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Mechanisms involved in phosphatidylinositol 3-kinase pathway mediated up-regulation of the mu opioid receptor in lymphocytes

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ABSTRACT

Despite the substantial progress made in understanding initiation expression of the MOR gene in lymphocytes, the signal pathway associated with MOR gene transcription remains to be better defined. As the phosphatidylinositol 3-kinase (PI3K)/AKT pathway can mediate diverse biological responses and is crucial for optimal immune responses and lymphocyte development, this study was undertaken to delineate the role of PI3K/AKT signaling in expression of the MOR gene in CEM ×174 cells. The data show that morphine treatment enhanced the level of phosphorylated, rather than un-phosphorylated, PI3K and AKT, which were synchronously recruited to membrane. The levels of PTEN and p53 which are negative regulators of these signal molecules were reduced, and as a result, the interaction between PTEN and p53 was completely interrupted. With morphine treatment, the levels of both cytoplasmic and nuclear E2F1 which is the downstream effecter of AKT were elevated and the interaction of E2F1with YY1, rather than Sp1, was also increased. Subsequently, E2F1 triggered the transcription of the MOR gene through its enhanced ability to bind the element in promoter region of the MOR gene. All responses to morphine were abolished by naloxone, which is an antagonist of MOR, or by LY294002, an inhibitor of PI3K, implying specific involvement of PI3K/AKT. These results strongly suggest that the PI3K/AKT pathway plays a critical role in the transfer of signal from morphine stimuli to the machinery by which MOR gene transcription is initiated.

1. Introduction

Although there has been progress in understanding mu opioid receptor (MOR) gene expression in neural and immune systems, transmission of signal from MOR onward into the nucleus and its affects on the expression of the MOR gene is not well defined [1–4]. This is of particular interest in lymphocytes as the MOR is known to mediate a variety of the physiological and pharmacological effects of morphine in lymphocytes via regulatory mechanisms which differ from those in neural cells. Changes in expression of the MOR gene in lymphocytes which results from opioid abuse has been shown to be of importance in the pathological progression of immune disorders such as AIDS [5].

MOR is a member of the G protein coupled receptor (GPCR) super-family and performs multiple immunological roles [6]. Although morphine mediated responses have been reported to involve signal molecule such as cAMP/PKA, PKC/Ca^{2+}, IP_3 and MAPK, the molecular machinery underlying such regulation of the MOR gene expression in lymphocytes and its biological significance are not well characterized [7–9].

Analysis of the sequence of the MOR promoter has shown that there are three transcription factor (Sp1, YY1 and E2F1) elements adjacent to each other, and these are responsible for the initiation of MOR gene transcription in lymphocytes. Although our previous work has verified there is interaction between transcription factors Sp1 and YY1, the role of E2F1, and its synergistic activation with other factors and relevant signal pathway in triggering MOR gene transcription mediated by these factors are unknown [1].

Recent studies with cardiac myocytes [10,11], dermal microvascular endothelial cells [12], the Jurkat leukemia cell line [13], and neuronal cells [14,15] have suggested there is involvement of the phosphatidylinositol 3-kinase (PI3K)/AKT pathway in morphine-induced cell survival and apoptosis. The PI3K/AKT cell survival pathway has been extensively studied. The regulation of PI3K activation has been linked to many normal and disease-related processes, including cell survival, cell growth and proliferation, cell differentiation, cell motility, and intracellular vesicle trafficking [16]. Various studies have

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suggested that *trans*-factor E2F1 functions as a downstream target of the PI3K/AKT pathway and contributes to the control of cellular proliferation [17]. AKT is a serine/threonine kinase and phosphorylates several cellular proteins including E2F1, thus serving to either inhibit or enhance their activities [18]. Therefore, the possibility that morphine-activation of the PI3K/AKT pathway is linked to the triggering of expression of the MOR gene by these three factors (Sp1, YY1 and E2F1) in lymphocytes became the focus of our interest.

This study was performed to evaluate the involvement of the PI3K/AKT pathway in morphine-induced MOR gene expression in lymphocytes, and determine what changes are produced by the interaction between these transcription factors and this pathway. Shedding light on these questions may help advance understanding of opioid abuse as a participating factor in the occurrence of dysfunction of immune cells, particularly in some diseases such as AIDS.

2. Materials and methods

2.1. Quantitative real-time reverse transcription PCR (RT-qPCR)

CEM ×174 cells, a hybrid of human T and human B cell lines, were treated with 10 μM morphine (Huabei Pharmacol Co., China) for various lengths of time (0, 4, 8, 12, 16, 20 and 24 h). Expression of the MOR gene was determined by RT-qPCR. Briefly, total cellular RNA was extracted at various treatment time points and subjected to RT-qPCR assay. Primer sequences for RT-qPCR detection of MOR and GAPDH mRNA levels are listed in 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 60 °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 relative contents of MOR mRNA were calculated as ratio = $2^{-CT(sample)}/2^{-CT(GAPDH)}$, where CT represents the threshold cycle number of the sample and control. Relative concentrations of MOR mRNA are represented as the mean *x*-fold change of the sample as compared to the control.

2.2. Immunoblotting assays

Western blotting was used for analysis of the level of MOR, phosphate and tension homology deleted on chromosome ten (PTEN), p53, phosphorylated PI3K and AKT, as well as translocation of cytoplasmic and nuclear E2F1 protein. CEM x174 cells were treated with 10 μM morphine or/and 10 μM naloxone for time intervals of 0, 4, 8, 12, 16, 20 and 24 h for analysis of expression of MOR, PTEN and p53; and for 0, 15, 30, 45 and 60 min for analysis of phosphorylated PI3K and AKT. For corroboration of the involvement of PI3K, 1.4 μM LY294002 ([2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one], Cell Signaling Technology, Inc., USA), a potent PI3K inhibitor, was added into the cell culture 15 min prior to treatment with morphine. Preparation of cell lysates and nuclear protein, and performance of SDS-PAGE and Western blot analysis were carried out as described previously [1]. Antibodies against MOR, PTEN, p53, E2F1, Topoisomerase I (Topo I) and β-actin were purchased from Santa Cruz Biotechnology, USA. Antibody against un-phosphorylated AKT was purchased from Abcom, UK. Antibodies against un-phosphorylated PI3K, phosphorylated PI3K (phosph-PI3K) and phosphorylated AKT (phosph-AKT) were purchased from Cell Signaling Technology Inc., USA.

2.3. Coimmunoprecipitation

Coimmunoprecipitation (CoIP) experiments were performed to evaluate the interaction between p53 and PTEN, and the interaction among transcription factors Sp1, YY1 and E2F1 under morphine exposure. CoIP has been described previously [1,8,19]. Antibodies against p53, PTEN, Sp1, YY1 and E2F1 were purchased from Santa Cruz Biotechnology, USA.

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

ChIP was performed to verify the impact of morphine on the capacity of E2F1 for binding to DNA. CEM ×174 cells were treated with 10 M morphine or/and 10 μM naloxone for 12 and 24 h, and the ChIP assay was performed as described previously [1]. LY294002 (1.4 μM) was added into the cell culture 15 min prior to treatment with morphine for corroboration of the involvement of PI3K/AKT. ChIP products were subjected to qPCR assay. The primers used for ChIP-qPCR are listed in 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). DNA levels from the ChIP-qPCR assay were calculated as ratio = $2^{-CTsample}/2^{-CTinput}$, where CT represents the threshold cycle number of sample and input. The relative concentration of the ChIP-qPCR product is represented as the *x*-fold change of the DNA–E2F1 complex level of the samples as compared to the input. Antibodies against Sp1, YY1 and E2F1 which were used in this experiment were purchased from Santa Cruz Biotechnology, USA.

2.5. Confocal microscopy

Confocal microscopy was used to observe the change of intracellular localization of PI3K and AKT after morphine treat-

Table 1
Sequences of oligonucleotides used as probe for EMSA and primers for quantitative real-time reverse transcription PCR (RT-qPCR), chromatin immunoprecipitation and quantitative real-time PCR (ChIP-qPCR).

Sequences of oligonucleotides			Product size (bp)
Probes for EMSA			
	Sense	5′-CTA CTC CTT GGA TCG CGC TTT GCG CAA AAT CCA CCC CTT TTC CCT CCT CCC TCC CTT CCA-3′	
	Antisense	5′-TGG AAG GGA GGG AGG AGG GAA AAG GGG TGG ATT TTG CGC AAA GCG CGA TCC AAG GAG TAG-3′	
Primers			
RT-qPCR			
MOR	Sense	5′-TAA AGC CTT GGT TAC AAT CCC AG-3′	264
	Antisense	5′-AGA CTG CGT ACC TGA TGA TTA GT-3′	
GAPDH	Sense	5′-TGA AGG TCG GAG TCA ACG GA-3′	232
	Antisense	5′-CCT GGA AGA TGG TGA TGG GAT-3′	
ChIP-qPCR			
	Sense	5′-ACT CCT TGG ATC GCT TTG C-3′	142
	Antisense	5′-CCT CCC ACC TTA GTA GTT CAC A-3′	

ment. CEM ×174 cells treated with morphine (10 μM) were fixed in ice-cold paraformldehyde solution (4%) followed by soaking in 0.1% Triton-X 100. Rabbit anti-phosphorylated PI3K and AKT antibodies (Cell Signaling Technology Inc., USA) were added separately into the cell culture for 0, 15, 30, 45 and 60 min. Secondary goat anti-rabbit IgG antibodies conjugated with rhodamine (TRITC) (Jackson Immuno Res Lab Inc., USA) were applied for 1 h followed by addition 10 μl of DAPI (100 μg/ml) (Sigma Inc., USA). Cells were viewed and captured with a Laser Confocal Microscope (Leica TCS-NT SP2, Germany).

2.6. Electrophoretic mobility shift assay (EMSA) and super-shift EMSA (sEMSA)

EMSA and sEMSA were used to confirm the binding of transcription factors Sp1, YY1 and E2F1 to their elements in the MOR gene promoter. CEM ×174 cells were treated with 10 μM morphine for 8 h as described above. Nuclear extracts were prepared by as previously described [1]. Oligonucleotides of different sizes containing Sp1, YY1 and E2F1 transcription factor binding sites were synthesized as probes for EMSA (Table 1). Radiolabeling and purification of these probes and EMSA were performed as described previously [1]. Super-shift analysis included the addition of 2 μg/ml of antibody against Sp1, YY1 and E2F1 (Santa Cruz Biotechnology, USA) to the reaction mixture for 30 min at room temperature before the addition of probes. All experiments were repeated at least three times.

2.7. Data analysis

All data are represented as mean ± S.D. Differences between two groups were evaluated by the student's *t*-test with SPSS 11.5 software.

3. Results

3.1. Evaluation of involvement of PI3K and AKT in morphine-induced change on the expression of the MOR gene

Expression of the MOR gene in CEM ×174 cells was evaluated at different time points after morphine administration (0, 4, 8, 12, 16, 20 and 24 h) by RT-qPCR and Western blotting. The data showed that levels of MOR mRNA and protein were elevated at 12 and 16 h, respectively, after morphine treatment (Fig. 1A and B). To determine whether the morphine-induced change in MOR gene expression was mediated by PI3K/AKT signaling, 1.4 μM LY294002, a specific inhibitor of PI3K, was added into the cell culture to block this pathway. The data showed that the effect of morphine on up-regulation of the MOR protein was abolished by LY294002, demonstrating the involvement of PI3K/AKT signaling molecules in morphine-induced up-regulation of MOR gene expression (Fig. 1C).

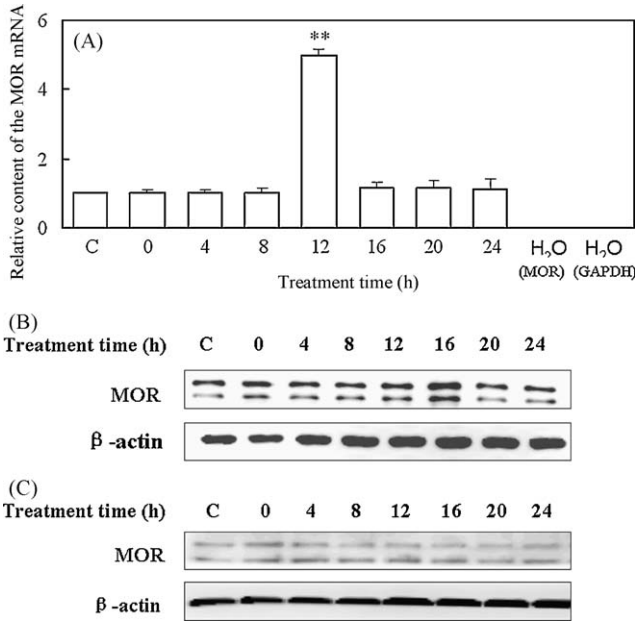

Fig. 1. Expression of the MOR gene in CEM ×174 cells. CEM ×174 cells were treated with morphine (10 μM) for 0, 4, 8 12, 16, 20 and 24 h. (A) Expression of the MOR gene in morphine treated CEM ×174 cells was analyzed by RT-qPCR. "H_2O (MOR)" and "H_2O (GAPDH)" representing the water controls with primers for MOR and GAPDH, respectively. (B) Effects of morphine with various treatment times on expression of the MOR gene were analyzed by Western blotting. (C) Western blot analysis of the effect of LY294002 on morphine-induced change of MOR gene expression. "C" represents the control group. $^{**}P < 0.01$ is considered statistically significant as compared with control.

3.2. Effect of morphine on activation and intracellular localization of PI3K and AKT

To further analyze whether morphine stimulation affected PI3K/AKT signaling in lymphocytes, CEM ×174 cells were treated

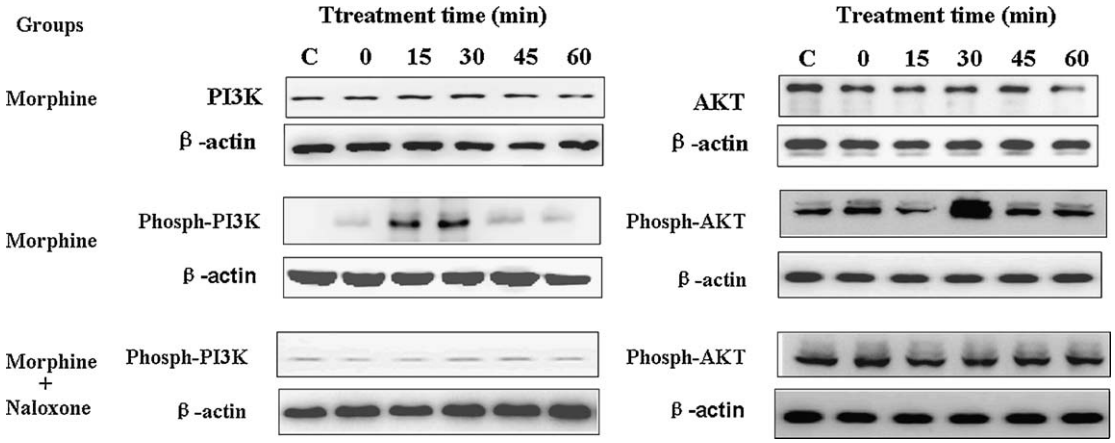

Fig. 2. Morphine-induced change of PI3K and AKT. CEM ×174 cells were treated with morphine (10 μM) for 0, 15, 30, 45 and 60 min. Western blot analysis was used for analysis of the levels of phosphorylated and un-phosphorylated PI3K/AKT. In the bottom panels, the result of naloxone blockade of morphine-induced changes on the levels of PI3K and AKT are shown. Phosph-PI3K and phosph-AKT represent antibodies against phosphorylated PI3K and AKT used in Western blot analysis.

with 10 μM morphine for various lengths of time and subsequently analyzed for activation and intracellular localization of PI3K and AKT with Western blotting and confocal microscopy. As PI3K is activated by recruitment to the phosphorylated effector and regulated by phosphorylation of its p85 regulatory subunit, the levels of both phosphorylated and un-phosphorylated PI3K/AKT were analyzed in this current study [20,21]. As shown in Fig. 2, the levels of un-phosphorylated PI3K and AKT were unchanged over all treatment time points (Fig. 2). However, it is noteworthy that phosphorylated PI3K was evidently up-regulated during the 15–30 min of morphine treatment. In addition, the level of phosphorylated AKT was also simultaneously elevated. These elevations of phosphorylated PI3K and phosphorylated AKT induced by morphine treatment were abolished by naloxone. These result indicated that phosphorylation of PI3K and subsequent phosphorylation of AKT mediated the activation of morphine-induced signaling pathway in lymphocytes. In view of the importance of membrane recruitment and localization of PI3K and AKT for their

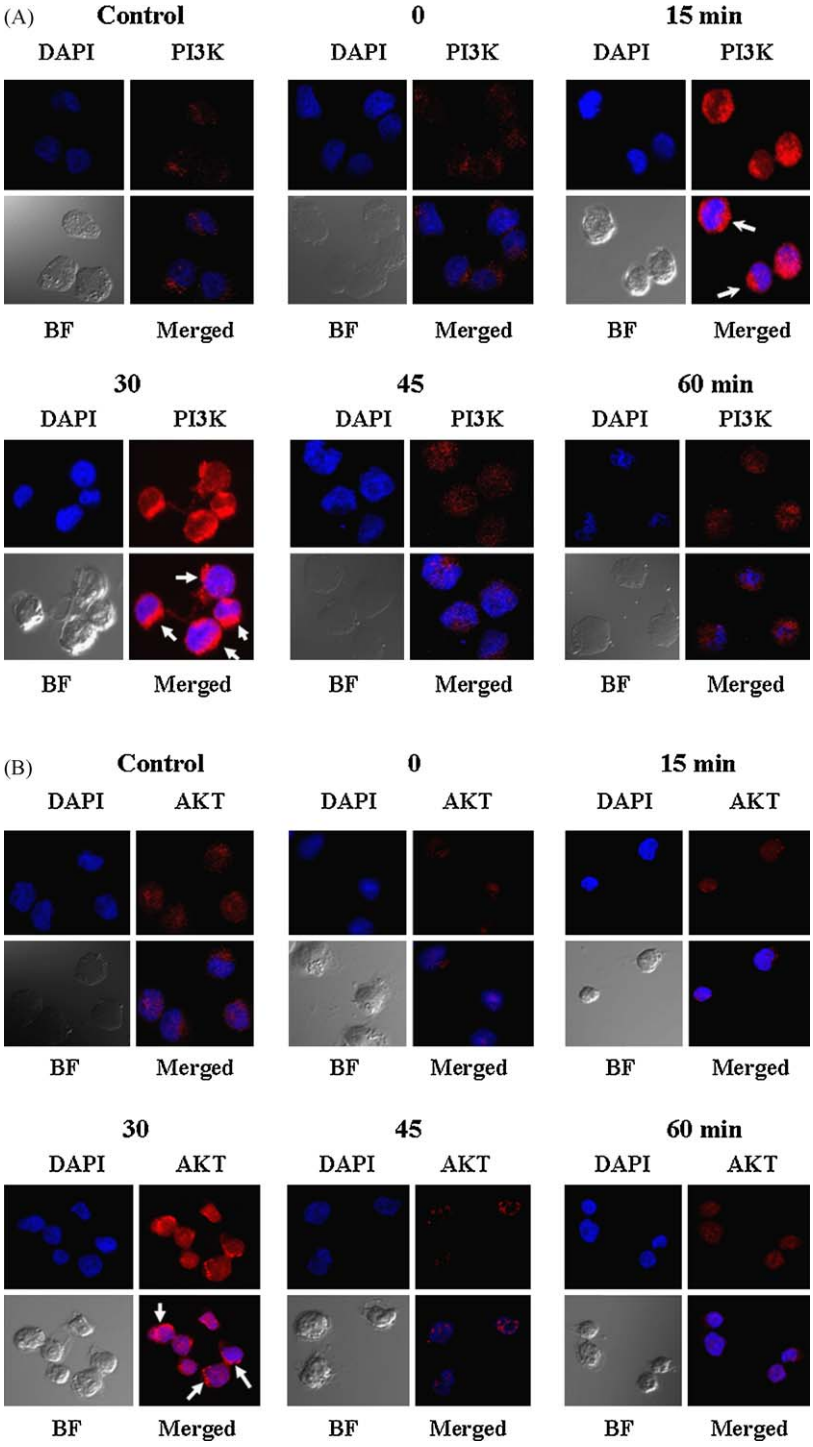

Fig. 3. Effect of morphine on membrane translocation of phosphorylated PI3K (A) and AKT (B). CEM ×174 cells were treated with morphine (10 μM) for 0, 15, 30, 45 and 60 min. Translocation of PI3K and AKT were observed and images were captured under laser confocal microscopy at various treatment time points. Nuclei were stained with DAPI (blue). PI3K and AKT were labeled with FRITC (red). The image is representative of three independent experiments. BF represents bright field. The arrows show the membrane congregation and localization of PI3K and AKT in CEM ×174 cells.

activation and subsequent onward signal transmission, the intracellular localization of PI3K and AKT were analyzed with laser confocal microscopy. Under laser confocal microscopy, PI3K was found to congregate and localize in the membrane of CEM ×174 cells during the 15–30 min of morphine treatment (Fig. 3A). Congregation and translocalization of AKT in the membrane of CEM ×174 cells was also apparent 30 min after morphine administration (Fig. 3B). The effect of morphine on membrane recruitment of PI3K and AKT was abolished by naloxone (data not shown). The morphine-induced changes in activation of PI3K and AKT were sustained for only a relative short time.

3.3. Observation of the level and interaction of PTEN and p53 proteins

In view of the effect of PTEN on negative regulation of PI3K activity and the stabilization of p53, the expression of PTEN and p53 and their interaction was assessed by Western blotting and CoIP assay. The data showed that the level of PTEN was moderately diminished 12 h after morphine treatment (Fig. 4A). The effect of morphine was more apparent on the level of p53 protein which was constitutively down-regulated during the treatment time period from 12 to 16 h. The interaction between PTEN and p53 was completely disrupted 12 h after morphine treatment (Fig. 4B). These results indicated that morphine promoted PI3K and AKT activity through destabilization of PTEN and p53, and consequently favored onward signal transmission.

3.4. Effect of morphine on translocation of transcription factor E2F1

Transcription factor E2F1, which is one of downstream effectors of PI3K/AKT, is a potential regulator of the expression of the MOR gene via binding to its element in the promoter region. Thus, E2F1 protein levels were analyzed for changes induced by morphine. Western blotting showed that morphine led to gradual elevation of cytoplasmic E2F1 during morphine treatment (Fig. 5A). The effect of morphine on elevation of E2F1 protein was abolished by naloxone and LY294002. At the same time, morphine-induced up-regulation of cytoplasmic E2F1. E2F1 was also translocated into the nucleus from the cytoplasm, which was particularly apparent at hours 8 and 16 of morphine treatment, and this effect was abolished by LY294002 (Fig. 5B).

3.5. Analysis of morphine-induced change on the interaction between Sp1, YY1 and E2F1

Our previous work has verified that there is an interaction between transcription factors Sp1 and YY1 in initiation of MOR gene transcription [1]. In view of the location of the E2F1 element adjacent to the Sp1 and YY1 elements, their interaction was analyzed by CoIP assay. Sp1-E2F1 and YY1-E2F1 protein complexes were immunoprecipitated with antibodies against Sp1, YY1 or E2F1 and then analyzed by Western blotting. The results showed that interaction between E2F1 and YY1 was undetectable at 0 h after morphine treatment, but was apparent at 12 h (Fig. 6). The result was similar for complexes immunoprecipitated with either E2F1 antibody or YY1 antibody. However, the interaction between E2F1 and Sp1 was detectable at all treatment time points and unaffected by morphine treatment. These results indicated that morphine promoted the translocation of E2F1 into the nucleus thus allowing its interaction with YY1, which is an important event in morphine-triggered initiation of the MOR gene expression.

3.6. Evaluation of the capability of Sp1, YY1 and E2F1 for binding to the MOR promoter

The capability of these transcription factors for binding to their elements in the MOR promoter region was further evaluated by EMSA and sEMSA. The results showed that all three Sp1-, YY1- and E2F1-complexes could be visualized. Addition of antibodies against Sp1, YY1 and E2F1 resulted in retardation of complex

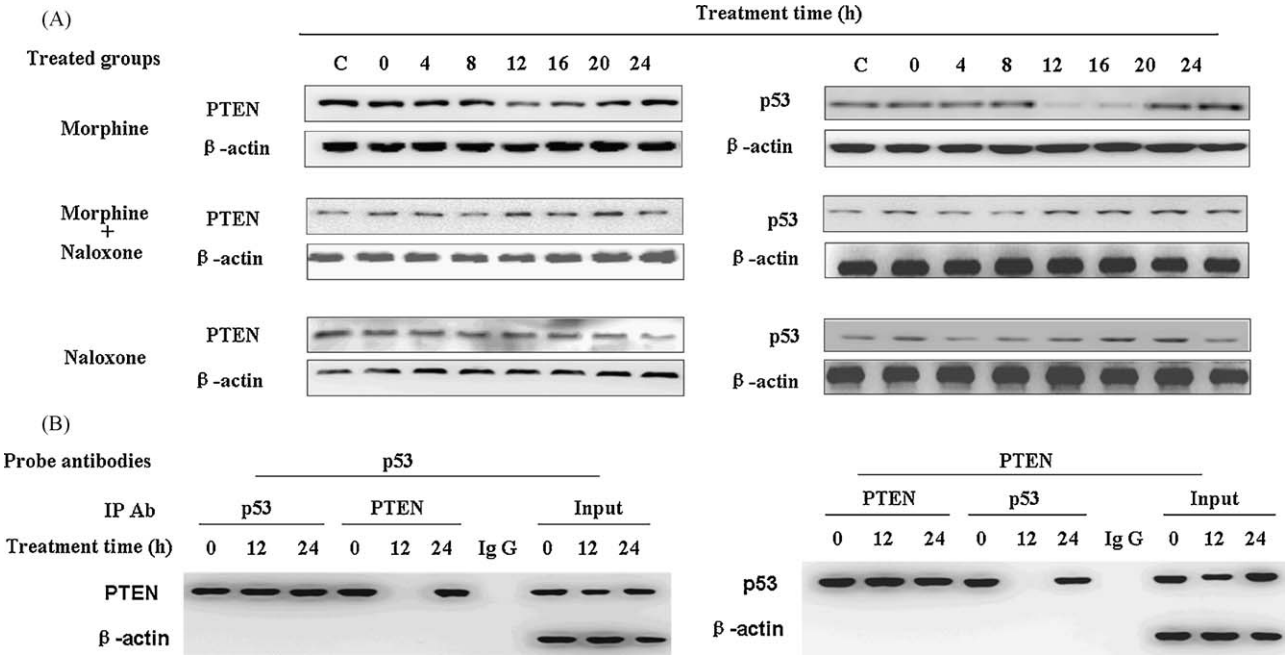

Fig. 4. Evaluation of the effect of morphine on expression of PTEN and p53, and their interaction. (A) CEM ×174 cells were treated with morphine (10 μM) for 0, 4, 8, 12, 16, 20 and 24 h. The level of PTEN and p53 were analyzed by Western blotting. 10 μM naloxone was used to antagonize the effect of morphine. (B) CoIP was used for evaluation of the interaction between PTEN and p53 in CEM ×174 cells treated with 10 μM morphine. Lysates from CEM ×174 cells treated with morphine for 0, 12 and 24 h were immunoprecipitated (IP) with antibodies against PTEN or p53 protein, or non-immune rabbit IgG (as indicated above each lane) and separated on an SDS/PAGE gel. Coimmunoprecipitated complexes were transferred to a nitrocellulose membrane, immunoblotted with anti-TPEN or anti-p53 antibody and X-ray film was exposed. Input represents coimmunoprecipitated PTEN–p53 complex with protein A-Sepharose beads precoated with non-immune rabbit IgG and probed with antibody to PTEN or p53. Images are representative of three repetitions of each experiment.

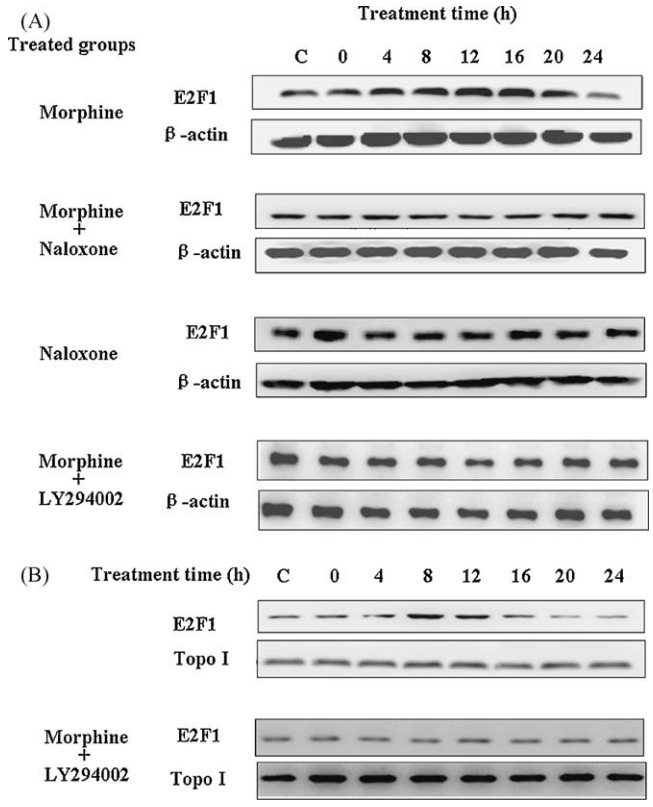

Fig. 5. Effect of morphine on expression and translocation of E2F1. CEM ×174 cells were treated with morphine (10 μM) for 0, 4, 8, 12, 16, 20 and 24 h. The levels of E2F1 in the cytoplasm (A) and nuclei (B) were analyzed by Western blotting. 10 μM naloxone was used to antagonize the effect of morphine. 1.4 μM LY294002 was used to inhibit PI3K. β-Actin and Topo I were used as loading controls for lysates from cytoplasm and nuclei respectively.

formation. These results demonstrated the capability of these proteins for binding to their elements, thus showing potential for regulation of expression of the MOR gene (Fig. 7A).

3.7. Impact of morphine on the capacity of E2F1 binding to the MOR promoter

The ChIP-qPCR assay was used to analyze the effect of morphine on the binding of E2F1 to the MOR promoter. The ChIP assay was performed to evaluate the binding of E2F1 to the MOR promoter. The level of E2F1 binding to its element was analyzed by pPCR. As shown in Fig. 5A, the level of E2F1 protein was remarkably elevated during morphine treatment and as a result the binding of E2F1 to the MOR promoter was also elevated at 12 h after morphine treatment (Fig. 7B). Both naloxone and LY294002 were able to abolish the effect of morphine. This result combined with the data in Figs. 5–7 further confirmed the involvement of E2F1 in morphine-triggered activation of PI3K/AKT signaling. Interruption of PI3K/AKT signaling either by a MOR antagonist, or by an inhibitor of AKT may lead to failure of E2F1 binding to the promoter region, with consequent depression of MOR gene expression.

4. Discussion

Earlier work regarding the immunological role of morphine was focused mainly on observation of the immunological effects of morphine. In humans MOR is the major molecular target of morphine and has been demonstrated to be expressed in immune cells. It has been shown that opioid receptors participate in the functioning of immune cells, with evidence suggesting that opioids modulate both innate and acquired immune responses, and this has been implicated as a cofactor in HIV infections leading to AIDS [7,8,22–24].

Nevertheless, the observations above have been derived mainly from pharmacological and immunological experiments, and the mechanism of expression of the MOR gene in lymphocytes at the level of transcription had not previously been worked out. Although there has been progress in the functional characterization of the mu opioid receptor gene 5′-flanking region in neural cells, neural cells and lymphocytes show substantial differences. For example, there is only one transcription initiation site in lymphocytes, rather than the multiple transcription initiation sites found in neural cells [4,25–28]. The cooperative interaction of Sp1 and YY1 transcription factors by binding to their elements in the promoter is the critical event triggering the initiation of transcription of the MOR gene in lymphocytes [1]. Although morphine-induced signaling has been widely investigated in neurons, the precise mechanism of morphine-triggered MOR expression in lymphocytes has been unclear up to this point. Previous studies showed that adenylyl cyclase was inhibited by the opioid receptor. In immune cells derived from multiple sources, the cAMP level and the activity of PKA were altered by opioid administration [29]. However, the information available from the limited number of studies which had been done was insufficient to account for the complicated regulation of MOR gene expression.

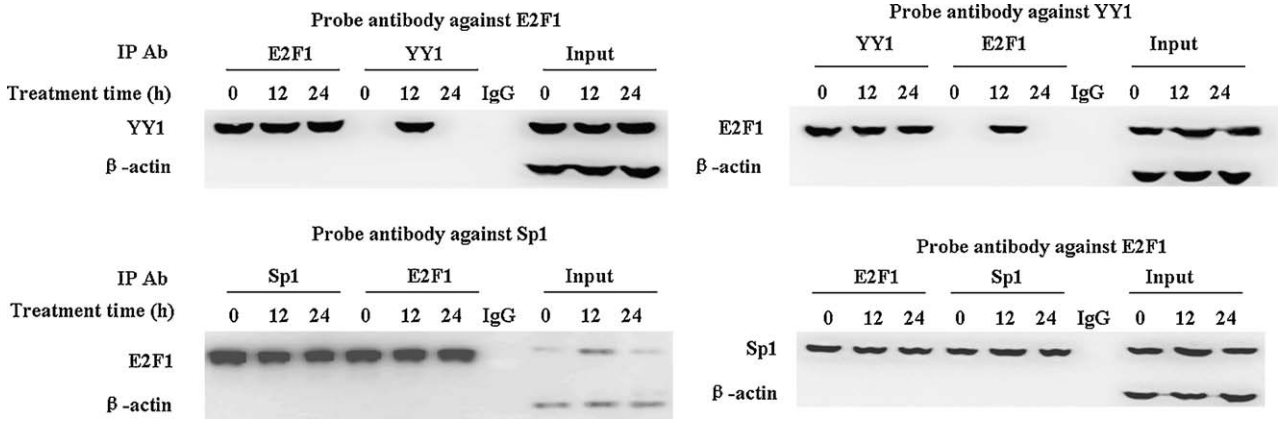

Fig. 6. Interaction of transcription factor E2F1 with Sp1 and YY1. CoIP was used for evaluation of the interaction between E2F1, Sp1 and YY1 in CEM ×174 cells treated with morphine. Lysates from CEM ×174 cells treated with morphine for 0, 12 and 24 h were immunoprecipitated (IP) with antibodies against E2F1, Sp1 or YY1 protein, or non-immune rabbit IgG (as indicated above each lane) and separated on an SDS/PAGE gel. Coimmunoprecipitated complexes were transferred to a nitrocellulose membrane, immunoblotted with anti-E2F1, -Sp1 or -YY1 antibody and X-ray film was exposed. Input represents coimmunoprecipitated PTEN–p53 complex with protein A-Sepharose beads precoated with non-immune rabbit IgG and probed with antibodies to E2F1, Sp1 or YY1. Images are representative of three repetitions of this each experiment.

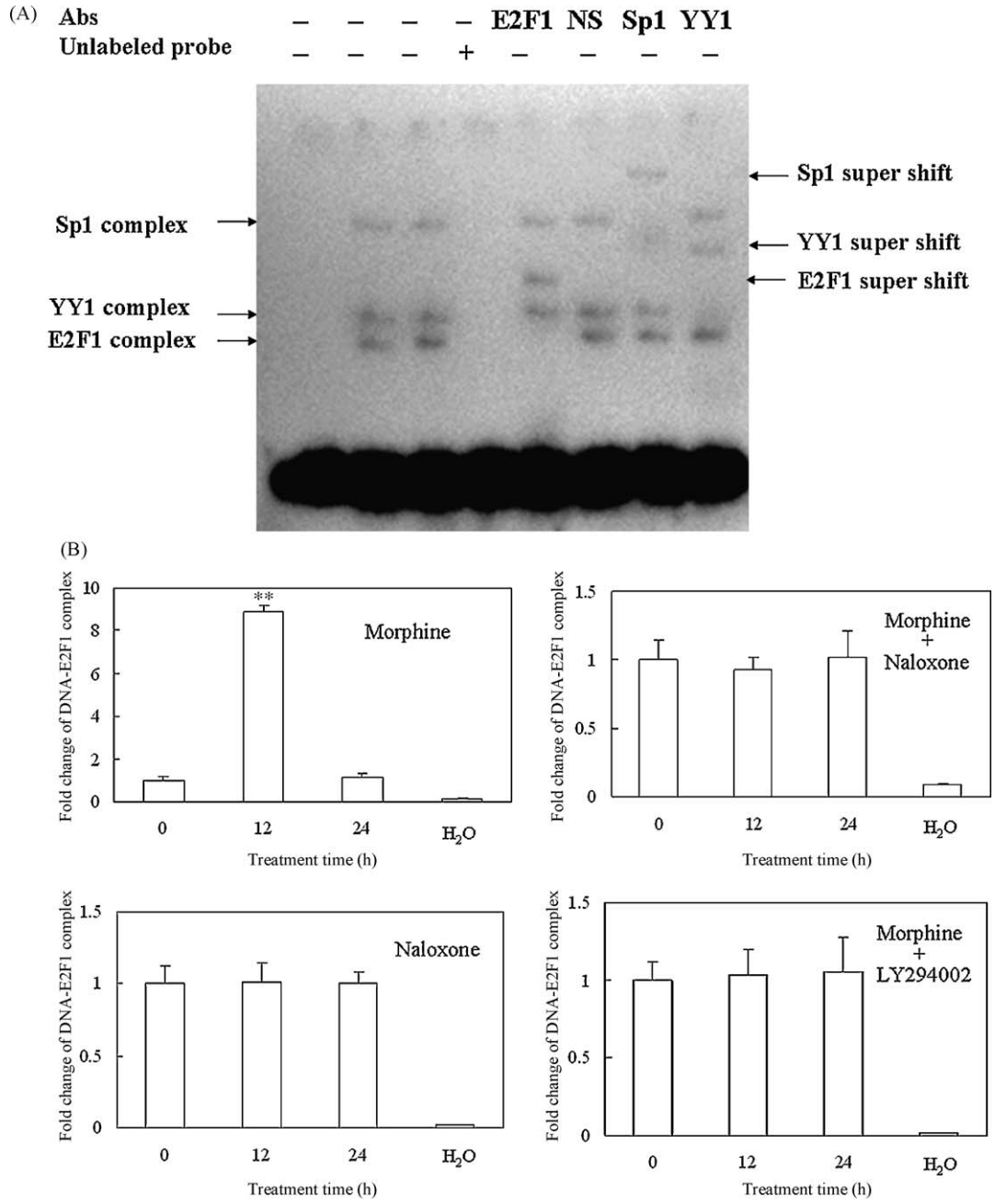

Fig. 7. Evaluation of the effect of morphine on binding of E2F1 to the promoter of the MOR gene. (A) EMSA and sEMSA were used to analyze the binding of E2F1, Sp1 and YY1 to their elements. Each complex is indicated by an arrow. The image is representative of an experiment that was repeated at least three times. (B) ChIP was used for analysis of E2F1 binding to the lymphocyte MOR promoter region at morphine treatment time points 12 and 24 h. 10 μM naloxone was used to antagonize the effect of morphine. 1.4 μM LY294002 was used to inhibit PI3K. $^{**}P < 0.01$ is considered statistically significant compared with treatment time at 0 h.

Taking all aspects of MOR gene expression in human lymphocytes into consideration, the details of regulation have been one of the least well understood features of this mechanism.

PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit that regulate a variety of cell responses, including cell division and survival. Some evidence has also been presented for the coupling of PI3K to GPCRs [30]. Moreover, overwhelming evidence suggests that the effects of morphine on the immune system are achieved through MOR, which is a member of the GPCR super-family. Recent studies have shown that morphine-induced reactions are dependent on the activation of PI3K/AKT in neural cells [11,13]. Under resting conditions, the p85 regulatory subunit serves to both stabilize and inactivate the p110 catalytic subunit. The inhibitory activity of p85 is released by occupancy of the NH_2-terminal SH2 domain of p85 by the phosphorylated tyrosine 688 [20]. In the current study, it was interesting to find that morphine stimulation was able to increase PI3K activity through phosphorylation at p85-Tyr458 based on results of Western blotting. AKT is one of the proteins recruited to the membrane by PIP3 where it is activated by other PIP3-activated proteins. Changes in PI3K and AKT activity were coincident with their congregation on the membrane, a process that we have shown can be reversed both by naloxone which is an antagonist of morphine, and LY294002, a specific inhibiter of PI3K.

Transcription factor E2F1 is a downstream effecter of AKT which operates via direct phosphorylation of E2F1 and indirect inhibition of TopBP1 [17,18]. With respect to the MOR gene, the elements for E2F1, as well as the elements for Sp1 and YY1, were

sequenced in the proximal promoter, and the interaction of these proteins in this region was crucial for initiation of MOR gene transcription [1]. The involvement of E2F1 in the expression of the MOR gene was confirmed by observation of the entrance of E2F1 into the nucleus following morphine exposure, this effect was abolished by administration of LY294002, indicating that this pathway is mediated by PI3K/AKT. Furthermore, our data showed that the effect of morphine on E2F1 functioning was achieved through two mechanisms: (1) promotion of the nucleus entrance of E2F1 and subsequent its interaction with YY1 and (2) enhancement of E2F1 gene expression. Thus, enhanced E2F1 interacted directly with Sp1 and indirectly with YY1 to bind the promoter as an integral part of PI3K/AKT signaling, bringing about initiation of MOR gene transcription. The enhanced level of E2F1 due to morphine exposure will further favor the expression of the MOR gene.

It is known that mechanisms of PTEN mediated responses include association of PTEN with p53 which increases stability of p53, and results in increased p53 protein levels and transcriptional activity [31,32]. Reduced levels of p53 will down-regulate PTEN levels allowing activation of AKT [33]. Since cooperation of p53 and PTEN is an essential factor for balancing the regulation of PI3K/AKT activation, the reduction of p53 12 h after morphine treatment as shown in the current study had a positive effect on onward transmission of the PI3K/AKT signal. In addition, the interaction between PTEN and p53 was thereby interrupted, which further favored PI3K/AKT activation.

Taken together, the data presented in this study serve to demonstrate that involvement of PI3K/AKT is necessary in morphine-induced initiation of MOR gene transcription. Although many other signal molecules have also been found to be involved in the regulation of the MOR gene, it is possible that PI3K/AKT signaling is the major pathway among several pathways in this mechanism. The pharmacological role of morphine is far from being completely explained by demonstration of the role of this one single pathway, however, these results will be beneficial for better understanding of the mechanisms involved.

Acknowledgments

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Appendix A. Supplementary data

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