Threefold Exposure to Moderate Hypobaric Hypoxia Decreases the Expression of Cu,Zn-Superoxide Dismutase in Some Regions of Rat Hippocampus

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The effect of moderate hypobaric hypoxia on the expression of a peptide antioxidant Cu,Zn-superoxide dismutase in rat hippocampal neurons was evaluated in an immunocytochemical study. The expression of Cu,Zn-superoxide dismutase decreased significantly in the dorsal hippocampus (CA1 and CA2) and tended to decrease in ventral regions (CA3 and dentate gyrus) by the 24th hour after 3-fold exposure to hypoxia.

Key Words: *Cu,Zn-superoxide dismutase; moderate hypoxia; preconditioning*

The molecular mechanisms of cell adaptation to hypoxia and oxidative stress include activation of enzymatic and nonenzymatic antioxidant systems, *e.g.* cytosolic Cu,Zn-superoxide dismutase (Cu,Zn-SOD, EC 1.15.1.1). According to modern views, the decrease in the expression and antioxidant enzyme activity (*e.g.*, Cu,Zn-SOD) induced by severe hypoxia/ischemia correlates with progression of pathological processes and nerve cell death [7-10]. By contrast, increased expression and activity of Cu,Zn-SOD under conditions of moderate hypoxia/ischemia attest to the neuroprotective response and prevents or reduces the severity of injury [10,15].

Our previous studies showed that Cu,Zn-SOD immunoreactivity increases significantly by the 24th hour after severe hypobaric hypoxia (3 h at 180 mm Hg) [2,14]. The increased expression is probably a molecular neuroprotective reaction. However, this reaction is delayed and cannot prevent massive apoptotic death of hippocampal neurons on days 3-7 of the experiment [1,13]. Published data show that the molecular mechanisms inducing neuronal death are triggered over the first 2-4 h after hypoxia [6].

Moderate hypoxic preconditioning (3-fold exposure to 360 mm Hg for 2 h) induces Cu,Zn-SOD expression by the 3rd hour after subsequent severe hypoxia (*i.e.*, critical period for the induction of apoptosis) [2,14]. The early increase in Cu,Zn-SOD expression and other antioxidants correlates with and, probably, causes a significant decrease in the number of dying neurons (days 3-7) [1,13]. These data suggest that the neuroprotective effect of preconditioning is related to significant increase in the expression of Cu,Zn-SOD and other antioxidants during the early period after subsequent severe hypoxia. The mechanism of these changes remains unclear.

Here we studied whether the increased content of Cu,Zn-SOD in preconditioned animals (as compared to non-preconditioned specimens) 3 h after hypoxia is determined by its accumulation during preconditioning (before severe hypoxia) or by modification of the response during severe hypoxia. Cu,Zn-SOD expression in the rat hippocampus was measured 3 and 24 h after 3-fold exposure to moderate hypoxic

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preconditioning (period between preconditioning and severe treatment).

MATERIALS AND METHODS

The experiments were performed on 2 groups of adult male Wistar rats weighing 200-250 g. Group 1 animals (n=6) were exposed three times to moderate hypobaric hypoxia in a flow altitude chamber at 360 mm Hg for 2 h (once daily). Group 2 animals (n=6) were placed in an altitude chamber at normal pressure (3 days for 2 h, control). Cu,Zn-SOD expression in hippocampal structures was measured by immunocytochemical methods 3 and 24 h after the last exposure.

For immunocytochemical study, the narcotized animals were transcardially perfused with 100 ml physiological saline and 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.3) for 4-5 min. The animals were decapitated after perfusion. The brain was removed and fixed with the same agent for 60 min. Before the study, brain samples were stored in 15% sucrose solution in PBS at 4°C.

The tissue was frozen in Tissue-Tek® O.C.T™ Compound (Sakura Finetek). Frontal brain slices (11 μ) at the level of the hippocampus and basolateral amygdala (-2.80 mm from bregma) were prepared immediately on a cryostat at -20°C. The slices were put on poly-L-lysine-coated slides (Sigma), preincubated in 1% BSA for 15 min (Boehringer Mannheim GmbH), and incubated with affinity-purified primary polyclonal rabbit antibodies to bovine Cu,Zn-SOD (diluted in PBS with 1% BSA ad 0.3% Triton X-100, 1:200) at 4°C for a night. After 3-fold washout (of 15 min each) in PBS, the slices were incubated with biotinylated goat secondary antibodies (dilution 1:300, Vector Labs) at room temperature for 30 min. After repeated 3-fold washing in PBS, these slices were incubated with avidin-biotin complex (Vector Labs) at room temperature for 30 min. Diaminobenzidine was used to visualize the immune reaction and to estimate the localization of Cu,Zn-SOD. The slices were dehydrated in alcohols with increasing concentrations and embedded into Entellan balsam.

Quantitative study of neuronal immunoreactivity was performed using a system, consisting of a

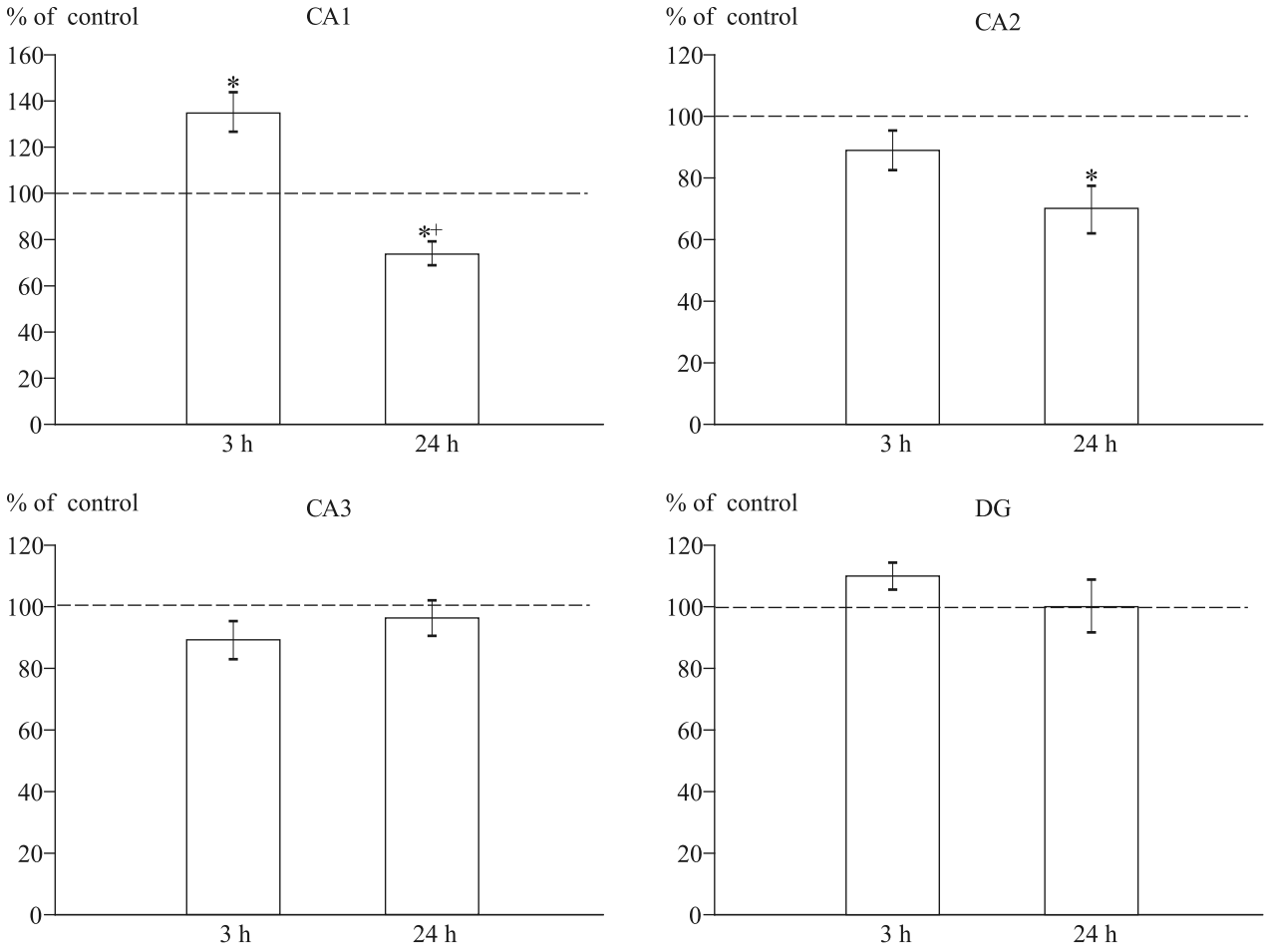

Fig. 1. Total number of Cu,Zn-SOD-immunoreactive cells (N_+) in various regions of rat hippocampus 3 and 24 h after 3-fold exposure to moderate hypobaric hypoxia. Here and in Fig. 2: control, 100%. p<0.05: *compared to the control; +compared to 24 h.

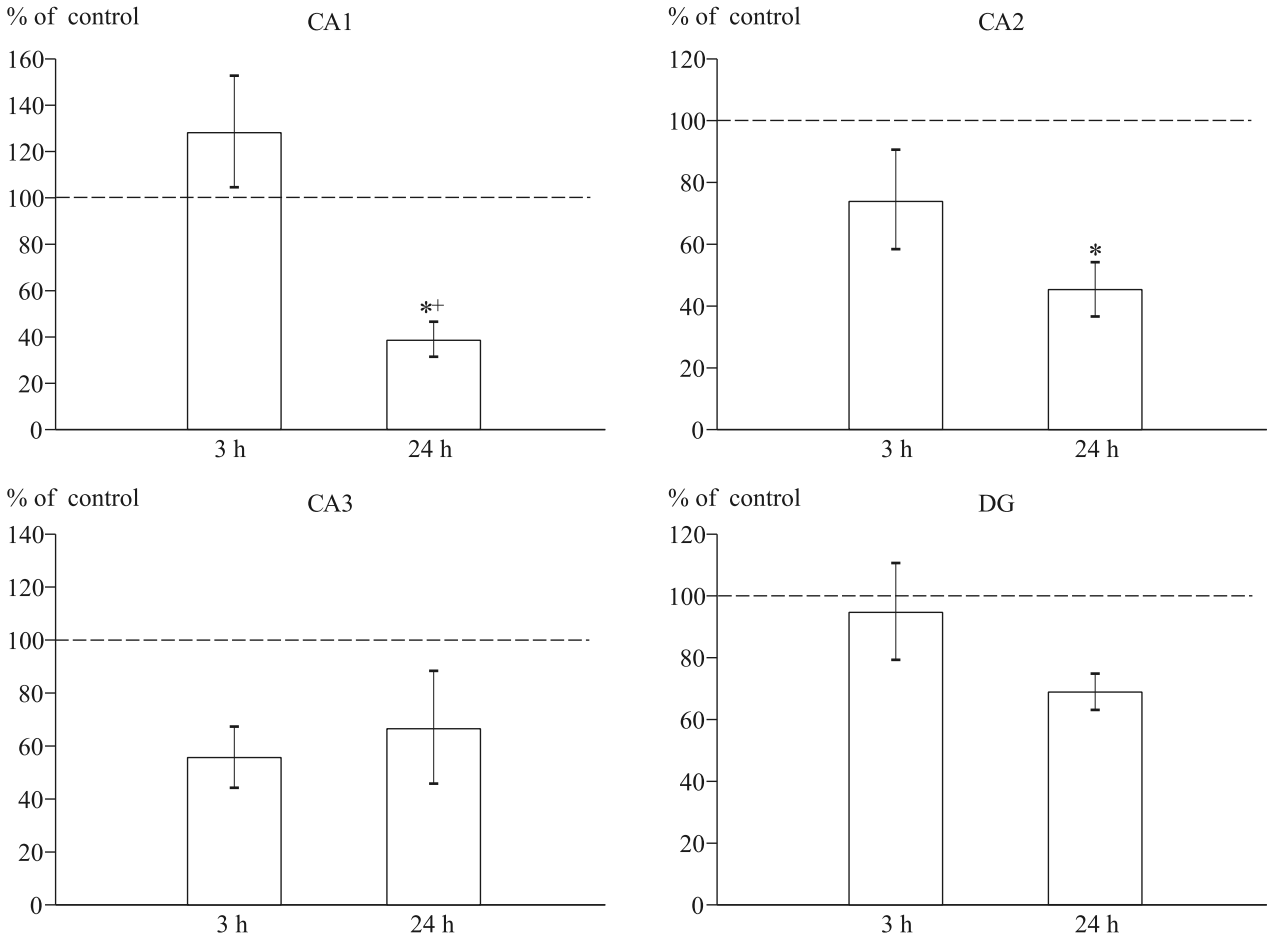

Fig. 2. Number of cells with intensive expression of Cu,Zn-SOD (Ni) in various regions of rat hippocampus 3 and 24 h after 3-fold exposure to moderate hypobaric hypoxia.

microscope (Nikon Microphot-FXA), camera (PCO Computer Optics GmbH), and IBM PC computer with Image-Pro Plus (Media Cybernetics) and Morphix software [4].

Cu,Zn-SOD expression was studied in neurons of Ammon's horn (CA1, CA2, and CA3 regions) and dentate gyrus (DG). The images were analyzed in a 500-μ region. Neuronal immunoreactivity in these structures was evaluated by examination of 6 slices from each brain. The degree of staining in digital images was expressed in arbitrary optical density units and varied from 0 (absolute white) to 100 (absolute black). Immunoreactive cells were conditionally divided into 2 classes of weakly stained cells (exceeding the baseline by 1-10 arb. units) and strongly stained cells (exceeding the baseline by more than 10 arb. units). The level of immunoreactivity was estimated by the following two criteria: total number of immunoreactive cells (percent of the control, N_+) and number of strongly intensively cells (percent of the control, Ni). The results were analyzed by one-way analysis of variance (ANOVA).

RESULTS

Immunocytochemical study showed that 3-fold moderate hypobaric hypoxic exposure (preconditioning) modulated the expression of Cu,Zn-SOD in the dorsal hippocampus of rats (CA1 and CA2 regions; Figs. 1 and 2).

The total number of Cu,Zn-SOD-expressing cells in CA1 increased significantly by the 3rd hour after treatment (N_+=135%). The number of these cells tended to decrease in CA2 (N_+=89%) and CA3 (N_+=89%), but increased in DG (N_+=110%; Fig. 1). Changes in the number of cells with intensive expression of Cu,Zn-SOD (Ni) were statistically insignificant in the test regions. The number of Ni tended to increase in CA1 (Ni=129%), decreased in CA2 (Ni=73%) and CA3 (Ni=56%), and remained practically unchanged in DG (Ni=95%; Fig. 2).

The total number of Cu,Zn-SOD-expressing cells in CA1 (N_+=74%) and CA2 (N_+=70%) decreased significantly by the 24th hour after treatment. The total number of Cu,Zn-SOD-expressing cells in CA3

(N_+=96%) and DG (N_+=100%) did not differ from the control (Fig. 1). The number of cells with intensive expression of Cu,Zn-SOD decreased significantly in CA1 (Ni=39%) and CA2 (Ni=45%). In CA3, the decrease in the number of these cells (Ni=67%) and DG (Ni=69%) was statistically insignificant (Fig. 2).

Hence, Cu,Zn-SOD expression in neurons of hippocampal regions CA1 and CA2 was significantly reduced 24 h after the last (third) session of preconditioning. The decrease in this parameter in CA3 and DG was statistically insignificant (Figs. 1 and 2).

These results were unexpected in view of our previous data on the effect of preconditioning on Cu,Zn-SOD expression after subsequent severe hypoxia and from the viewpoint of general agreement that only severe hypoxia and ischemia decrease the expression and enzyme activity of SOD, while moderate hypoxia and ischemia have an inducing effect on Cu,Zn-SOD. However, published data show that administration of exogenous antioxidants before and during preconditioning abolishes the preconditioning-induced cytoprotective effect [5,11,12]. It is related to the fact that the cytoprotective effect is triggered by increased concentration of reactive oxygen species. Our experimental findings can be explained by the existence of a similar feedback system in the reciprocal regulation of Cu,Zn-SOD gene and its protein product. Variations of functional activity contributing to rapid and intensive expression of Cu,Zn-SOD by preconditioned cells during severe hypoxia are probably determined by increased amount of superoxide. The reduced level of Cu,Zn-SOD expression after preconditioning provides optimal conditions for this signal.

Our previous investigations on the same experimental model revealed similar changes in the expression of another antioxidant, thioredoxin-1 [1]. These data suggest that the paradoxical effect of hypoxic preconditioning on the expression of a peptide antioxidant Cu,Zn-SOD in the dorsal hippocampus is not the exception.

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