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Carnosine protects against NMDA-induced neurotoxicity in differentiated rat PC12 cells through carnosine-histidine-histamine pathway and H_1/H_3 receptors

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ABSTRACT

Since the histidine-containing dipeptide carnosine (β-alanyl-L-histidine) is believed to have many physiological functions in the brain, we investigated the neuroprotective effects of carnosine and its mechanisms of action in an *in vitro* model of neurotoxicity induced by N-methyl-D-aspartate (NMDA) in differentiated PC12 cells. Pretreatment with carnosine increased the viability and decreased the number of apoptotic and necrotic cells measured by MTT and Hoechst 33342 and propidium iodide (PI) double staining assays. Carnosine also can inhibite the glutamate release and increase HDC activity and the intracellular and extracellular contents of carnosine, histidine and histamine detected by high-performance liquid chromatography (HPLC). The protection by carnosine was reversed by α- fluoromethylhistidine, a selective and irreversible inhibitor of histidine decarboxylase (HDC). Pyrilamine and thioperamide, selective central histamine H_1 and H_3 antagonists also significantly reversed the protection of carnosine. Further, the inhibition of glutamate release by carnosine was reversed by thioperamide. Therefore, the protective mechanism of carnosine may not only involve the carnosine-histidine-histamine pathway, but also H_1/H_3 receptors and the effective inhibition of glutamate release. This study indicates that carnosine may be an endogenous protective factor and calls for its further study as a new antiexcitotoxic agent.

1. Introduction

The N-methyl-D-aspartate (NMDA) receptor (NMDAR) is a type of ionotropic glutamate receptor that contributes to excitatory postsynaptic potentials in neurons. Excessive activation of NMDAR is lethal to neurons [1], and this excitotoxic response has been proposed to play a role in the pathology of many diseases, especially cerebral ischemia [2]. Thus, effective inhibition of the neuronal death induced by NMDAR is clinically significant.

Clinical investigations and behavioral studies have shown that histaminergic neurotransmission plays an important role in the development of cerebral ischemia, and has a protective effect on the delayed neuronal death mediated by NMDAR [3,4]. Either histamine or the H_2 receptor agonist, dimaprit, can suppress the increase in dopamine and glutamate levels during ischemia and the neuronal degeneration after ischemia [5]. In *in vitro* experiments, our previous results indicated that histamine protects against NMDA-induced necrosis in cultured cortical neurons [6]. Further, the

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antihistamine terfenadine potentiates NMDAR-mediated calcium influx, oxygen radical formation, and neuronal death [7]. Therefore, specific histaminergic compounds have potential clinical uses in preventing and treating cerebral ischemia, although it has been reported that histamine can selectively facilitate NMDA-evoked currents through coactivation of NMDAR in hippocampal neurons [8]. However, histamine itself cannot cross the blood–brain barrier and it is involved in brain inflammation. For example, Vizuete et al. [9] reported that the infusion of histamine into rat substantia nigra results in an acute inflammatory response manifested by a loss of glial fibrillary acidic protein-immunolabeled astrocytes.

Carnosine (β-alanyl-L-histidine) is a naturally occurring dipeptide, widely distributed in tissues including the animal and human brain, at concentrations up to 20 mM, and can easily enter the central nervous system from the periphery [10]. Carnosine has been assigned many putative roles such as anti-inflammatory agent, free radical scavenger [11], and protein glycosylation inhibitor [12], and may serve as a neurotransmitter in the olfactory bulb [13]. However, so far, the physiological functions of carnosine in the brain remain obscure, and a unifying concept has not yet emerged. On the other hand, previous studies have suggested that carnosine can be metabolically transformed into histamine by carnosinase and histidine decarboxylase enzyme that exist in the brain [14,15]. There seems to be a relationship between carnosine and histamine, and carnosine is proposed as a new histaminergic drug that can replace histamine and avoid inflammation in clinical therapeutics. For example, we recently reported that, like histamine, carnosine decreases seizure stage, afterdischarge duration and generalized seizure duration *via* the H_1 receptor [16]. Yet, few reports have illustrated a significant relationship between carnosine and histamine, especially in NMDAR-mediated injury.

To understand the action of carnosine on NMDA-induced neurotoxicity at the cellular and molecular levels, we used the rat pheochromocytoma cell line PC12, an *in vitro* model that is widely used to study oxidative stress, chemical hypoxia, and other toxic insults. Differentiated PC12 cells express histamine receptors [17,18] and functional NMDAR, including the NMDAR1 and NMDAR2 subtypes [19]. Thus, the present study was undertaken to elucidate the pharmacological functions and actions of carnosine on NMDA-induced neurotoxicity in differentiated rat PC12 cells.

2. Materials and methods

2.1. Materials

Carnosine, N-methyl-D-aspartate (NMDA), histamine, histidine, pyrilamine, cimetidine, thioperamide, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), hoechst 33342, and propidium iodide (PI) were from Sigma (St. Louis, MO, USA). L-Glutamine, penicillin, streptomycin, trypsin, poly-L-lysine, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum and horse serum were from GIBCO-BRL (Grand Island, NY, USA). (S)-α-Fluoromethylhistidine (α-FMH) was from Merck Sharp & Dohme Research Laboratory (Rahway, NJ, USA).

2.2. Cell culture

Differentiated PC12 cells were obtained from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai). Cells were grown in pH 7.4 growth medium consisting of DMEM supplemented with 10% horse serum, 5% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin, and maintained at 37 °C and 5% CO_2 in a humidified incubator. Subculture was performed when the cells reached confluence. Cells were trypsinized at a ratio of 1:3 after confluence using 0.25% trypsin in Hank's containing 0.02% EDTA. Subcultured cells were seeded onto poly-L-lysine-coated 96-, 24- or 6-well plates at densities of 1×10^4, 1×10^5 or 5×10^5 cells/well, respectively. Experiments were carried out 24 h after cells were seeded.

2.3. Drug treatment

As a general rule, drugs were dissolved in sterile purified water and pH was adjusted to approximately 7.4 prior to dilution into culture medium. Excitotoxicity was induced by various NMDA concentrations for different incubation times, during which the medium was supplemented with 10 μmol/L glycine. For restoration, at the end of NMDA exposure, cells were rinsed twice with Earle's balanced salt solution (EBSS) with the following composition (in mmol/L): 116.4 NaCl, 5.4 KCl, 2.6 NaH_2PO_4, 26.2 $NaHCO_3$, 1.8 $CaCl_2$, 0.8 $MgSO_4 \cdot 7H_2O$, 5.5 glucose, 20.1 HEPES, 0.01 glycine, pH 7.4, and incubated with new medium for an additional 24 h. For drug treatment, various concentrations of carnosine were applied to the cultures for different incubation times until the end of NMDA exposure. Pyrilamine, cimetidine or thioperamide was added 15 min before carnosine. α-FMH was added 3 h before carnosine. Control groups were treated only with vehicle medium.

2.4. Cell viability assay

Cell viability was monitored by the colorimetric MTT assay as described with slight modification [6]. Briefly, PC12 cells were cultured on 96-well plates and there were 6 wells in each group. At the end of experiments, the cells were incubated with 0.5 mg/ml MTT for 2 h at 37 °C. Then, the supernatant layer was removed, and 100 μL of dimethyl sulfoxide was added into each well. MTT metabolism was quantitated spectrophotometrically at 490 nm in a Biorad microplate reader. Results were expressed as the percentage of MTT reduction, taking the absorbance of control cells as 100%. Apoptosis and necrosis were distinguished using combined staining with the chromatin dye, Hoechst 33342, and propidium iodide (PI) (Molecular Probes, USA) [6].

2.5. Neurochemical analysis of carnosine, histidine, histamine, glutamate and gamma-aminobutyric acid (GABA) levels

Sample preparation: PC12 cells were plated onto poly-L-lysine-coated 24-well plates for 2 days. For carnosine, histidine and histamine analysis, the cultures were exposed to carnosine (5 mmol/L) for 18 h, and the supernatant and cell pellet disrupted by 1% Triton X-100 werecollected separately. For

glutamate and GABA analysis, carnosine was applied to the cultures for 18 h and then NMDA (750 μmol/L) supplemented with 10 μmol/L glycine was added for the indicated times. α-FMH or thioperamide was added 3 h or 15 min before carnosine, respectively. The samples were deproteinized with 0.4 mol/L perchloric acid and centrifuged at 15,000 × *g* for 20 min at 4 °C. Then the supernatant was removed and filtered with a 0.22 μm polyvinylidene difluoride membrane.

Chromatographic conditions: Carnosine, histidine, histamine, glutamate and GABA concentrations were determined by high-performance liquid chromatography (HPLC) combined with electrochemical detection using the technique developed in our laboratory [16]. The system consisted of a model 582 pump, a model 540 autosampler and a four-channel CoulArray electrochemical detector. The HPLC was controlled and the data acquired and analyzed using CoulArray® software. All of the above equipment was from ESA (Chelmsford, MA, USA). After reacting with the derivate *o*-phthalaldehyde, analysates were separated on a 3 μm, 3 × 50 mm Capcell Pak MG C18 column from Shiseido (Tokyo, Japan). A two-component gradient elution system was used, with component A of the mobile phase being 100 mM Na_2HPO_4, 13% acetonitrile, and 22% methanol, pH 6.8, and component B being similar to A except with 5.6% acetonitrile and 9.4% methanol. A gradient elution profile was used as follows: 0–3.5 min, isocratic 100% B; 3.5–20 min, linear ramp to 0% B; 20–22 min, isocratic 0% B; 22–23 min, linear ramp to 100% B; 23–30 min, isocratic 100% B. The flow rate was set to 0.75 mL/min. The temperature of the column was maintained at 38 °C. The first cell was set at 250 mV, whereas the second cell was set at 350 mV. All standards were obtained from Sigma (St. Louis, MO, USA). Under these conditions, the retention times of glutamate, histidine, carnosine, GABA and histamine were 5.58, 8.70, 10.84, 15.16 and 18.36 min, respectively. The detection limits (signal/noise ≥3) were 0.1 μg for glutamate, 5 ng for carnosine, histidine and GABA, and 1 ng for histamine. Reproducibility was assessed by analyzing a standard mixture six times a day for four consecutive days. The average coefficients of variation of within-day and between-day assays were 0.5–2.3% and 0.7–3.6%, respectively, for all analyzed substances.

2.6. Histidine decarboxylase (HDC) activity

On Day 2, carnosine (5 mmol/L) was applied to the cultures plated in six-well plates. HDC activity was determined 18 h after incubation with carnosine as described [20] with slight modification. The cells were washed twice with PBS, harvested and briefly sonicated with 50 μl of HDC buffer [0.1 mol/L potassium phosphate buffer (pH 6.8) containing 0.1 mmol/L dithiothreitol, 0.01 mmol/L pyridoxal 5′-phosphate, 1% polyethylene glycol (average molecular weight 300), and 20 μg/ml phenylmethanesulfonyl fluoride]. The sonicate was centrifuged at 10,000 × *g* for 15 min and 40 μl of the supernatant was transferred to a microcentrofilter tube. After centrifugation at 5000 × *g* for 40 min, 40 μl of phosphate buffer was added to the pellet, which was gently suspended. This process was repeated twice to remove intrinsic histamine, then the enzymatic reaction was stopped with 8 μl of ice-cold 20% perchloric acid. The histamine concentration in each aliquot was measured by HPLC. HDC activity was defined as picomoles of histamine produced in each well per hour (pmol HA/well/h).

2.7. Statistical analysis

Statistical analyses were performed with SPSS 12.0 Software (SPSS Inc.). All data are

presented as mean ± S.D. of three to six experiments. Percentage inhibition of the protection by drugs was calculated from values of percent MTT metabolism and determined by the formula:

$$\frac{(\text{protective drug} + \text{NMDA}) - (\text{inhibitory drug} + \text{protective drug} + \text{NMDA})}{(\text{protective drug} + \text{NMDA}) - (\text{NMDA alone})} \times 100\%^{*}$$

(*Protective drug means carnosine, inhibitory drug means histamine receptor antagonists in this study.)

Statistical significance was estimated with one-way or two-way analysis of variance (ANOVA) followed by LSD or Dunnett's T3 *post-hoc* test (where equal variances were not assumed) to determine the significance of differences between groups. Significance was established at $P < 0.05$.

3. Results

3.1. Neurotoxic effect of NMDA on differentiated PC12 cells

Exposure of differentiated PC12 cells to NMDA caused time- and concentration-dependent cell death. Under our experimental conditions, approximately 25 or 50% of PC12 cells died after incubation for 12 or 24 h with 750 μmol/L NMDA, respectively, whereas less than 5% of PC12 cells died after incubation for 12 h with 50 or 250 μmol/L NMDA (Fig. 1). PC12 cells were therefore treated with 750 μmol/L NMDA for 12 h and reperfused for 24 h in subsequent experiments to induce an injury level of 76%. To verify the type of cell death, we stained the cells with Hoechst 33342 and PI, a sensitive assay both for apoptosis and necrosis. Without NMDA treatment, the nuclei of control cells showed uniform blue fluorescence, indicating that they were healthy and the nuclei were intact (Fig. 2a). In contrast, after treatment with NMDA, a high percentage of nuclei were swollen and stained red by the membrane-impermeant dye PI, indicating necrotic cell death. Some displayed apoptotic characteristics with fragmented nuclei stained by Hoechst 33342 (Fig. 2b). These data showed that NMDA-induced delayed cell death occurred *via* both necrosis and apoptosis

3.2. Time- and concentration-dependent neuroprotection by carnosine against NMDA insult

Carnosine increased cell survival with time of exposure and concentration, compared with control groups (Fig. 3). Carnosine at concentrations of 10^{-4}, 10^{-3} and 5×10^{-3} mol/L significantly protected against NMDA-induced injury. At the concentration of 5 mmol/L and preincubation for 18 h, carnosine induced maximal protection and almost completely reversed the NMDA-induced neurotoxicity. On the basis of nuclear morphology by Hoechst 33342 and PI staining, 18 h

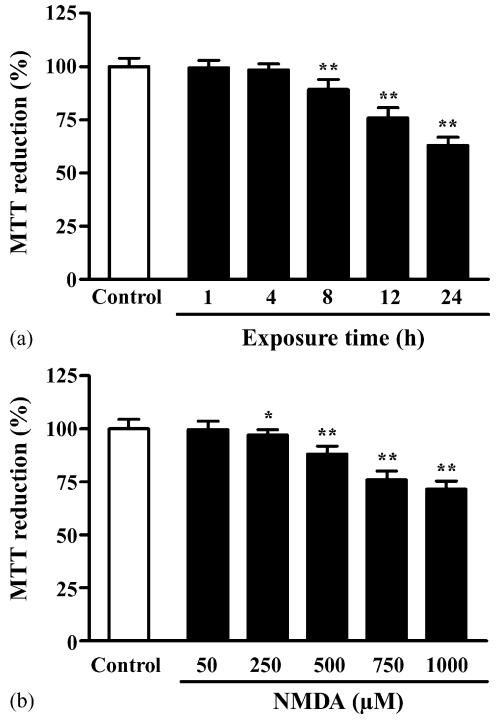

Fig. 1 – Time- and concentration-dependent neurotoxicity of NMDA in differentiated PC12 cells. Cells were exposed to NMDA (750 μmol/L) for various durations (a) or were exposed to various concentrations of NMDA for 12 h (b). Cell viability was assayed 24 h later using the MTT reduction assay. Results expressed as percentage of control. Data are mean ± S.D. of three to six independent experiments with four replicates for each condition. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared to control (one-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test).

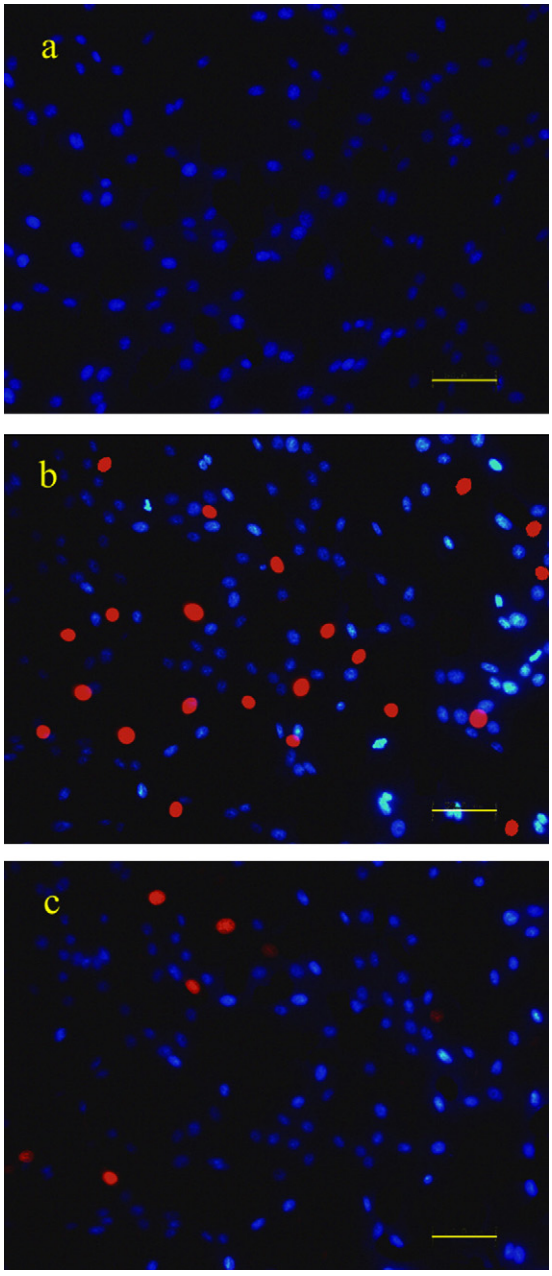

Fig. 2 – Necrotic and apoptotic cell death of differentiated PC12 cells as revealed by supravital double nuclear staining. Cells were exposed to either (a) vehicle medium (control) or (b) 750 μmol/L NMDA for 12 h followed by 24 h reperfusion or (c) preincubation with 5 mmol/L carnosine for 18 h, then treatment with 750 μmol/L NMDA for 12 h, and 24-h reperfusion followed immediately by double-staining with cell membrane-permeable (Hoechst 33342; blue) and -impermeable (propidium iodide; red) DNA labeling fluorochromes. Scale bars, 50 μm.

preincubation with 5 mM carnosine inhibited both NMDA-induced necrotic and apoptotic PC12 cell death (Fig. 2c).

3.3. *Effects of H_1, H_2 and H_3 antagonists on the protective effect of carnosine on NMDA-induced neurotoxicity*

The expression of H_1, H_2 and H_3 receptors in PC12 cells was confirmed by immunocytochemistry (data not shown). To investigate the mechanism of carnosine-induced neuroprotection against NMDA insult, the effects of H_1, H_2 and H_3 antagonists were measured. Treatment with pyrilamine, a selective central H_1 antagonist, reversed the protective effect of carnosine in a concentration-dependent manner (Fig. 4). In addition, thioperamide, a H_3 antagonist, at high concentration of 10^{-5} and 10^{-4} mol/L, also significantly inhibited this protection. The H_3 agonist (*R*)-α-methylhistamine, at 10^{-5} and 10^{-4} mol/L, significantly reversed the effect of thioperamide (data not shown). On the other hand, the selective central H_2 antagonist, cimetidine, did not affect the protective action of carnosine. In addition, these histamine receptor antagonists alone did not have appreciable effects on PC12 cells at the concentrations used (data not shown).

3.4. *Changes of intracellular and extracellular levels of carnosine, histidine and histamine induced by carnosine in PC12 cells*

To confirm the mechanism of protective action of carnosine, we studied its time-dependent effects on intracellular or

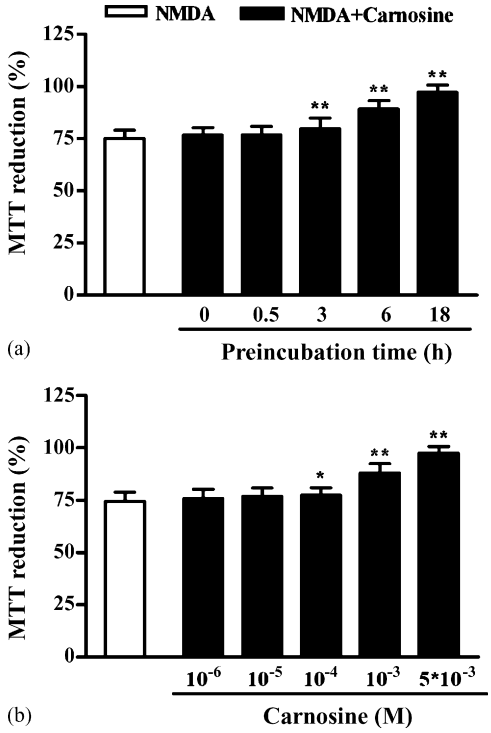

Fig. 3 – Time- and concentration-dependent neuroprotection by carnosine against NMDA insult in differentiated PC12 cells. Cell cultures were pretreated with 5 mmol/L carnosine for the indicated times, and then cultures were exposed to 750 μmol/L NMDA for 12 h (a) or cells were pretreated with indicated concentrations of carnosine for 18 h, and then cultures were exposed to 750 μmol/L NMDA for 12 h (b). Neuronal viability was determined 24 h after NMDA treatment using the MTT reduction assay. Results expressed as percentage of control. Data are mean ± S.D. of three to six independent experiments with 4 replicates for each condition. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with NMDA group (one-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test).

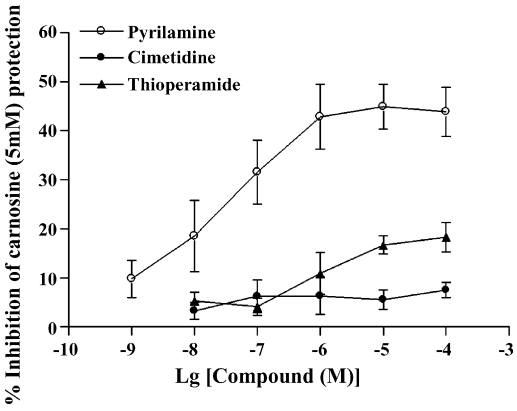

Fig. 4 – Effects of H_1, H_2 and H_3 receptor antagonists on the protection by carnosine. Cell cultures were exposed to vehicle or the indicated drugs. Each histamine receptor antagonist was added 15 min before carnosine. Carnosine (5 mmol/L) was added 18 h before NMDA (750 μmol/L). Cell viability was determined by MTT assay 24 h after application of NMDA for 12 h. Values expressed as percentage inhibition of the protection by carnosine and are from three to six independent experiments with four replicates for each condition.

extracellular levels of carnosine, histidine and histamine. In our culture system, PC12 cells were able to synthesize carnosine at a concentration of 2.6 nmol/well (Fig. 5). Preincubation with 5 mmol/L carnosine for 30 min produced a significant increase of carnosine content in PC12 cells. When the cultures were pretreated with carnosine for 3 h, the intracellular carnosine content reached a maximal level, 26.5 nmol/well. However, after 6 and 18 h, the intracellular carnosine levels were lower than those measured after preincubation for 3 h. On the other hand, pretreatment with carnosine induced a significant increase of intracellular and extracellular levels of histidine and histamine in a time-related manner (Fig. 6). When preincubated with carnosine for 18 h, the intracellular and extracellular histamine content reached 523 and 145 pmol/well, respectively. In addition, pretreatment with EBSS did not alter the levels of histidine and histamine at any time during the experiments (data not shown).

3.5. *Effect of α-FMH on the protective action of carnosine against NMDA-induced neurotoxicity*

α-FMH, a selective and irreversible inhibitor of histidine decarboxylase (HDC), which is the key enzyme for the synthesis of histamine from histidine, significantly reversed carnosine-related protective effects on NMDA-induced cell death in PC12 cells at concentrations of 10^{-6}, 10^{-5} and 10^{-4} mol/L (Fig. 7). However, the antagonistic effect of α-FMH was less than that of pyrilamine, a selective central H_1 antagonist (Figs. 4 and 7). In addition, α-FMH markedly inhibited the increase of intracellular and extracellular

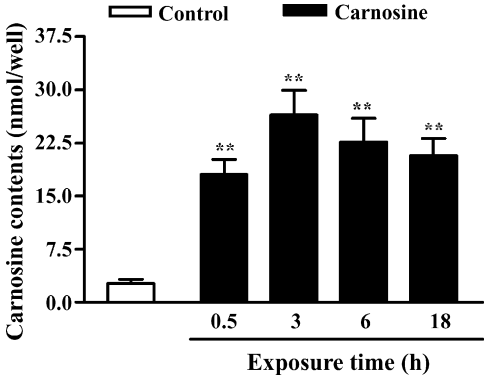

Fig. 5 – Changes of intracellular carnosine content after preincubation with carnosine (5 mmol/L) for the indicated times. The level of intracellular carnosine was quantified by HPLC at the indicated times after exposure. Data represent the mean ± S.D. of three to six independent experiments with six replicates for each condition. $^{**}p < 0.01$, compared with control group (one-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test).

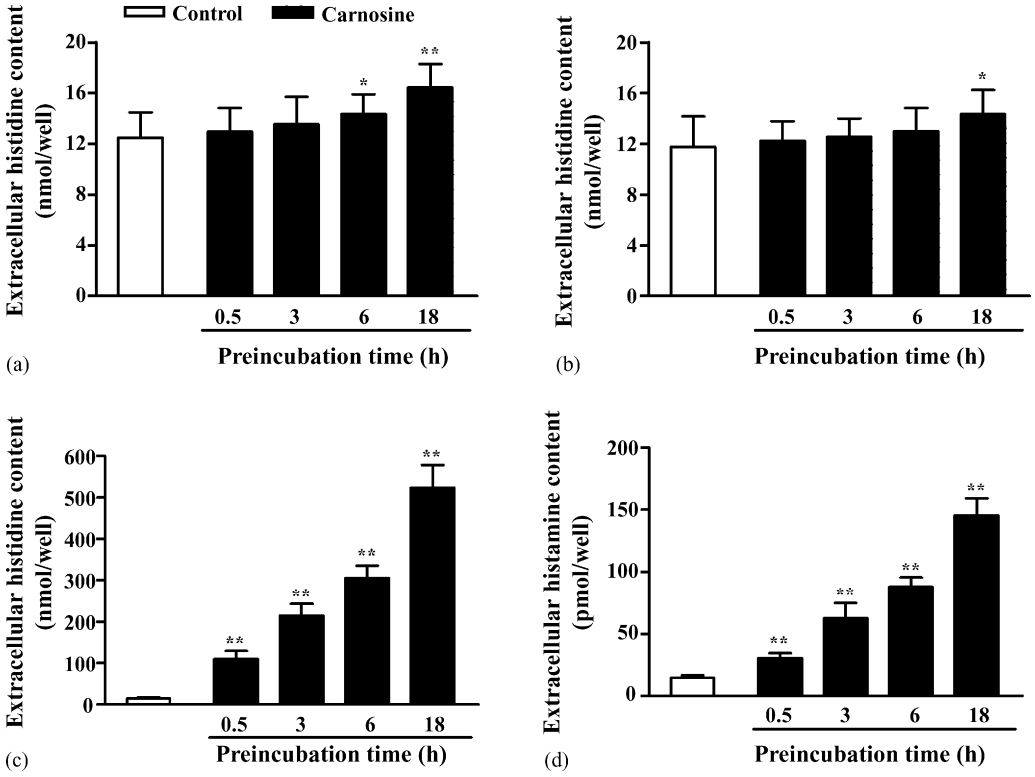

Fig. 6 – Changes of extracellular and intracellular histidine and histamine concentrations induced by carnosine (5 mmol/L) for the indicated times. The levels of extracellular and intracellular histidine and histamine were quantified by HPLC at the indicated times after exposure. Data represent the mean ± S.D. of three to six independent experiments with four replicates for each condition. *$p < 0.05$ and **$p < 0.01$, compared with control group (one-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test).

histamine induced by 18 h incubation with 5 mmol/L carnosine (Table 1).

3.6. *Increase of HDC activity by carnosine treatment in PC12 cells*

The effect of carnosine on HDC activity was investigated in differentiated PC12 cells after 18 h incubation with 5 mmol/L carnosine. The control value of HDC activity was 4.54 ± 0.39 pmol histamine/well/h. Carnosine significantly increased the HDC activity, to 5.07 ± 0.43 pmol histamine/well/h (data not shown).

3.7. *Effect of carnosine and thioperamide on glutamate and GABA release*

We also tested whether the protective action of carnosine against NMDA insult involved other neurotransmitters, such as the principal excitatory neurotransmitter glutamate and the main inhibitory neurotransmitter GABA. NMDA significantly increased the glutamate release, while pretreatment with 5 mmol/L carnosine significantly inhibited glutamate release in the presence or absence of excitotoxic concentrations of NMDA (Fig. 8). Thioperamide (10 μmol/L), a H_3 antagonist, significantly reversed the inhibition of glutamate release induced by carnosine (Fig. 9). On the other hand,

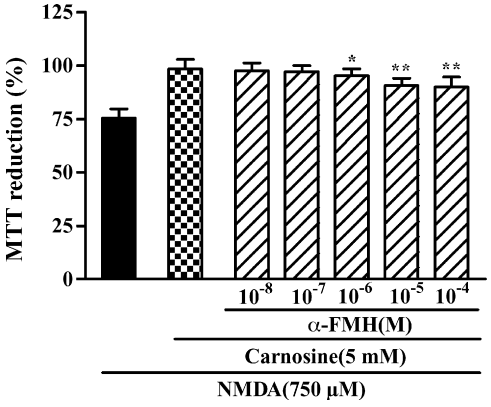

Fig. 7 – Effects of α-FMH on the protective action by carnosine on NMDA-induced neurotoxicity in differentiated PC12 cells. α-FMH was added 3 h before carnosine. Carnosine (5 mmol/L) was added 18 h before NMDA (750 μmol/L). Cell viability was determined by MTT assay 24 h after application of NMDA for 12 h. Values expressed as percentage of control. Data are mean ± S.D. of three to six independent experiments with four replicates for each condition. *$p < 0.05$ and **$p < 0.01$, compared with carnosine group (one-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test).

Table 1 – Effect of α-FMH on intracellular and extracellular histidine and histamine concentrations induced by carnosine

	Histidine (nmol/well)		Histamine (pmol/well)	
	Intracellular	Extracellular	Intracellular	Extracellular
Control	11.40 ± 2.52	12.02 ± 1.18	14.87 ± 1.82	15.04 ± 2.10
Carnosine	14.36 ± 2.14[a]	16.81 ± 2.07[a]	149.26 ± 15.58[a]	574.95 ± 22.79[a]
Carnosine + α-FMH	15.51 ± 3.46	18.30 ± 2.32	40.93 ± 6.8[b]	57.57 ± 9.00[b]

PC12 cells were treated with carnosine (5 mmol/L) for 18 h. α-FMH (10^{-5} mol/L) was added 3 h before carnosine. The amounts of intracellular and extracellular histidine and histamine were quantified by HPLC. Data represents the mean ± S.D. of three to six independent experiments with four replicates for each condition. (One-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test.)

[a] $p < 0.01$, compared with control group.

[b] $p < 0.01$, compared with carnosine group.

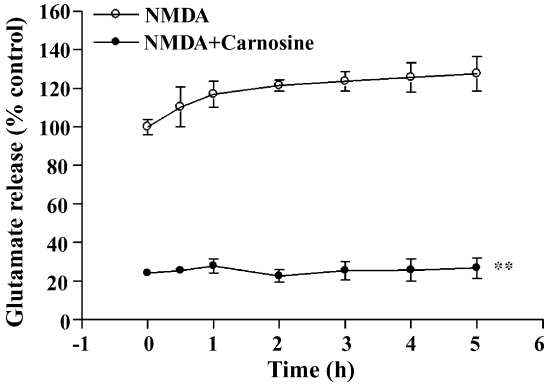

Fig. 8 – Effect of carnosine on glutamate release in differentiated PC12 cells. PC12 cells were pretreated with carnosine (5 mmol/L) for 18 h, and then cultures were exposed to NMDA (750 μmol/L) for the indicated times. The glutamate level was quantified by HPLC. Data represent the mean ± S.D. of three to six independent experiments with four replicates for each condition. A two-way ANOVA assessed a significant difference between groups. $^{**}p < 0.01$, compared with NMDA group.

carnosine treatment did not significantly influence GABA levels in differentiated PC12 cells (data not shown).

4. Discussion

Carnosine, a histidine dipeptide, has been known for about a century. A variety of roles and functions have been suggested for this agent, which is present in high concentrations in brain tissue and skeletal muscles [21]. Its specific localization in neurons and glia, its high millimolar concentrations, as well as its abilities to scavenge many ROS and inhibit glycosylation, suggest that it is a neuroprotective agent in the brain [22]. This idea is supported by our present study in which the neuroprotective effect of carnosine against NMDA-induced necrosis and apoptosis was measured in differentiated PC12 cells. Usually, the protective function of carnosine is related to its action as an antioxidant, metal chelator, free radical scavenger, and inhibitor of protein glycosylation. On the other hand, carnosine has been reported to serve as a non-mast-cell reservoir for histidine, utilized for histamine synthesis [15,23]. In the present study, we found that carnosine induced a significant increase of intracellular and extracellular histidine and histamine levels in a time-dependent manner, and the increase of histamine was reversed by the selective and irreversible HDC inhibitor, α-FMH. We also showed that the protection by carnosine was significantly reversed by the H_1 antagonist pyrilamine. These findings indicate that the carnosine-histidine-histamine pathway is involved in the carnosine-mediated protection against NMDA-induced neurotoxicity in PC12 cells, while further confirming the existence of this pathway in brain as shown in our previous study [16]. Direct infusion of histamine into brain evokes an acute inflammatory response [24], while carnosine can transform into histamine to play a protective function while avoiding long-term exposure of tissue to histamine. Moreover, it has been demonstrated that carnosine can easily cross the BBB and has few side-effects since it is normally present in the brain [25]. Therefore, carnosine holds promise as an anti-excitotoxic drug.

Several studies have shown that histamine can modulate NMDAR-mediated synaptic transmission, and the H_1 receptor

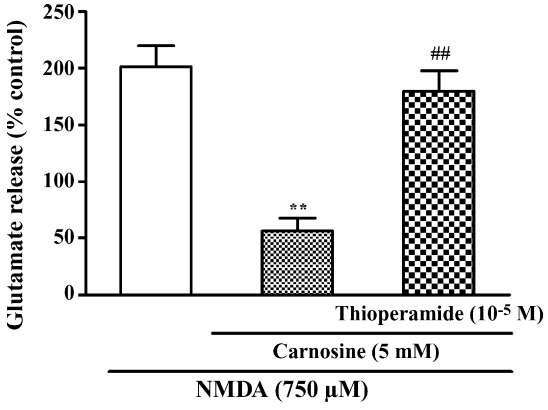

Fig. 9 – Effect of thioperamide on glutamate release in differentiated PC12 cells. PC12 cells were pretreated with carnosine (5 mmol/L) for 18 h, and then cultures were exposed to NMDA (750 μmol/L) for 12 h; thioperamide (10 μmol/L) was added 15 min before carnosine. The amount of glutamate was quantified by HPLC. Data represent the mean ± S.D. of three to six independent experiments with four replicates for each condition. [a]$p < 0.01$, compared with NMDA group; [b]$p < 0.01$, compared with NMDA + Carnosine group (one-way ANOVA followed by LSD or Dunnett's T3 *post-hoc* test).

plays an important role in this process. For example, Diaz-Trelles et al. reported that the H_1 receptor antagonist terfenadine potentiates NMDAR-mediated neuronal death, and this is completely reversed by histamine [7]. H_1 receptor blockade aggravates NMDA-induced neuronal damage in rats [4]. In this study, the protection by carnosine was markedly reversed by the H_1 antagonist pyrilamine, but not by the H_2 antagonist cimetidine. This suggests that the H_1 receptor plays a critical role in the protective mechanism of carnosine.

Interestingly, we also found that intracellular carnosine reached maximum after preincubation with carnosine for 3 h, but the maximal neuroprotection by carnosine was observed after preincubation for 18 h. Boldyrev et al. [11] reported that carnosine accumulates within the cells and immediately acts to quench intracellular ROS generated by NMDA insult, or acts as an anti-glycating agent. Therefore, the maximal neuroprotection that occurs long after the accumulation of carnosine in the cell is probably due to histamine synthesis. Furthermore, time-dependent mechanisms may also exist, since the antioxidation occurs early and the histamine-related neuroprotection occurs later. These results also provide further evidence to support the proposal that carnosine can be metabolically transformed into histamine in differentiated PC12 cells.

Carnosine enhanced histidine and histamine levels. However, the increase in histamine obviously exceeded that of histidine. In addition, α-FMH markedly blocked histamine synthesis, but only partly elevated the levels of the precursor histidine. Therefore, we checked whether HDC activity was promoted by carnosine. Interestingly, we found that HDC activity was significantly increased by carnosine in differentiated PC12 cells. Previous workers have reported that carnosine can inhibit the activity of HDC, but this can also be increased by substances like compound 48/80 [26,27]. Our data support the idea that carnosine plays an important role in histaminergic neurons. The potential effect of carnosine on HDC further suggests that carnosine may be a histaminergic agent. In addition, the seemingly uncoordinated changes in histidine and histamine levels may be due to their normally unbalanced distribution in PC12 cells.

On the other hand, however, as shown in Fig. 4, the H_1 antagonist did not completely inhibit the protection by carnosine. Further, we were surprised to find that α-FMH, at the high dose of 10^{-4} mol/L, only partly reversed the neuroprotective effect of carnosine but markedly decreased intracellular histamine content by 90%. These data suggest that the elevated histamine level in PC12 cells induced by carnosine is not the only mechanism contributing to its protection against NMDA insult, and other potential mechanisms may exist. Recently, O'Dowd and Miller [28] reported that, like histamine, carnosine directly activates smooth muscle H_1 receptors to evoke vasoconstriction, with greater efficacy than noradrenaline. The agent-receptor binding test also showed that carnosine can directly bind to H_1 receptors in the CNS. Therefore, it is likely that carnosine can significantly inhibit NMDA-induced neurotoxicity through its own actions, like antioxidant activity, and also by directly acting at H_1 receptors and evoking a histamine-like response.

In this study, the protection by carnosine was also inhibited by thioperamide at high concentration, and this was reversed by the H_3 agonist (*R*)-α-methylhistamine, indicating the involvement of H_3 receptors in the action of carnosine. H_3 receptors are exclusively presynaptic, where they not only provide negative feedback to restrict histamine synthesis and release, but also inhibit the release of other neurotransmitters such as glutamate and GABA [29]. Recently, Kurata et al. [30] reported that carnosine exerted a significantly renoprotection against ischemia/reperfusion-induced renal injury through activating the H_3 receptors in the central nervous system and then suppressing increased renal sympathetic nerve activity during the renal ischemia. In this study, we found that the glutamate release level was significantly decreased after 18 h preincubation with carnosine. Moreover, the inhibition of glutamate release by carnosine was still effective even with externally applied excitotoxic concentrations of NMDA. However, the inhibition of glutamate release was significantly reversed by the H_3 antagonist, thioperamide. Therefore, the glutaminergic system may be suppressed by the elevated histamine derived from carnosine through the H_3 receptor, and the neuroprotection by carnosine may partially be due to the suppression of excessive glutamate release during excitotoxity in PC12 cells. On the other hand, treatment with carnosine did not significantly change the release of GABA, the major inhibitory neurotransmitter in the brain. In conclusion, the data presented in this study demonstrate that carnosine can protect against NMDA-induced neurotoxicity in differentiated PC12 cells. The mechanism of this protection may not only involve the carnosine-histidine-histamine pathway, but also H_1/H_3 receptors and the effective inhibition of glutamate release. This study indicates that carnosine may be an endogenous protective factor and calls for its further study as a new antiexcitotoxic agent.

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