Neuroscience and Behavioral Physiology, Vol. 40, No. 7, 2010

Prevention of Neurodegenerative Damage to the Brain in Rats in Experimental Alzheimer's Disease by Adaptation to Hypoxia

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Translated from Rossiiskii Fiziologicheskii Zhurnal imeni I. M. Sechenova, Vol. 95, No. 7, pp. 706–715, July, 2009. Original article submitted December 12, 2008. Revised version received May 15, 2009.

We report here studies addressing the possibility of preventing neurodegenerative changes in the brain using adaptation to periodic hypoxia in rats with experimental Alzheimer's disease induced by administration of the neurotoxic peptide fragment of β-amyloid (Ab) into the basal magnocellular nucleus. Adaptation to periodic hypoxia was performed in a barochamber (4000 m, 4 h per day, 14 days). The following results were obtained 15 days after administration of Ab. 1. Adaptation to periodic hypoxia significantly blocked Ab-induced memory degradation in rats, as assessed by testing a conditioned passive avoidance reflex. 2. Adaptation to periodic hypoxia significantly restricted increases in oxidative stress, measured spectrophotometrically in the hippocampus in terms of the content of thiobarbituric acid-reactive secondary lipid peroxidation products. 3. Adaptation to periodic hypoxia completely prevented the overproduction of NO in the brains of rats with experimental Alzheimer's disease, as measured in terms of increases in tissue levels of stable NO metabolites, i.e., nitrites and nitrates. 4. The cerebral cortex of rats given Ab injections after adaptation to periodic hypoxia did not contain neurons with pathomorphological changes or dead neurons (Nissl staining), which were typical in animals with experimental Alzheimer's disease. Thus, adaptation to periodic hypoxia effectively prevented oxidative and nitrosative stress, protecting against neurodegenerative changes and protecting cognitive functions in experimental Alzheimer's disease.

KEY WORDS: beta-amyloid, Alzheimer's disease, nitric oxide, oxidative stress, brain neurons, adaptation to hypoxia.

Alzheimer's disease is a neurodegenerative disease of the central nervous system, characterized by progressive loss of memory and other impairments to cognitive functions. Beta-amyloid (Ab) plays a major role in the pathogenesis of Alzheimer's disease. One of the key processes initiating the deposition of aggregated Ab is activation of free-radical processes, including overproduction of active forms of oxygen and nitric oxide (NO) which, along with other factors, ultimately leads to irreversible damage and death of brain neurons [20].

Almost 100 years of attempts to fight Alzheimer's disease using medicinal agents have shown that their potential is limited. Pharmacotherapy produces only a degree of slowing of the progression of cognitive losses [25]. In addition, the development and use of agents normalizing cognitive functions in patients with Alzheimer's disease raise many ethical questions linked with general wellbeing and the quality of life of these patients [15]. About ten agents are currently used in the treatment of Alzheimer's disease, most of which have marked cholinomimetic actions. The

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main pharmacological effects of these compounds are directed to improving cognitive capacities, which are impaired in Alzheimer's disease as a result of reductions in the functional activity of the cholinergic neurotransmitter system; their neuroprotective properties are quite weak. Several classes of drugs have now been developed which are used in the treatment of Alzheimer's disease. These include acetylcholinesterase inhibitors, agents acting on NMDA and glutamate receptors, agents restricting Ab synthesis, agents which counter the formation of neurofibrillary tangles, anti-inflammatories, etc. [1, 25]. However, the efficacy of these agents is limited by the fact that each agent affects only one component of the pathogenesis of Alzheimer's disease and, furthermore, acts on both damaged and healthy cells.

At the same time, it is already clear that the endogenous defense systems of the brain can limit the progression of Alzheimer's disease over prolonged periods of time after disease onset. These systems include the antioxidant, heat shock protein (HSP), NO, and other so-called stress-limiting systems [7, 10]. These systems are the most sensitive sensors for unfavorable extra- and intracellular conditions and are the first to respond to changes at the earliest stages of Alzheimer's disease. Thus, much attention is currently being paid to approaches able to increase adaptive potential and mobilize the body's endogenous protective systems. These approaches include natural adaptogens, the most active of which is *Gingko biloba*, an agent which can improve a number of neuropsychological measures [30], dietary restrictions [38], cognitive-linguistic mental activity training [14], and physical exercise [32].

The protective effects of adaptation to hypoxia have been demonstrated in lesions to and diseases of the cardiovascular, immune, and respiratory systems [10]. The possibility of preventing central lesions by adaptation to hypoxia has received less study. Adaptation to periodic hypobaric hypoxia created in a barochambers was found to have protective effects in experimental epilepsy [11], while adaptation to altitude hypoxia in mountains improved the formation and retention of a conditioned reflex in mice [10]. In humans, adaptation to hypobaric hypoxia has been used in the treatment of paranoid forms of schizophrenia and alcoholism [10]. Finally, adaptation to normobaric hypoxia was effective in preventing the behavioral impairments associated with neurodegenerative processes in experimental Parkinson's disease [27]. Adaptation to hypoxia or hypoxic preconditioning had neuroprotective effects in cerebral ischemia [4, 5, 16]. Thus, the possibility of preventing neurodegenerative brain damage on exposure to Ab by adaptation to hypoxia has potential.

The present report describes studies of the effects of prior adaptation of rats to periodic hypobaric hypoxia on neurodegenerative brain damage and cognitive functions in rats with experimental Alzheimer's disease induced by administration of Ab.

METHODS

Experiments were performed on male Wistar rats weighing 320 ± 50 g.

Alzheimer's disease was modeled by bilateral stereotaxic administration into the basal magnocellular nucleus of a neurotoxic peptide fragment of β-amyloid (25–35) at a dose of 2 μl of $0.4 \cdot 10^{-9}$ M Ab (25–35) solution. Experiments were started 30 days after Ab administration. As we have previously demonstrated that administration of physiological saline to rats in place of Ab has no harmful effects [13], this control was not used in the present study.

Adaptation of rats to hypobaric hypoxia was performed in a barochamber to reduce air density to a level corresponding to 4000 m above sea level, with exposure for 4 h per day for 14 days. The last adaptation session was performed 24 h before modeling Alzheimer's disease.

The extent of neurodegenerative brain damage was assessed in terms of changes in memory in rats by testing a conditioned passive avoidance reflex (CPAR) 14 days after administration of β-amyloid. Rats were placed in a special apparatus in a space with low illumination and the latent period of transfers into a dark chamber (LP_1) was measured over a period of 3 min. After entering the dark chamber, the rat received an "unavoidable" painful electrical shock via the floor (current 0.7 mA, 12 sequential shocks each lasting 1 sec, separated by 2-sec intervals). The test was repeated 24 h later. Rats were again placed in the apparatus and the latent period of transfer into the dark chamber (LP_2) was measured over a period of 3 min. When mental processes were intact, the animals remembered the painful electric shock applied in the dark chamber and did not enter it or entered it later, producing an increase in the latent period of the transfer into the dark area. Thus, the better the animal retained information about the electric shock in memory, the longer the latent period LP_2 and the greater the difference $LP_2 - LP_1$, i.e., ΔLP, or the memory level.

The level of oxidative stress was assessed in terms of the content of secondary thiobarbituric acid-reactive (TBA) lipid peroxidation products, which were measured spectrophotometrically [37] in brain structures, including the cortex, cerebellum, and hippocampus. The absorption (optical density) of extracts was measured at wavelengths of 535 nm (the absorption peak of malondialdehyde, MDA) and 515 and 550 nm (to account for nonspecific absorption) on a KFK-3 photometer (Russia).

NO production in the brain was assessed in terms of the total concentration of NO metabolites, i.e., nitrites and nitrates (NO_x), which was measured spectrophotometrically in cerebral cortex tissue homogenates using the Griess reaction. Nitrates were reduced to nitrites using Nitralyzer reactors (World Precision Instruments Inc., USA).

Neurohistological studies were performed by anesthetizing the animals with chloral hydrate and fixing the brain by transcardiac perfusion with a mixture of 40% for-

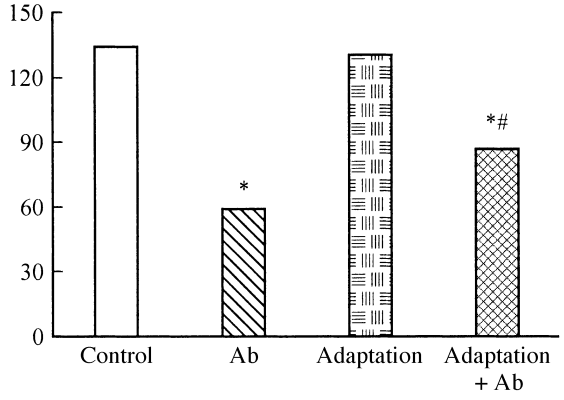

Fig. 1. Effects of prior adaptation to hypoxia on Ab-induced memory impairments in rats. The abscissa shows changes in the latent period of transfer to the dark sector of the chamber in the CPAR test (ΔLP, sec). *Significant differences from controls, $p < 0.05$; #significant difference from the Ab group, $p < 0.05$.

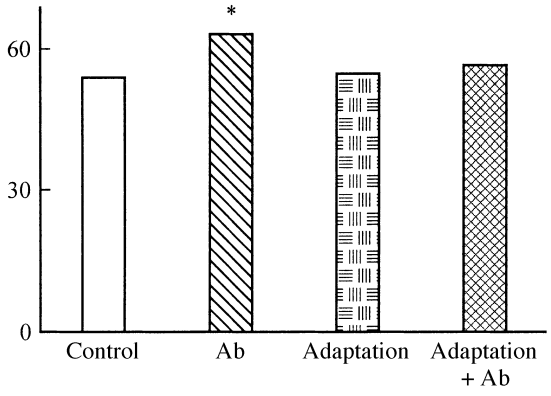

Fig. 2. Effects of adaptation to hypoxia on measures of Ab-induced oxidative stress in the hippocampus of Wistar rats (M ± m). The abscissa shows contents of thiobarbituric acid-reactive substances (malondialdehyde), nmol/g tissue. *Significant differences from controls, $p < 0.05$.

malin, glacial acetic acid, and 96% ethanol (2:1:7). Paraffin brain sections of thickness 10 μm were stained with 0.3% fast cresyl violet by the Nissl method. Light microscopy was used to identify dead and pathologically altered (darkened, pyknotic) neurons.

Statistical analysis of results was performed using the paired Wilcoxon *t* test for comparison of LP_1 and LP_2 and ΔLP values in each animal in the CPAR and the nonparametric Wilcoxon–Mann–Whitney U test for comparing CPAR results from different series.

RESULTS

Results obtained from the CPAR test showed that 89% of the rats remembered the electric shock, which produced an increase in the LP of entering the dark section of the chamber. After injections of Ab, the difference between LP_2 and LP_1 was significantly smaller than in control rats, indicating deterioration of memory (Fig. 1, *A*, *B*). The proportion of rats remembering the electric shock decreased to 75% ($p < 0.05$). Adaptation to hypoxia itself had virtually no effect on memory, though ΔLP was longer in adapted rats after Ab administration than in non-adapted animals, i.e., adaptation significantly prevented Ab-induced deterioration of memory in the rats. The proportion of rats remembering the electric shock returned to the control level, i.e., 87%.

The plot in Fig. 2 shows that Ab induced oxidative stress in the hippocampus. The quantity of TBA-reactive products in rats with experimental Alzheimer's disease increased from 53.8 ± 2.2 to 63.1 ± 2.7 nmol/g tissue ($p < 0.05$). Adaptation to hypoxia itself had no effect on the level of TBA-reactive products, though it significantly limited the increase induced by Ab, to 56.1 ± 2.1 nmol/g tissue ($p < 0.05$), which was not significantly different from controls.

Administration of Ab led to overproduction of NO, as shown by the significant increase in the tissue content of stable NO metabolites, i.e., nitrites and nitrates (Fig. 3). Adaptation to hypoxia had no significant effect on this parameter but completely prevented NO overproduction in the brains of rats with experimental Alzheimer's disease.

The protective effect of adaptation was supported by morphological studies (Fig. 4). No pathologically altered neurons were seen in control rats (Fig. 4, *A*) or rats adapted to hypoxia (Fig. 4, *B*). In rats given Ab injections (Fig. 4, *C*), the temporal-parietal cortex showed numerous hyperchromic, pyknotic (dead) neurons (arrows) in the injected area. The brains of rats given Ab injections after adaptation to hypoxia, in contrast to those of injected rats without adaptation, lacked neurons with these pathomorphological changes and no dead neurons were seen (Fig. 4, *D*).

DISCUSSION

The main result of the present study was that adaptation of rats to hypobaric hypoxia effectively prevented the marked activation of free-radical processes which accompanies the development of neurodegenerative brain damage with concomitant memory impairments in animals with experimental Ab-induced Alzheimer's disease, thus protecting neurons from oxidative and nitrosative stress and preserving cognitive functions.

Active forms of oxygen and NO accompanying the process of neuroinflammation represent important factors in the development and progression of Alzheimer's disease. Oxidative stress can impair the metabolism of β-amyloid precursor protein, while deposition of β-amyloid in the walls of cerebral arteries and capillaries is one of the characteristic features of Alzheimer's disease, and the neurotox-

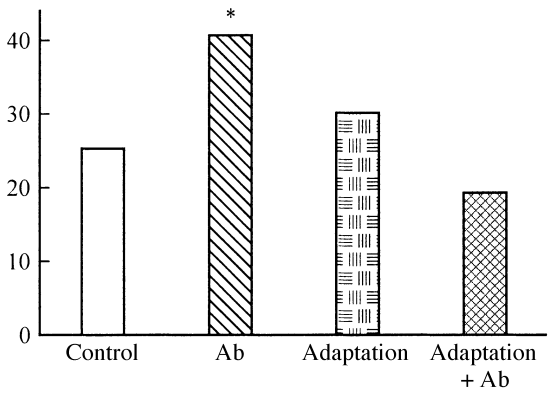

Fig. 3. Effects of prior adaptation to hypoxia on the total content (μM) of stable NO metabolites, i.e., nitrites and nitrates, in rat brain tissue after administration of Ab. *Significant differences from controls, $p < 0.05$.

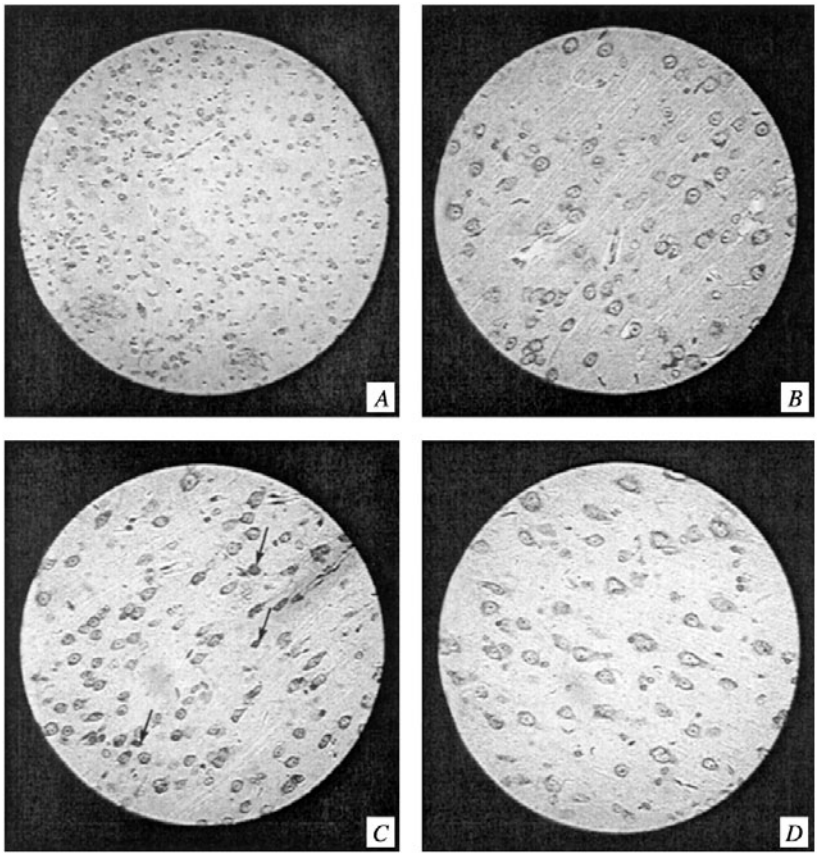

Fig. 4. Sections of the temporal-parietal cortex of a rat. Stained by the Nissl method (×400). *A*) Controls; *B*) adaptation; *C*) influence of Ab (arrows show dead neurons); *D*) effects of prior adaptation on the actions of Ab.

icity itself can lead to lead to increases in free radical formation. Increases in oxidative stress in Alzheimer's disease could also result from inherited mitochondrial membrane pathology [26, 39].

Nitric oxide is formed in neurotoxic quantities by inducible NO synthase in microglia and astrocytes and neuronal NO synthase in neurons [21]. The neurotoxic action of excess NO is mediated by mitochondrial dysfunction and

depletion of ATP, which ultimately leads to apoptotic death of neurons [29]. Damaged brain areas show large quantities of 3-nitrotyrosine, a marker for the toxic product of the reaction of NO with the superoxide anion, i.e., peroxynitrite [33]. The direct estimation of stable NO metabolites, i.e., nitrates and nitrites, in the rat brain performed here showed that administration of Ab leads to marked NO overproduction.

The brain is particularly susceptible to oxidative stress, as its tissues are rich in unsaturated fatty acids and are quite lacking in protective antioxidant factors. Thus, for example, catalase activity in the brain is only about 10% of that in the liver [34]. At the same time, some parts of the brain have high iron contents, while brain tissue overall has high ascorbate levels, creating significant potential for activation of lipid peroxidation in cell membranes [44]. Our experiments revealed significant increases in TBA-reactive lipid peroxidation products in the brains of rats with experimental Alzheimer's disease, which is consistent with published data [19].

The brains of patients with Alzheimer's disease show loss of synaptic contacts even at the early stages of illness [22, 40]. This process correlates quite closely with impairment to cognitive functions [23]. Neuron damage and pyknosis then begin to occur, to be followed by selective neuron death in the hippocampus, cerebral cortex, and limbic structures [18, 43].

Cholinergic neurons in the basal nucleus of Meinert, which project directly to the cerebral cortex [43], are affected to a greater extent in Alzheimer's disease, as are analogous neurons located in the diagonal band of Broca and medial septum and projecting to the hippocampus [18, 43]. Thus, our experiments used a widely accepted model of Alzheimer's disease in rats – administration of Ab into the structures analogous to Meinert's nucleus, i.e., the basal magnocellular nucleus [17], followed by morphological analysis of neurodegenerative damage to neurons in the temporal-parietal cortex. Our study provided the first demonstration that prevention of impairment to cognitive functions in rats occurring after administration of Ab in rats undergoing prior adaptation to hypoxia, as also seen in our previous experiments [12], is associated with reductions in the susceptibility of brain neurons to neurodegeneration and, thus, decreases in the proportion dying.

These results suggest possible mechanisms for the protective effects of adaptation to hypoxia. The first consists of restriction of oxidative stress due to the known effect consisting of activation of endogenous antioxidant defense, which has been demonstrated both in experiments and in clinical conditions. In fact, previous studies have demonstrated that this mechanism of adaptation to periodic hypoxia prevents oxidative stress and concomitant brain damage induced by chronic ethanol consumption in rats [28] or iron citrate [31]. In patients with chronic cerebral ischemia, 10 days of hypoxic training led to decreases in the content of TBA-reactive substances, while superoxide dismutase, catalase, and glutathione peroxidase activities in erythrocytes increased, with the result that patients experienced decreases in the frequency of episodes of headache and nocturnal sleep and short-term memory improved [3]. The experiments reported here yielded analogous data providing evidence supporting the important role of restriction of oxidative stress and the neuroprotective effect of adaptation to hypoxia.

Another protective mechanism may be associated with preventing Ab-induced NO overproduction in the brains of adapted animals, as seen in the present experiments. One of the adaptive reactions directed to protecting the body from the toxic actions of excess NO is an increase in the binding of NO into complexes forming so-called depot NO in vessel walls [2, 8]. Our previous experiments showed that the volume of depot NO in brain vessels in rats with experimental Alzheimer's disease previously adapted to hypoxia was significantly greater than that in unadapted rats [9].

There is no doubt that the protective adaptation mechanisms underlying prevention of neurodegenerative brain damage are not limited to those described above. Thus, for example, the literature contains data showing that transcription factor HIF-1, which is induced by hypoxia, can protect nerve cells from the toxic actions of Ab [42]. It has been suggested that HIF-1, by virtue of its rapid synthesis in conditions of hypoxia and degradation on reoxygenation, is responsible for long-term adaptation to periodic hypoxia at the level of gene transcription [36, 41]. Thus, our further studies will focus on the possible role of HIF-1 in the neuroprotective effect of adaptation to hypoxia. Identification of the protective mechanisms of adaptation to hypoxia may in future be useful in developing new and clinically useful pharmacological methods for the prophylaxis and treatment of Alzheimer's disease.

This study was supported by the Russian Foundation for Basic Research (Grant No. 07-04-00650).

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