Transcriptional and Epigenetic Regulation of Interleukin-2 Gene in Activated T Cells by Morphine*

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Chronic morphine inhibits interleukin-2 (IL-2) at both the transcriptional and protein synthesis levels. The molecular mechanisms by which morphine decreases IL-2 are not fully understood. The production of IL-2 is tightly regulated by several transcription factors that bind to the IL-2 promoter. Herein, we show that chronic morphine treatment results in an increase in cAMP levels with a concurrent up-regulation of the cAMP inducible repressor inducible cAMP early repressor (ICER)/cAMP response element modulator (CREM) and down-regulation of p-cAMP-response element-binding protein (CREB) in activated T cells. Furthermore, ICER competes for p-CREB binding to the cAMP-responsive elements (CREs) site. This leads to the uncoupling of CBP/p300 thereby abrogating IL-2 transcription. Overexpression of either antisense CREM or CREB plasmid rescued morphine-induced inhibition of IL-2 promoter activity and protein production. In addition, we also found that chronic morphine treatment inhibited the acetylation and trimethylation of histones and decreased both DNA demethylation and accessibility of the IL-2 promoter. These findings suggest that chronic morphine treatment may function through both transcriptional and epigenetic mechanisms to inhibit IL-2 production.

Transcriptional and epigenetic mechanisms of IL-2 gene repression by morphine include down-regulation of CREB binding protein and CREM isoforms to abrogate IL-2 promoter activity and protein synthesis. In vitro, chronic morphine exposure increases cAMP levels and down-regulates CREB activity, which is necessary for full IL-2 production. In vivo, chronic morphine exposure leads to similar biological effects. This study supports a role for CREM and CREB in the regulation of IL-2 gene expression and provides new insights into the mechanisms by which morphine inhibits IL-2 production.

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‡The abbreviations used are: IL-2, interleukin-2; CRE, cAMP-response element; CREB, cAMP-response element-binding protein; CREM, cAMP response element modulator; ICER, inducible cAMP early repressor; CBP, CREB binding protein; MOR, μ-opioid receptor knockout; WT, wild type; Chip, chromatin immunoprecipitation analysis; Ab, antibody; CD28RE, CD28-responsive element; RT, reverse transcriptase.
quent increase in intracellular cAMP (20, 21). It is well-established that cAMP signaling is inhibitory to T cell proliferation and effector functions. In particular, cAMP has been shown to inhibit the expression of T helper-1 cytokine genes (22). Transcriptional regulation coupled to the cAMP-dependent signal transduction pathways is mediated through a related family of DNA-binding proteins comprising the CREB, CREM (23). The CREM gene contains two alternatively utilized promoters, P1 and P2. The internal P2 promoter is activated by cAMP signaling to produce mRNAs encoding the ICER repressor isoforms, whereas the P1 promoter that encodes CREM isoforms is unresponsive to cAMP. These proteins encoded by CREM gene act as both activators and repressors of cAMP-mediated gene transcription. ICER is the most potent repressor (24). The ICER protein consists mainly of the basic leucine zipper domain and, therefore, represses transcription either by heterodimerization with activating forms of CREB/CREM/ATF or other basic leucine zipper-containing transcription factors or by competition with these proteins for DNA binding (25). ICER induced by cAMP is a typical early response gene, with maximal expression levels being attained within 3–6 h of stimulation. In turn, ICER inhibits both its own transcription and the expression of other CRE-containing promoters (26). Induced expression of ICER through the cAMP signaling pathway has been implicated in several responses, including the suppression of T lymphocyte function, attenuation of Fas ligand expression in T and NK lymphocytes, and glucagon-mediated suppression of insulin gene expression in pancreatic B cell (27–29). In this study, we show for the first time that morphine produces a dramatic increase in ICER expression in both naïve and activated T cells. Morphine-induced ICER competes with CREB in the context of CD28RE motif of IL-2 promoter. Since ICER lacks a transactivating domain, it fails to recruit CBP and p300. The CBP/p300 proteins, in addition to acting as co-activators, can also modulate histone acetylation, promote DNA demethylation, and increase accessibility to transcription factors. Since ICER expression fails to recruit CBP/p300 proteins, its expression results in a failure to maintain a transcriptionally competent conformation of chromatin state thus inhibiting activation of IL-2 transcription. These studies, therefore, indicate the existence of a potentially novel pathway for morphine-mediated suppression of IL-2 gene transcription.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals—**μ-Opioid receptor knock-out (MORKO) mice (129S × C57BL/6 genetic background) were produced as described previously by Loh and co-workers (30). Wild type (WT) mice (B6129SF2) were obtained from Jackson Laboratory. A maximum of four mice were housed per cage. Laboratory. A maximum of four mice were housed per cage. Wild type (WT) mice (B6129SF2) were obtained from Jackson produced as described previously by Loh and co-workers (30). (10 nM to 10

**Chromatin Immunoprecipitation (ChIP) Analysis—**Binding of transcription factor CREM and CREB to the IL-2 promoter was investigated using the ChIP assay according to the protocol of Upstate Biotechnology, Inc. (Lake Placid, NY). Briefly, T lymphocytes were treated with 1% formaldehyde in tissue culture medium for 10 min at 37 °C. Cell samples were washed twice in ice-cold phosphate-buffered saline containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Cells were pelleted and lysed in SDS lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, supplemented with protease inhibitor mixture). Samples were sonicated to shear DNA to lengths between 200 and 1000 bp. Subsequently, an aliquot of sheared DNA was analyzed by agarose gel electrophoresis to confirm sheared DNA length and standardize protein-DNA complex input for immunoprecipitation. The chromatin samples were incubated overnight at 4 °C with rabbit antibody directed to CREM and p-CREB, CBP, p300, acetyl-histone H3, acetyl-histone H4 (Upstate Biotechnology), and trimethyl-histone H3 (lys4) (Abcam). Immune complexes were precipitated with salmon sperm DNA-bovine serum albumin-Sepharose beads. DNA was prepared by treatment with DNaise- and RNase-free proteinase K and extraction with a QiaAmp DNA extraction kit. The following primer was used for PCR analysis of immunoprecipitated DNA as well as input samples: 5′-CTGTTGGCAGAGCATATCACCT-3′ (forward) and 5′-CCTAAATCCATTAGTCTATTGAC-3′ (reverse). This primer amplified a 93-bp region.
including the −180 and −160 site within −198 to −107 of IL-2 promoter.

Western Blot—T lymphocytes were treated with morphine or vehicle for 24 h before stimulation with anti-CD3 and anti-CD28. Stimulation with anti-CD3 and anti-CD28 lasted for 2 h. The cells were then washed with phosphate-buffered saline and lysed in lysis buffer. After one freeze/thaw cycle, lysates were centrifuged. Protein concentration was determined by a Bradford assay (Bio-Rad) using bovine serum albumin as the standard. A quantity amounting to 5 µg of protein sample was subjected to SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to an ECL nitrocellulose membrane (Amersham Biosciences) by electroblotting. Incubating the membrane in Superblock (Pierce) for 1 h blocked nonspecific binding. Membranes were then incubated overnight at 4 °C in primary Ab, CREM, CREB, and p-CREB. The blots were washed three times with TBST buffer and then incubated for 1 h at room temperature with anti-rabbit secondary Ab conjugated with horseradish peroxidase. Western blot analysis was conducted according to standard procedures using Supersignal chemiluminescence detection substrate (Pierce).

Enzyme-linked Immunosorbent Assay—T lymphocytes from each mouse were adjusted to a final concentration of 2 × 10⁶ cells/ml in 24-well plates and stimulated with anti-CD3 (10 µg/ml) and anti-CD28 (2.5 µg/ml) and a rabbit anti-mouse cross-linking Ab (25 µg/ml). The microtiter plates were then incubated for 24 h at 37 °C in a humidified 5% CO₂ incubator. Culture supernatant was analyzed using cytokine-specific enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Quantification of ICER mRNA Transcripts by Reverse Transcriptase-PCR and Real-time PCR—ICER gene expression in T cells treated with morphine or vehicle was examined by RT and real-time PCR. The RT-PCR was conducted as described previously (20). Real-time, SYBR Green PCR analysis was performed for ICER mRNA and 18 S rRNA, and relative quantification was performed using the ABI PRISM 7500 sequence detection system (Applied Biosystems). ICER transcript levels were normalized to 18 S rRNA transcript levels from the same preparations of cDNA.

Methylation-sensitive PCR Assay—Methylation-sensitive PCR assay was performed by using the promoter methylation PCR kit (Panomics, Redwood City, CA) following the manufacturer’s instructions (31). Briefly, genomic DNA from T cells was prepared using the DNeasy tissue kit (Qiagen). Genomic DNA was digested with MseI (New England Biolabs). The methylated DNA fragments were isolated by a spin column affinity purification using MeCP2 and were amplified by PCR for subsequent visualization by agarose gel electrophoresis. The following primer was used for PCR analysis of methylated DNA: 5′-CCAGAGAGTCATCAGAAGGGGA-3′ (forward) and 5′-AATAATATGCGGGTGTCACGA-3′ (reverse). This primer amplified a 109-bp region including the −68 CpG islands within −157 to −49 of IL-2 promoter.

DNA Accessibility Assay—T cells were either treated with various concentrations of morphine or vehicle. Twenty-four hours after morphine treatment, T cells were stimulated with CD3 and CD28 for 18 h. T cells were harvested, resuspended in ice-cold PBS, and lysed in buffer A (10 mM HEPES-KOH, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) with addition of Nonidet P-40. Nuclear pellets were harvested and washed in restriction enzyme buffer. Digestion was conducted at suggested temperatures with Dral for 1 h. DNA was extracted using a QIAMP DNA micro kit and amplified with primers flanking the IL-2 promoter that contained the digestion sites.

Statistical Analysis—Results were expressed as mean ± S.E. They were analyzed statistically by the unpaired t test between two groups and analysis of variance among multiple groups. Statistical significance was accepted at p < 0.05.

RESULTS

Chronic Morphine Treatment Induced Transcriptional Activation of IL-2 Promoter and IL-2 Protein Production—Prior studies have shown that IL-2 secretion and IL-2 mRNA expression can be inhibited in antigen-stimulated T cells by chronic treatment with morphine (10). The mechanism for this mode of repression has never been defined, although previous work has established that c-fos and AP-1 was selectively inactivated in morphine-treated thymocytes (32). Primary T cells respond to molecular signals that are initiated by the ligation of the T cell receptor and the CD28 class of co-receptors on its cell surface. These cell surface-initiated signaling events can be mimicked in vitro in primary T cells by the addition of anti-CD3 in the presence of antibodies that cross-link the CD28 surface receptor (33). Stimulation of primary T cells with anti-CD3 and anti-CD28 leads to a significant increase in IL-2 gene activation and secretion as determined by transcriptional reporter assays and IL-2 enzyme-linked immunosorbent analysis, respectively. We initially investigated whether morphine treatment modulated IL-2 protein synthesis and gene transcription following activation of T cells with anti-CD3/CD28. As shown in Fig. 1, A and B, morphine treatment inhibited both IL-2 promoter activity and IL-2 protein synthesis in activated T cells derived from WT mice in a dose-dependent manner. The effects of morphine were completely abolished in T cells derived from MORKO mice, suggesting that the effect of morphine is mediated through the μ-opioid receptor.

Evaluation of the Effect of Exposure to Morphine on Intracellular cAMP Level—To address the possibility that the elevations of cAMP in morphine treated T lymphocytes induced the inhibition of IL-2 transcription, the effect of morphine on intracellular cAMP levels were examined. T lymphocytes were treated with morphine, the adenylyl cyclase agonist forskolin, or morphine plus forskolin for 24 h, then cAMP was tested in naïve and 24-h anti-CD3/CD28 activated T cells. Morphine treatment resulted in detectable elevation of the cAMP levels in naïve T cells and markedly stimulated cAMP levels in activated T cells derived from WT mice. Morphine treatment also synergized with forskolin to result in a significant increase in intracellular cAMP (Fig. 1C).

Chronic Morphine Treatment Induced ICER Expression in Both Naïve and Activated T Cells—We next investigated whether morphine-induced elevation in cAMP levels results in the induction of ICER expression in both naïve and activated T lymphocytes derived from WT mice. In untreated naïve T lymphocytes, ICER mRNA levels were undetectable. Treatment of
naïve T lymphocytes with morphine for 24 h resulted in a modest increase in levels of ICER I-specific mRNA signal. However, morphine treatment in activated T lymphocytes induced both stronger ICER I and II specific mRNA signals (Fig. 2, A and B). Morphine treatment resulted in a parallel increase in ICER protein levels, which migrated at the expected band (13.5 kDa) (Fig. 2C; Ref. 34). In contrast, morphine treatment of activated T cells resulted in a significant decrease in phospho-CREB protein levels. Activation of T cells in the absence of morphine, however, resulted in time dependent increase in the phospho-CREB. Total CREB, however, was not altered following morphine treatment (Fig. 2D).

Chronic Morphine Treatment Inhibited p-CREB and Enhanced CREM to Bind to the IL-2 Promoter in Activated T Cells—Because ICER/CREM was up-regulated and p-CREB was down-regulated by morphine in stimulated T cells, we wanted to determine whether morphine modulated the recruitment of these transcriptional factors to the IL-2 promoter in activated T cells. T cells derived from WT mice were treated with morphine (10 nM or 1 μM) for 24 h, then stimulated with or without anti-CD3 (10 μg/ml) and anti-CD28 (2.5 μg/ml) for 6 h. Total RNA was subjected to RT-PCR using primers for ICER1/II, CREM II, and β2-microglobulin or conducted to real-time PCR using primers for ICER/I, ICER II, and 18 S rRNA. C. Western blot analysis of proteins from whole cell extracts of T cells treated with morphine or vehicle and stimulated with or without anti-CD3 (10 μg/ml) and anti-CD28 (2.5 μg/ml) for 24 h. The position of the 13.5-kDa ICER protein is shown. D. morphine treatment inhibited CREM phosphorylation. All the results are representative of at least three independent experiments. **, significant at level p < 0.01 compared with the vehicle treatment group.
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**FIGURE 3.** Chronic morphine treatment decreased recruitment of p-CREB and increased recruitment of CREM to the IL-2 promoter and decreased the binding of CBP/p300 in activated T cells derived from WT mice. Overexpression of CREB and antisense CREM rescued the inhibitory effect of morphine on IL-2 promoter activity and protein production. A and B, ChIP assays were performed using cross-linked DNA from T cells treated with morphine or vehicle and stimulated with CD3 and CD28 Abs. T cells were fixed at 6 h after stimulation. The DNA-protein complexes were immunoprecipitated with anti-p-CREB, anti-CREM, anti-p300, and anti-CBP. The DNA was purified and amplified with primers including the −180 and −160 site within −198 to −107 of IL-2 promoter and visualized in 1.5% agarose gel. C and D, T cells derived from WT mice treated with morphine (1 μM) or vehicle were transiently co-transfected with IL-2 firefly luciferase reporter plasmid (10 μg) and 5 μg of transcriptional factor plasmids (CREB, antisense-CREM, NF-κB p65, NFAcT1) by electroporation. Anti-CD3 and CD28 stimulation followed as described under “Experimental Procedures.” C, lysates were harvested at 6 h and assayed for firefly and Renilla luciferase activity using a dual luciferase reporter assay system with a luminometer. Western blot is shown as an insert to confirm the overexpression of CREB and knockdown of CREM in transfected cells. Lane 1, transfected with 10 μg of CREB or antisense CREM plasmid; lane 2, transfected with 5 μg of CREB or anti-antisense CREM plasmid DNA; lane 3, transfected with 1 μg of CREB or antisense CREM plasmid; lane 4, transfected with 5 μg of vector plasmid DNA. D, after transfection, T lymphocytes were stimulated with anti-CD3 (10 μg/ml) and anti-CD28 antibodies (2.5 μg/ml) and a rabbit anti-mouse cross-linking Ab (25 μg/ml) for 24 h. Culture supernatant was harvested and analyzed using cytokine-specific enzyme-linked immunosorbent assay kits. The data are presented as mean ± S.E. for three independent experiments. *,** Significantly different from the vehicle treatment group.

The recruitment of p-CREB to the IL-2 promoter but promoted the recruitment of CREM to the IL-2 promoter (Fig. 3A). Although the CREM antibody recognizes all CREM isoforms, however, since only the ICER isoform is inducible by cAMP, it is very likely that the CREM antibody used for immunoprecipitation in the ChIP assay corresponds to ICER.

Overexpression of CREB and Antisense CREM Achieved by Transfection of CREB and Antisense CREM-encoding cDNAs in Activated T Cells Abrogates the Morphine-induced Inhibition of IL-2 Transcription and Production—To further evaluate whether CREB and ICER/CREM were involved in the suppression of IL-2 transcription and IL-2 protein production, we transfected activated T cells derived from WT mice with either a CREB overexpression plasmid or a CREM antisense plasmid. The empty vector containing plasmids served as controls. Transfection of T cells with either the CREB overexpression plasmid or the CREM antisense plasmid resulted in enhanced transcriptional activity of IL-2 promoter and significant increase in IL-2 protein production. Overexpression of either CREB or antisense CREM/ICER significantly rescued morphine-induced inhibition of IL-2 promoter activity (Fig. 3C) and protein production (Fig. 3D). In contrast overexpression of NFAT and NF-κB p65 did not rescue the effect of morphine (Fig. 3, C and D). Overexpression of CREB and CREM knockdown was confirmed following transfection using Western blot analysis (Fig. 3C).

**Chronic Morphine Treatment Disrupted Recruitment of the CBP and p300 to the IL-2 Promoter**—To further evaluate that the ICER isoform of CREM was associated with the −180 CRE site of IL-2 promoter, we investigated whether recruitment of CBP and p300 to the IL-2 promoter was disrupted following morphine treatment. Chronic morphine treatment resulted in a dose-dependent decrease in the binding of CBP and p300 to the IL-2 promoter (Fig. 3B). These studies suggest that chronic morphine treatment enhances binding of ICER to the CRE. Since ICER lacks a transactivating domain, binding of ICER to the CRE leads to the uncoupling of CBP or its homologue, p300 (CBP/ p300), abrogating early stages of transcriptional initiation of IL-2 promoter in activated T cells derived from WT mice.

**Morphine Treatment Decreased the Binding of Acetylated and Methy-lated Histones, thus Reducing Accessibility of IL-2 Promoter in Activated T Cells**—Treatment of T cells with morphine has been shown to inhibit CD3 and CD28 activation of IL-2 gene transcription, which occur through the alteration of specific transcription factors, ICER and CREM. ICER and CREM, in turn, may function to recruit chromatin remodeling complexes. Since chromatin remodeling has been shown to be dependent on the modification of histone proteins by acetylation followed by trimethylation at K4 sites, we next examined the effect of morphine treatment on histone acetylation and trimethylation. Our results show that morphine treatment decreased binding of acetylated H3 and H4 and reduced binding of trimethylated H3K4 to the IL-2 promoter in T cell when compared with vehicle treated activated T cells (Fig. 4, A and B). Since histone acetylation of chromatin modulates the accessibility of a gene, we next investigated whether morphine treatment will inhibit the accessibility of the IL-2 gene to restriction endonucleases. T cells were treated with morphine and stimulated with anti-CD3 and CD28 for 24 h. Nuclei were prepared from these samples and incubated with restriction enzymes. As shown Fig. 4C, CD3 and CD28 leads to an increase in Drl
induced by CD3 and CD28 within the IL-2 promoter. Treatment prevented the demethylation of CpG dinucleotides; therefore, we next tested whether morphine gene expression is methylation of genomic DNA at CpG dinucleotides; therefore, we next tested whether morphine treatment decreased histone acetylation and methylation induced by CD3 and CD28. T cells were treated with morphine (10 nM or 1 μM) or vehicle for 24 h and stimulated for 18 h with CD3 and CD28 Abs, and then a ChIP assay was performed using anti-acetylated H3, anti-acetylated H4, and anti-trimethylated H3K4. The extracted DNA was amplified with primers flanking −198 to −107 the CRE site of the IL-2 promoter by RT-PCR, and the products were electrophoresed on agarose gels. C, chronic morphine treatment inhibited restriction enzyme accessibility of the IL-2 gene. Intact nuclei isolated from T cells treated with morphine or vehicle and stimulated with anti-CD3 and anti-CD28 were incubated with or without 100 units of DraI for 60 min at 37 °C. One-hundred nanograms of genomic DNA was subjected to PCR analysis with the primers flanking −198 to −107 the CRE site of the IL-2 promoter. The DraI accessibility was expressed as a percentage of uncut DNA and plotted for each treatment. D, morphine treatment inhibited the DNA demethylation induced by CD3 and CD28. At 48 h after CD3 and CD28 stimulation, T cells treated with morphine or vehicle were collected, and genomic DNA was purified. The methylation status of −68 CpG sites was determined by methylation-sensitive PCR assay. **, significant at level p < 0.01 compared with the vehicle treatment group. The data presented are representative of at least three experiments.

accessibility for the primer set spanning the −180 and −160 site within −198 to −107 of IL-2 promoter. Morphine treatment of activated T cells significantly inhibited accessibility of IL-2 promoter to DraI (20–50% compared with morphine-un treated T cells) (Fig. 4C).

Morphine Treatment Decreased the Demethylation of CpG Dinucleotides Induced by CD3 and CD28 within the IL-2 Promoter—Another epigenetic modulation that can affect IL-2 gene expression is methylation of genomic DNA at CpG dinucleotides; therefore, we next tested whether morphine might influence the level of DNA methylation at the IL-2 locus. We examined the DNA methylation status of the CpG island flanking the −68 region within the IL-2 promoter using methylation-sensitive PCR assay. As shown in Fig. 4D, morphine treatment prevented the demethylation of CpG dinucleotides induced by CD3 and CD28 within the IL-2 promoter.

DISCUSSION

Both innate and adaptive immune function requires careful regulation. In most cases, regulation of immune response is mediated by cytokines. The majority of reports, including our previous studies, indicate that morphine treatment inhibits T helper 1 (Th1) immune responses, usually starting with the inhibition of IL-2 (35–37). The mechanism by which morphine down-regulates IL-2 expression remains to be established. Understanding the precise mechanisms by which morphine modulates IL-2 expression may lead to several important paradigms with relevant application to the developing of new approaches to the treatment of the devastating infection caused by drug abuse. Here we are first to demonstrate that ICER/CREM is a critical transcriptional factor for the negative regulation of IL-2 transcription following morphine treatment in CD3- and CD28-activated T cells.

Intracellular cAMP accumulation induced by chronic morphine treatment has been reported in certain types of cells, including immune cells (20, 21). Consistent with the previous findings, we found that intracellular cAMP levels were elevated following chronic morphine treatment in both naive and activated T cells. Moreover, we also found that the cAMP-mediated inhibition of IL-2 transcription induced by chronic morphine treatment correlated with ICER expression in both naive and activated T cells. ICER is produced from the CREM gene using an alternative promoter located internally and the translated protein product structurally contains only the leucine zipper domain. ICER heterodimerizes with the CREB/ATF-1 family and the AP-1 family of transcription factors and represses their activity (38–40). Previous findings indicated that the CD28-responsive element (CD28RE), a composite DNA binding element consisting of NFAT and CRE-like motifs in position −180 of IL-2 promoter has a high affinity for ICER binding as well as NFAT/ICER complexes, suggesting that ICER directly mediates transcriptional attenuation of IL-2 expression (20). In addition, a direct role of ICER in cAMP-mediated inhibition of cytokine and chemokine gene expression has been demonstrated in transgenic mice expressing ICER under the control of the lymphocyte-specific proximal lck promoter. On stimulation, transgenic thymocytes overexpressing ICER exhibited reduced levels of IL-2 and IFN-γ and failed to express MIP-1α and MIP-1β. Moreover, splenic T cells from ICER transgenic mice showed a defect in proliferation and lacked a mixed lymphocyte reaction response, suggesting that ICER-mediated transcriptional attenuation of cytokine and chemokine gene expression may compromise the T cell response (41). As we have shown here, chronic morphine treatment induced high levels of cAMP and with concurrent increase in ICER mRNA expression in both naive and activated T cells. Western immunoblot analysis confirmed that in these conditions the ICER mRNA is translated efficiently into ICER protein. Furthermore, chronic morphine treatment inhibited p-CREB binding to IL-2 promoter at the −180 and −160 site and disrupted recruitment of CBP and p300 to the IL-2 promoter. These results, therefore, reveal that ICER may play an important role in morphine-induced transcriptional inhibition of IL-2.
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Significant chromatin remodeling occurs selectively across the IL-2 promoter upon T cell activation in both murine primary CD4+ T cells and EL-4 T cells. There appears to be an interdependence between chromatin remodeling and the formation and maintenance of stable transcription factor complexes on the promoter. Two models of chromatin remodeling on inducible IL-2 promoters have been presented by some groups. In the first model chromatin remodeling is dependent on the presence of specific transcription factors that recruit remodeling complexes to the promoter region (42). In the second model recruitment of remodeling complexes is preceded by other events on the chromatin such as post-translational modifications, for example by acetylation or phosphorylation, of the histone tails of nucleosomes on the IL-2 promoter (43). The CBP/p300 proteins in addition to acting as co-activators can also modulate histone acetylation. Although cAMP has been shown to inhibit transcription by preventing the binding of NFAT and NF-κB factors to the IL-2 promoter (44), we show here that overexpression of NFAT and NF-κB did not rescue chronic morphine-induced suppression of IL-2 transcription and production, but overexpression of CREB could rescue the inhibitory effects of morphine. Furthermore, ChIP assays demonstrate that chronic morphine treatment decreased the recruitment of CBP/p300 to the IL-2 promoter and reduced acetylated H3 and H4 associated with the IL-2 promoter. Since histone acetylation in the regulatory region of the IL-2 gene results in chromatin remodeling, promotes accessibility, and is closely correlated with IL-2 gene expression, from our data we conclude that chronic morphine treatment modulates IL-2 gene transcription both at the transcription and epigenetic levels.

DNA methylation is inversely related to IL-2 transcriptional activity (45). T cell activation results in the demethylation of CpG sites within IL-2 promoter (43). The epigenetic changes, including active demethylation of a specific CpG site, recruitment of Oct-1, and changes in histone modifications are necessary and sufficient to enhance the transcription of IL-2 promoter stimulated by CD3 and CD28 (46). Our results show that morphine treatment decreased DNA demethylation at the −68 CpG islands within the IL-2 promoter following CD3 and CD28 activation. This site has been shown by several studies to be demethylated following T cell activation (43, 47). Furthermore, recent studies by Murayama et al. (46) show that methylation of this critical site decreased DNA accessibility and inhibited T cell activation. Our studies suggest that modulation of CpG demethylation at the −68 CpG island is a potential epigenetic mechanism by which morphine regulates IL-2 gene.

In summary, chronic morphine treatment results in an increase in cAMP levels with a concurrent up-regulation of the cAMP-inducible repressor ICER/CREM and a down-regulation of p-CREB expression in activated T cells. Furthermore, ICER competes for p-CREB binding to the CREs. This leads to the uncoupling of CBP/p300, thereby abrogating IL-2 transcription. In addition, chronic morphine treatment decreased the acetylation of histones 3 and 4, decreased H3K4 trimethylation, prevented demethylation of a critical CpG site, and decreased accessibility of the IL-2 promoter to endonuclease digestion. These studies show for the first time a transcriptional and epigenetic regulatory mechanism involved in the inhibition of IL-2 by morphine.

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