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Carnosine protects rats under global ischemia

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ABSTRACT: Rat brain subjected to 45-min global ischemia is characterized by decreased activity of K-*p*-nitrophenyl phosphatase and monoamine oxidase B and a disordering of the membrane bilayer by reactive oxygen species attack, the latter being monitored by the fluorescence of the membrane fluorescent probe, 1-anilino,8-naphtalene sulphonate (ANS). Ischemic injury resulted in 67% mortality of the animals. In the group of animals pre-treated with the neuropeptide carnosine the mortality was only 30%. At the same time, carnosine protected both the activity of the above-mentioned enzymes and the brain membrane disordering, which was also tested by ANS fluorescence. The conclusion was made that carnosine protects the brain against oxidative injury and thereby increases the survival of the animals. © 2000 Elsevier Science Inc.

KEY WORDS: Brain ischemia, Enzymes, Neuronal membranes, Oxidative injury, Antioxidants, Carnosine.

INTRODUCTION

Neuronal damage due to ischemic injury is a multiple process. Disordering of the blood supply results in cellular acidosis and the release of free ferrous ions, a rapid increase in ionized calcium ions within the intracellular space and accelerated reactive oxygen species (ROS) generation resulting in neuronal cell death [17,19, 30]. The well-known consequence of ROS attack is oxidative modification of membrane bound lipids resulting in membrane bilayer damage [12] as well as inhibition of the membrane bound enzymes responsible for the regulation of cellular metabolism [30].

At present, the protecting effect of several synthetic antioxidants and ROS scavengers, like *idebenone* [14] or N-*tert*-butyl-α-phenylnitrone is described [9]; their side effects in living organism restricts the clinical trials of these compounds. In our current research we have evaluated the effect of the natural brain constituent, the dipeptide *carnosine* (β-alanyl-L-histidine) under global rat brain ischemia. Carnosine is known to act as potent hydrophilic antioxidant protecting from ROS attack both cellular enzymes like Na/K-ATPase [8] and guanylate cyclase [27], and membrane lipids [6]. Carnosine protects cells and tissues under unfavorable conditions accompanied by increased ROS generation [3,4]. In this study, we have characterized the protecting effect of carnosine on rats under 45-min global ischemia analyzing the survival of animals as well as the activity of key membrane bound brain enzymes and the properties of brain membrane preparations.

Recently, using magnetic resonance imaging and magnetic resonance spectroscopy we have demonstrated that after 45-min four-vessel ischemia the brain of the rats is characterized by a higher level of lactic acid and a dramatic decrease in adenosine triphosphate (ATP) and creatine phosphate [28]. These data suggested that 45-min occlusion of common carotid arteries is followed by changes in the biochemical parameters of the brain. The animals pre-treated with carnosine contain a (two times) lower level of lactate compared to that of control animals, thus suggesting a protective effect of the compound under study on brain metabolism and survival of the animals. In addition, protection of neuronal cells against oxidative stress by carnosine *in vitro* was recently demonstrated [5].

MATERIALS AND METHODS

In the experiments adult male Wistar rats of 200–250 g weight were used. Global brain ischemia was produced by four-vessel occlusion as described elsewhere [24]. Under halothane anesthesia both vertebral arteries were cauterized and a loop of floss was placed around each common carotid artery without occlusion. Sixteen hours after surgery, global ischemia was induced by 45-min occlusion of the carotid arteries. At this step of the experiment, the rectal and brain temperature was measured. The former was practically unchanged through the whole experimental procedure, at 36.0 ± 0.6°C while the latter decreased from 38.0 ± 0.6°C to 35.0 ± 0.5°C starting 15 min from the beginning of the experiment. During the occlusion period, the animals were characterized by constant coma and autonomic breath. The experiment was ended 45 min after occlusion of the carotid arteries with animal decapitation.

Carnosine dissolved in physiological solution was administrated intraperitoneally in a single dose of 150 mg/kg of body weight 30 min before carotid occlusion.

Because the death in the control (carnosine non-treated) group of rats was two to three times higher than that in carnosine-treated group, the initial amount of animals in the control group exceeded that of the carnosine treated group. Thus, 24 control rats versus 10 carnosine-treated rats underwent surgical operation. Another group of 10 animals (controls) were used to measure the parameters

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being characterized for normal conditions. The whole brain of animals alive after ischemic attack was quickly removed and the cortex dissected on ice. Synaptosomal and mitochondrial fractions were isolated from rat brain gray matter using a discontinuous sucrose gradient as described elsewhere [11].

Ouabain-inhibited K-*p*-nitrophenyl phosphatase (K-pNPPase) used as a test for brain Na/K-ATPase was measured in the synaptosomal fraction [2,16]. Incubation medium used for activity determination contained 100 mM KCl, 20 mM $MgCl_2$, 7.5 mM *p*-nitrophenyl phosphate, and 30 mM imidazole (pH 7.4 at 37°C). The part of phosphatase not being related to Na/K-ATPase enzyme was measured in the presence of 1 mM ouabain, a specific inhibitor of Na/K-ATPase and subtracted from the total phosphatase activity, which was monitored during 15 min at 410 nm (absorbance due to release of *p*-nitrophenol from *p*-NPP hydrolysis [17]) using an LKB Spectrophotometer (Ultrospec III; Pharmacia LKB, Sweden) and analyzed by "Enzyme Kinetics Program" for Microsoft Windows [18]. The molar extinction coefficient for *p*-nitrophenol was taken as 6,880 $M^{-1} \cdot cm^{-1}$.

Monoamine oxidase B (MAO B) was measured in rat brain mitochondria using benzylamine as a substrate and monitoring the reaction by benzaldehyde (BA) accumulation [23]. A suspension containing 0.25 mg mitochondrial protein was added to 0.9 ml of 0.2 M Na/K-phosphate buffer (pH 7.4 at 37°C) and the reaction was initiated by benzylamine addition (1 mM final concentration). After subsequent incubation, the reaction was stopped by 5% trichloroacetic acid. To the control sample, trichloroacetic acid was added before benzylamine addition. Samples were treated by hexane (3 ml each) and BA accumulation was measured by absorption at 241 nm using the LKB Spectrophotometer (Ultrospec III; Pharmacia LKB). Molar extinction coefficient of BA was taken as 13,080 $M^{-1} \cdot cm^{-1}$.

Fluorescence of 1-anilino,8-naphtalene sulphonate (ANS) was measured in both membrane fractions used in order to characterize rat brain membrane packaging [10]. In our experiments, samples were excited at 365 nm and the fluorescent signal was measured at its fluorescent maximum (480 nm) 2 min after ANS addition to the membrane suspension (0.1 mg protein in 2 ml incubation medium). The incubation medium for fluorescence measurements contained 100 mM KCl and 15 mM imidazole (pH 7.4 at 25°C). Measurements were made using a Shimadzu Spectrofluorophotometer. Successive titration of each sample by ANS was performed within the concentration range of 10–100 μM. From the experimental data the maximal values of ANS fluorescence, F_{max} and ANS binding constant, K_{bind} were calculated from the Lineweaver-Burk plot. The Student's *t*-test was used to estimate the significance of differences in the parameters measured, and $p < 0.05$ was taken as the limit of significance. To estimate the statistical significance of mortality of the different groups of animals the non-parametric statistics and chi-square criterion were used.

RESULTS

Rat brain ischemia induced by 45-min occlusion of carotid arteries resulted in 67% death of animals; 16 out of 24 rats in this group were deceased by the end of experiment. Administration of carnosine to rats 30 min before experimental ischemia prevented their mortality: in the carnosine-treated group only 3 from 10 rats were deceased (Fig. 1).

Synaptosomal and mitochondrial fractions of rat brain were used in biochemical experiments. In the former, ouabain-dependent K-pNPPase and in the latter—MAO B was measured, in addition membranes of both fractions being tested on the bilayer packaging using ANS as a fluorescent probe. It was found that both enzymes were significantly inhibited by 45-min ischemia: K-pNNPase was found to decrease from 3.98 ± 0.51 (control) to 2.67 ± 0.21 (ischemia) μmoles pNP/mg protein per h ($p < 0.05$), and MAO B in the mitochondrial fraction—from 167.5 ± 17.0 to 127.3 ± 12.4 nmole BA/mg protein per h ($p < 0.05$). F_{max} for ANS being equal to 104.2 ± 7.3 and 88.9 ± 11.0 for synaptosomal and mitochondrial fractions was decreased in these fractions after ischemic attack by 28% and 42% respectively. K_{bind} for ANS was not changed by ischemic attack in both synaptosomal and mitochondrial membranes being equal to 53.2 ± 6.6 μM and 31.5 ± 3.3 μM, respectively.

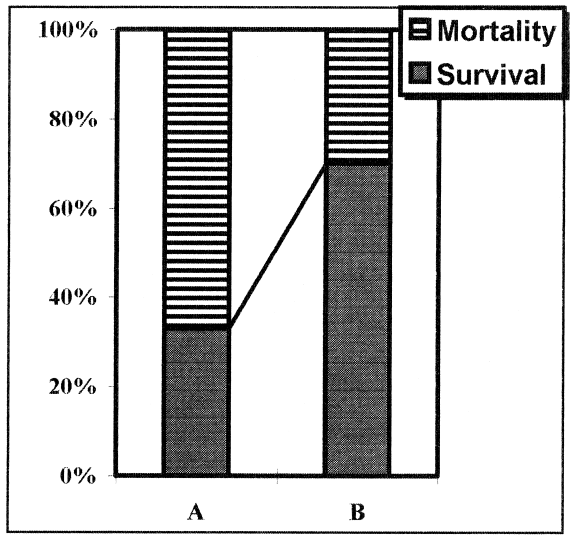

FIG. 1. Distribution between dead and survived rats after global ischemia induced by 45 min 4-vessel occlusion. (A) Control group of animals; (B) group of animals pre-treated with carnosine in a dose of 150 mg/kg body weight 30 min before carotides occlusion. The data of survival of animals between (A) and (B) are statistically significant ($p < 0.05$).

The protecting effect of carnosine on rat mortality was accompanied by prevention of K-pNPPase and MAO B from inhibition by ischemic attack; F_{max} for ANS fluorescence in the membrane preparations was also not decreased (Fig. 2). Thus, based on the parameters measured, membrane preparations isolated from the ischemic brain of carnosine pre-treated rats were distinct from those obtained from non-treated animals and close to the control ones not being subjected to global ischemia.

DISCUSSION

In order to characterize the biochemical modifications of ischemic brain metabolism, K-pNPPase in synaptosomes and MAO B in mitochondria were measured simultaneously with fluorescence of the membrane bilayer probe, ANS. ANS is an amphiphilic molecule with a positive charge at its hydrophilic moiety; it is located in the membrane near the area of lipid-protein contacts but is not bound by membrane glycoproteins [10]. When the probe is accumulated within the bilayer its fluorescence increases but disordering of the membrane bilayer by free radicals generated by the ischemic process increases the water accessibility to the membrane and to successive quenching of ANS fluorescence [25]. Thus, the fluorescent signal of ANS characterizes the packaging of the membrane core and is usually suppressed after oxidative modification of membranes.

In our study more than 60% mortality of rats was demonstrated as a result of 45-min global brain ischemia, which is known to be

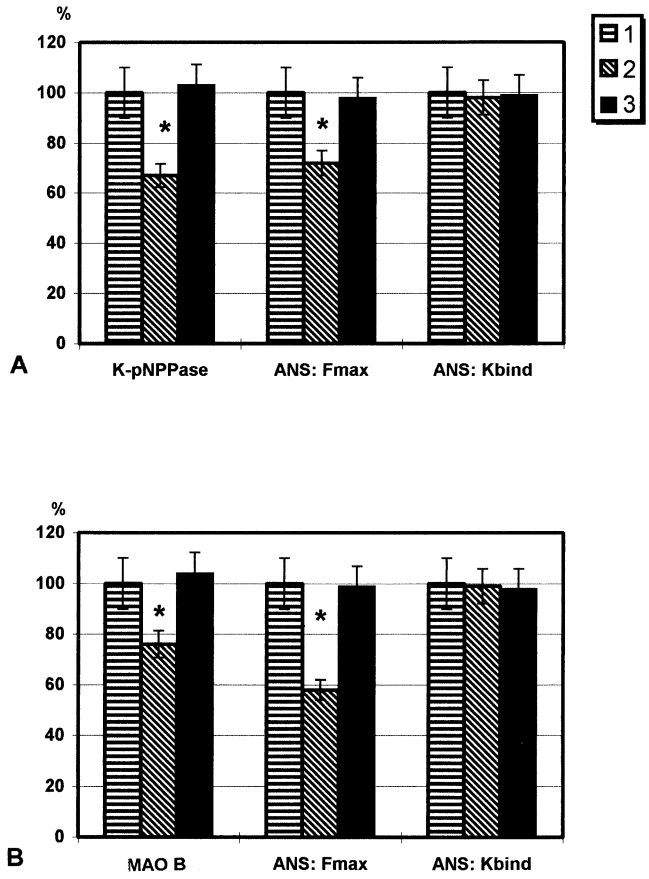

FIG. 2. Effect of global ischemia on *p*-nitrophenyl phosphate (K-pNPPase) and monoamine oxidase B (MAO B) activity and parameters characterizing 1-anilino,8-naphtalene sulphonate (ANS) fluorescence in synaptosomal (A) and mitochondrial (B) membrane fractions prepared from intact (1) and ischemic (2) rat brain as well as from ischemic brain of rats preliminary treated with carnosine (3) as described in the legend to Fig. 1. All values are expressed as % to control. *Significantly different from control.

accompanied with increased ROS generation [30]. Ouabain-dependent K-pNPPase and MAO B were also suppressed. These data are in a good agreement with the known data that Na/K-ATPase is inhibited by 15-45 min ischemia in rats [28], gerbils [22] and mice [15]. Mitochondrial MAO B is also involved in the mechanisms accompanying brain ischemic injury [1]. Both enzymes are important for the normal function of neuronal cells. The former supports the electrochemical gradient of monovalent cations on the cellular membrane used in the electrical activity of neurons and in reuptake of mediator molecules. The latter enzyme performs metabolic control of biogenic amine turnover. It is located preferably in astrocytes but supports neuronal viability because of its important functions [21].

Thus, inhibition of the enzymes studied is in close correlation with animal death induced by ischemic attack. Such disordering in enzyme function is obviously induced by oxidation of membrane lipids [6,12,15,26,29]. Direct confirmation of this point of view was found in our experiments. Actually, a decrease in F_{max} for ANS fluorescence with no change in K_{bind} directly reflects disordering of the membrane bilayer and increased access of the hydrophobic membrane core to surrounding water, which is fully consistent with increased lipid membrane peroxidation during ischemic injury [12].

Thus, our data are in agreement with the oxidative damage of neuronal cells by increased ROS generation, which usually accompanies ischemic injury. On the other hand, another possibility exists—direct oxidative modification of membrane-bound enzymes could not be excluded. Thus, inhibition of K-pNPPase activity may reflect a direct SH-group oxidation of Na/K-ATPase protein under ROS attack [18].

The ability of carnosine to protect Na/K-ATPase against ROS attack in several models was shown recently [4,8,28]. Its protection of mitochondrial MAO B, which is demonstrated in our study is also important for the neurons viability under ischemic attack because of the known excitotoxic effect of biological amines [13,21]. Carnosine is known to prevent the accumulation of the end products of lipid peroxidation (malonic dialdehyde) under disordered brain microcirculation [6]. Moreover, carnosine demonstrated direct protection of Na/K-ATPase being administered before brain ischemic injury in gerbils [28]. These properties of carnosine presumably provide the basis for the potent protecting effect under oxidative damage of isolated brain neurons *in vitro* [7] or hypobaric hypoxia of rats *in vivo* [8].

In the experiments presented, carnosine was shown to decrease the mortality of rats and to protect their brain against ischemic injury. Its effect at the cellular level consists of both protection of the enzyme's activities and the membrane bilayer itself from ROS attack. It is known from previous experiments that carnosine penetrates the blood—brain barrier of rodents and is accumulated within the brain tissues 30–120 min after injection [4,6]. We may conclude that carnosine interferes with the brain metabolism and protects the brain tissue under stroke conditions. We did not follow the delayed cell death in brain areas damaged by the experimental ischemia and thus cannot elucidate the precise mechanism of brain protection by carnosine, though our previous data demonstrated the ability of this dipeptide to serve as a protector of brain neurons against oxidative injury [5]. However protection of the astrocytic enzyme, MAO B demonstrated in this study suggests that carnosine may provide an indirect effect on the neuronal stability via regulation of biogenic amine turnover. The molecular mechanisms of brain protection by carnosine have to be the aim of further study but there is no doubt that carnosine may be a promising agent in the therapy of brain stroke.

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