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Effects of Dopamine and Haloperidol on Morphine-induced CREB and AP-1 DNA Binding Activities in Differentiated SH-SY5Y Human Neuroblastoma Cells

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In the present study, we first examined whether the changes in the DNA binding activities of the transcription factors, cAMP response element binding protein (CREB) and activator protein-1 (AP-1) mediate the long-term effects of morphine in differentiated SH-SY5Y human neuroblastoma cells. The increases in CREB and AP-1 DNA binding activities were time-dependent up to 6 days of morphine treatment (1, 4, and 6 days). However, the significant reduction in the DNA binding activities of CREB and AP-1 was observed after 10 days of chronic morphine (10 μM) administration. Secondly, we examined whether the changes of CREB and AP-1 DNA binding activities could be modulated by dopamine and haloperidol. Dopamine cotreatment moderately increased the levels of the CREB and AP-1 DNA binding activities induced by 10 days of chronic morphine treatment, and haloperidol cotreatment also resulted in a moderate increase of the CREB and AP-1 DNA binding activities. However, dopamine or haloperidol only treatment showed a significant increase or decrease of the CREB and AP-1 DNA binding activities, respectively. In the case of acute morphine treatment, the CREB and AP-1 DNA binding activities were shown to decrease in a time-dependent manner (30, 60, 90, and 120 min). Taken these together, in differentiated SH-SY5Y cells, morphine tolerance seems to involve simultaneous changes of the CREB and AP-1 DNA binding activities. Our data also suggest the possible involvement of haloperidol in prevention or reversal of morphine tolerance at the transcriptional level.

Key Words: CREB, AP-1, Morphine tolerance, SH-SY5Y Cells

INTRODUCTION

Recent research efforts have proven that the neurobiological mechanisms underlying opiate action are mediated at the level of gene expression (Nestler et al, 1993). Human neuroblastoma SH-SY5Y cell line (Ross et al, 1983), derived by Biedler et al (1973) from SK-N-SH line, expresses both *mu* and *delta* receptors. SH-SY5Y cells differentiate in the presence of retinoic acid (RA) (Pahlman et al, 1984), dibutyryl cyclic AMP (dBcAMP) (Schulze & Perez-Polo, 1982), nerve growth factor (NGF) (Schulze & Perez-Polo, 1982; Spinelli et al, 1982), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Schulze & Perez-Polo, 1982). The differentiated SH-SY5Y cells predominantly express *mu* receptors and fewer *delta* opioid receptors in a ratio of 5 to 1 (Kazmi & Mishra, 1986; Yu et al, 1986). Although pharmacological and biochemical actions are exhibited *via* multiple opioid receptor subtypes, considerable evidence suggests that *mu* opioid receptors play a key role in mediating the analgesic effect of narcotics (Rosenbaum et al, 1984; Carr et al, 1984). However, the mechanisms underlying opioid tolerance and dependence remain elusive because of a lack of well defined experimental

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models. The SH-SY5Y cells have been estabilished as a suitable model system for studying the biochemical correlation of narcotic analgesic efficacy and tolerance (Yu et al, 1986; Yu & Sadee, 1988).

We focused first on the activities of the transcription factors, cyclic AMP response element binding protein (CREB) and activator protein-1 (AP-1), which can regulate downstream gene expression *via* cAMP and dimers of *fos* and *jun* proteins upon chronic morphine treatment. CREB has been shown to be involved in important brain functions including the action mechanism of drugs of abuse (Gutart et al, 1992; Nestler, 1992; Nestler et al, 1993; Widnell et al, 1994), process of learning and memory (Bourtchuladze et al, 1994; Yin et al, 1994), and circadian rhythms (Ginty et al, 1993). The biochemical changes that accompany drug abuse are mediated, at least in part, by long-term changes in neuronal gene expression. Dopamine is a possible candidate to transmit the reinforcement properties of drugs of abuse (Acquas et al, 1991; Koob, 1992; Steiner & Gerfen, 1995). Therefore, we secondly examined the effects of dopamine and haloperidol on the changes of CREB and AP-1 DNA binding activities upon induction of morphine tolerance.

In this study, we demonstrated that chronic administration of morphine induces a decrease in CREB and AP-1 DNA binding activities. This reduction in the CREB and AP-1 binding activities were shown to be reversed by haloperidol. Our data indicate that morphine tolerance might be a neural plasticity mediated by the decreased gene expression *via* regulation of CREB and AP-1 transcription factors, and might be prevented by haloperidol.

METHODS

Cell culture

SH-SY5Y human neuroblastoma cells were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2, humidified atmosphere. SH-SY5Y cells at low density were initiated and differentiated with 10 μM of all *trans*-retinoic acid (RA; Sigma, USA) for 6 to 10 days until formation of monolayer confluence.

Drug treatment

All experiments were conducted in culture medium containing 5% FBS. Medium was replaced with fresh medium before each treatment. For acute morphine treatment, 20 μM of morphine (Jeil pharm. company, Korea) was added to the culture medium for 30, 60, 90, and 120 minutes. For induction of morphine tolerance, SH-SY5Y cells were exposed to 10 μM of morphine for 12 hours a day for 1 to 10 days. Dopamine (100 μM for 1 hr; Sigma, USA) or haloperidol (100 μM for 1 hr; Sigma, USA) were added to the cells that had been treated with morphine for 10 days.

Preparation of nuclear protein extracts

Cells were washed three times in ice-cold phosphate-buffered saline and harvested on ice. Cell pellets were resuspended in 5 volumes of buffer A [10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF], and were incubated on ice for 10 minutes. The subsequent centrifugation at $300\times g$ for 10 minutes, 4°C was done. The cell pellets were resuspended in 3 volumes of modified buffer A [buffer A+0.05% IGEPAL CA-630 (Sigma, USA)]. Cells were homogenized with 20 strokes of a tight-fitting Dounce homogenizer to release the nuclei. And then cells were centrifuged at $300\times g$ for 10 min, 4°C to pellet the nuclei. The pellet was resuspended in buffer C [5 mM Hepes (pH 7.9), 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.3 mM NaCl] and mixed well by inversion. After incubation on ice for 30 minutes cells were centrifuged at $24,000\times g$ for 20 min, 4°C. The extracts were aliquoted and stored at −70°C until the time of use. Protein concentration was determined with Bicinchoninic acid (BCA) protein assay kit from Sigma, USA.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using nuclear extracts prepared from RA-induced SH-SY5Y cells. Consensus oligonucleotides (CREB and AP-1; Promega, USA) were [γ-^{32}P] ATP (Amersham, USA) radiolabeled with T4 polynucleotide kinase for 1 hour at 37°C. Nuclear extracts prepared from control or stimulated cells (5 μg) were incubated with 10,000 cpm of the radiolabeled oligonucleotides for 20 min at room

temperature in a 5X binding buffer [20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5)]. One microgram of poly (dI-dC) · poly (dI-dC) (Pharmacia Biotech) was added as competitor for nonspecific DNA-protein interaction in each reaction. The reaction mixture was subjected to electrophoresis on a nondenaturing 6% polyacrylamide gel. Gels were run at 100 V in 0.5 X TBE buffer, dried, and autoradiographed. For a specific competition study, a 100-fold molar excess of unlabeled CREB or AP-1 probe was used, respectively. The oligonucleotide sequence of CREB was 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ and that of AP-1 was 5′-CGCTTGATGAGTCAGCCGGAA-3′.

RESULTS

Time course of CREB and AP-1 binding activities by morphine

When the differentiated SH-SY5Y human neuroblastoma cells were treated with 20 μM of morphine, the increase in CREB and AP-1 binding activities was observed at 30 min of morphine treatment, but it was gradually attenuated at 60, 90, and 120 min time points compared with control group (Fig. 1). The chronic exposure to morphine for 1, 4, and 6 days resulted in a time-dependent increase in the CREB and AP-1 DNA binding activities (Fig. 2). However, the chronic morphine treatment of 10 days reduced the CREB and AP-1 DNA binding activities in comparison with those of 1, 4, and 6 days of mor-

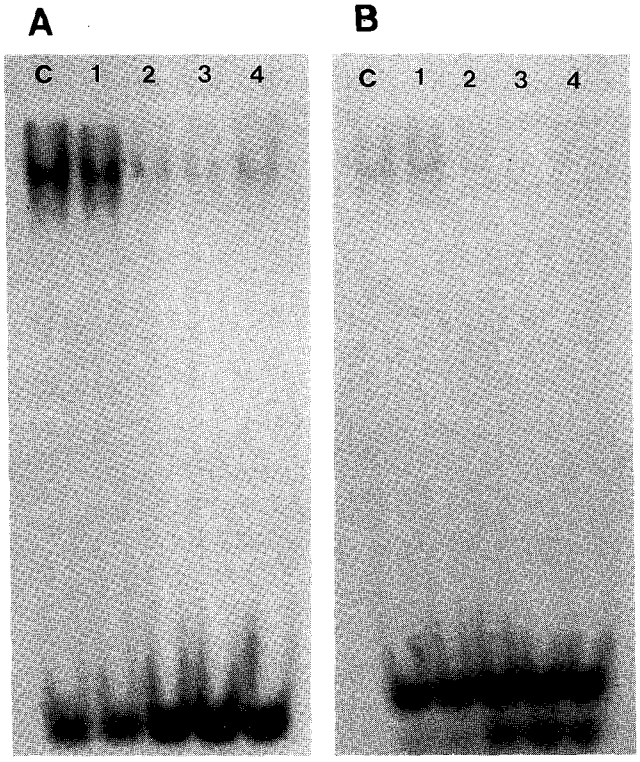

Fig. 1. EMSA showing the effects of acute morphine treatment on CREB (A) and AP-1 (B) DNA binding activities in SH-SY5Y human neuroblastoma cells differentiated with RA for 6 days. Lane C, SH-SY5Y human neuroblastoma cells differentiated with RA for 6 days; Lane 1, 30 min; Lane 2, 60 min; Lane 3, 90 min; Lane 4, 120 min exposure to 20 μM of morphine. These data are representative of at least three sets of individual experiments.

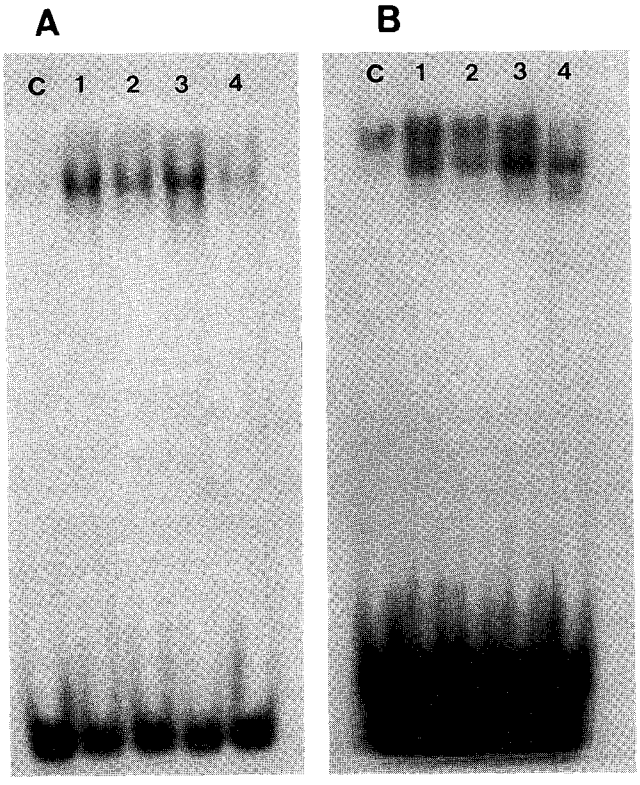

Fig. 2. EMSA showing the time course of CREB (A) and AP-1 (B) DNA binding activities upon chronic morphine administration in SH-SY5Y human neuroblastoma cells differentiated with RA for 10 days. Lane C, SH-SY5Y human neuroblastoma cells differentiated with RA for 10 days; Lane 1, 1 day; Lane 2, 4 days; Lane 3, 6 days; Lane 4, 10 days exposure to 10 μM of morphine for 12 hours daily.

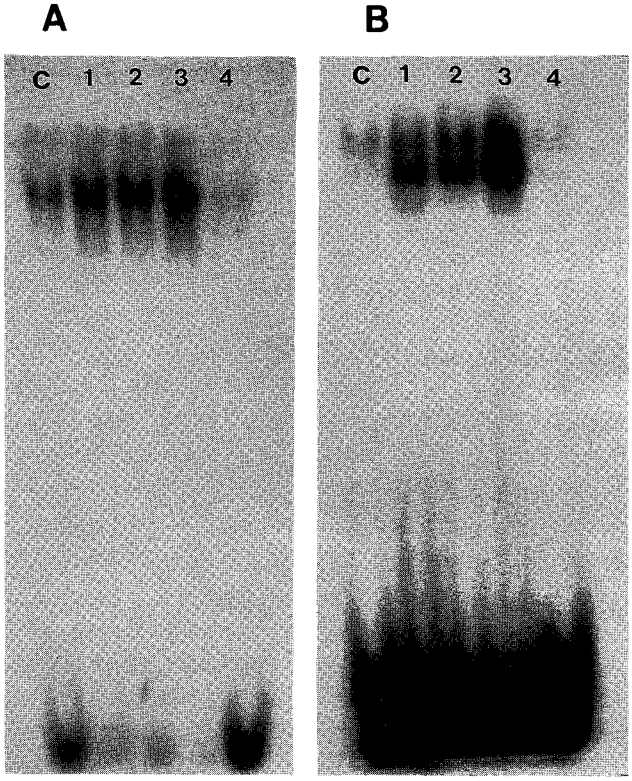

Fig. 3. EMSA showing the effects of dopamine (DA) and haloperidol (HAL) on CREB (A) and AP-1 (B) DNA binding activities upon chronic morphine administration. Lane C, SH-SY5Y human neuroblastoma cells differentiated with RA for 10 days; Lane 1, morphine + DA; Lane 2, morphine + HAL; Lane 3, DA only; Lane 4, HAL only. SH-SY5Y cells were exposed to 10 μM of morphine for 12 hours a day for 10 days. DA (100 μM for 1 hr) or HAL (100 μM for 1 hr) were added to the cells that had been treated with morphine for 10 days.

phine induction (Fig. 2A, lane 4 and Fig. 2B, lane 4).

Effects of dopamine and haloperidol on the CREB and AP-1 binding activities upon chronic morphine treatment

Dopamine cotreatment, the addition of 100 μM of dopamine to the cells that had been under repeated exposure to morphine for 10 days, increased the CREB DNA binding activity exerted by chronic morphine administration (Fig. 3A). When the differentiated SH-SY5Y cells were treated with dopamine only, our data showed CREB binding activity was also increased (Fig. 3A, lane 3). On the other hand, although 100 μM of haloperidol cotreatment increased the CREB binding activity haloperidol only treatment resulted in an inhibition of CREB DNA binding activity (Fig. 3A, lane 2). The similar patterns were obtained regarding the effects of dopamine and haloperidol on the changes of AP-1 DNA binding activity (Fig. 3B).

DISCUSSION

Administration of a drug initiates transient acute physiological effects on intracellular messengers, and also initiates a set of gene transcription leading to posttranslational changes of transcription factors and associated proteins. Here we report the corelated simultaneous changes of CREB and AP-1 DNA binding activities upon induction of morphine tolerance. In this study, we first examined the CREB and AP-1 binding activities at various time points of 30, 60, 90, and 120 min after acute morphine treatment. And the long-term effects of morphine on the CREB and AP-1 DNA binding activities were investigated at 1, 4, 6, and 10 days of exposure to morphine.

Since CREB is phosphorylated by cAMP-dependent protein kinase, regulation of CREB is consistent with the known effects of opiates on the activity of the cAMP system. Many studies have reported the elevation of cAMP upon withdrawal or precipitation by an opioid antagonist in differentiated SH-SY5Y cells after a prolonged treatment with morphine and other selective μ opioid receptor agonists (Yu et al, 1990; Carter & Medzihradsky, 1993). We previously have reported the increase of cAMP levels following abrupt cessation after chronic morphine treatment for 6 days in differentiated SH-SY5Y cells (Kim et al, 1997). Regulations of CREB and cAMP system by morphine have proven to occur in the specific brain regions, locus coeruleus (LC), the major noradrenergic nucleus in brain (Guitart et al, 1992; Nestler, 1992; Nestler et al, 1993; Widnell et al, 1994) and nucleus accumbens (NA), a dopaminergic nucleus (Widnell et al, 1996). The LC is closely involved in physical opioid dependence, but plays little role in opiate reinforcement (Rasmussen et al, 1990; Koob, 1992; Nestler, 1992; Nestler et al, 1993; Maldonado & Koob, 1993). On the other hand, the NA mediates the reinforcement of many abused drugs such as opiates, cocaine, amphetamine, ethanol, nicotine, cannabinoids, but plays little role in physical dependence

(Kuhar et al, 1991; Koob, 1992; Nestler, 1992; Nestler et al, 1993). Many animal studies have reported that acute morphine administration decreases the extent of phosphorylation of CREB, and it returns to control level upon chronic exposure to morphine (implantation of 75 mg of morphine pellets for 5 days) in LC (Guitart et al, 1992; Nestler, 1992; Nestler et al, 1993; Widnell et al, 1994) and NA (Widnell et al, 1996). In contrast, chronic morphine treatment increases the levels of adenylyl cyclase, cAMP-dependent protein kinase, and CREB in the LC (Duman et al, 1988; Nestler & Tallman, 1988; Gutart et al, 1992; Nestler, 1992; Nestler et al, 1993; Widnell et al, 1994). Recent experiments support that CREB is responsible for mediating the morphine-induced upregulation of specific components of the cAMP system, type 1 and type VIII adenylyl cyclase, in the LC (Matsuoka et al, 1994; Lane-Ladd et al, 1997). According to our data, the decreases in the CREB and AP-1 DNA binding activities were observed upon 10 days of chronic morphine treatment implying the induction of morphine tolerance (Fig. 2).

Most of the studies on the opiate regulation of gene expression have focused on gene products related to the opioid receptor system. AP-1 mediated gene expression via *fos* and *jun* proteins is regulated in response to morphine dependence. Morphine treatment has been shown to induce region-specific expression of *fos* and *jun* genes in brain. Our unpublished data indicated a marked reduction of *c-fos* mRNA levels by chronic morphine treatment of 10 days. The present study revealed inhibition on CREB and AP-1 binding activities upon 10 days of morphine treatment in SH-SY5Y cells (Fig. 3). Thus, we could draw a conclusion that morphine tolerance can be characterized as a simultaneous and synergistic phenomenon mediated through direct gene products related to the opioid receptor system as well as transcriptional regulation of downstream gene expressions.

The mesolimbic dopaminergic system has been implicated as an important neural system on the reinforcing properties of abused drugs including cocaine, amphetamine, ethanol, nicotine and cannabinoids. The biochemical adaptation of these drugs of abuse has also been a major focus of recent research efforts, especially mesolimbic dopaminergic system. According to the data obtained from this study, haloperidol seemed to reverse the reduction of CREB and AP-1 DNA binding activities exerted by chronic morphine administration (Fig. 3).

In conclusion, chronic morphine treatment induced a decrease in the CREB and AP-1 DNA binding activities implying the morphine tolerance phenomenon in differentiated SH-SY5Y cells. This reduction was restored by haloperidol cotreatment. These findings suggest that morphine tolerance may be a phenomenon, which results in the alteration of the signal transcription pathways involving CREB and AP-1 transcription factors. The observed DNA binding activities of these transcription factors presumably reflect more complex changes than merely increasing the transcriptional activity. CREB and AP-1 DNA binding activities might be directly related to morphine-induced gene expression, and morphine tolerance could be prevented by haloperidol. Furthermore, the specific binding sites on these transcription factors could be plausible targets of the development of pharmacological agents that prevent or reverse the actions of morphine tolerance on specific target neurons.

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