according to the manufacturers’ instructions. Data are presented as standardized luciferase activity and was determined by the ratio between firefly luciferase and Renilla reniformis luciferase.

**Statistics**

Each cytokine supernatant protein concentration was expressed as mean ± SEM, and comparisons between group means were assessed using an unpaired Student’s t test. Standardized luciferase activity ± SEM was plotted according to treatment group and differences were assessed using an unpaired Student’s t test. Significance was set at p < 0.05.

**Results**

**In vitro morphine withdrawal decreases IL-12p70 and p40 production from primary splenocyte-derived LPS-stimulated macrophages**

Splenic macrophages were purified as described in Materials and Methods and subjected to in vitro withdrawal treatment paradigm as described above. Following the treatment period, both cell and supernatant fractions were harvested, and supernatant fractions were used to assess IL-12p70 and p40 protein concentrations via ELISA. Fig. 1 demonstrates that in vitro morphine withdrawal treatment results in a significant reduction in LPS-induced IL-12p70 and IL-12p40 protein levels, respectively, when compared with vehicle-withdrawn cells treated with LPS (Fig. 1, ***, p < 0.001). Interestingly, although chronic morphine treatment (washed in the presence of morphine and incubated with morphine) resulted in some inhibition of IL-12p70 and p40 protein levels (Fig. 1; *, p < 0.001), the effect was significantly different from the morphine-withdrawal group (Fig. 1; #, p < 0.002).

**In vitro morphine withdrawal decreases IL-12 p40 protein and message levels from the LPS-stimulated CRL-2019 mouse alveolar macrophage cell line**

One of the major goals of these studies was to understand the underlying molecular mechanisms by which morphine withdrawal regulates the production of IL-12. To accomplish this goal, an in vitro model system was initially established that replicated what was observed using primary macrophages. The CRL-2019 mouse alveolar macrophage cell line was chosen to be used in these studies for several reasons. First, these cells were demonstrated to express the μ opioid receptor (data not shown), the cells are highly responsive to both morphine and LPS treatment, and finally these cells were generated from a genetically similar background to the mice used in previous experiments (13). The CRL-2019 cells were cultured and treated as described above, and following the treatment period supernatants and cells were harvested to assess both IL-12p40 protein and message levels. Fig. 2A demonstrates that in vitro morphine withdrawal plus LPS significantly decreased (***, p < 0.001) the protein levels of IL-12p40 compared with vehicle withdrawal plus LPS (***, p < 0.001). In Fig. 2, B and C, we show that message levels of IL-12p40 (Fig. 2, B, gel-based
PCR, and C, real-time PCR) are similarly decreased in the morphine-withdrawal plus LPS group when compared with vehicle-withdrawal plus LPS group in the CRL-2019 cells. Once again morphine withdrawal resulted in a more robust inhibition of IL-12 compared with chronic morphine treatment similar to what was observed with primary splenic macrophages (Fig. 2; #, \( p < 0.01 \)). It can be concluded from these data that this cell line can be used as a model to understand how morphine withdrawal may affect signaling, ultimately resulting in a decrease in IL-12 production.

**FIGURE 2.** In vitro morphine withdrawal decreases both IL-12p40 protein and message levels from LPS-stimulated CRL-2019 macrophages. A, CRL-2019 macrophages were cultured and treated as described above and stimulated overnight with 5 \( \mu \)g/ml LPS. Cell supernatants were collected and protein levels of IL-12p40 were assessed by ELISA. ***, \( p < 0.001 \) represents morphine withdrawal + LPS compared with vehicle withdrawal + LPS; *, \( p < 0.01 \) represents chronic morphine + LPS compared with vehicle withdrawal + LPS; #, \( p < 0.01 \) represents chronic morphine + LPS compared with morphine withdrawal + LPS. Data presented are the mean ± SEM of three independent experiments and comparisons between groups were assessed using an unpaired Student’s \( t \) test. B, CRL-2019 macrophages were cultured and treated as described above and stimulated overnight with 5 \( \mu \)g/ml LPS. Total RNA was isolated and RT-PCR experiments were performed to assess mRNA levels of IL-12. The housekeeping gene \( \beta_2 \)-microglobulin was used to monitor equivalence of loading. Data presented are representative of three independent experiments. C, CRL-2019 macrophages were cultured and treated as described above and stimulated overnight with 5 \( \mu \)g/ml LPS. Total RNA was isolated and real-time RT-PCR experiments were used to assess mRNA levels of IL-12p40. Total cellular RNA was isolated and DNase I treated. RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The established primers for IL-12 and 18S were used at a final concentration of 300 nM and 18S at a final concentration of 100 nM. All samples were performed in triplicates or quadruplicates and normalized to ribosomal 18S mRNA in the same cDNA set. Data are expressed as fold change over control, untreated cells by the cycle threshold method. Each experiment was performed independently and at least three times. ***, \( p < 0.001 \) represents morphine withdrawal + LPS compared with vehicle withdrawal + LPS; *, \( p < 0.01 \) represents chronic morphine + LPS compared with vehicle withdrawal + LPS; #, \( p < 0.01 \) represents chronic morphine + LPS compared with morphine withdrawal + LPS. Data presented are the mean ± SEM of three independent experiments and comparisons between groups were assessed using an unpaired Student’s \( t \) test.
Morphine withdrawal decreases NF-κB binding to response elements in LPS-treated CRL-2019 cells

As mentioned, morphine withdrawal decreased the message levels of the transcriptionally regulated IL-12 subunit p40. Therefore, the ability of morphine withdrawal to mediate transcription factor binding to response elements was assessed. Several transcription factors are involved in the transactivation of IL-12p40 including AP-1, C/EBP, PU.1, and NF-κB. All of these transcription factor interactions were measured following withdrawal periods of 4 and 8 h. Earlier time periods were used in an attempt to reveal what was occurring at the time of transcription of IL-12. Fig. 3A displays that morphine withdrawal greatly reduced NF-κB binding to response elements at both the 4- and 8-h withdrawal time points. Fig. 3B reveals that PU.1 binding is decreased following morphine withdrawal of both 4 and 8 h. In contrast, morphine withdrawal did not significantly alter binding of either AP-1 or C/EBP to their consensus oligonucleotide (data not shown). The results obtained for PU.1 binding were interesting given the findings that PU.1 acts as a repressor at the μ opioid receptor (MOR) gene promoter through a PU.1 binding site (17). Under the current circumstances, morphine withdrawal by decreasing binding of PU.1 should remove the repression exerted by PU.1 on the MOR gene and possibly lead to an increase in MOR expression. This possibility has not been explored, but this mechanism may serve as a cellular adaptation to morphine withdrawal in immune cells. In conclusion, these findings that morphine withdrawal decreases the ability of the key IL-12 transcription factors NF-κB and PU.1 to bind to DNA response elements.

Morphine withdrawal inhibits translocation of the NF-κB subunit p65 into the nuclear compartment of LPS-stimulated CRL-2019 macrophages

Given the findings that morphine withdrawal decreased binding of NF-κB to DNA response elements, the effect of withdrawal on translocation of NF-κB subunits into the nucleus was assessed. CRL-2019 cells were treated as described above and, after 24 h of in vitro withdrawal, cells were stimulated with LPS for 10, 30, and 60 min. Following the stimulation, the cells were lysed, nuclear protein was harvested and separated on SDS-PAGE gels, and finally probed with anti-p65 Abs as described. Fig. 4A demonstrates the effects of in vitro morphine withdrawal on nuclear translocation of the p65 NF-κB protein subunit. LPS treatment alone resulted in an increased translocation of the NF-κB p65 subunit into the nucleus. This increase was observed at all LPS treatment time points examined (10, 30, and 60 min) when compared with baseline activity. In contrast, in the morphine-withdrawal plus LPS groups, the expression of p65 was markedly reduced when compared with the matched vehicle-withdrawn LPS-treated cells. These results provide support for the idea that morphine withdrawal may be inhibiting the production of IL-12p40 by effecting the transcriptional regulation of this cytokine through a mechanism that involves NF-κB.

Morphine withdrawal modulates NF-κB inhibitory protein IκBα levels in the cytosolic compartment of LPS-stimulated CRL-2019 macrophages

Translocation of NF-κB to the nucleus is regulated by the inhibitory protein IκB and the associated IκB kinases (IKKs). When cells are in a stimulus-free environment, NF-κB is sequestered to the cytoplasm by IκB. Following stimulation, IKKs become activated and phosphorylate IκB, which triggers the ubiquitination and degradation of IκB and ultimately allows for the translocation of NF-κB to the nucleus where it transactivates NF-κB-responsive genes. Given the observations that the p65 subunit translocation into the nucleus is blunted following withdrawal, the levels of IκB in the cytoplasm were examined. It can be expected that if the NF-κB subunit expression is blunted in the nucleus there would be increased detection of IκB levels in the cytoplasm facilitating this blunted response. CRL-2019 cells were treated as described above and, after 24 h of in vitro withdrawal, cells were stimulated with LPS for 5, 10, and 20 min. Following the stimulation, the cells were lysed, whole cell lysates were prepared and separated on

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1 Abbreviations used in this paper: MOR, μ opioid receptor; IKK, IκB kinase; PKA, protein kinase A.
Given the results observed following in vitro morphine withdrawal with regard to NF-κB DNA response element binding and expression of subunits in the nuclear compartment, we next investigated the effects of withdrawal on IL-12p40 promoter activity. CRL-2019 macrophages were transfected as described above and underwent morphine withdrawal for 24 h followed by 4 h of LPS stimulation. Following treatment, cells were lysed and luciferase activity was measured as described previously. Standardized luciferase activity measurements were calculated and are indicative of IL-12p40 promoter activity. Fig. 5 demonstrates that in the vehicle-withdrawal plus LPS treatment group a significant increase in promoter activity was observed when compared with baseline activity (*, p < 0.001), whereas morphine withdrawal plus LPS treatment resulted in a significant decrease in LPS-induced IL-12p40 promoter activity when compared with vehicle treatment plus LPS group (**, p < 0.001). These results provide evidence that morphine withdrawal decreases the promoter activity of IL-12p40 following LPS stimulation and adds further support to the idea that withdrawal is acting to suppress IL-12p40 production by inhibiting transcriptional regulation presumably provided by NF-κB.

Overexpression of the p65 subunit of NF-κB partially reverses morphine-withdrawal-mediated decreases in IL-12p40 promoter activity

Given the results described with regard to the contribution of NF-κB to this system, the effects of the overexpression of the p65 subunit of NF-κB on IL-12p40 promoter activity were examined. CRL-2019 cells were treated and transfected as described above and Fig. 6 displays the results. As was seen in the previous figure, vehicle withdrawn plus LPS treatment alone significantly increased (#, p < 0.001) IL-12p40 promoter activity when compared with baseline activity. In contrast, morphine withdrawal plus LPS stimulation significantly reduced this activity when compared with cells treated with vehicle withdrawn plus LPS (*, p < 0.001). Overexpression of p65 enhanced IL-12p40 promoter activity as would be expected given that there are increased levels of this transcription factor which can also serve to transactivate the IL-12p40 promoter-luciferase reporter (***, p < 0.01). Vehicle withdrawn plus LPS.
MORPHINE WITHDRAWAL AND IL-12 SYNTHESIS

Cyclic AMP (cAMP) antagonists have been shown to play a role in the regulation of IL-12 synthesis in macrophages (27). It was of interest to determine whether the cAMP antagonist rescued morphine-induced inhibition of IL-12p40 promoter activity (Fig. 6). These results provide further support for the idea that morphine withdrawal impairs IL-12p40 production by impeding the transcriptional regulation of the IL-12p40 promoter by NF-κB.

Morphine withdrawal increases intracellular cAMP levels

cAMP has been shown to be elevated following periods of chronic morphine treatment or morphine withdrawal, a phenomenon known as adenylyl cyclase superactivation (28). The contribution of this phenomenon to morphine-withdrawal-mediated reductions in IL-12p40 seemed to be a logical path to examine given the combined observations that withdrawal increases cAMP, which can act to decrease IL-12 40 (8). To initially determine whether morphine withdrawal results in an increase in intracellular cAMP, CRL-2019 cells were treated with morphine and withdrawn as described above. Our results show a time-dependent increase in cAMP following morphine withdrawal (Fig. 7), with almost a 3-fold increase in cAMP levels seen at 30 min following withdrawal. We next compared intracellular cAMP levels following chronic morphine with that observed following morphine withdrawal. Interestingly, the increase in cAMP following withdrawal was almost 3-fold greater when compared with the vehicle treatment plus withdrawal group and 2-fold greater than the chronic morphine treatment group (Fig. 7B).

To expand on these findings and to provide further evidence that it is the ability of morphine withdrawal to elevate cAMP, which is the ultimate causative factor in impairing IL-12p40 production, the effect of the cAMP inhibitor Rp cAMP was investigated on morphine-withdrawal-induced decrease in IL-12 synthesis. CRL-2019 cells were pretreated with varying doses of Rp cAMP at the time of withdrawal for 1 h and then stimulated with LPS for 24 h. IL-12p40 was measured as described above. Data shows that the cAMP antagonist rescued morphine-induced inhibition of IL-12 synthesis in a concentration-dependent manner, providing strong support for our hypothesis that morphine withdrawal modulates IL-12 synthesis through a mechanism that involves cAMP (Fig. 7C).

The protein kinase A (PKA) inhibitor H-89 reverses morphine withdrawal-mediated decreases in IL-12p40 promoter activity

To further examine the signaling mechanisms involved in the observed reductions in IL-12p40 as a result of morphine withdrawal, the effects of several protein kinase inhibitors on promoter activity were tested. Specifically, both H-89 (PKA inhibitor, 1 μM) and SB203580 (p38 MAPK inhibitor, 1 μM) were examined by treating the CRL-2019 cells with the particular inhibitor at the stated concentration for 1 h immediately after withdrawal treatment. Fig. 8A demonstrates the results, which once again displays that LPS significantly increases promoter activity, whereas morphine withdrawal significantly reduces the activity as compared with LPS treatment alone. Pretreatment with the PKA inhibitor H-89 was able to reverse morphine withdrawal-induced reduction in IL-12 promoter activity, demonstrated by the nonsignificant difference between LPS alone and morphine-withdrawal plus LPS plus H-89 treatment groups. Finally, the p38 MAPK inhibitor

stimulation of cells that overexpressed p65 only marginally increased promoter activity over NF-κB p65 expression alone, indicating that a ceiling in promoter activity may have been reached.

FIGURE 7. Role of cAMP in morphine-withdrawal-mediated effects on IL-12 40 promoter activity. A, Effect of morphine withdrawal on intracellular cAMP in CRL-2019 macrophages. B, Comparison of cAMP levels in morphine-withdrawn cells to cells chronically treated with morphine. C, Effect of the cAMP antagonist Rp cAMP on morphine-withdrawal-mediated decreases in IL-12p40 protein synthesis. A, CRL-2019 macrophages were subjected to morphine withdrawal as described above. At 5, 10, and 30 min following withdrawal, cells were washed in PBS and spun and resuspended at a final density of 1 × 10⁷ cells/ml and lysed in cell lysis buffer. The supernatant of lysed cells was analyzed using cAMP assay kits (**, p < 0.01). B, CRL-2019 macrophages were subjected to either morphine withdrawal or chronically treated with morphine for 24 h. At 30 min following morphine withdrawal or 24 h following morphine treatment, cells were washed in PBS and spun and resuspended at a final density of 1 × 10⁷ cells/ml and lysed in cell lysis buffer. The supernatant of lysed cells was analyzed using cAMP assay kits (*, p < 0.01). C, CRL-2019 macrophages were subjected to either vehicle withdrawal or morphine withdrawal and immediately treated with varying concentrations of Rp cAMP (10–100 μM) for 1 h. Cells were then stimulated with LPS (5 μg/ml) for 24 h. Culture supernatants were collected and IL-12p40 protein levels were determined using ELISA. Each treatment group was tested in triplicate and results are the mean of three independent experiments. Comparisons between groups were assessed using an unpaired Student’s t test.

Overexpression of p65-NF-κB in cells that were morphine withdrawn plus LPS- treated partially but significantly rescued morphine withdrawal-induced inhibition of IL-12p40 promoter activity (***, p < 0.01). These results indicate that a more robust effect may not have been observed given the idea that morphine withdrawal is preventing the degradation of IκB (Fig. 4B) and therefore sequestering NF-κB p50, a necessary binding partner for p65 that is required for appropriate promoter activation. Overexpression of both p65 and p50 significantly and completely rescued morphine withdrawal-induced inhibition of IL-12p40 promoter activity (Fig. 6). These results provide further support for the idea that morphine withdrawal impairs IL-12p40 production by impeding the transcriptional regulation of the IL-12p40 promoter by NF-κB.
with 5 macrophages were cultured and treated as described above and stimulated. Blots were probed with anti-rabbit secondary Ab and visualized using ECL detection reagents. A rabbit anti-mouse primary mAb, followed by incubation with a goat antibody, was used to detect IkB-α. Blots were probed with the anti-actin primary Ab to ensure for equivalence of protein levels.

**FIGURE 8.** The PKA inhibitor H-89 reverses morphine-withdrawal-mediated decreases in IL-12p40 promoter activity (A), returns nuclear expression of p65 (B), and restores the degradation of IkB-α from LPS-stimulated CRL-2019 macrophages (C). A, CRL-2019 macrophages were cultured, transfected, and subjected to withdrawal as described above. Immediately after withdrawal, cells were treated with either the PKA inhibitor H-89 (1 μM) or SB203580 (p38 MAPK inhibitor, 1 μM) for 1 h and then stimulated for 4 h with 5 μg/ml LPS. Data are presented as standardized luciferase activity, which are values that were calculated by the ratio between firefly and Renilla luciferase and represents IL-12p40 promoter activation. Each treatment group was tested in triplicate and results are mean ± SEM of three independent experiments (*, p < 0.01). CRL-2019 macrophages were cultured and treated as described above and stimulated with 5 μg/ml LPS for 30 min. Nuclear extracts (B) or whole cell lysates (C) were prepared and separated by SDS-PAGE. Blots were then probed with a rabbit anti-mouse primary mAb, followed by incubation with a goat anti-rabbit secondary Ab and visualized using ECL detection reagents. Blots were probed with anti-β-actin primary Ab to ensure for equivalence of protein levels.

(SB203580) had no effect as evidenced by the observation that there still was a significant difference between the vehicle-withdrawal plus LPS group compared with the morphine-withdrawal plus LPS group that was pretreated with SB203580 cells. In addition, it is also important to note that these compounds alone had no effect on IL-12p40 promoter activity (data not shown). Taken together, these results suggest that morphine withdrawal is able to increase cAMP which acts as a second messenger that signals through PKA to reduce IL-12p40 promoter activity and ultimately results in a decrease in IL-12p40 cytokine levels.

Given all of the results described thus far, it seemed appropriate to examine the effects of the PKA inhibitor on the expression of the p65 subunit of NF-κB. Appropriate to the studies described within, reports have demonstrated that both PKA and cAMP inhibit the NF-κB pathway (29, 30). Figs. 3A and 4A demonstrated that morphine withdrawal decreased DNA binding and nuclear compartment expression levels of NF-κB, respectively; therefore, the effects of the PKA inhibitor on p65 expression were examined. CRL-2019 cells were treated as described above and, after 24 h of in vitro withdrawal, cells were stimulated with LPS for 30 min. Fig. 8B demonstrates that treatment of the cells with H-89 (1 μM) at the time of withdrawal restores expression in the morphine-withdrawal group to levels that were observed in LPS treatment alone. In addition, as Fig. 8C demonstrates, H-89 also prevents the morphine-withdrawal-mediated decrease in IkB-α degradation, as evidenced by greater degradation of IkB-α in cultures treated with H-89 to levels that are similar to those observed following LPS treatment alone.

These results suggest that PKA is involved in the morphine withdrawal-mediated signaling pathway that results in the reduction in IL-12p40 production. Taken together, it can be concluded that morphine withdrawal results in increased concentrations of cAMP, which then activates PKA and inhibits NF-κB signaling, ultimately leading to a decrease in IL-12p40 cytokine production (Fig. 9).

**FIGURE 9.** Proposed signaling mechanism by which morphine withdrawal suppresses IL-12p40 production.

**Discussion**

Given our laboratory’s previous findings that morphine withdrawal inhibits IL-12 production, it was the goal of these studies to examine the mechanism by which withdrawal affects the generation of IL-12. Initially, a paradigm in which to study morphine withdrawal in vitro had to be established to closely examine signaling mechanisms that may be involved in this process. The in vitro paradigm closely replicates conditions that are present in vivo by...
maintaining the same treatment time course and using plasma concentrations of morphine that were observed following in vivo manipulations, i.e., surgical s.c. implantation of 75 mg of morphine pellets. This treatment paradigm referred to throughout as in vitro morphine withdrawal was a successful recapitulation of in vivo conditions, given the evidence that withdrawal decreased both IL-12p70 and p40 cytokine levels when compared with vehicle-withdrawal plus LPS treatment. This model was further extended to the mouse alveolar macrophage cell line CRL-2019 due to the fact that transfection experiments needed to be performed and this cell line provided an accessible and efficient transfection model. Once again, in vitro morphine withdrawal decreased both the protein and message levels of IL-12p40 as compared with vehicle-withdrawal plus LPS treatment. Taken together, these results indicate that the in vitro model developed was adequate and replicated what had been observed in vivo.

Using this model, we show that transcription of IL-12p40 was significantly reduced following withdrawal and that this reduction correlated with decreased binding of both NF-kB and PU.1 to consensus oligonucleotide sequences. Furthermore, we also show decreased translocation of the active form of NF-kB, a p65 subunit into the nucleus of cells that are morphine withdrawn. Interestingly, this decrease in p65 translocation in the morphine-withdrawal treatment group was accompanied by a concurrent increase in cytosolic levels of IκBα, suggesting that withdrawal may be inhibiting the degradation of IκBα (Fig. 4B). Hindering degradation of IκBα would therefore prevent the dissociation between IκBα and p65 and would therefore sequester p65 in the cytoplasmic compartment. This hypothesis was validated since overexpression of p65 only partially rescued morphine-withdrawal-induced inhibition of IL-12 promoter activity; however, overexpression of both p50 and p65 completely reversed the observed inhibition by morphine withdrawal. The idea that morphine withdrawal is hindering the degradation of IκBα and ultimately resulting in a decrease in IL-12p40 production is a worthwhile explanation given the observations that both vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide act via a similar mechanism to reduce IL-12p40 production (31). These authors demonstrated that treatment of IFN-γ-primed and LPS-stimulated RAW 264.7 macrophages with these compounds resulted in decreased IκBα degradation accompanied by decreased expression levels of p65 in the nuclear compartment. It was concluded that this may be one of the mechanisms by which these compounds act to decrease IL-12p40 production.

Given the expression pattern results for NF-kB and IκBα following morphine withdrawal, we sought to determine the mechanism by which morphine withdrawal is effecting these alterations. We show that, following both chronic morphine treatment and morphine withdrawal, there is an increase in the second messenger cAMP (32). This phenomenon known as adenyl cyclase super-activation is well characterized in the CNS, and it is speculated that it is a process that works to counteract the effects of acute morphine treatment and is also considered the cellular hallmark of morphine tolerance (28). Furthermore, this process has been demonstrated to occur in cells of the immune system following chronic morphine treatment (32). We further show that Rp cAMP, a cAMP antagonist, significantly reversed morphine-induced inhibition in a concentration-dependent manner. It is also well documented that cAMP and cAMP-elevating agents are known to decrease the production of IL-12 (11, 19, 27). Taken together, it can be speculated that it is the ability of morphine withdrawal to increase the intracellular concentrations of cAMP, which ultimately decreases IL-12 production possibly via a NF-kB pathway. It can be concluded that the cAMP-modulating effects of morphine withdrawal may be decreasing IL-12p40 promoter activity and ultimately resulting in decreased cytokine levels of IL-12p40.

Next, we sought to link the effects morphine withdrawal has on cAMP with what was observed with regard to the NF-kB pathway. Previous studies have demonstrated that cAMP is a potent inhibitor of the NF-kB pathway by blocking the phosphorylation and subsequent degradation of IκB and that PKA is a downstream effector in this process (16). In addition, it has also been demonstrated that increases in cAMP activate PKA which decreases the transcriptional potential of the p65 subunit of NF-kB. Interestingly, however, these studies demonstrated that PKA did not affect IκBα phosphorylation, degradation, nor NF-kB/DNA binding (26). Given these previous findings, the role of PKA in morphine-withdrawal-mediated IL-12p40 suppression was investigated. It was demonstrated that the PKA inhibitor H-89 pretreatment before withdrawal was able to restore both IL-12p40 promoter activity, as well as p65 translocation into the nucleus. Furthermore, it was demonstrated that H-89 was able to prevent the decrease in IκBα degradation, as evidenced by a return of expression levels of IκBα similar to those observed following LPS treatment alone. The mechanism by which PKA is acting is yet unknown but it can be speculated that there are several possible mechanisms. First, PKA may be activating a phosphatase which prevents the phosphorylation and hence degradation of IκBα. Second, PKA may alter the activity of IKKs, the kinase which phosphorylates IκBα. Finally, PKA may interrupt the ubiquitination of IκBα, which is a crucial signal ultimately resulting in the degradation of the protein. Taken together, it can be concluded that morphine withdrawal acts to increase cAMP, which activates PKA, thus activating a yet unknown protein or series of proteins which ultimately prevents the degradation of IκBα. The increased stabilization of the IκBα/NF-kB-p65 complex prevents nuclear translocation of p65 and thus decreases the transactivation of IL-12p40 and ultimately hinders the production of IL-12p40 following LPS stimulation (Fig. 9).

In conclusion, it was the goal of these studies to understand the molecular mechanisms by which morphine withdrawal decreases IL-12 production. It was demonstrated using an in vitro model of withdrawal that the increases in cAMP generated following withdrawal may be acting through PKA to inhibit the NF-kB pathway and thus IL-12p40 production. Understanding the mechanisms by which morphine withdrawal brings about immune system failure is a worthwhile endeavor given the wide use and abuse of morphine.

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References


