—— **Original Article** ——

Lithium Chloride Protects PC12 Pheochromocytoma Cell Line from Morphine-Induced Apoptosis

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Background: Opioid drugs are considered as important members of drugs of abuse. Opioid abusers are more likely to be infected which may be due to apoptotic effects of the drugs on immune cells. Furthermore, there are some reports on the apoptotic effect of morphine on neural cells. In the present study, the effect of morphine and lithium on apoptosis in PC12 cell line (as a model of neural cells) was examined.

Methods: We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, annexin V-fluorescein isothiocyanate test and quantitative real time RT-polymerase chain reaction for detection of necrosis and apoptosis (programmed cell death).

Results: PC12 cells were exposed to different concentrations of morphine for six, 12, 24, 48, and 96 hours. Quantitative real-time RT-polymerase chain reaction revealed that mRNA expression of BAX (proapoptotic element) increased while a decrement in the mRNA expression of BCL-2 (protective element) was observed after six hours (but not after 12 or 24 hours) exposure to morphine. Furthermore, the results of MTT assay and annexin V-fluorescein isothiocyanate test indicated that morphine exposure causes an increase in the percentage of apoptotic and necrotic cells, respectively. Interestingly, the results of MTT assay and annexin V-fluorescein isothiocyanate test were observed 12 and 24 hours after morphine exposure. Thus, it can be concluded that alteration in mRNA expression is an early event rather than as a consequence of apoptosis or necrosis.

On the other hand, lower concentrations of lithium elicit protective effect against apoptosis in some of mammalian cells while the higher concentrations are toxic. Despite large body of evidences on the protective effect of lithium, elucidation of downstream events are still unknown. In the present study, 72-hour preincubation of PC12 cells with 1.2 mM lithium chloride reversed the effects of morphine on the mRNA expression of BAX and BCL-2. Furthermore, the results of real time RT-polymerase chain reaction were supported by annexin V-fluorescein isothiocyanate test and MTT assay.

Conclusion: The protective effect of lithium on the morphine-induced cytotoxicity is mediated via down-regulation of BAX and up-regulation of BCL-2 mRNA expression.

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Keywords: Annexin V test • apoptosis • lithium • mRNA expression • MTT assay

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Introduction

Apoptosis (programmed cell death) is a normal phase of cell homeostasis and considered as a physiologic form of cell death. Several apoptotic pathways have been identified which are activated by extrinsic or intrinsic stimuli.[1–3] Furthermore, apoptosis is characterized by cytoplasmic membrane blebbing,

cell shrinking, and DNA fragmentation into nucleosome-sized fragments and requires ATP or new biosynthesis of RNA and protein.[4]

Lithium chloride is a monovalent ion which has a variety of biochemical and physiologic properties.[5–8] Apparently, the cation exerts a dual effect on cellular proliferation. Some of investigations have shown the antiapoptotic property of lithium,[9–11] while others have indicated the pro-apoptotic effect.[12–15] The factors responsible for these opposite effects of lithium may be due to differences in the underlying genetic factors of different cell lines and the applied concentrations of the drug.[16,17]

On the other hand, opioid drugs are an important class of analgesics.[18–20] It seems that most cells of the immune system are susceptible to apoptosis induced by the drugs of abuse.[21–23] Furthermore, mu (μ) receptor agonists modulate neural and tumor cells survival and proliferation.[19,24–27] On the other hand, it has been shown that morphine improves cell growth and viability. According to the report of Meriney et al.[28] morphine prevents apoptosis of ciliary ganglion neurons which usually die during the period of embryogenesis synapse formation. Morphine also reverses peroxynitrite-induced apoptosis in different cell lines.[29,30]

In summary, despite of numerous investigations on the interrelationship between morphine and lithium, their interaction on apoptosis is still unclear. Thus, in the present study, the effect of low concentration of lithium (1.2 mM) was investigated against the morphine-induced apoptosis.

Materials and Methods

Drugs

The drugs used in this study were morphine sulfate (Temad Co., Tehran, Iran), and lithium chloride (Sigma-Aldrich Co., UK). Both drugs were dissolved in phosphate-buffered saline (PBS) and were added to cell culture. Both drugs were stocked at the highest concentration and then were diluted as needed.

Cell culture

PC12 cells were obtained from Pasteur Institute (Tehran, Iran), maintained and subcultured at 37°C in a humidified incubator containing 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. The concentration of glucose in DMEM was 4.5 mg/mL. The cells were used for the experiments at about 70% of confluency.[31] The cells were further incubated for six, 12, 24, 48, and 96 hours in DMEM containing 4% serum in the presence or absence of different concentrations of morphine and lithium. The use of culture medium with 4% serum concentration has previously been shown not to affect the viability PC12 cells.[32]

Drug treatment

For adding drugs to PC12 cell culture, serum was removed by washing the media by PBS (pH 7.4), then was replaced by DMEM containing serum 4% supplemented with different concentrations of morphine and lithium. The cells were then placed in the incubator at 37°C with 5% CO_2. The control cells were treated with equivalent concentrations of sodium chloride to control for changes in osmolarity.

Cell viability measurement by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay introduced by Mosmann[33] is used to determine the number of viable cells in culture. The assay is based on the cleavage of the yellow tetrazolium salt [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals by mitochondrial reductases. Briefly, the cells were plated into 96-well microtiter plates and exposed to different concentrations of morphine. Following morphine exposure, 10 μL of MTT labeling reagent was added to each well and the plates were incubated at 37°C for six hours. The cultures were then solubilized by PBS and the spectrophotometric absorbance of the samples was detected by a microtiter plate reader. The wavelength to measure the absorbance of formazan product was 570 nm, with a reference wavelength of 750 nm.[31]

Total RNA extraction

At first, PC12 cells were counted. Then, about 10^6 cells were collected and lyzed in guaninidium thiocyanate (GITC) and homogenized by Silent Crusher S (Hidolf) and were subjected for total RNA extraction by High Pure Tissue RNA Extraction Kit (Roche) as stated in the manufacturer's instructions. The quality of extracted RNA was checked on denatured agarose gel and quantified by Biophotometer (Eppendorf).

Reverse transcription

Reverse transcription was carried out by using 1 µL total RNA as template, 1 µL expand reverse transcriptase (Roche), 4 µL buffer, 1 µL dNTPs (10 mM), 1 µL DTT, and 1 µL oligo-(dT)$_{15}$ (20 pM) in a total volume of 20 µL at 42°C for 60 minutes.

Real-time polymerase chain reaction

The polymerase chain reaction (PCR) was used for amplification of cDNA for β-actin (as internal standard) BAX and BCL-2 separately by using the following primers:

β-actin forward primer: 5' TGA AGT ACC CCA TTG AAC ATG 3'.

β-actin reverse primer: 5' GAT GGC TAC GTA CAT GGC TG 3'

BAX forward primer: 5' GCA GGG AGG ATG GCT GGG GAG 3'

BAX reverse primer: 5' TCC AGA CAA GCA AGC AGC CGC TCA CG 3'

BCL-2 forward primer: 5' CAC CCC TGG CAT CTT CTC CT 3'

BCL-2 reverse primer: 5' GTT GAC GCT CCC CAC ACA CA 3'

PCRs were carried out in a reaction volume of 25 µL consisting of 20 pM of each primer, 2.5 IU Taq DNA polymerase (Roche), 5 µL cDNA, 0.5 µL dNTPs (10mM), 2.5 µL PCR buffer, 2.5 mM MgCl$_2$,and 1/10,000 SYBR®Green as fluorogenic dye. Thermal cycling was initiated with a first denaturation step of 94°C for three minutes and followed with the thermal profile as 94°C (20") +55°C (30") +72°C (40") for 40 cycles in a Stratagen real-time system.

Data analysis

A suitable threshold was applied on amplification plots and the resultant threshold cycles (Ct) values were used for relative quantification. The Ct values of BAX and BCL-2 were normalized regarding to the Ct values for β-actin and the resultant values were compared with respective control (saline-saline) group using $2^{-\Delta\Delta Ct}$ method.[34]

Annexin V affinity assay

A fluorescein isothiocyanate-conjugated (FITC) annexin V assay was used as a sensitive method of membrane disruption detection utilizing a fluorescent microscope according to Sigma company protocol. Apoptotic cells expose phosphatidyl-serine (PS) at their outer membrane early after the onset of the execution phase of the apoptosis until the final stage at which the cells have broken into apoptotic bodies. Phosphatidyl-binding protein annexin V, which can be coupled to biotin or fluorescein for visualization, is used for detection of apoptosis. Annexin V is usually used in combination with propidium iodide (PI) which stains cells with disrupted membrane integrity.

Statistical analysis

All calculations were performed by SPSS (version 10; SPSS Inc.). Differences among treatment groups were tested using one-way ANOVA and Student's *t*-test. $P<0.05$ was considered statistically significant.

Results

Detection of the effects of morphine and lithium on PC12 cell viability by MTT assay

At first, PC12 cells were exposed to different concentrations of lithium chloride (1.2, 2.4, 4.8, and 9.6 mM) or the same concentrations of sodium chloride (as the control group). Then, cell viability was measured by MTT assay at 36, 72, and 144 hours after the exposure. The results of this experiment showed that higher concentrations of lithium chloride (4.8 and 9.6 mM) decreased the cell viability. While, exposure of the cells to different concentrations of sodium chloride or the lower concentrations of lithium chloride (1.2 and 2.4 mM) did not affect the cell viability (Figure 1). In the present study, lithium chloride 1.2 mM was selected for the rest of the experiment. The application of lithium chloride 1.2 mM is therapeutically relevant and was previously reported by Tseng and Lin-Shiau.[17]

In the other parts of experiments, PC12 cells were exposed to different concentrations of morphine (0.2, 0.4, and 0.8 mM) and then cell viability was measured by MTT assay at six, 12, 24, 48, and 96 hours after morphine exposure. Morphine exposure for six or 12 hours did not change viability of the cells (data not shown). The results of exposure of PC12 cells to different concentrations of morphine for 24 hours are shown in Figure 2. Morphine exposure did not affect the number of vital cells after 24 hours ($P>0.05$). It is worth noting that 0.8 mM of morphine decreased the viability of the cells by almost 15% ($P>0.05$).

The results of 72 hours preincubation of PC12 cells with 1.2 mM of lithium chloride or sodium chloride followed by 48 hours exposure to
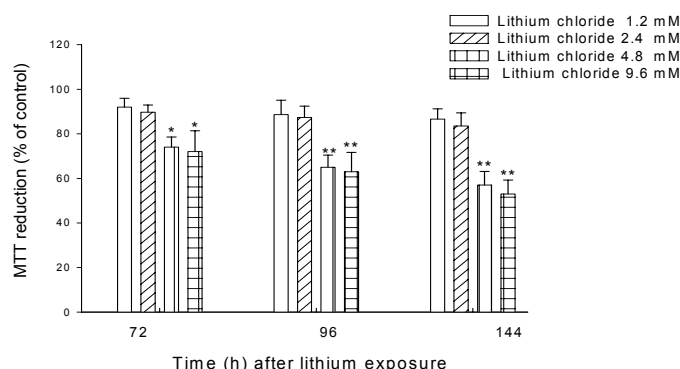
Figure 1. The effects of 72-, 96- and 144-hour exposure of different concentrations of lithium chloride on PC12 cell viability determined by MTT assay.
PC12 cells were treated with different concentrations of lithium chloride (1.2, 2.4, 4.8, and 9.6 mM) in 96-well plates for 72, 96 and 144 hours as described in materials and methods section. The viability of cells was expressed as the absorbent value at 570 nm. Data are presented as mean±SEM of 10 – 12 samples.
*P<0.05, **P<0.01 different from the respective saline control group.

different concentrations of morphine (0.2, 0.4, and 0.8 mM) are shown in Figure 3A. Morphine decreased cell viability (P<0.001) in a dose-dependent manner. Furthermore, 72 hours preincubation with lithium chloride (but not sodium chloride) significantly reduced the cell death rate induced by different concentrations of morphine (P<0.05).

The results of 72 hours preincubation of PC12 cells with 1.2 mM of lithium chloride or sodium chloride followed by 96 hours exposure to different concentrations of morphine (0.2, 0.4, and 0.8 mM) are presented in Figure 3B. Morphine decreased the number of vital cells (P<0.001) in a dose-dependent manner. Meanwhile, 72 hours pre-incubation with lithium chloride (but not sodium chloride) significantly attenuated the cell death rate induced by different concentrations of morphine (P<0.001).
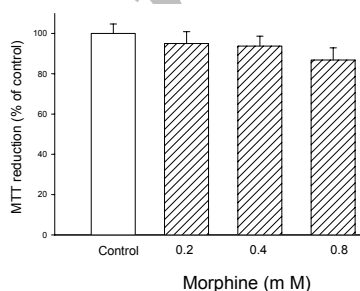

Figure 2. The effects of 24-hour exposure of different concentrations of morphine on PC12 cell viability determined by MTT assay.
PC12 cells were treated with different concentrations of morphine (0.2, 0.4, and 0.8 mM) in 96-well plates for 24 hours as described in materials and methods section. The viability of cells was expressed as the absorbent value at 570 nm. Data are presented as mean±SEM of 10 – 12 samples.

Detection of apoptosis induced by morphine and lithium in PC12 cell line by annexin V staining test

According to the results of MTT assay, the higher concentration of morphine (0.8 mM) was selected for the rest of experiments. Figure 4 shows the effects of morphine and lithium treatment on PC12 cells apoptosis. The cells were preincubated with 1.2 mM of lithium chloride or sodium chloride for 72 hours. Then, PC12 cells were exposed to 0.8 mM of sodium chloride or morphine for six, 12, and 24 hours. Administration of 0.8 mM of morphine for 12 and 24 hours (but not six hours) significantly increased the percentage of apoptotic cells compared with the control group (P<0.001). Furthermore, 72 hours preincubation of PC12 cells with lithium chloride (but not sodium chloride) decreased the percentage of apoptotic cells after 12 hours (P<0.05) and 24 hours (P<0.01) exposure to 0.8 mM of morphine.

Detection of mRNA expression of BAX and BCL-2 in the presence of morphine and lithium in PC12 cell line by quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed to determine the relative gene expression of BAX and BCL-2 in PC12 cell line. Figure 5A presents the alterations in relative mRNA expression of BAX in different groups of PC12 cells. In the first step, the cells were preincubated with 1.2 mM of sodium chloride for 72 hours and then were exposed to 0.8 mM of sodium chloride or morphine for six, 12, and 24 hours. Administration of 0.8 mM of morphine for six hours (but not 12 or
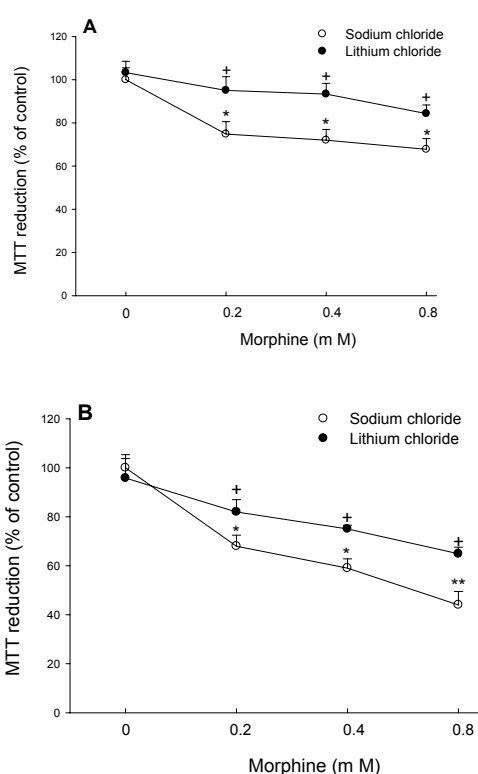
Figure 3. The effects of 72-hour preincubation of PC12 cells with sodium chloride or lithium chloride followed by morphine exposure for 48 or 96 hours on cell viability determined by MTT assay.
PC12 cells were preincubated with 1.2 mM of sodium chloride or lithium chloride for 72 hours. Then, the cells were exposed to different concentrations of morphine (0.2, 0.4, and 0.8 mM) in 96-well plates for 48 hours (Figure 3A) or 96 hours (Figure 3B) as described in materials and methods section. The viability of cells was expressed as the absorbent value at 570 nm. Data are presented as mean±SEM of 10 – 12 samples.
*$P<0.05$, **$P<0.01$ different from the respective saline control group.
+$P<0.05$ different from the respective morphine control group.

24 hours) significantly increased the level of mRNA expression of BAX compared with the control group ($P<0.01$). In the second part of the experiments, PC12 cells were preincubated with 1.2 mM of lithium chloride for 72 hours and then were exposed to 0.8 mM of morphine or sodium chloride for six, 12, and 24 hours. Preincubation of cells with lithium chloride reversed the 0.8 mM morphine-induced elevation of BAX mRNA expression ($P<0.001$). This property of lithium was observed only six hours (but not 12 or 24 hours) after morphine exposure.

Figure 5B presents the alterations in relative mRNA expression of BCL-2 in different groups of PC12 cells. In the first step, the cells were pre-
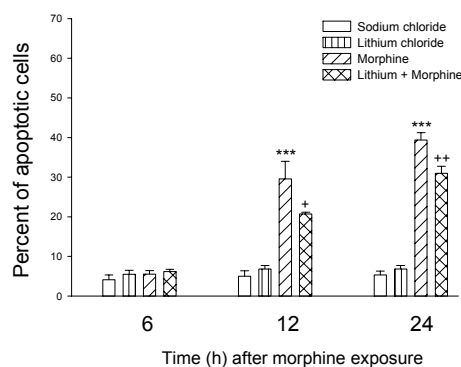

Figure 4. The effects of 72-hour preincubation of PC12 cells with sodium chloride or lithium chloride followed by morphine exposure on apoptosis determined by FITC annexin V test.
PC12 cells were preincubated with 1.2 mM of sodium chloride or lithium chloride for 72 hours. Then, the cells were exposed to 0.8 mM of morphine or sodium chloride for six, 12, and 24 hours. At different times of morphine exposure, about one million cells from each group were stained with FITC annexin V and PI and then analyzed by flowcytometry. Data are presented as mean±SEM of three samples.
***$P<0.001$ different from the respective saline-saline control group.
+$P<0.05$, ++$P<0.01$ different from the respective saline-morphine control group.

incubated with 1.2 mM of sodium chloride for 72 hours and then were exposed to 0.8 mM of sodium chloride or morphine for six, 12, and 24 hours. Administration of 0.8 mM of morphine for six hours (but not 12 or 24 hours) significantly decreased the level of mRNA expression of BCL-2 compared with the control group ($P<0.05$). In the second part of the experiments, PC12 cells were preincubated with 1.2 mM of lithium chloride for 72 hours and then were exposed to 0.8 mM of morphine or sodium chloride for six, 12, and 24 hours. Preincubation of cells with lithium chloride (but not sodium chloride) reversed 0.8 mM morphine-induced attenuation of BCL-2 mRNA expression ($P<0.05$). This property of lithium was observed only six hours (but not 12 or 24 hours) after morphine exposure.

Discussion

Opioid drugs exert a variety of biological effects which may be mediated through mechanisms other than the classic opioidergic receptors. The exact mechanism whereby opioids modulate cell survival and death is not clearly understood.[19] Several evidences have revealed that
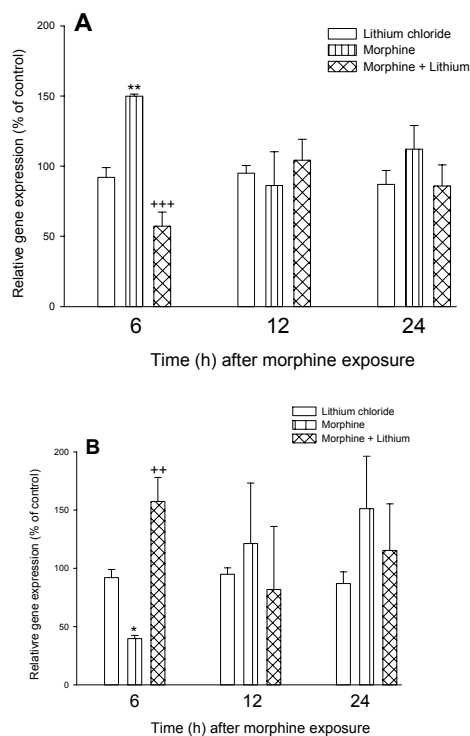
Figure 5. The effects of 72-hour preincubation of PC12 cells with sodium chloride or lithium chloride followed by morphine exposure on mRNA expression of BAX or BCL-2 determined by quantitative real time RT-PCR.
PC12 cells were preincubated with 1.2 mM of sodium chloride or lithium chloride for 72 hours. Then, the cells were exposed to 0.8 mM of sodium chloride or morphine for six, 12, and 24 hours. Gene expression was measured by quantitative real time RT-PCR. The amount of mRNA of BAX (5A) and BCL-2 (5B) was normalized to the housekeeping gene (β-actin)+ and expressed as relative gene expression vs. the respective saline-saline control group. Data are presented as mean±SEM of three samples.
*P<0.05, **P<0.01 different from the respective saline-saline group.
++P<0.01, +++P<0.001 different from the respective saline-morphine group.

immunosuppression occurs during chronic opioid treatment. It seems that the immunosuppression is due to induction of apoptosis in the cells of the immune system (see introduction).

Meanwhile, it has been suggested that tolerance to morphine is the result of apoptosis in GABAergic neurons of dorsal horn regions of the spinal cord.[20] Increased synaptic glutamate availability and N-methyl-D-aspartate (NMDA) receptors stimulation play an important role in the morphine-induced apoptosis within the dorsal horn of the spinal cord.[35–39] Furthermore, morphine up-regulates proapoptotic Fas receptors and down-

regulates BCL-2 proteins in the rat brain.[40]

PC12 cell line is extensively used for cell signaling investigations.[31] Cell viability was measured by MTT assay. On the other hand, apoptosis was detected by annexin V staining test (specific test for apoptosis) and mRNA expression of BAX and BCL-2. In the first step of the present study, PC12 cells were exposed to different concentrations of morphine. The proapoptotic and necrotic effects of morphine have been shown in multiple cell lines.[27,30,41–43] In agreement with the other investigations, the results of MTT assay and annexin V FITC test showed that morphine decreased the cell viability and increased the number of apoptotic cells, respectively, in a dose-dependent manner.[41,42] Furthermore, according to the results of real time RT-PCR morphine exposure increased the mRNA expression of BAX and decreased that of BCL-2. In summary, it can be concluded that morphine induces apoptosis in PC12 cell line in a dose-dependent manner.

The intrinsic pathway of apoptosis is regulated by the members of BCL-2-related proteins. These proteins are categorized into two groups based on their ability to either induce or inhibit apoptosis.[44,45] Proapoptotic members (BAX and Bak) induce mitochondrial membrane permeability while the antiapoptotic elements (BCL-2 and BCL-x_L) prevent it.[46] Interestingly, the antiapoptotic BCL-2 proteins inhibit oligomerization and prevent possible pore formation.[47]

Furthermore, BAX protein induces mitochondria to release cytochrome C[48] which is translocated from cytoplasm to the mitochondria upon induction of apoptosis. It has been shown that BCL-2-related proteins may regulate cytosolic calcium levels and are associated with the inositol triphosphate receptor type 1 (InsP3R).[45] voltage-dependent anion channels (VDACs) which act as a positive regulator of apoptosis, are also controlled by BCL-2 family proteins.[49]

Moreover, BCL-2 attenuates the activity of transcription factor NF-kB which is upregulated during the apoptosis.[50] In addition, following oxidative stress, BCL-2 decreases the release of calcium from the endoplasmic reticulum.[51] On the other hand, mitochondrial changes mediated by BAX are calcium dependent and are prevented by cyclosporine A.[52] Moreover, BCL-2 seems to have the ability to reduce the calcium uptake into isolated nuclei.[53]

In the second part of this investigation, the protective effect of lithium was investigated. In

1949, Cade discovered the new psychoactive property of lithium.[54,55] But, the exact mechanism underlying its pharmacologic action is not completely understood.[56] Increasing evidences support the idea that lithium exerts neuroprotective[57-61] and neurogenetic effects.[62,63]

There are common grounds for the interaction between lithium and morphine. This interaction has been reported in animal models of morphine-induced addiction,[64,65] tolerance,[64] self-stimulation,[66] and antinociception.[67,68] Furthermore, lithium may change the opioid receptor conformation [69] and stimulate the release of endogenous opioids in the rat brain.[70] Moreover, lithium decreases the cellular storage of inositol and inositol (1,4,5)-triphosphate (IP3)[71–73] and also blocks calcium-induced hydrolysis of inositol phosphates.[71,74] On the other hand, morphine affects phosphoinositide turnover.[71,75–78] Thus, it can be considered as a basis for the interaction between morphine and lithium.

It has been well documented that the pharmacologic effects of morphine are at least partly mediated through inhibition of adenylyl cyclase activity.[18] On the other hand, lithium directly inhibits catalytic subunit of adenyl cyclase[55,79] and the formation of cAMP.[71,80,81] The other similarities of mechanism of action between morphine and lithium are cellular nitric oxide (NO)-cGMP pathway[82,83] and dopamine receptors sensitivity.[69,84]

We aimed to elucidate the molecular mechanisms underlying lithium-induced neuroprotection in PC12 cell line. Thus, the effect of lithium pre-incubation on mRNA expression of BAX and BCL-2 was investigated. According to previous studies, three to five days of pretreatment with lithium is essential for complete protection while three hours (short-term) or six to seven days are ineffective or even toxic.[17] Our data using quantitative real time RT-PCR showed that lithium pretreatment reversed the morphine-induced alterations in mRNA expression of BAX and BCL-2. Lithium pretreatment decreased the level of mRNA expression of BAX and increased that of BCL-2. Lithium-induced alterations in pro- and anti-apoptotic agents improved cell viability. Furthermore, the results of genetic studies were supported by MTT assay and annexin V FITC test.

The exact mechanism whereby lithium exerts its neuroprotective effect has not been clearly uncovered. Numerous biochemical actions have been attributed to *in vivo* and *in vitro* lithium treatment, including inhibition of phosphoinoside turnover, alteration of intracellular concentration of calcium, and effects on G-proteins and protein kinase C.[56] Previous investigations had revealed that therapeutic concentrations of lithium protect cells from apoptosis induced by glutamate excitotoxicity.[11,85,86] p53 can be considered another explanation for the obtained results. This protein positively regulates BAX but negatively controls BCL-2 expression. Thus, inhibition of p53 expression may be a crucial point in the regulation of BAX and BCL-2 expression by lithium.[87–89]

In summary, lithium is able to reverse the apoptosis induced by morphine via down-regulation of BAX and/or up-regulation of BCL-2. Yet, the exact mechanism by which lithium interferes with the apoptotic effect of morphine remains to be fully understood.

Acknowledgment

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