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PHYSIOLOGY

Carnosine Prevents the Development of Oxidative Stress Under the Conditions of Toxic Action of Cadmium

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Abstract—Protective effect of the natural dipeptide carnosine on the antioxidant system of rats under conditions of oxidative stress caused by chronic cadmium administration was investigated. Oxidative status of experimental animals were evaluated based on a number of informative parameters of iron-induced chemiluminescence. It was shown that the introduction of cadmium for 7 days reduces the duration of the latent period of chemiluminescence in the brain, liver, and blood plasma suggesting the depletion of endogenous antioxidant defense. Coexposure to carnosine and cadmium led to significant increase in the level of antioxidant protection in plasma, liver, and brain of animals. Carnosine also prevented the increase of lipid hydroperoxides in the brain and prevented the development of lipid peroxidation content in liver and plasma of animals. Mechanism of the protective effect of carnosine under conditions of oxidative stress induced by cadmium administration was shown on human neuroblastoma SH-SY5Y cell culture. Addition of the cadmium to the incubation medium to a final concentration of 5 μM reduced cell viability of a culture, as was determined by MTT assay; simultaneous addition of carnosine (0.25 mM final concentration) with cadmium resulted in increased cell viability during 24 hours of incubation. Thus, carnosine in a final concentration of 1 mM effectively prevented the development of necrotic lesions of neuroblastoma cells, inhibiting the formation of reactive oxygen species as measured by flow cytometry. The results indicate the ability of carnosine to prevent the development of oxidative stress under the toxic action of cadmium.

Keywords: carnosine, cadmium, oxidative stress, Wistar rats, iron-induced chemiluminescence, human neuroblastoma SH-SY5Y, ROS, viability, cell death.

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INTRODUCTION

Cadmium is one of the most toxic and carcinogenic heavy metals. Cadmium is considered as a serious environmental and industrial pollutant, and it can pose a significant risk to human and animal health, even in low doses, since it accumulates in body tissues and has a long half-life [1]. Cadmium is present in the air around industrial plants, various emissions from production, in food, and cigarette smoke [2]. Exposure to cadmium may cause damage of many organs and tissues, including the CNS, leading to various pathologies [3, 4]. Cadmium is a causative factor of neurodegenerative diseases, such as Alzheimer's and Parkinson's disease [5]. It causes neurological changes in humans and animals, leading to impaired concentration, olfactory dysfunction, memory loss [6], causes irritability, and reduces learning ability [7]. Chronic exposure to cadmium in rats leads to a change in the number of blood cells and induces processes of lipid peroxidation (LPO) in blood plasma [8]. The potential mechanism of cadmium toxicity consists in the ability to induce the production of reactive oxygen species (ROS), resulting in depletion of reserves of the antioxidant systems [9–11] and development of oxidative stress (OS), mitochondrial dysfunction, and apoptosis [12, 13]. These processes are particularly important for the nervous system; it is associated with the selective sensitivity of cells of excitable tissue to oxidative degradation [14, 15].

Many low molecular weight antioxidants (ascorbic acid, alpha-tocopherol, glutathione, histidine-containing dipeptides, etc.) are not only able to capture active radicals but also can chelate metal ions, reducing their catalytic activity in the processes of ROS and nitrogen production [11].

Carnosine (β-alanyl-L-histidine) is an effective protector of cells and tissues from OS, combining both direct antioxidant effect, and indirect effect, expressed in modulating of the activity of enzymes and NMDA-receptors involved in the development of OS [16]. It normalizes physiological parameters impaired by acute hypobaric hypoxia [17]. Carnosine and its ana-

logs can form complexes with bivalent metals: copper, cobalt, magnesium, zinc, and cadmium [18]. Among natural antioxidants, carnosine is different by its ability to cross the blood-brain barrier and penetrate into brain tissue [19].

At the same time, in the literature, there is no data showing the system protective effect of carnosine under cadmium intoxication of experimental animals and mechanisms of the protective action of carnosine under the toxic effect of cadmium on cell cultures. In reference to the above, the investigation of the ability of carnosine to prevent the development of OS in tissues most subjective to the oxidative damage under the toxic action of cadmium is an urgent task.

The goal of this study was the investigation of the protective effect of natural antioxidant carnosine on the endogenous antioxidant status and the state of LPO in blood plasma, liver, and brain of Wistar rats under the toxic action of cadmium, with an assessment of the protection mechanisms in in vitro experiments on human neuroblastoma SH-SY5Y cell culture.

MATERIALS AND METHODS

In vivo experiments. Twenty-seven Wistar male rats of the age of 8 months, weighting 280–300 g, kept under standard vivarium conditions with a 12-hour daily cycle "day/night" and free access to water and food were used for the study.

The rats were intraperitoneally administered for 7 d with cadmium chloride (1 μM, 1 mL/kg body weight) and carnosine (100 mg/mL, 1 mL/kg) (Hamari Chemicals Ltd., Japan), prepared using physiological solution.

Experiment protocol.

The rats were divided into four groups:

(1) control animals: two normal saline injections with an interval of 3 h;

(2) carnosine: first injection of normal saline and second of carnosine after 3 h;

(3) cadmium: first injection of cadmium and second of normal saline after 3 h;

(4) cadmium + carnosine: first injection of cadmium and second of carnosine after 3 h.

On the seventh day, the animals were decapitated and blood plasma, liver, and cerebral hemisphere samples were taken. Heparin solution (1%) was added to the blood in order to prevent coagulation; the blood was then centrifuged for 10 min at 3000*g* and plasma was collected. All the samples were frozen in liquid nitrogen and stored at –80°C for further use.

Iron-induced chemiluminescence (CL) method of biological samples. The state of LPO processes and the level of endogenous antioxidant protection were investigated by the method proposed by Y.A. Vladimirov and adapted for a Luminometer-1251 (LKB, Sweden) [20]. The following parameters were analyzed: quick flash of the CL (h), whose intensity characterizes the level of modified products of LPO, latency period (τ), indicating the resistance of the substrate to further oxidation, reflecting the endogenous antioxidant capacity and the highest possible intensity of CL (H) associated with the further oxidation of ferrous ions and accumulation of LPO products.

Preparation of samples for analysis. Blood plasma was thawed in the thermostat at 37°C. Phosphate buffer (0.85 mL) was added to 0.05 mL of plasma. Tissue samples (cerebral hemispheres, liver) were homogenized in the presence of phosphate buffer (60 mM KH_2PO_4 and 105 mM KCl at pH 7.45); 0.8 mL phosphate buffer was added to 0.1 mL of 10% tissue homogenate.

Registration of parameters. The cuvette with the obtained suspension was placed in a measuring chamber (preheated to 37°C with constant stirring) and baseline values were recorded. For the initiation of chemiluminescence, 0.1 mL of ferrous iron solution ($FeSO_4 \cdot 7H_2O$) at a final concentration of 2.5 mM was added using a dispenser into a cuvette and luminescence curve was recorded.

In vitro experiments. Experimental studies were conducted on human neuroblastoma SH-SY5Y cell culture (ATCC, United States).

Cells were cultured on a mixture of minimal essential medium with Earle's salts and glutamine (PanEco, Russia) and F-12 without glutamine (PanEco, Russia) in a ratio of 1 : 1 with the addition of 1% penicillin-streptomycin solution (PanEco, Russia) and 10% fetal calf serum (PAA Laboratories, United States). The cell culture was kept in incubator (ShelLab) at 37°C, 90% humidity, 5% CO_2. Medium was replaced every 3 d. Cells were passaged every 7–8 d depending on the growth rate.

The dose-dependent effect of cadmium toxicity was determined by the addition of cadmium chloride in concentrations of 10 μM–10 mM to cells 3 d after passage. The protective effect of carnosine was determined by the addition of carnosine in the concentration of 0.25–5 mM (MTT test) and 1 mM (flow cytometry) to the selected concentration of cadmium. In all cases, the cells were incubated for 24 h.

MTT-test. The number of viable cells was determined in 96-well plates with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) using a Synergy H4 microplate spectrophotometer at wavelengths of 570 and 660 nm [21]. The signal level in the control wells was taken as 100%.

Determination of the proportion of dead cells and ROS level. The proportion of dead cells and ROS level in the cell population was determined by a FACSCalibur flow cytometer (BDBiosciences, United States) using propidium iodide, PI (Invitrogen), and 2',7'-dichlorodihydrofluorescein diacetate, DCFN2-DA

(Invitrogen), respectively. Propidium iodide and DCFN2-DA were added to final concentrations of 10 μM 3 min and 30 min before the measurement, respectively.

The data obtained from histograms were evaluated; the ordinate axis represented the number of events and the horizontal axis represented the distribution of propidium iodide and DCFN2-DA fluorescence. The data were processed using CellQuestPro program (BD, United States).

Statistical processing of the obtained results was performed using the Mann–Whitney *U* test.

RESULTS AND DISCUSSION

Effect of cadmium on LPO and antioxidant status in the blood plasma, liver and brain tissue of animals. In the brains of rats treated with cadmium, an increase in the level of lipid hydroperoxides (h, mV) by 9% was observed, and the level of lipid hydroperoxides was in the range of control values in the blood plasma and liver. A significant increase in the maximum intensity of lipid peroxidation (H, mV) was observed in blood plasma (41%) and rat liver (15%); significant changes in the brain were not observed. The duration of the latent period of CL (τ) in the blood plasma was decreased by 40%, by 49% in the liver, and by 41% in the brain (Fig. 1).

Evaluation of the protective effect of carnosine on LPO processes and antioxidant status in the blood plasma, liver, and brain tissue under prolonged administration of cadmium. The administration of carnosine to intact animals had no significant effect on the studied parameters in blood plasma. In livers, carnosine increased the duration of CL latency by 11% and reduced the level of lipid hydroperoxides in the brain by 10% relative to the control group.

Daily administration of carnosine 3 h after cadmium during 7 d contributed to the reduction of the level of lipid hydroperoxides in the brain to control values. The maximum intensity of LPO in the liver decreased to control levels, while this value in blood plasma reduced by 13% relative to animals treated with cadmium, but remained increased relative to intact animals. The duration of CL latent period in blood plasma increased up to control values: up to 92–93% in the brain and in the liver.

Evaluation of the protective effect of carnosine on cell viability. In preliminary experiments, assessing the dose-dependent effect of cadmium on cell viability, the concentration of cadmium was 5 μM, since this concentration decreased the number of surviving cells by 25% as was measured by MTT test under conditions of 24 h incubation [23]. The addition of carnosine in incubation medium containing cadmium led to an increase in the number of viable cells. The most significant protective effect of carnosine was shown for a concentration of 0.25 mM.

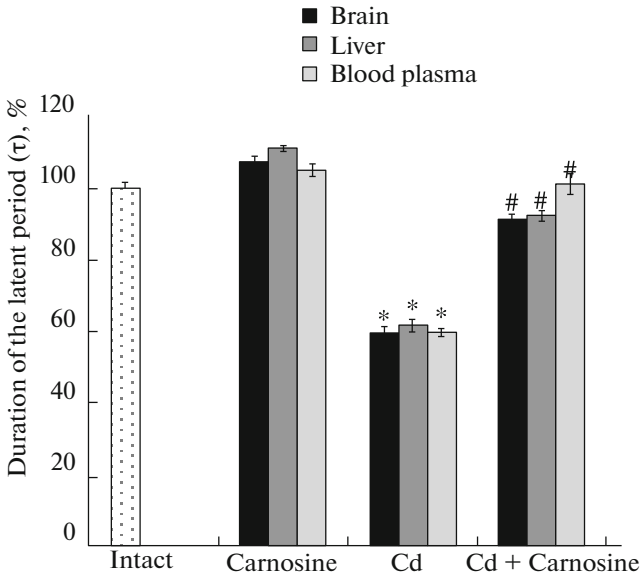

Fig. 1. Effect of cadmium and carnosine on the duration of the latent period of chemiluminescence in blood plasma and in brain and liver tissues during the chronic cadmium administration to rats. Values in the control group were taken as 100%. *$p < 0.05$ relative to the control group; *$p < 0.05$ relative to the group of animals treated with cadmium.

Evaluation of the protective effect of carnosine on cell death and ROS level. It was found that, in the presence of 10 μM cadmium, the proportion of dead cells increased, on average, by 64% (Fig. 2a), and ROS level increased by 40% (Fig. 2b). The addition of 1 mM carnosine to the culture medium effectively prevented the development of necrotic damage and decreased cell death by 51%. Under these conditions, carnosine also provided suppression of ROS production. The basis for the selection of effective protective dose of carnosine were data of previous studies [24, 25], which, based on CL, demonstrated that carnosine at a final concentration of 1 mM decreased the content of preformed lipid hydroperoxides by 50%.

The obtained data indicate a significant decrease in the activity of the endogenous antioxidant defense system and the development of oxidation processes in animals during the systemic administration of cadmium. These processes were manifested as increased maximum intensity of lipid peroxidation in plasma and liver of animals and increased level of lipid hydroperoxides in the brain of animals. The selective increase in the level of lipid hydroperoxides in the brain indicates its higher sensitivity to oxidative damage caused by the toxicity of cadmium. Administration of carnosine under these conditions promoted a significant increase in the level of antioxidant defense system in the plasma, liver, and brain of the animals and the prevention of the increase of lipid hydroperox-

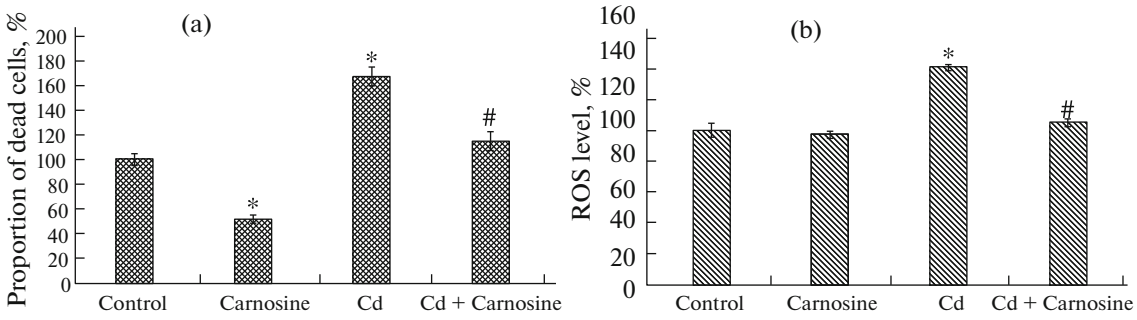

Fig. 2. Effect of 1 mM of carnosine on the proportion of (a) dead cells and (b) and ROS level in human neuroblastoma SH-SY5Y cell culture under intoxication with 10 mM cadmium chloride. Values obtained for intact cells were taken as 100%. *p < 0.05 relative to intact cells; #relative to cells to which cadmium was added.

ides in the brain and the development of LPO in liver and blood plasma.

An important aspect of the action of carnosine is not only the ability to prevent the development of OS under pathological conditions but also the regulation of the oxidative balance in healthy animals. Thus, the administration of carnosine to intact animals contributed to increased level of endogenous antioxidant defense by 8%, 11%, and 5% in the brain, liver, and blood plasma, respectively.

The addition of 0.25 mm carnosine to the culture medium of human neuroblastoma SH-SY5Y cell culture significantly increased the cell viability as determined by MTT assay, and carnosine in a concentration of 1 mM prevented the death of cells, inhibiting the formation of ROS, as was established by flow cytometry. The obtained data suggest direct antioxidant effect of carnosine under conditions of cadmium toxicity.

In general, the obtained results indicate the ability of carnosine to prevent the development of oxidative stress caused by the chronic cadmium administration at the level of the whole organism and individual organs. This protective effect of carnosine is determined by its ability to prevent the formation of ROS and cell death caused by necrotic processes.

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