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Expression of Early Gene Proteins, Structural Changes in Brain Neurons in Hypobaric Hypoxia, and the Correcting Effects of Preconditioning

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The Nissl method and immunocytochemistry were used to study the effects of severe hypobaric hypoxia and its actions in combination with the preconditioning actions of moderate hypoxia on the expression of the early gene proteins c-Fos and NGFI-A as well as structural changes in hippocampal and neocortical neurons in the rat brain. Severe hypoxia was found to suppress c-Fos and NGFI-A synthesis (3–24 h after exposure) and to induce delayed (days 3–7) structural damage to neurons, of the "light" and predominantly the "dark" types, which appear to reflect the development of necrotic and apoptotic processes respectively. Preconditioning with the regime used here corrected these derangements, resulting in increases in the expression of early gene proteins and significant reductions in structural damage to neurons after severe hypoxia.

KEY WORDS: hypobaric hypoxia, preconditioning, hippocampus, neocortex, early gene proteins.

Severe hypoxia (circulatory hypoxia-ischemia, hypoxemic hypoxia, hypobaric hypoxia, etc.) induces profound disturbances to the structure and function of brain neurons, with neurological and behavioral disorders. The extent of the disturbance depends on the type and duration of hypoxia [6, 13]. The best studied structural damage is to neurons in various brain formations (hippocampus, neocortex, striatum, etc.) using models of experimental global or focal ischemia in Mongolian gerbils and rats [1, 6, 13]. These experiments demonstrated the phenomenon of "delayed" neuron death in the most ischemia-sensitive brain formations, particularly the hippocampus, neocortex, and striatum [10, 15].

"Delayed" neuron death occurs 48–72 h after ischemia, and its induction in hippocampal field CA1 requires hypoxia lasting only 3 min (in Mongolian gerbils) or 10 min (in rats). Neuron death in different formations may be delayed by periods ranging from a few days to several weeks after hypoxia, depending on the model used [13]. Ischemia-induced death of brain neurons occurs by necrosis or apoptosis. Neuron death delayed by several days or more is generally associated with the process of apoptosis [14]. Post-ischemic selective neuron death in these brain formations links the development of severe impairments in cognitive processes, learning, and memory [6].

However, studies at the beginning of the last decade demonstrated the phenomenon of "ischemic tolerance" of the brain, whereby transient (sublethal) ischemia significantly increases the structural resistance of neurons in these brain formations (especially in hippocampal field CA1) to subsequently experienced severe (lethal) ischemia [11, 12]. These moderate treatments responsible for the development of brain tolerance are termed ischemic/hypoxic preconditioning, and are regarded as a type of adaptation of cells to hypoxia [1, 2].

Hypobaric hypoxia, not infrequently encountered in medical practice, provides a convenient model for studying

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the effects of preconditioning, as it has identical influences on all brain formations and the regimes for creating severe and moderate forms of this type of hypoxia are easily controlled and standardized. However, there are few data on the effects of severe hypobaric hypoxia on structural changes in brain neurons, and there are no data on the effects of preconditioning on these changes.

It has been established that the development of a valuable protective effect of preconditioning (by morphological criteria) requires a fairly long period of time (24 h or more) between the preconditioning and severe hypoxic treatments [5, 7, 12]. This fact suggests involvement of the neuron genome in forming the mechanisms of hypoxic tolerance [1, 13]. It would appear that an important role in this process is played by families of so-called early genes (c-fos, jun, krox, etc.), whose products are transcription factors which regulate the expression of the phenotype-specific late genes [1, 3].

The aim of the present work was to study the effects of direct severe hypobaric hypoxia and its application in combination with preconditioning on the expression of early gene proteins of the c-fos and krox-24 families (nerve growth factor I-A (NGFI-A) and zif268) and structural changes in the neurons of a series of brain formations.

MATERIALS AND METHODS

Experiments were performed in accord with the Rules for Studies Using Experimental Animals on 40 male Wistar rats weighing 200–220 g, which were subjected to hypobaric hypoxia in different regimes in a barochamber of the flow type. Rats of one group (A) were subjected to severe hypoxia (the pressure in the barochamber was maintained for 3 h at 160 mmHg, which corresponds to an altitude of 11,000 m). The second group (B) underwent this procedure after three 2-h sessions of moderate (preconditioning) hypoxia separated by 24-h intervals (the barochamber pressure was maintained at 360 mmHg, which corresponds to an altitude of 5000 m). One day after the last preconditioning session, these animals were subjected to severe hypoxia.

This method of preconditioning significantly decreased the lethality of severe hypoxia (mean survival rates were 15% in group A and 85% in group B).

An immunocytochemical method was used in 4–6 rats of groups A and B to measure the level of expression of the early protein genes c-Fos and NGFI-A in the frontoparietal area of the cortex and various areas of the hippocampus (CA1, CA2, CA3, CA4, and the dentate fascia) 3 and 24 h after severe hypoxia.

Anesthetized animals (Nembutal, 50 mg/kg) underwent brain fixation with 4% paraformaldehyde by transcardiac perfusion. Animals were then decapitated, brains were removed, and were additionally fixed in paraformaldehyde for 1 h, after which they were stored at 4°C in 20% sucrose. Fragments were frozen in Tissue Tek and a cryocat was used to cut sections at the level of hippocampus; sections were 12 μm thick. Sections were mounted on microscope slides coated with poly-L-lysine. Frozen sections were preincubated for 15 min in 1% bovine serum albumin, which was followed by overnight incubation with monoclonal antibodies to c-Fos (Nova Castra) or NGFI-A (Santa Cruz) (diluted 1:100) in a moist chamber at 4°C. The immunocytochemical procedure was continued from this point using a standard SuperStain ABC System kit (Novo Castra), containing polyclonal secondary anti-mouse antibodies, an avidin-biotin complex, and diaminobenzidine chromogen.

The general morphological assessment of neurons in study brain areas of animals of groups A and B was addressed using the classical Nissl method 3 and 7 days after severe hypoxia (using four rats from each experimental group).

Immunoreactivity and structural changes to neurons were assessed using a computer image analysis system consisting of a microscope and a computer running the Videotest Morfologiya 5.0 program.

RESULTS

After severe hypoxia, rats of group A (not preconditioned) showed large numbers of neurons making responses of the "light" (early degree of chromatolysis, cytoplasmic vacuolization, ghost cells) and "dark" (hyperchromatosis, shrinkage, glial nodes at sites of dead cells) types.

On day 3, the most marked changes were seen in the hippocampus. Fields CA1, CA2, and CA4 showed significant numbers of hyperchromic pyknotic cells (Fig. 1*a*, *b*). Pericellular edema was seen quite often. Some neurons showed chromatolysis and cytoplasmic vacuolization. However, attention is drawn to the good preservation of cell structure in the dentate gyrus.

The olfactory, prepiriform, and entorhinal cortex showed diffusely distributed hyperchromic and pyknotic neurons. Chromatolysis, ghost cells, and pericellular edema were also seen frequently.

In the frontoparietal neocortex, there was a large number of unchanged pyramidal cells in all layers, along with relatively small numbers of cells with reactions of the "light" type. Foci of hyperchromic and shrunken neurons were seen.

On day 7 after severe hypoxia, there were significant increases in the numbers of pyknotic hyperchromic neurons in layers II, V, and VI of the frontoparietal cortex (see Fig. 1*c*, *d*). Areas CA1, CA2, and CA3 contained dead cells along with large numbers of pyknotic hyperchromic neurons.

In preconditioned animals (group B), structural damage to neurons after severe hypoxia was significantly less marked than in rats of group A. This was manifest primari-

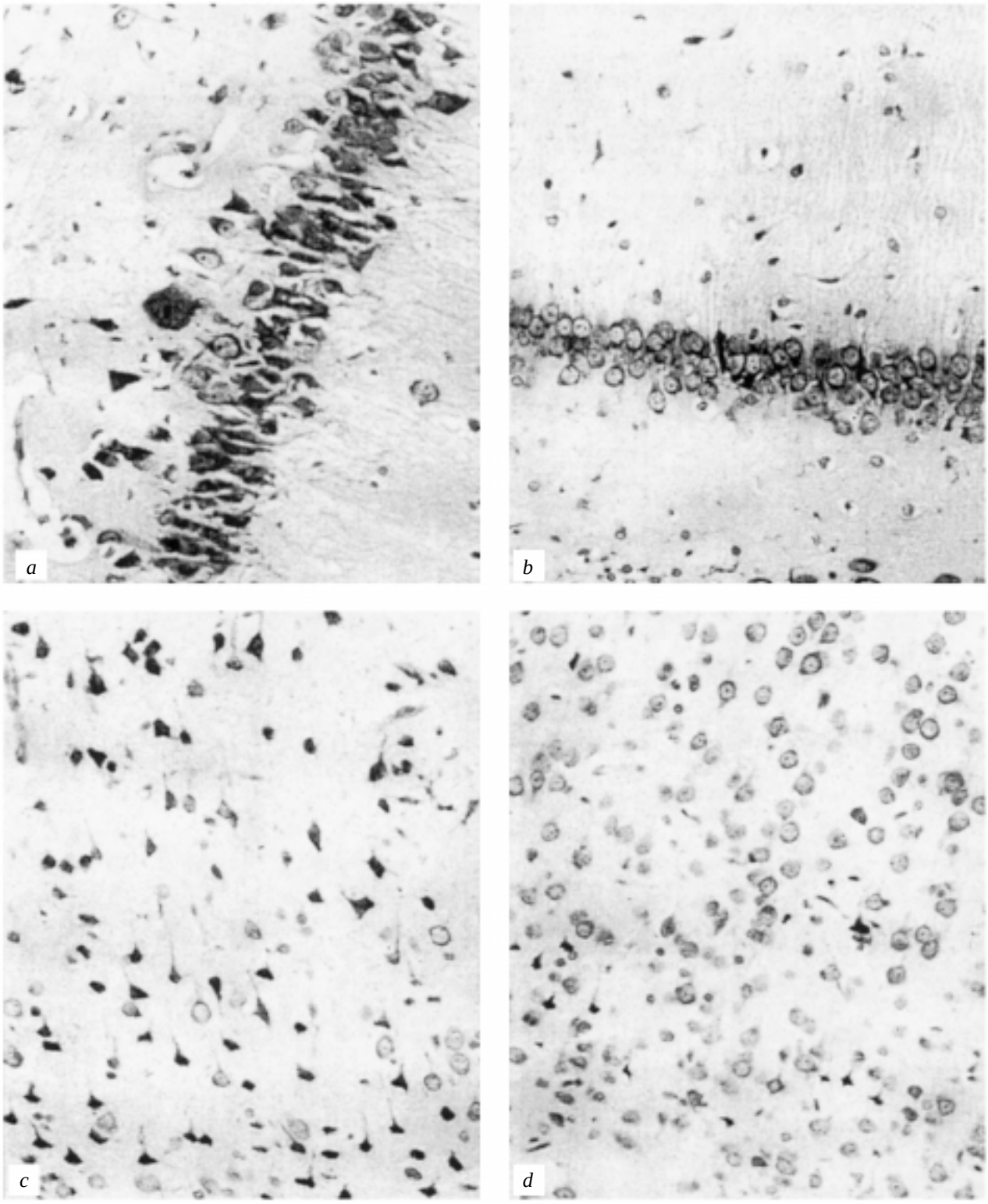

Fig. 1. Structure of neurons in hippocampal field CA1 on day 3 (*a*, *b*) and the frontoparietal area of the neocortex on day 7 (*c*, *d*) after severe hypobaric hypoxia in non-preconditioned (*a*, *c*) and preconditioned (*b*, *d*) rats. Stained with toluidine blue by the Nissl method. Objective ×40; ocular ×8.

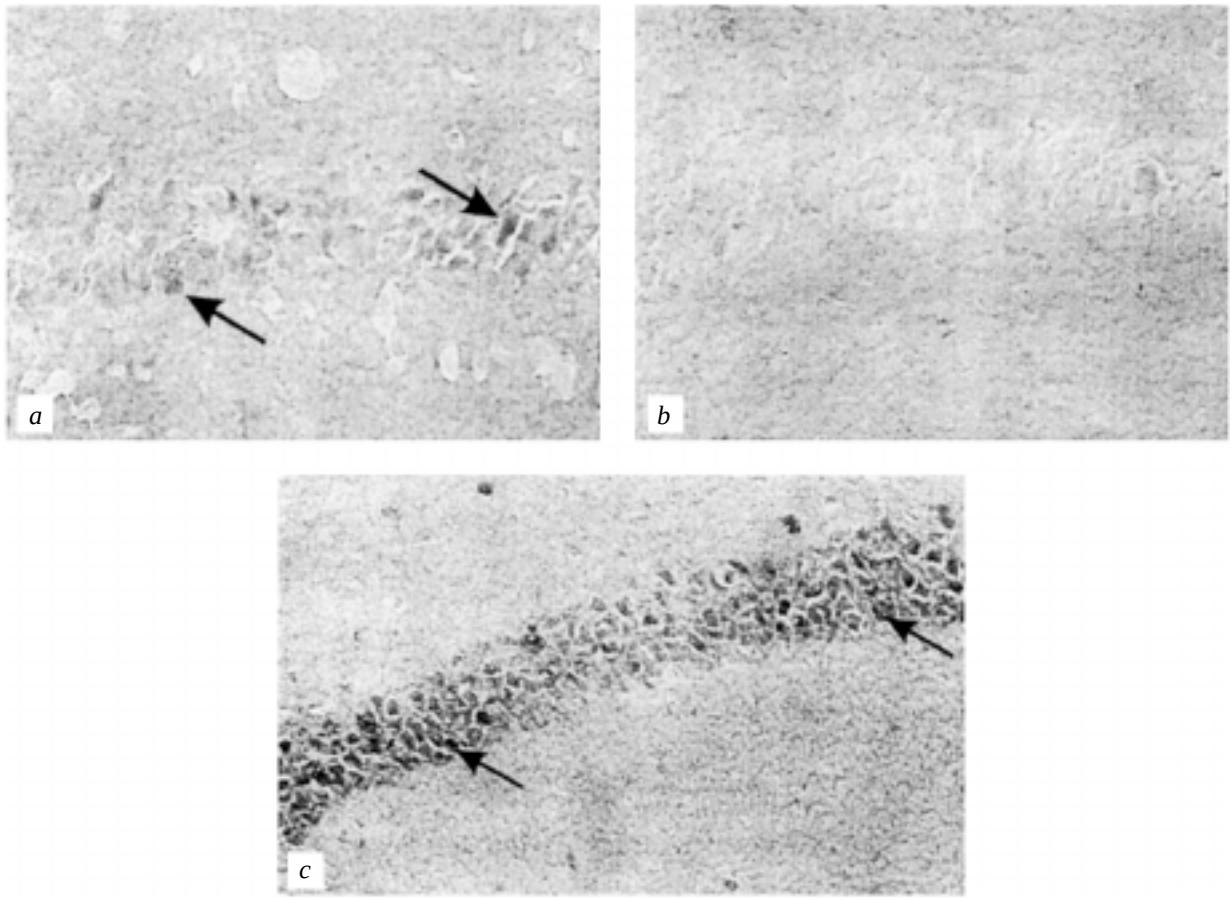

Fig. 2. Immunoreactivity for NGFI-A protein in hippocampal field CA1 in controls (*a*), 3 h after severe hypobaric hypoxia in non-preconditioned (*b*) and preconditioned (*c*) rats. Arrow shows immunopositive cells. Objective ×40; ocular ×8.

ly as significant decreases in the numbers of damaged and dead neurons in all the brain formations studied. However, both the hippocampus and neocortex contained occasional diffusely distributed hyperchromic and pyknotic neurons (see Fig. 1). The numbers of neurons making reactions of the "light" type in these animals also decreased as compared with those in rats of group A.

Control intact animals showed moderate reactivity to the NGFI-A protein by hippocampal and neocortical neurons (in terms of the numbers of immunopositive cells and their staining intensities). In non-preconditioned rats (group A), severe hypoxia suppressed immunoreactivity for NGFI-A in the brain formations studied at 3 and 24 h after exposure. Immunopositive cells were virtually absent (or were generally found only occasionally, in hippocampal areas CA1, CA2, and CA4 and the neocortex (Fig. 2). Conversely, these brain formations in preconditioned animals (group B) showed clear increases in the expression of NGFI-A 3 h after severe hypoxia as compared with controls (see Fig. 2). At 24 h, the increased immunoreactivity persisted, though it was less marked than at 3 h after exposure. Immunoreactivity to c-Fos protein in the hippocampus and neocortex in control animals was either absent or minor. c-Fos immunopositive cells were either absent or very rare in the hippocampus and neocortex of animals of group A, at 3 and 24 h after severe hypoxia, and were mainly seen in the medial part of area CA1. However, animals of group B at these times after severe hypoxia showed significantly increased levels of expression of c-Fos in the neurons of these brain formations. Attention is drawn to the prolonged (up to 24 h) persistence of increased immunoreactivity to c-Fos in some formations, especially hippocampal area CA4 and the neocortex.

DISCUSSION

The experiments reported here showed that severe hypobaric hypoxia ("elevation" of animals to an altitude of 11,000 m for 3 h) leads to marked structural changes, developing by the "light" and "dark" types in neurons in hippocampal fields CA1, CA2, and CA4, the frontoparietal area of the neocortex, and other areas of the rat cortex. Some neu-

rons, characterized by extensive chromatolysis, cytoplasmic vacuolization, and formation of ghost cells, evidently underwent necrosis. However, most damaged neurons (pyknotic, chromophilic cells) showed signs of apoptosis.

A similar but less marked pattern was seen with long-term (four days) "elevation" of rats to an altitude of 6400 m – this is a level of hypobaric hypoxia at which chronic exposure in humans produces neurological impairments [8, 17]. In our experiments, more severe acute conditions were used, leading to the death of more than 50% of the animals. Judging from the results obtained, this procedure initiates significantly stronger neurodegenerative damage in various brain formations than the hypoxia model cited above.

Attention is drawn to the diffuse selective localization of neuron damage in different brain formations both in the paleocortex and the neocortex, similar to that seen after ischemia [13]. Thus, the most affected structures in the hippocampus were fields CA1/CA2 and CA4, and the least affected was the dentate gyrus. It was also interesting to note the different course of development of damage to neurons in different brain formations. In all probability, the neocortex showed signs of damage later than the hippocampus.

The model of hypoxic preconditioning used here (three sessions of 2-h "elevation" to an altitude of 5000 m) was quite effective in terms of increasing the structural resistance of brain neurons to severe hypoxia. In accord with clinical observations, "elevation" to this altitude, although a powerful stimulus to the body, does not induce neurological disturbances [17].

It follows from our results that preconditioning significantly but not completely prevents the development of the structural damage to brain neurons induced by severe hypoxia. This effect has been reported by other authors when sublethal ischemia was applied as preconditioning [11, 12].

The mechanism of the protective effect of preconditioning, although subjected to intense study over the last decade, remains far from understood. The suggestion that the expression of adaptive genes may have a role in this effect has been mentioned above. Previous studies have demonstrated that preconditioning with moderate hypobaric hypoxia modifies the expression of mRNA both for the early genes (NGFI-A, junB, c-jun) and late genes, particularly genes for peptide antioxidants, in a variety of brain formations after severe hypoxia [3, 4, 16].

Observations in the present study showed that preconditioning significantly increased the expression of early gene products, i.e., NGFI-A and c-Fos proteins, in the hippocampus and neocortex 3–24 h after severe hypobaric hypoxia. These proteins, members of transcription factor families, bind to specific DNA sites in their late-acting target genes to induce their expression. The multiplicity of these genes include adaptive genes (neurotrophins, antioxidants, proenkephalin, etc.) involved in protective reactions of brain neurons. Thus, the transcriptional regulation of these genes by the protein products of the early-acting genes is apparently needed for the development of neuroprotective mechanisms defending brain neurons from structural and functional damage induced by hypoxia [9].

Comparison of the pattern of expression of early gene mRNA and protein immediately after severe hypoxia in non-preconditioned and preconditioned animals is of particular interest. Previous studies have demonstrated that the hippocampus and neocortex of non-preconditioned rats show clear increases in the expression of early gene mRNA (NGFI-A, junB) 3 h after severe hypobaric hypoxia, this being suppressed by 24 h [3, 16]. However, the results of the present study indicate that increased early gene transcriptional activity 3 h after treatment was not accompanied by increases synthesis of their proteins, i.e., transcription factors.

Similar data were obtained in a model of severe brain ischemia in Mongolian gerbils [9]. The authors raised the question of the differential mechanisms of transcription and translation of early genes after brain ischemia. Their view was that the main reason for the non-correspondence between early gene mRNA and protein expression was the post-ischemic inhibition of the process of protein synthesis. It is likely that severe hypobaric hypoxia leads to the same sequelae and that preconditioning corrects these changes.

Attention is also drawn to the fact that in preconditioned animals, the increased expression of early genes is evident by 24 h but not 3 h after severe hypoxia [3, 16]. However, our results revealed a significant increase in the synthesis of the NGFI-A and c-Fos proteins 3 h after hypoxia. This appears to provide evidence for early post-hypoxia modification of the process of early gene protein synthesis at the post-transcriptional level, induced by preconditioning. This question requires further study.

Thus, these experiments showed that severe hypobaric hypoxia suppresses the synthesis of early gene proteins (NGFI-A and c-Fos) in the hippocampus and neocortex, as well as delayed (3–7 days) structural damage to neurons in these brain formations in rats, this being manifest as the "light" and predominantly "dark" types, apparently reflecting the development of necrotic and apoptotic processes. Preconditioning with moderate hypobaric hypoxia as used here corrects these lesions, resulting in a marked increase in the expression of early gene proteins and significant reductions in structural damage to neurons after severe hypoxia.

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