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Carnosine Protects the Brain of Rats and Mongolian Gerbils Against Ischemic Injury: After-Stroke-Effect*

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Carnosine, a specific constituent of excitable tissues of vertebrates, exhibits a significant antioxidant protecting effect on the brain damaged by ischemic-reperfusion injury when it was administered to the animals before ischemic episode. In this study, the therapeutic effect of carnosine was estimated on animals when this drug was administered intraperitoneally (100 mg/kg body weight) after ischemic episode induced by experimental global brain ischemia. Treatment of the animals with carnosine after ischemic episode under long-term (7–14 days) reperfusion demonstrated its pronounced protective effect on neurological symptoms and animal mortality. Carnosine also prevented higher lipid peroxidation of brain membrane structures and increased a resistance of neuronal membranes to the *in vitro* induced oxidation. Measurements of malonyl dialdehyde (MDA) in brain homogenates showed its increase in the after brain stroke animals and decreased MDA level in the after brain stroke animals treated with carnosine. We concluded that carnosine compensates deficit in antioxidant defense system of brain damaged by ischemic injury. The data presented demonstrate that carnosine is effective in protecting the brain in the post-ischemic period.

KEY WORDS: Brain ischemia; oxidative stress; antioxidant activity; carnosine; Mongolian gerbils; rats.

INTRODUCTION

Carnosine (β-alanyl-L-histidine) is a specific constituent of excitable tissues of vertebrates possessing significant antioxidant properties (1–4). As it was found in model experiments, carnosine inhibits Fe^{2+}-induced oxidation of membrane lipids (5), oxidative modification of proteins (6), or DNA fragmentation induced by reactive oxygen species (ROS) (7,8). Carnosine was found to suppress ROS generation taking place under activation of ionotropic glutamate receptors *in vitro* and to prevent neuronal cell death both by apoptosis and necrosis (9). When carnosine was injected into rats intraperitoneally before experimental ischemia it supported microcirculation (10) and normalized a number of biochemical parameters in the brain including synaptosomal Na/K-ATPase (11,12) and mitochondrial monoamine oxidase B (12). Moreover, using the same application (intraperitoneally) of carnosine before ischemic injury, it prevented also brain membrane lipid disordering induced by oxidative stress, and decreased animal mortality at the same time (10,12). More recent studies demonstrated that carnosine can

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protect both *in vitro* and *in vivo* activity of some important antioxidant enzymes like ceruloplasmine (13) or superoxide dismutase (14,15)

In this study, the antioxidant ability of carnosine was estimated on animal models when it was administered intraperitoneally following free radical brain damage induced by experimental brain ischemia. We were able to demonstrate the pronounced protective effect of carnosine on neurological symptoms, animal viability and reparation of brain membranes damaged by oxidative stress under experimental brain ischemia.

EXPERIMENTAL PROCEDURE

Animals. Two different kinds of animals were used in our experiments: Wistar rats and Mongolian gerbils. The animals were purchased from The Institute of Experimental Pharmacology, Slovak Academy of Sciences, Slovak Republic. Animals were treated and handled according to the guidelines of the European Union Council. This was controlled by the Jessenius Faculty of Medicine, Comenius University Ethics Committee and approved by the State Veterinary Commission of the Slovak Republic (No 3669/00–220). The animals were housed in a well ventilated animal room under a light/dark cycle of 12:12 h (light on from 06:00 to 18:00 h). The animals had free access to food (MP 30, Dobra Voda, Slovak Republic) and water.

Surgical Methods. Two experimental brain ischemia models (Models 1 and 2), induced by a short-term occlusion of the head arteries with subsequent long-term reperfusion, were used in this study.

Model 1: Wistar rats (280–300 g body weight, n = 20) were used in this set of experiments. Modification to the 4-vessel occlusion model (16) was made and animals were exposed to 3-vessel occlusions (both common *carotid arteries* and the left *arteria vertebralis*) for 15 min. One group of animals (n = 7) was treated with i.p. injection of 1 ml saline, 1 min before clip removal; injections were repeated after 3, 8, 24 h, and each day during the entire 6-day reperfusion period. The second group of animals (n = 8) were treated with carnosine dissolved in saline solution in amount corresponding to dose of 100 mg/kg body weight) using the same protocol. From the second day, both groups of animals were treated with 3-nitropropionic acid (3-NP) (30 mg/kg body weight during 2–4 days and 20 mg/kg body weight during the 5th and 6th days after the surgical operation). The morbidity of the rats of both groups was analyzed throughout the experimental period using the 6-point neurological symptoms scale to classify "Huntington's disease" induced by 3-NP (17,18). At the end of experiment (7th day) the rats were decapitated and the gray matter was used for biochemical analysis. Remaining animals (n = 5) were not operated and used as a control.

Model 2: (Mongolian gerbils). Two-vessel occlusion (left and right *arteria carotis communis*) producing brain ischemia was followed by long-term reperfusion in this animal group (19,20). Mongolian gerbils of 65–75 g body weight (n = 29) were subjected to 30-min brain ischemia followed by 13-day reperfusion. The animals of the first group (n = 15) were treated with an i.p. injection of 0.5 ml saline fifteen minutes before reperfusion. The injections were repeated at 3, 8, and 24 h and each day of the experiment. The animals of the second group (n = 8) were treated with carnosine (100 mg/kg, dissolved in 0.5 ml saline) using the same protocol. Morbidity was estimated using the McGrow Stroke Index (21). After 13 days of reperfusion the living animal from both groups were decapitated and gray matter was used for biochemical analysis. The data from biochemical analysis were compared with that obtained from control gerbil brain samples (n = 6) that were not subjected to surgery.

Biochemical Methods. Brain gray matter membrane fractions were obtained and Fe^{2+}-induced chemiluminescence (CL) was measured as described in detail earlier (22) using LKB–1251 chemiluminometer. Fast CL response (*h*, mV) was used as an index of lipid hydroperoxide's stationary level in the sample analyzed; lag period (τ, s)—between addition of 2.5 mM $FeSO_4$ and slow CL flash—as a factor characterizing oxidative resistance of the membrane structures. The CL yield during 15 min of measurement (H) was used as an index characterizing susceptibility of the samples to oxidation (23). In addition to this procedure, the lipid peroxidation end products in the brain homogenate were measured with 2-thiobarbituric acid taking the amount of malonic dialdehyde (MDA) measured as the primary parameter indicating damage to brain lipids (24).

Chemicals. Carnosine was purchased from Hamary Chemicals Ltd. (Japan). 3-Nitropropionic acid, 2-thiobarbituric acid, butanol, glacial acetic acid and other bench reagents were purchased from Sigma Chemical Co., St.Luis, Mo. (USA).

RESULTS

Estimation of Clinical Status of the Animals

The rats that survived ischemic attack do not demonstrate in general neurological symptoms until day 7 of reperfusion. This hampers quantification of both the individual response of animals to the damaging effect of ischemia and the possible protection of the animals by drugs injected after the ischemic episode. In order to increase post-ischemic injury we used 3-NP, which was injected according to the protocol described in Materials and methods. As a result of the combination of ischemia and 3-NP treatment, strong neurological symptoms developed over time (starting from day 3 of the experiment), the level of neurological defects being notably milder in the group of animals that had been treated with carnosine during the period directly following ischemic injury (Fig. 1). There was no mortality during the observation period in either group of animals.

Mongolian gerbils subjected to experimental ischemia demonstrated both progressive mortality and increased neurological symptoms during the first days post surgery (see Fig. 2a and b). Early cases of mortality in the group of un-treated animals were noted on the 2nd day after ischemia and progressively increased until the 7th day (80%). In the group of animals treated with carnosine, mortality was noted

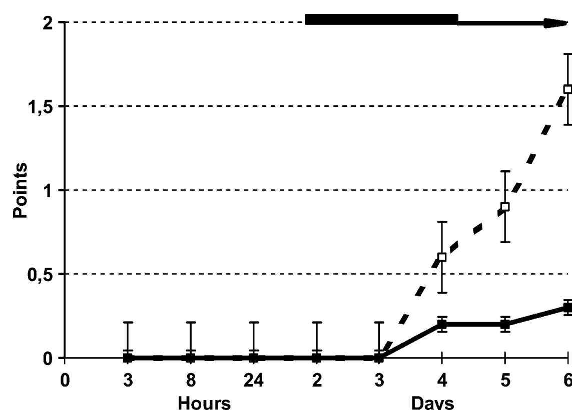

Fig. 1. Neurological symptoms in rats after experimental ischemia. 3-NP administration is shown by thick (30 mg/kg body weight, 2–4 days) and thin (20 mg/kg body weight, 5–6 days) lines with arrow: full black line – animals treated with carnosine, dashed line – untreated animals.

starting from the 3rd day after ischemia; the number of dead animals was significantly lower (42%). Neurological symptoms in this group were also markedly less. While the Stroke Index in both groups of animals (untreated and treated with carnosine) decreased with time to nearly the same level. This level was achieved at a much faster rate in the carnosine treated animals (2 days after the operation); in untreated animals this process took 7 days. Further recovery was slower and indistinguishable for both ischemic groups of animals (Fig. 2b).

Biochemical Results

Following the acute post-ischemic period (7th day in rats and 14th day in gerbils) the animals were decapitated and the brain homogentates were used to determine MDA and resistance of neuronal membranes to *in vitro* Fe^{2+}-induced lipid peroxidation. In Tables I and II the results of these experiments are presented for rats and Mongolian gerbils respectively.

As seen in Table 1, the stationary level of lipid hydroperoxides (*h*) is greatly increased after brain stroke in rats. However, when the animals were treated with carnosine during the post stroke period, the hydroperoxides accumulated to the significantly lower rate. The CL lag-period (τ) is reversed: it was shortened after ischemic injury, but restored nearly to the control level when carnosine treated animals were analyzed. The parameter *H* (oxidizability characteristic of membrane lipids) was similar in all three cases, which demonstrates that membrane lipid pattern is conservative and unchanged during the experimental period. Thus, CL parameters showed pronounced rat brain membrane oxidative damage after an ischemic episode, which was not repaired until the 7th day of ischemia. This is consistent with severe neurological symptoms during this period (Fig. 1). In the group of carnosine treated rats, the CL parameters are close to control level, which correlates with less severe neurological symptoms.

Measurements of MDA in brain homogenates of the same animals demonstrate a tendency similar to the above mentioned lipid hydroperoxides with an increase in MDA level in the ischemic brain and a decrease in that of animals treated with carnosine (Table I). However, stationary levels of brain MDA are very low and individual variations between the animals are relatively high, therefore the differences obtained are not statistically significant. It should be noted that lipid hydroperoxides are more objective parameters for evaluation of the brain membranes damage due to ischemic-reperfusion injury than MDA is.

Also the above parameters were found to be similar in the Mongolian gerbils living to the 14th day after-stroke injury (Table II). The stationary level of lipid hydroperoxides (*h*) increased by ischemic injury was normalized by carnosine. A lag-period (τ) of lipid peroxidation, decreased by brain ischemic injury, was not only restored to previous level, but exceeded that after carnosine treatment. At the same time, total

Table I. Chemiluminescence Parameters of the Fe^{2+}-Induced Brain Membrane Oxidation Isolated from Control [Ischemic Rats Untreated and Treated with Carnosine–Model 1]

Groups of animals	n	Parameters of Fe^{2+}-Induced Chemiluminescence			MDA nmoles/g tissue
		h, mV	τ, s	*H*, mV	
1 (Ischemia)	6	127.5 ± 17.5*	67 ± 8*	442 ± 40	24.4 ± 3.5
2 (Ischemia + Carnosine)	8	90.0 ± 13.8**	110 ± 12**	489 ± 40	21.8 ± 3.4
3 (Control)	5	69.5 ± 10.6	98 ± 7	458 ± 26	21.6 ± 2.6

*$P < 0.05$ with respect to group 3 animals.
**$P < 0.05$ with respect to group 1 animals.

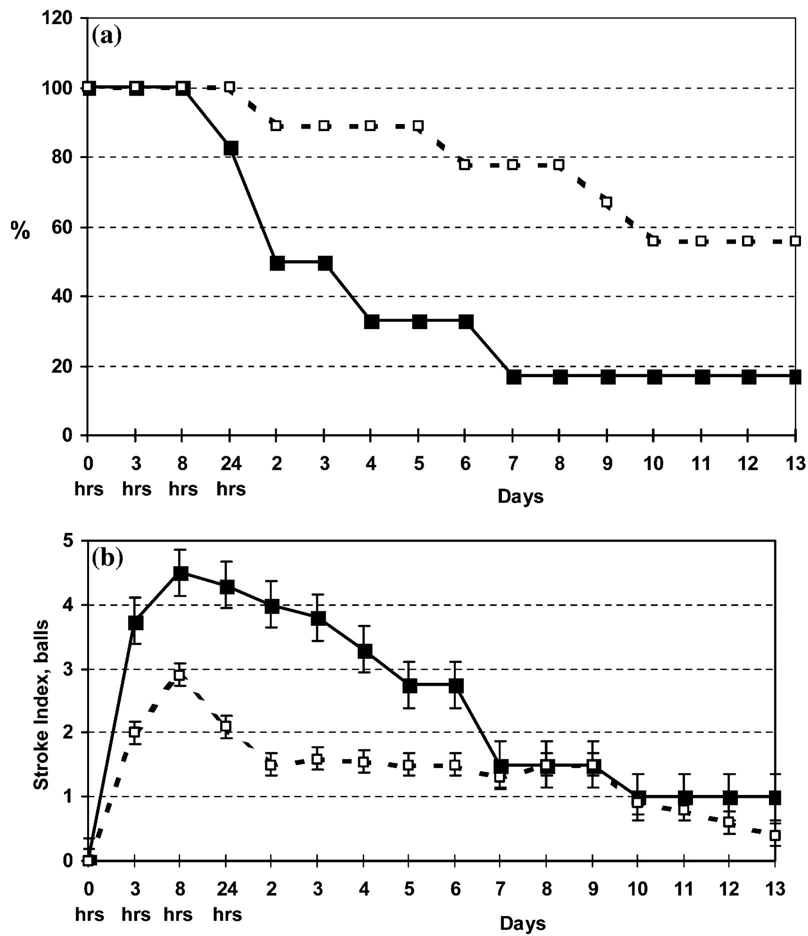

Fig. 2. Mortality (a) and morbidity (b) of Mongolian gerbils at different periods following experimental ischemia. 1 – untreated animals (full line). 2 – animals treated with carnosine as described in Materials and Methods (dashed line).

oxidizability of membrane lipids (*H*) and MDA level changed non-significantly again (Table II).

DISCUSSION

The both approaches to the brain ischemia-reperfusion injury used in our study, the modified Pulsinelli model conducted by 3-NP injections in rats and two-vessel occlusion of the carotides in Mongolian gerbils, allowed us to estimate neurological deficit development in time. Clinical symptoms of the brain damage in Mongolian gerbils are expressed very well in comparison to the rats; thus in these cases, one can characterize the gravity of ischemic injury by the mortality of the animals.

Table II. Chemiluminescence Parameters of the Fe^{2+}-Induced Brain Membrane Oxidation Isolated from Control [Ischemic Mongolian Gerbils Untreated and Treated with Carnosine–Model 2]

		Parameters of Fe^{2+}-Induced Chemiluminescence			
Groups of animals	n	*h*, mV	τ, s	*H*, mV	MDA nmoles/g tissue
1 (Ischemia)	3	127 ± 22.2*	66 ± 8*	552 ± 39	21.8 ± 2.6
2 (Ischemia + Carnosine)	5	94.5 ± 35.1	120 ± 23*, **	583 ± 58	17.0 ± 5.1
3 (Control)	6	78 ± 13.9	87 ± 6	677 ± 69	23.4 ± 2.6

*$P < 0.05$ with respect to group 3 animals.
**$P < 0.05$ with respect to group 1 animals.

Therefore, neurological deficit accumulation after an ischemic episodes in rats can be only elucidated when 3-NP was used in combination with ischemia itself. This compound in the *in vitro* experiments suppresses glucose transport into the neural cells and glutamate re-uptake by brain synaptosomes. It irreversibly inhibits mitochondrial succinate dehydrogenase and stimulates ROS formation by mitochondria. Therefore the administration of 3-NP results in a chronic deficit in cellular energy supply (25). Simultaneously 3-NP induces persistent NMDA-receptors activation with calcium ions influx into the neuronal cells and increase generation of superoxide radicals (26). It is also known that 3-NP administration leads to GSH depletion and diminution of glutathion peroxidase activity (27). All these factors reinforce the oxidative stress induced by ischemic injury and stimulate neuronal cell death (28). The evidence exist that several antioxidants like *N*-acetylcysteine prevent brain damage induced by 3-NP; when brain protein carbonyle amounts were decreased, the striatum area specifically damaged by 3-NP was decreased (29).

Using both approaches, we were able to demonstrate the protective effect of natural antioxidant carnosine, injected into the blood of animals after ischemic injury. The concentration (100 mg/kg) which was previously effective in the experiments with radioprotection (30) or prophylactic carnosine injection 30 min before ischemic injury (12), was also effective in experiments where carnosine was injected into the blood stream after an ischemic episode.

It is well known that initially, after ischemic injury, brain neurons are subjected to ROS attack that can induce immediate (necrotic) cell death; later, delayed apoptotic cell death can also take place (31). 3-NP exhausts the energy supply of the cells and thus stimulates necrotic processes; in our experiments this resulted in pronounced neurological symptoms after the injection (Fig. 1). Carnosine not only decreases the antioxidant stationary ROS level in the cells (10), but it is also the compound that prevents neurodegeneration by both necrotic or apoptotic mechanisms (9). Carnosine in physiological concentrations (2–20 mM) is a more effective protector than *N*-acetylcystein, (-tocopherol, ascorbic acid (32), or cavinton (33). It could be that the above mentioned properties of carnosine, in combination with its ability to penetrate through the blood-brain barrier and to be accumulated in the brain, might result in neurological symptom improvement (rats and gerbils) and in viability (gerbils) when it is injected into blood after an ischemic episode.

Simultaneously, in a neurological situation, carnosine improves biochemical parameters. Injection results in decreased lipid hydroperoxide levels (which are very toxic for brain) and an increased brain membrane resistance to ferrous ion induced oxidation. Taken together these data demonstrate that carnosine compensates deficits in the antioxidant defense system damaged by ischemic injury, and effects the process even when carnosine is used after ischemic conditions. These results are especially important when considering the possibility of using carnosine for treatment of the patients after a stroke.

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