Effect of Carnosine on the Prevention of High-Dose Morphine-Induced Apoptosis of PC12 Cells

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Abstract—It has been reported that carnosine provide protection against various neurotoxic insults. In the previous study, we demonstrated that high dose morphine can induce apoptosis in PC12 cells possibly by the mitochondrial pathway through higher expression of Bax pro-apoptotic protein. The present study was designed to investigate the protective effect of carnosine on morphine-induced apoptotic death in PC12 cells. The activity of lactate dehydrogenase (LDH) and the levels of malondialdehyde (MDA), intracellular reactive oxygen species (ROS), cell viability and DNA fragmentation were measured in morphine-treated PC12 cells with and without carnosine pre-treatment. Morphine caused concentration-dependent cell death and pretreatment with carnosine was associated with a marked diminution in DNA fragmentation, intracellular ROS and MDA levels. Carnosine also increased cell viability as measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) and LDH assays. These findings indicate that carnosine might be useful as potential agent to protect against morphine neurotoxicity.

Index Terms—carnosine, morphine, apoptosis, PC12 cells

I. INTRODUCTION

Morphine, a widely used opioid as an analgesic agent as well as a drug of abuse, has been reported to cause apoptosis in neuronal cells when used at relatively high concentrations [1]. This neurotoxicity might be associated with various unwanted side effects such as developmental abnormalities, hyperalgesia and tolerance that greatly limit the clinical applications of morphine [2]. Although the exact mechanism underlying neuronal damage remains unknown, it has been suggested that the oxidative stress elicited by a high morphine concentration is one of the major factors contributing to the neuronal cell death [3]. In order to reduce the risk of such neuronal damage, it is therefore crucial to explore tools by which one can reduce oxidative stress in neurons to ameliorate the impaired neuronal function produced by morphine and possibly control morphine side effects.

Carnosine (b-alanyl-L-histidine), an endogenous dipeptide, is widely distributed in the nervous tissues. It is well tolerated and commonly used as a dietary supplement [4]. It has been demonstrated that carnosine can scavenge reactive oxygen species (ROS) and exert neuroprotective effects through suppression of many biochemical changes such as protein oxidation, glycation, AGE formation and glutamatergic excitotoxicity [5]. These diverse pharmacological activities led us to speculate that treatment with carnosine may diminish neuronal damage triggered by high morphine concentrations.

We used the rat pheochromocytoma cell line PC12, an in vitro model that is extensively used to study neurotoxicity [6]. In the previous study, we demonstrated that morphine can induce apoptosis in PC12 cells possibly by the mitochondrial pathway through higher expression of Bax pro-apoptotic protein [7]. In this study, we investigated for the first time the effect of carnosine in PC12 cells subjected to high concentrations of morphine.

II. METHODS

A. Cell Culture and Drug Treatment

PC12 cells were obtained from Pasteur Institute (Tehran, Iran) and maintained in RPMI1640 medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum, 10% (v/v) horse serum and 1% (v/v) penicillin–streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) in a 90% humidified incubator of 5% CO_2 in air at 37 °C. The culture medium was changed every 48 h, and the cells were subcultured every 2–3 days.

After cultured in RPMI1640 medium for 24h, the cells were pre-incubated with carnosine (1, 5, 10mM) for 18 hours and then co-treated with carnosine and morphine (1mM) for 96 hours. Control cells were treated only with vehicle medium. The selected concentrations of carnosine were according to previous studies [8].

B. Determination of Cell Viability

The cells were plated at the density of 5000/well in a 96micro plate well and cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay [9]. In brief, MTT solution (5 mg/ml) was added to the 96-well plates and incubated for 1h at 37◦C and then was replaced with DMSO to solubilise the precipitated dye. Absorbance was measured at 570 nm by an ELISA reader.

C. Measurement of LDH Release

The plasma membrane damage of the PC12 cells was assessed by measuring the release of LDH into culture medium. The LDH activity was quantified using a LDH diagnostic kit (STANBIO Laboratory, USA) according to the manufacturer's protocol. PC12 cells were cultured in 6-well plates and treated as described above. At the end of the drug treatment, the medium was collected and 100 μL of it was added to 1mL of LDH reagent. The absorbance was recorded at 340nm. To determine the intra-cellular LDH activity, the cells were washed with D-Hanks solution and then detached from the plates into 500 μL of ice-cold PBS (0.1 M, containing 0.05mM of EDTA) and homogenized. The homogenate was centrifuged (4000×g) at 4°C for 30 min and the supernatant was used for the LDH activity assay. LDH leakage was expressed as a percentage (%) of total LDH activity (LDH in the medium+LDH in the cell), according to the equation % LDH released = (LDH activity in the medium/total LDH activity) ×100.

D. Measurement of Intracellular ROS Level

The production of intracellular ROS was measured by using a cell-permeable oxidation-sensitive fluorescent probe 2, 7-dichlorofluorescein diacetate (DCFH-DA) [10]. In the presence of ROS, DCFH-DA has been shown to be oxidized to the high fluorescent 2, 7-dichlorofluorescin (DCF), which is retained within the cells. Briefly, PC12 cells were cultured in a 96-well plate at a density of 5×10^3 cells/well. At the end of the drug treatment, culture medium was removed and cells were washed with phosphate buffered saline three times. DCFH-DA, diluted to a final concentration of 10 μM with RPMI1640, was added and incubated in the dark at 37 °C for 20 min. Then the relative levels of fluorescence were quantified by a fluorescence microplate reader.

E. Quantification of DNA Fragmentation

To examine DNA fragmentation as a marker of cell death, we quantified the levels of monooligonucleosome fragments using Cell Death Detection ELISA[Plus] kit (Roche Applied Sciences, Germany) according to the manufacturer's instructions, except that cells were cultured in 96-well plates at a density of 2×10^4 cells/well for the ELISA analysis.

F. MDA Assay

After drug treatment, the cells were washed with D-Hanks, scraped from the plates into 1mL of ice-cold PBS (0.1 M, containing 0.05mM of EDTA), and homogenized. The homogenate was centrifuged at 4000×g for 30 min at 4 °C and the supernatant was stored at −80 °C. The MDA content was measured as previously described [11]. Briefly, 100 μL of the supernatant was mixed with 1.5mL of acetic acid (20%, v/v, pH 3.5), 1.5mL of thiobarbituric acid (0.8%, w/v), and 200 μL of sodium dodecyl sulphate (8%, w/v). Each reaction mixture was heated for 60 min at 95 ∘C and cooled to room temperature. Then, 5mL of n-butanol was added. After mixing and centrifugation at 3000×g for 10 min, the organic layer was collected and the absorbance measured at 532 nm.

G. Statistical Analysis

Data were derived from 3–8 independent experiments and presented as means±S.E.M. Statistical differences between control and treated groups were determined by non-paired Student's t-test. The criterion for significance was a P value < 0.05.

III. RESULT

A. Effect of Carnosine on Morphine-Induced Cytotoxicity

Morphine (1mM for 96 hours) significantly reduced cell viability compared to control cells (P < 0.01). However, incubation of cells with carnosine (1-10mM) concentration-dependently reversed morphine-induced cell death (Fig. 1A).
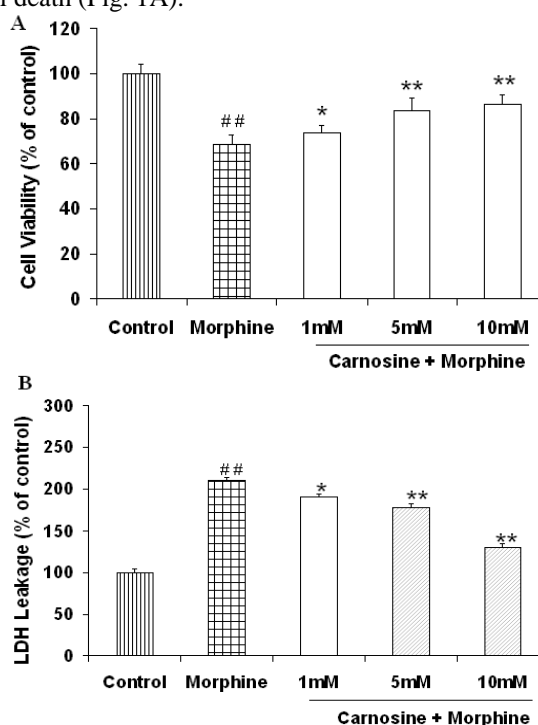

Figure 1. Protective effect of carnosine on morphine-induced cytotoxicity in PC12 cells. Cells were incubated with 1mM morphine for 96 h. Carnosine was added to the culture 18 h prior to morphine. Cell viability was determined by measuring MTT reduction (Panel A) and LDH assay (Panel B).

Data are expressed as percent of values in untreated control cultures, and are mean±S.D. from three separated experiments with triplicates. [##]$P < 0.01$ compared with control. *$P < 0.05$, **$P < 0.01$ compared with the group treated by morphine alone.

B. Effect of Carnosine on LDH Release

To further investigate the protective effect carnosine in morphine-induced PC 12 cells injury, LDH assay, another indicator of cell toxicity, was performed. The results were similar to those determined by MTT assay. Treatment with morphine (1mM) resulted in significant increase of LDH release into the medium compared to control cells (P < 0.01) (Fig. 1B). Pre-incubation with various concentrations of carnosine (1-10mM) remarkably blocked LDH leakage in the PC12 cell system under high morphine concentration.

C. Effect of Carnosine on Intracellular ROS Content

To examine whether the inhibitory effect of carnosine on the toxicity of morphine is mediated by the antioxidant ability, PC12 cells were treated with 1mM morphine for 96 h, and the level of ROS was measured by DCF fluorescence. As shown in Fig. 2, when PC12 cells were exposed to high morphine concentration, the intracellular ROS level was significantly increased compared to control (P < 0.01), revealing that morphine enhanced ROS concentration in PC12 cells. However, simultaneous treatment with carnosine and morphine effectively reduced ROS generation, and the suppressing effect was strengthened with the increase of carnosine concentration.
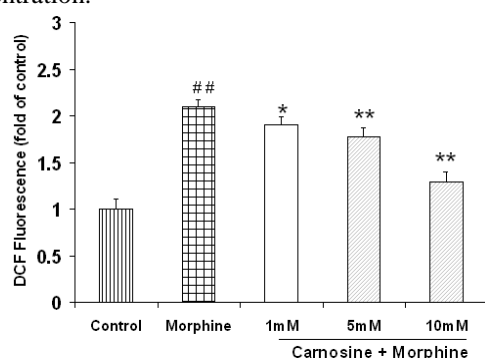

Figure 2. Effects of carnosine on the formation of ROS due to high morphine concentration.

PC12 cells were treated with 1mM morphine with/without carnosine (1-10mM) for 96 h. Formation of ROS was assayed by measuring fluorescence of DCF. Each value represents the mean±S.D. from three separated experiments with triplicates. *P < 0.05, *P < 0.01 compared with the group treated by high morphine concentration. ##P < 0.01 compared with control.

D. Effect of Carnosine on the DNA Fragmentation in Morphine-Treated PC12 Cells

To determine whether carnosine protect neuronal PC12 cells from morphine-induced apoptosis, we measured DNA fragmentation, a marker of apoptosis. When PC12 cells were exposed to 1mM morphine for 96h, there was a 2.3-fold increase in DNA fragmentation vs untreated controls (Fig. 3). This increment was efficiently prevented by pretreatment and then coadministration of carnosine (1-10mM) with morphine.
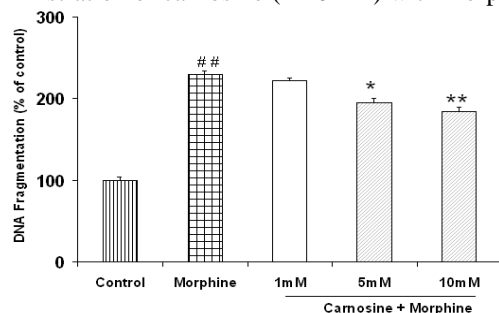

Figure 3. Effect of carnosine on the DNA fragmentation in morphine-treated PC12 cells.

Values given are the mean±S.E.M. (*n* = 6). ##P < 0.01 as compared with control group; *P < 0.05, **P < 0.01 as compared with morphine group.

E. Effects of Carnosine on MDA Level

After exposure of PC12 cells to 1mM morphine for 96h, intracellular MDA was significantly raised (#P< 0.05 vs. control, Table I). When the PC12 cells were incubated with different concentrations of carnosine (1-10mM) in the presence of 1mM of morphine for 96 h, the MDA level significantly decreased (*P< 0.05) compared with the morphine-treated PC12 cells. These results may prove that carnosine counteracts the oxidative stress elicited by morphine.

TABLE I.   EFFECTS OF CARNOSINE ON THE LEVEL OF MDA IN RAT PC12 CELLS UNDER HIGH MORPHINE CONCENTRATION

Group	MDA level (% of Control)
Control	100±6.8
Morphine (1mM)	161.2±7.3#
Carnosine (1mM)+ Morphine	155.7±9.8
Carnosine (5mM)+ Morphine	141.7±7.5*
Carnosine (10mM)+ Morphine	130.5±7.9*

Cultured PC12 cells were exposed to 1mM morphine. Carnosine was added 18 h prior to morphine addition and the level of MDA was measured 96 h later. Data were the means ± SEM expressed as percentages of the corresponding untreated control (n = 3-7). #P< 0.05 vs. control, *P< 0.05 vs. Morphine.

IV.   DISCUSSION

Our present results clearly demonstrated that carnosine, a free radical scavenger, had inhibitory effect on high-dose morphine-induced apoptosis of PC12 cells. Pretreatment with carnosine not only increased cell viability, but also attenuated oxidative stress as evidenced by a marked diminution in intracellular ROS and MDA levels. Additionally, carnosine efficiently prevented DNA fragmentation, a marker of apoptosis, in PC12 cells exposed to high concentration of morphine. These results are in agreement with previous studies on the protective effect of carnosine on neurons [12], [13] and confirm that carnosine can enhance cell viability and inhibit apoptotic cell death by decreasing oxidative stress. Oxidative stress, which is defined as a disturbance in the balance between the production of ROS and antioxidant defense systems, may contribute to neuronal injury induced by high dose morphine [14]. Excessive ROS levels are known to trigger damage to major macromolecules in cells, including lipids, proteins, and nucleic acids, leading to neuronal dysfunction and cell death. MDA, which is a by-product of lipid peroxidation, is produced under oxidative stress and reflects oxidative damage to the plasma membrane. To diminish oxidative stress, cells use antioxidant defense systems and scavenge ROS [15].

Although the mechanisms of neuronal injury induced by high dose morphine are still poorly defined, there are growing evidences showing that several molecules may play roles in the etiology. Reactive oxygen species (ROS) has been shown to be elevated by morphine in neuronal cells [16]. As a potent hydrophilic antioxidant, carnosine has been reported to reduce the intracellular ROS

production in neuronal cells. Therefore, it is also possible that carnosine may exert its effect via its powerful antioxidative pathway [17], [18].

Carnosine can be metabolically transformed into histamine by enzymes that occur in the brain and the protective effect of carnosine may be partly mediated by activating the histaminergic system [19]. In fact, several studies reported that selective central histamine H_1 and H_3 antagonists significantly reversed the protection of carnosine against neurotoxic insults in PC12 cells [20], [8]. On the other hand, in animal models morphine administrations decreased the histamine content and reduced the number and size of histaminergic neurons [21]. Therefore, it is likely that through directly acting on H_1 receptors and evoking a histamine-like response, carnosine can inhibit the effects of high dose morphine in PC12 cells.

In summary, this study showed that pretreatment with carnosine can protect against high-dose morphine-induced neurotoxicity in PC12 cells. The neuroprotective effect of carnosine may be possibly attributed to its antioxidant actions and direct action on H_1 receptors. Further studies will be needed to identify the mechanisms of attenuation of neuronal PC12 cell death by carnosine.

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