Biochemical Pharmacology 77 (2009) 1553–1560

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Involvement of STAT5a signaling in morphine-induced up-regulation of the cyclin D1

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ARTICLE INFO

Article history:
Received 26 December 2008
Accepted 12 February 2009

Keywords:
Morphine
Cyclin D1
STAT5a
Signaling
Lymphocytes

ABSTRACT

Opioid receptors and cytokine receptor have been verified to have a functional link and interaction. However, the pathway by which opioid receptor in lymphocytes is linked to cytokine signaling is not well defined. Using confocal microscopy and Western blotting this study showed that morphine treatment was able to activate cytoplasmic STAT5a in CEM x174 cells, which then translocated into the nucleus and bound to elements of the cyclin D1 promoter. As a consequence the expression of the cyclin D1 was apparently up-regulated. The data from EMSA–superEMSA and ChIP-qPCR further confirmed that morphine was capable of promoting the binding of STAT5a to its elements (proximal and distal), and this was abolished by the antagonist naloxone. As shown by transient transfection assay, activity of the cyclin D1 promoter was significantly reduced by 82% (distal) and 65% (proximal) after two STAT5a elements were mutated in comparison with wild type STAT5a elements. Moreover, knockdown of STAT5a was associated with a concurrent silencing of morphine-induced expression of cyclin D1, demonstrating involvement of STAT5a in morphine-triggered signaling in the regulation of cyclin D1 expression. The finding provides evidence which demonstrates that there is cross-talk between the mu opioid receptor and cytokine signaling in lymphocytes. Thus, we conclude that morphine may modulate cyclin D1 gene expression via signal transducers and activators of transcription (STATs) signaling, which will be beneficial for further understanding of the pharmacological effect of morphine on immune regulation.

1. Introduction

Opioid compounds such as morphine produce powerful analgesia that is effective in treating various types of pain. In addition to their therapeutic efficacy, opioids can produce several well-known adverse effects, and, as has recently been recognized, can interfere with the immune response. The immunomodulatory activities of morphine have been characterized in animal and human studies. Besides its role in modulation of antibody and cytokine secretion [1], T helper cell balance [2,3], the debilitating effects of a variety of stressors on immune cell function [4] and monocyte migration [5], morphine has a capacity to promotion of cell proliferation. For instance, morphine is able to stimulate glomerulopathy and mesangial cell proliferation [6] and reduce apoptosis of lymphocytes infected with simian immunodeficiency virus (SIV) by disturbing the profile of cell cycle, which leads to accumulation of cells in G1 phase [7]. Based on accumulated evidence, it appears that alteration of the cell cycle and temporarily increased survival of infected cells by morphine may favor pathological progression of diseases, such as AIDS and cancer [8–10]. However, the precise intracellular mechanisms involved have not been fully evaluated.

In mammalian cells the cyclin Ds play an essential role in promoting cell cycle progression from G to S phase. The level of cyclin Ds is controlled by the extracellular environment. For this reason, cyclin Ds are believed to serve as a "linker" between the extracellular environment and the core cell cycle machinery [11]. Cyclin D1 is a pivotal cell cycle-regulatory molecule and a well-studied therapeutic target for cancer. Although many publications have emphasized the importance of cyclin D1 in cell growth and its involvement in opioid function, the precise mechanism in morphine-regulated proliferation of lymphocytes remains

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doi:10.1016/j.bcp.2009.02.007

uncharacterized, despite the fact that the profile of cell cycle progression is changed by morphine in SIV-infected lymphocytes [12]. In addition, a recent study indicated there is involvement of signal transducers and activators of transcription (STATs) in the up-regulation of cyclin D1, suggesting a novel role for these molecules in the regulatory mechanism of cell cycle [13].

Although previous studies have demonstrated that several signal effectors mediate responses of the mu opioid receptor (MOR), new information has continued to accumulate [7,9]. In general, STATs are activated by cytokine receptors. Ligand-induced receptor dimerization results in activation of Janus tyrosine kinases (Jak), which in turn phosphorylate the receptors of specific tyrosines that serve as docking sites for STATs. However, several studies have shown that STATs are also activated upon activation of certain G protein coupled receptors (GPCRs), including opioid receptor [14,15]. A recent study indicated there is cross-talk between the cytokine receptor and MOR [16]. Considering there are functional links and interaction between these two receptors, it is possible that they share a common signal pathway. To gain insight in detail into the mechanism by which morphine modulates cell growth, we have here extended our studies to investigate whether morphine, through activation of the STAT signal pathway, affects expression of cyclin D1 and in turn affects cell growth. Clarifying the molecular mechanism involved will be beneficial for further understanding of the pharmacological effects of morphine on immune regulation.

2. Materials and methods

2.1. Cell culture and determination of cells growth

CEM x174 cells, a hybrid of a human T cell line and human B cell line, were adjusted to a density of 1×10^5 per ml and were seeded into 96-well plates and maintained in RPMI-1640 medium (GIBCO, Invitrogen Corporation, USA) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. 10 μM morphine chloride was used in all experiments, which has been proven to be an optimal dose according to dose response curves as reported in our previous experiments [9]. In the blocking assay, the CEM x174 cells were preincubated with 10 μM naloxone for 30 min before subsequent treatment with 10 μM of morphine. CEM x174 cells were treated with 10 μM morphine and/or 10 μM naloxone for various lengths of time (0, 24, 48 and 71 h). Cell growth was determined by the dimethylthiahzolyl-2,5-diphenyl-tetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics, Switzerland) as previously described [17]. Four hours prior to measuring absorption values, 16 μl of the 5 mg/ml MTT stock solution was added to the samples. After washing three times with PBS, the reaction was terminated by adding 100 μl of DMSO. The crystallized MTT was dissolved, and the absorption was measured on a Universal Microplate Reader (EL x800) at 570 nm wavelength. Proliferation of cells in each different treatment was expressed as a ratio of absorption at 570 nm of sample to control (0 h).

2.2. Observation of localization and translocation of STAT5a

CEM x174 cells treated with morphine (10 μM) were fixed in ice-cold paraformaldehyde solution (4%) followed by soaking in 0.1% Triton-X 100. Rabbit anti-STAT5a antibody (Santa Cruz Biotech Inc, USA) was added for 12 h. Secondary goat anti-rabbit IgG antibodies conjugated with rhodamine (TRITC) (Jackson Immuno Res Lab, Inc, USA) were applied for 2 h followed by addition 10 μl DAPI (100 μg/ml) (Sigma, Inc, USA). Cells were viewed and captured with a Laser Confocal Microscope (Leica TCS-NT SP2, Germany).

2.3. Immunoprecipitation

CEM x174 cells were stimulated with 10 μM morphine, washed with PBS and solubilized with lysis buffer consisting of 1% Triton X-100, 10 mM Tris pH 7.6, 5 mM Na_2EDTA, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 100 mM Na_3VO_4, 1 mM phenylmethyl sulphonyl fluoride (PMSF) and protease and phosphatase inhibitor cocktail. In the blocking assay, the CEM x174 cells were preincubated with 10 μM naloxone for 30 min and subsequently treated with 10 μM of morphine for 15 min. Polyclonal antibodies against STAT5a (Santa Cruz Biotechnology, USA) was used to immunoprecipitate the corresponding proteins from cell lysates. After overnight incubation, protein A sepharose was added for 8 h and the immunoprecipitated immune complexes were washed with PBS, resuspended in Laemmli loading buffer, boiled and analyzed by Western blotting.

2.4. Western blotting

Western blotting was used for analysis of expression of the STAT5a and cyclin D1. Proteins were subjected to SDS-PAGE (10%, w/v) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with the appropriate primary antibody (1:1000) and incubated overnight. Blotting primary antibodies against STAT 5a (Santa Cruz Biotechnology, USA), phosphated STAT5 (p-STAT5) (Santa Cruz Biotechnology, USA), cyclin D1 (Ebioscience, USA), β-actin (Santa Cruz Biotechnology, USA) and Topo I (Santa Cruz Biotechnology, USA) were used for different experiments. The appropriate secondary antibodies conjugated to horseradish peroxidase were incubated with the PVDF membrane. Immunoreactive bands were detected by enhanced chemiluminescence according to the manufacturer's instructions.

2.5. Electrophoretic mobility shift assay (EMSA) and supershift EMSA (sEMSA)

Nuclear extracts from 10 μM morphine and/or 10 μM naloxone treated CEM x174 were prepared with the method as described by Koga et al. [18]. Synthetic oligonucleotides (BR-1 and BR-2) containing two separate STAT5a transcription factor binding regions (proximal and distal respectively) were used as probes for EMSA and are listed in Table 1. Double-stranded oligonucleotides were generated by annealing the synthetic oligonucleotides with respective complementary sequences. Complementary oligonucleotides of equal quantity (2.5 mM each) were annealed in a thermocycler (Techgene, UK) at the following temperatures: 88 °C, 2 min; 65 °C, 10 min; 37 °C, 10 min and 25 °C, 5 min. EMSA was carried out with the method as described before [19]. Competitors including unlabeled probe BR1 and BR2, mutated probe $BR1^m$ and $BR2^m$, and non-specific probe were used for verification of the binding between STAT5a and specific probe. Supershift EMSA analysis included the addition of 2 μg antibody against STAT5a (Santa Cruz Biotechnology, USA) to the reaction mixture for 30 min at room temperature before the addition of probes.

2.6. STAT5a RNA interference

Silencing hairpin DNA oligonucleotides for STAT5a were cloned into pGUP6/Neu (Shanghai Genepharma Co. Ltd, China) according to the manufacturer's instructions by using the primers Sh-1, -2, -3 and -4 (Table 1). After CEM x174 cells were transfected with constructs for silencing STAT5a by Lipofectamine 2000 (Invitrogen Co. USA) for 24 h, knockdown of STAT5a was evaluated by Western blot analysis.

Table 1
Sequences of oligonucleotides used as primers or probes for Plasmid construct, ChIP-qPCR amplification and EMSA. The mutated bases are in boldface.

Constructs		Sequences
Probes for EMSA		
BR-1	Sense	5′-GGC GTT CTT GGA AAT GCG CC-3′
BR-1	Antisense	5′-GGC GCA TTT CCA AGA ACG CC-3′
BR-1^{m}	Sense	5′-GGC G**G**T CTT G**TG G**AT GCG CC-3′
BR-1^{m}	Antisense	5′-GGC GCA T**CC A**CA AGA **C**CG CC-3′
BR-2	Sense	5′-GCA TTT CTA TGA AAA CCG GA-3′
BR-2	Antisense	5′-TCC GGT TTT CAT AGA AAT GC-3′
BR-2^{m}	Sense	5′-GCA T**G**T CTA T**TG G**AA CCG GA-3′
BR-2^{m}	Antisense	5′-TCC GGT T**CC A**AT AGA **C**AT GC-3′
Primers for Plasmid construct and site-directed mutagenesis		
WT	Sense	5′-CTA GCT AGC TAG GCG CCG GAA TGA AAC TT-3′
WT	Antisense	5′-CCC AAG CTT GGG CTC GGC TCT CGC TTC T-3′
M-635	Sense	5′-GGT GCC CTC GTG GCG **G**TC TTG **TGG** ATG CGC CCA TTC TGC C-3′
M-635	Antisense	5′-GGC AGA ATG GGC GCA T**CC A**CA AGA **C**CG CCA CGA GGG CAC C-3′
M-401	Sense	5′-CAG CGG GGC GAT TTG CAT **G**TC TAT **TGG** AAC CGG ACT ACA GGG GC-3′
M-401	Antisense	5′-GCC CCT GTA GTC CGG TT**C CA**A TAG A**C**A TGC AAA TCG CCC CGC TG-3′
Primers of RNA interference against STAT5a		
Sh-1	Sense	5′-GCG CTC AAC ATG AAA TTC AAG-3′
Sh-1	Antisense	5′-CTT GAA TTT CAT GTT GAG CGC-3′
Sh-2	Sense	5′-GCG CAG AAA CTG TTC AAC AAC-3′
Sh-2	Antisense	5′-GTT GTT GAA CAG TTT CTG CGC-3′
Sh-3	Sense	5′-AGG CCG AAG TGC AGA GCA A-3′
Sh-3	Antisense	5′-TTG CTC TGC ACT TCG GCC T-3′
Sh-4	Sense	5′-CTA AAG CTG TTG ATG GAT A-3′
Sh-4	Antisense	5′-TAT CCA TCA ACA GCT TTA G-3′
Primers for ChIP/qPCR		
BR-1	Sense	5′-CGT CCT TGC ATG CTA AA-3′
BR-1	Antisense	5′-CGA GAA GGG GTG ACT GG-3′
BR-2	Sense	5′-CTG CAC CCC TCT TCC CT-3′
BR-2	Antisense	5′-GAG CCC AAA AGC CAT CC-3′

2.7. Plasmid promoter constructs and site-directed mutagenesis

A 740 bp fragment encoding bases -794 to -55 of the cyclin D gene was amplified by PCR assay from CEM x174 cells using primers WT (Table 1). The amplified fragment containing two STAT5a binding sites in promoter region was subcloned into the vector pGL3-Basic (Promega, USA) at the NheI and HindIII sites to create the wild type construct (WT) with the Luciferase reporter gene. The mutated cyclin D1 promoter-reporter constructs (M-401 and M-635) that included individual mutations in binding region -635 to -645 bp (distal) and -401 to -410 bp (proximal) upstream of the start codon were created by PCR using WT construct as template and the mutation primers M-401 and M-635 (Table 1). All constructs were verified by DNA sequencing.

2.8. Transient transfection and reporter expression assays

All the plasmids used in these transfection experiments were prepared by the Large-scale Purification Kit (Vigorous, China) following the manufacturer's recommended protocol. CEM x174 cells were transfected with Lipofectamine 2000 Reagent (Invitrogen, USA) following the manufacturers recommended protocol as described in our previous report [20]. Transfection efficiency was monitored by cotransfection of the pRL-SV 40 promoter driven Renilla luciferase (Promega, USA). After standardization with Renilla luciferase activity, a relative luciferase activity was obtained and the mean and standard deviation from triplicate wells was calculated. All procedures were performed in triplicate.

2.9. Effect of STAT5a knockdown on cell growth

The MTT assay was carried out to observe the effect of STAT5a knockdown on morphine-induced cell growth. CEM x174 cells were transfected with Sh-4 for 24 h and then treated with 10 μM morphine for various periods of time (0, 24, 36 and 48 h). The MTT assay was carried out as described above.

2.10. Chromatin immunoprecipitation and quantitative real-time PCR (ChIP-qPCR)

CEM x174 cells were treated with 10 μM morphine and/or 10 μM naloxone for 15 min as described above. Antibody against STAT5a used for immunoprecipitation was purchased from Santa Cruz Biotechnology. The ChIP assay was performed as described by Li et al. [19]. ChIP products were subjected to quantitative real-time PCR using primers specific to two STAT5a binding sites in the cycling D1 promoter and performed using SYBR Green Realtime Master Mix (TOYOBO, Japan) in triplicate (Table 1). The reaction conditions were as follows: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s (denaturation); and 55 °C for 1 min (annealing and elongation). Threshold cycle numbers (CT) were determined with the ABI PRISM 7000 sequence detection system (Version 1.1 software). The DNA levels from ChIP-qPCR assay were calculated as: ratio = $2^{-CT\text{-}CTsample}/2^{-CTinput}$, where CT represents threshold cycle number of sample and input. Relative concentration of ChIP-qPCR product was presented as the fold change of DNA-STAT5a complex level of samples to control.

2.11. Statistical analysis

All data were statistically analyzed by the *t*-test with SPSS 11.5 software and expressed as mean ± S.D. (standard deviation) from at least three independent experiments unless otherwise stated. A value of $P < 0.05$ was considered significant.

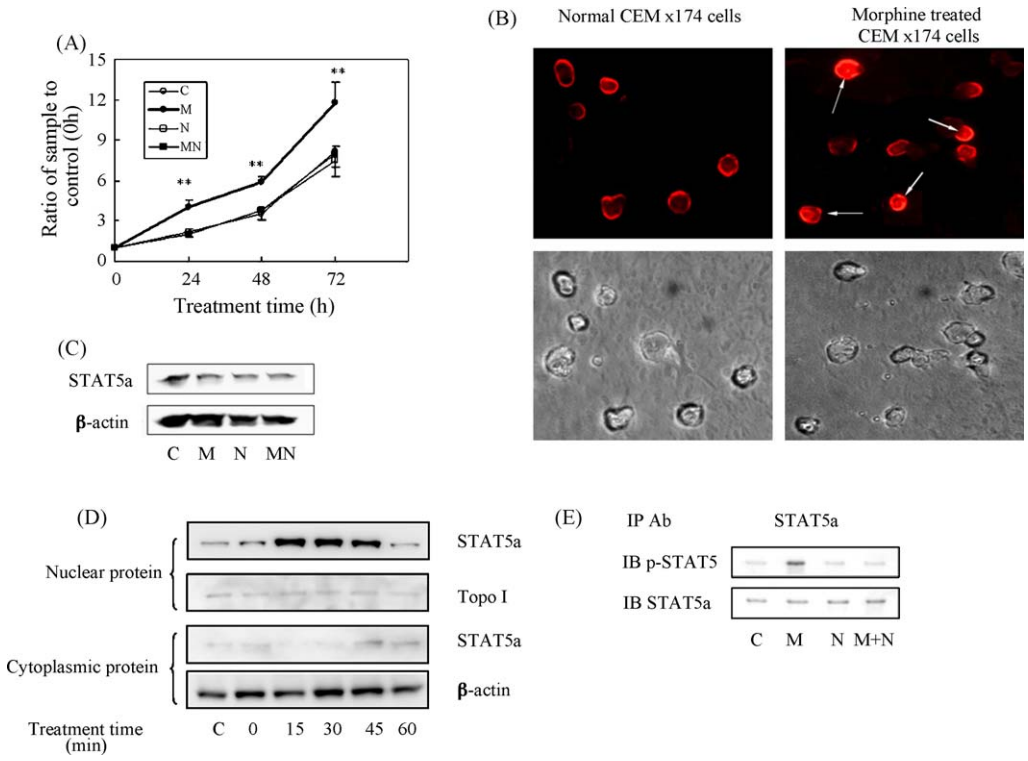

Fig. 1. Morphine-stimulated cell proliferation and activation of STAT5a. (A) The effect of morphine on proliferation of CEM x174 cells analyzed by MTT. $^{*}P < 0.05$ and $^{**}P < 0.01$ are considered statistically significant between experimental groups and control group. (B) Observation of translocation of cytoplasmic STAT5a into nuclei under confocal (upper) and light (lower) microscopy. The arrows show STAT5a in nuclei. (C) Western blot analysis of intracellular total STAT5a protein. (D) Western blot analysis for expression of cytoplasmic and nuclear STAT5a at different time points. Topoisomerase I (Topo I) and β-actin are used to balance the loading of proteins from nuclei and cytoplasm, respectively. (E) CoIP analysis for the effect of morphine on phosphated STAT5a. M: 10 μM morphine treated group; N: 10 μM naloxone treated group; MN: morphine plus naloxone treated group. All images are representative of an experiment that was repeated at least three times.

3. Results

3.1. Impact of morphine on cell proliferation and activation of STAT5a

The MTT assay was used to evaluate the effect of morphine on proliferation of CEM x174 cells. The results showed that 10 μM morphine moderately promote cell growth during 72 h incubation, which was consistent with our previous study (Fig. 1A) [9]. The effect of morphine could be abolished by pretreatment with 10 μM naloxone, which specifically antagonizes morphine.

To investigate whether morphine stimulation influences STAT5a activity in immune cells, CEM x174 cells were treated with 10 μM morphine for various lengths of time and subsequently analyzed for intracellular localization of STAT5a. By observation with laser confocal microscopy, it was found that STAT5a was localized in the cytoplasm of untreated CEM x174 cells and was undetectable in nuclei. After treatment with morphine for 15 min, cytoplasmic STAT5a began to move from the cytoplasm into nuclei (Fig. 1B). Treatment with morphine did not affect the level of total STAT5a (Fig. 1C). However, the level of STAT5a in nuclei was elevated and accompanied by a synchronous decrease in the cytoplasm 15 min after morphine administration (Fig. 1D). This change in the subcellular localization of STAT5a was sustained for 45 min after morphine administration.

To examine whether the increased nuclear STAT5a is activated STAT5a, the phosphorylation of STAT5a was examined 15 min after morphine administration. 10 μM naloxone was added in CEM x174 cells at 30 min prior to morphine stimulation to specifically antagonize the effect of morphine. As shown in Fig. 1E with a specific phosphotyrosine antibody, the level of activated STAT5a (p-STAT5a) was obviously elevated 15 min after morphine administration, and this effect was abolished by naloxone (Fig. 1E).

3.2. Effects of morphine on the formation of the STAT5a-elements complex

EMSA and superEMSA were used to demonstrate the binding of STAT5a to the proximal and distal elements in the cyclin D promoter. The results showed that nuclear extracts from CEM x174 cells could form a STAT5a complex which was abolished by competition (Fig. 2A). Antibody against STAT5a resulted in super shift bands. To evaluate the effect of morphine on the level of nuclear STAT5a, EMSA with nuclear extracts from CEM x174 cells treated with morphine was performed. The result showed by using probes for the distal (Fig. 2B) or proximal (Fig. 2C) binding site that a complex with STAT5a was visualized 15 min after morphine administration, and this effect could be abolished by specific unlabeled probe, but not by mutated or non-specific probe. The effect of morphine was further confirmed by observation that its effect was reversed by treatment with naloxone.

3.3. Up-regulation of cyclin D1 by morphine is STAT5a dependent

After activation, STAT5a becomes phosphorylated on tyrosine and enters the nucleus to regulate transcription of many different genes. Our primary experiment with quantitative real-time reverse transcription PCR showed the involvement of STAT5a in the regulation of some genes including cyclin D1 after morphine administration (data not shown). Western blotting showed that the level of cyclin D1 expression in CEM x174 cells was promoted in comparison with the control at 12 h after morphine stimulus,

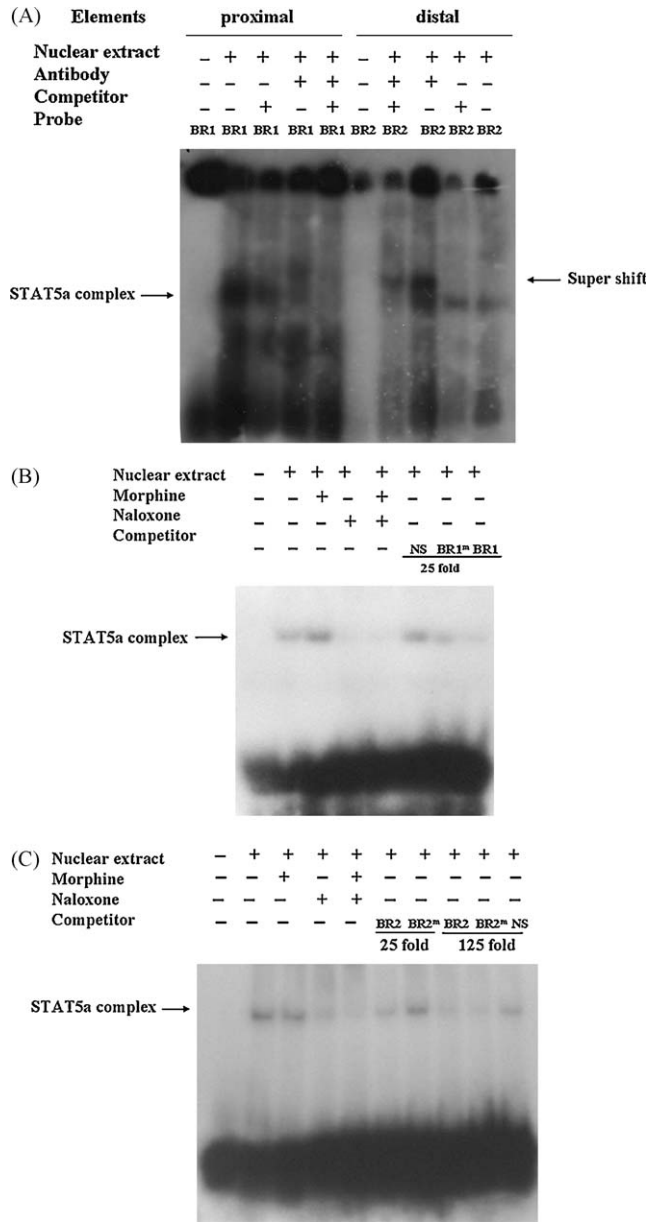

Fig. 2. Verification of putative STAT5a binding sites in the proximal and distal promoter of the cyclin D1 gene in CEM x174 cells. (A) EMSA and super-EMSA analysis of binding of STAT5a in the proximal and distal promoter of cyclin D1. STAT5a complex and super shift band are indicated by horizontal arrows. (B) EMSA analysis for the effect of morphine on binding of STAT5a to its distal (B) or proximal (C) element in the cyclin D1 promoter. Competitors and probes used for EMSA are listed in Table 1. All images are representative of an experiment that was repeated at least three times.

and then gradually declined (Fig. 3A). The increase in cyclin D1 with morphine treatment was abolished by naloxone (Fig. 3B). To examine the involvement of STAT5a, CEM x174 cells were transiently transfected with shRNA expression vectors against STAT5a. We constructed four anti-specific shRNA-expressing vectors targeted to the regions 2169–2189 (Sh-1), 2241–2261 (Sh-2), 2188–2206 (Sh-3) and 2704–2722 (Sh-4) of the STAT5a mRNA coding sequence. Of the four constructs, the anti-STAT5a shRNA construct directed to the 2704–2722 region (Sh-4) was most effective as judged by the mean intensity of STAT5a bands in Western blotting, and it was therefore chosen for further experiments (Fig. 3C). As shown in Fig. 3D, knockdown of STAT5a by Sh-4 has the capability of inhibiting morphine-induced up-regulation of cyclin D1 expression in comparison with control and vector groups (Fig. 3D). At the same time, morphine-induced growth of CEM x174 cells transfected with Sh-4 was obviously reduced during the culture period in comparison with the group without knockdown (Fig. 3E).

3.4. Impact of morphine on the binding of STAT5a to elements in the cyclin D1 promoter

To identify the underlying role of the two STAT5a elements of the cyclin D promoter in lymphocytes, we introduced constructs mutated in two individual STAT5a elements (Fig. 4A). Constructs with wild type and two mutated sites for STAT5a binding (M-401 and M-635) were transfected into CEM x174 cells separately. The effect of these mutated STAT5a elements was determined by measuring luciferase activity in transfected cells, which was normalized with cotransfected pRL-null Renilla luciferase activity. Data showed that CEM x174 cells transfected with wild type (WT) constructs expressed a higher level of luciferase activity (Fig. 4B). However, the promoter activity of M-401 and M-635 was respectively decreased by approximate 82% and 65% in comparison with WT ($P < 0.01$).

The impact of morphine stimulus on the capability of STAT5a for binding the cyclin D1 promoter was evaluated by ChIP assay linked to quantitative real-time PCR (ChIP-qPCR). The results showed that DNA fragments pulled down by Ab against STAT5a in the morphine treatment group were elevated up to approximate 2.8-fold for the proximal site and 6.4-fold for the distal site (Fig. 4C). All increases which resulted from morphine treatment in these two STAT5a binding elements was capable of being reversed by treatment with naloxone. These results provided further evidence for involvement of STAT5a in morphine-stimulated up-regulation of cyclin D gene expression.

4. Discussion

The mu opioid receptor is generally coupled with G protein which mediates multiple biological responses through activating downstream signal effectors including adenyl cyclase [21], Ca^{2+} channels [22], K^+ channels [23], phosphatidylinositol 3-kinase/Akt [24], mitogen-activated protein kinase [25] and Nuclear factor-kappaB (NF-kappaB) [26]. However, most of the reported MOR-mediated intracellular itineraries have been investigated in neuronal cells. It has definitively emerged that not all cells share the same regulation profile under exposure to morphine. The MOR-mediated response is very diverse and is often cell-type-dependent. For instance, the amount of the induced MOR mRNA in the immune cells was 15–200 times less than those in neuronal cells [27]. This diversity of response of immune cells under exposure to morphine underscores the complexity of the MOR signaling. Therefore, further investigation of the intrinsic mechanism which is involved is of importance for understanding and evaluation of the effect of morphine in immune cells.

Cytokines are a group of modulatory proteins that bind their specific receptors in response to a variety of stimuli, resulting in the activation of second messengers and signal transduction pathways within a cell. STAT protein family receptors are downstream of many cytokines, and their activation thereby mediated signal transduction from the plasma membrane to the nucleus [28,29]. The network involved in morphine signaling is a complicated and highly regulated system. It has been documented that receptors for cytokine and morphine are closely related and together may form heterodimers or oligomers, which differ in their pharmacological profiles, ligand binding affinity and/or intracellular pathways [30]. In addition, these receptors possess the capacity to cross-regulate receptor function through the process of heterologous desensitization, wherein activation of one type of

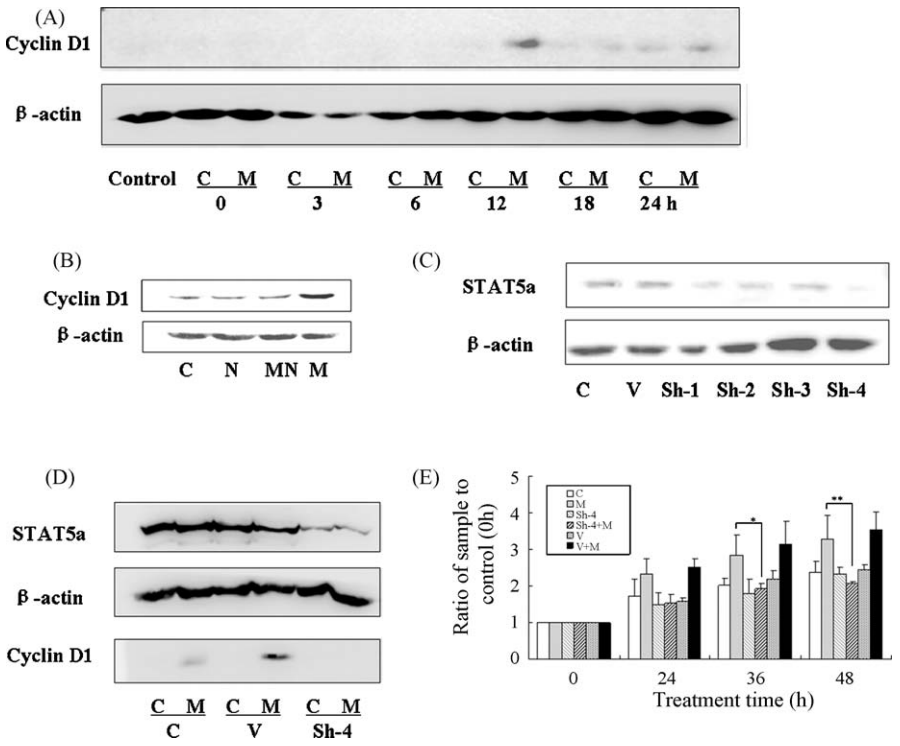

Fig. 3. The effect of morphine on expression of cyclin D1 and impact of STAT5a knockdown. (A) The effect of morphine in expression of cyclin D1 at different time points. (B) Blockade of the effect of morphine by naloxone on expression of cyclin D1. (C) Comparison of four shRNA expression vectors against STAT5a for silencing of STAT5a expression. Sh-1 to Sh-4 represent four shRNA expression vectors. (D) Impact of silencing STAT5a on the expression of cyclin D1. M: 10 μM morphine treated group; N: 10 μM naloxone treated group; MN: morphine plus naloxone treated group; C: Control group; V: empty vector. All images are representative of an experiment that was repeated at least three times. (E) The effect of STAT5a knockdown on morphine-induced cell growth. Sh-4 + M: CEM x174 cells were transfected with Sh-4 for 24 h and then treated with 10 μM morphine; V: transfected with empty vector; V + M: transfected with empty vector for 24 h and then treated with 10 μM morphine. $^{*}P < 0.05$ is considered statistically significant for comparison of the Shi-4 + M and 10 μM morphine treated groups or groups transfected with empty vector and treated with 10 μM morphine. These data were reproducible in three independent experiments.

GPCRs (e.g. the opioid receptor) may result in phosphorylation and inactivation of the other GPCR (e.g. a chemokine receptor). The activation of MOR may induce the expression of CCL2, CCL5, and CXCL10, as well as CCR5 and CXCR4 [31]. These findings imply that there is evolutionary conserved cross-talk between cytokines and opioids.

In view of the fact opioid receptors activate cytokine related-signaling [32], it is possible that STAT-involved signal pathways activated by cytokine are also shared by opioid receptors [16]. Some evidence from previous studies supports this hypothesis. For example, many GPCRs agonists may bring about activation of STATs and changes in gene transcription [33,34], indicating that there are variations in the mechanism of activation among the different receptors including opioid receptors. Moreover, the consensus motif YXXL present in the opioid receptor may interact with STAT5 which in turn can be phosphorylated by Src kinase [33,35]. In accordance with these findings, we propose the possibility that the diverse biological function of MOR may be achieved by transferring downward signal via cross-talk between intracellular signal pathways. Nevertheless, definitive evidence demonstrating that the effect of morphine is coupled to STAT signaling in immune cells is lacking.

Previous studies demonstrated that short-term exposure to morphine could result in proliferation of lymphocytes [7]. Specifically, the results in current study provide further evidence that the STAT5a signal pathway contributes to the proliferative response of lymphocytes to morphine administration. Given the involvement of STAT5a signaling in the morphine-induced change of cyclin D1 gene expression, it is not surprising to learn that there is a functional interrelationship between opioids and cytokines in the regulation of cell growth. A recent study provides further evidence for the involvement of STAT signaling in pancreatic cell proliferation via regulation of cyclin Ds, demonstrating a novel aspect of regulatory mechanism of the cell cycle [13].

Although previous studies have shown that morphine is only a weak promoting agent for lymphocyte proliferation during short exposure, this is of special significance in the progression of HIV infection to AIDS associated with morphine addiction [7,36]. In mammalian cells, cyclin D1 plays an essential role in promoting cell cycle progression from G0 to G1 phase and cell proliferation. It has been documented that morphine promotes cell cycle progression by increasing cyclin D1 [37]. Proliferative responses of lymphocytes were significantly suppressed during morphine withdrawal [38]. Despite these observations, the precise signaling pathway that regulates cyclinD1 expression and leads to proliferation in morphine treated lymphocytes remains unclear. It is intriguing that the STAT5a signal cascade is specifically activated by morphine administration, and its role in cyclin D1 expression as demonstrated in the current study provides a novel finding in morphine-induced proliferation of lymphocytes.

Although further studies are necessary for full evaluation of the precise effect of morphine exposure on cell growth, the morphine-STAT5a-cyclin D pathway may represent a heretofore unknown, but fundamental immunoregulatory mechanism. Thus, this molecular mechanism provides not only a new explanation of how morphine induces change in the profile of cell growth, but also offers insight into the functional link between MOR and chemokine signaling in lymphocytes.

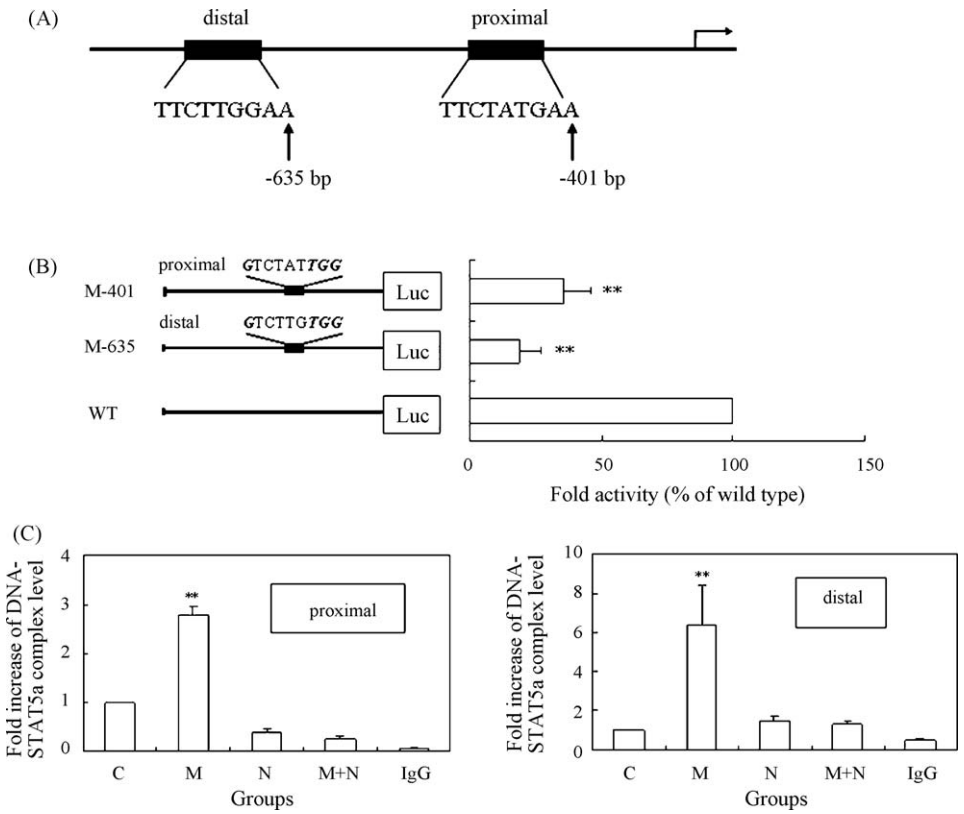

Fig. 4. The effect of morphine on activation of the cyclin D1 promoter. (A) The schematic shows the sequence of two STAT5a binding elements (proximal and distal). (B) Impact of site mutation of STAT5a binding elements on activation of the cyclin D1 promoter. Mutated bases in the sequence of STAT5a elements are presented in italics. $^{**}P < 0.01$ is considered statistically significant in comparing the mutated group and wild type group. (C) ChIP-qPCR analysis for the effect of morphine on the capacity of STAT5a binding to proximal and distal elements. The bars show the mean ± S.D. of the results from four independent transfection experiments. $^{**}P < 0.01$ is considered statistically significant for comparison of the morphine treated group and control group. M: 10 μM morphine treated group; N: 10 μM naloxone treated group; MN: morphine plus naloxone treated group; WT, M-401 and M-635 and represent respectively a wild type construct and constructs mutated at sites -401 (proximal element) and -635 (distal element) bp of the cyclinD1 promoter.

Acknowledgments

The authors express their gratitude to the National Natural Science Foundation of China (No. 30621002, 30671856, 30772536) and The Foundation of National Education Ministry for graduate program (No. 20070001735) for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.02.007.

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