Induction of NO-Synthase and Acetaldehyde Dehydrogenase in Neurons of Human Cerebellar Cortex during Chronic Alcohol Intoxication

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Induction of NO-synthase and acetaldehyde dehydrogenase in Purkinje cells, basket-like neurons, and microvascular endothelium of human cerebellar cortex was detected in patients with chronic alcohol intoxication. It was hypothesized that the neuromodulating effect of NO is associated with mechanisms protecting neurons from toxic effects of ethanol and acetaldehyde.

Key Words: *nitrogen oxide; ethanol; cerebellum*

Among the pathogenetic mechanisms of alcohol dependence, NOergic mechanisms, whose activity is modified by exogenous ethanol, received recently special attention [7,9]. Injection of NO-synthase (NOS) inhibitors to experimental rats produced an inhibitory (synergic with ethanol) effect on neurons [14]. The sensitivity of neurons to ethanol under this conditions increased and its cytotoxic effects were intensified [11,14]. The damaging effect of ethanol is realized via the formation of acetaldehyde (intermediate ethanol metabolite) and is most pronounced in neurons expressing no acetaldehyde dehydrogenase (AlDH) [3,4]. We studied cerebellar cortical NOergic neurons responsible for acetaldehyde oxidation in humans.

MATERIALS AND METHODS

Human brain samples from subjects aged 25-56 years with a history of alcoholic disease dead from craniocerebral injury were obtained in forensic medical autopsies. Specimens of the cerebellum from subjects dead from conditions not associated with narcological and neurological diseases served as the control. The material was collected 6 h postmortem.

The severity of ethanol intoxication was evaluated at chemical laboratory of forensic medical morgue. Arbitrary degrees of ethanol intoxication were determined depending on the level of ethanolemia by the moment of death and classified as compensated (0.5-2.9‰), decompensated (3-5‰), and conditionally lethal (more than 5‰) [2,3].

Histochemical analysis of AlDH (EC 1.2.1.3) activity was carried out by the method of S. M. Zimatkin *et al.* [1]. Brain samples were frozen in a cryostat and 15-25-μ sections were made. After drying the sections were placed into incubation medium containing 0.1 M Na-pyrophosphate buffer (pH 7.4), 5 mM NAD (Calbiochem), 2 mM amital, 0.1 mM pirasole (Sigma), 5 mM magnesium chloride, 0.025% nitroblue tetrazolium (NBT), 4% polyvinylpyrrolidone, and 30 mM acetaldehyde (Sigma). The sections were incubated for 20-30 min at 37°C, washed in isotonic sodium chloride solution, dehydrated, and embedded in balm.

For evaluation of NOS activity (NADPH diaphorase; EC 1.6.99.1), the material was fixed for 2 h at 4°C in 4% paraformaldehyde in 0.1 M Na-phosphate

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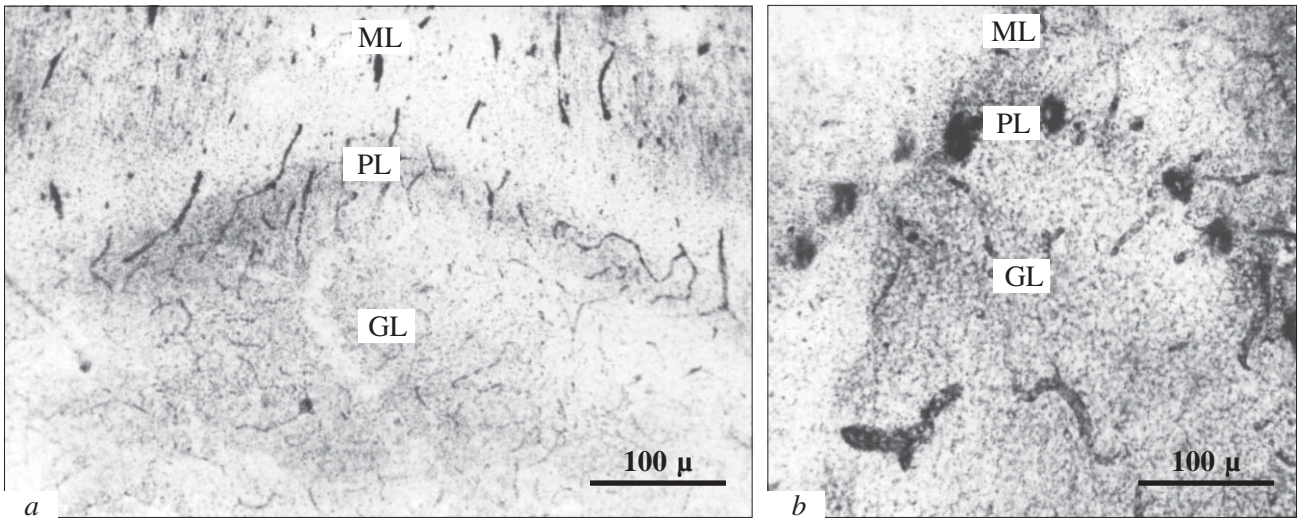

Fig. 1. Human cerebellar cortex acetaldehyde dehydrogenase (AlDH). *a*) location of AlDH in controls; *b*) inductive form of AlDH in Purkinje neurons in chronic alcoholic intoxication. AlDH reaction. Here and in Fig. 2: ML: molecular layer; PL: Purkinje cells layer; GL: granular layer.

buffer (pH 7.4) and washed in 15% sucrose for 24 h. Cryostat sections (25 μ) were incubated for 1 h at 37°C in a medium ocontaining 50 mM Tris buffer, 0.2% Triton X-100, 0.8 mg/ml β-NADPH (Sigma), and 0.4 mg/ml NBT (pH 8.0) [10].

Enzyme activities in cerebellar cortical neurons were evaluated by the density of histochemical precipitate on a Vickers cytodensitometer and expressed in optical density units.

RESULTS

Fine ramified capillary network penetrating all cortical layers was detected by the AlDH test in control samples (Fig. 1). The granular layer was diffusely colored bright violet due to formazan reaction with the substratum in mossy fiber glomerular terminals. Only solitary Purkinje cells expressed moderate or low AlDH activity (37-40 opt. dens. units), while most of them were unstained (Fig. 1, *a*). In decompensated alcoholemia the number of positively stained Purkinje neurons increased by 38.0±3.4% (Fig. 1, *b*).

Constitutive NOS in human cerebellar cortex is expressed in Purkinje neurons, molecular layer neurons, and granular cells (Fig. 2, *a*). They were detected by NADPH diaphorase test due to deposition of finely grained precipitate stained from pale to dark blue, which presumably reflected different activity of neuronal NOS isoform. In subjects with decompensated ethanol intoxication these neurons contained large intensely stained bluish black diformazan granules penetrating into the processes (Fig. 2, *b*). Narrow cytoplasm rim and optically empty nucleus in the center were seen in granular cells.

Inductive reactivity of NOS was detected in capillary endotheliocytes of all cortical layers: the vessels were stained from sites of ramification of the descending cortical arterioles to precapillary sphincters (Fig. 2, *b*). Optical density of formazan precipitate in the neuronal cytoplasm and endothelium detected by photometry was much higher than in the diaphorase test in the control group (Table 1).

Nerve cells with low AlDH activity are regarded as critical, in which acetaldehyde is accumulated irrespective of its content in the blood [4]. Among these cells are Purkinje cells which, unlike other brain cells, are most susceptible to ethanol, which fact is believed to be responsible for ataxia in 55% alcoholics [5]. Changes in AlDH activity in Purkinje cells detected in our study should be regarded as a manifestation of

TABLE 1. NADPH Diaphorase Activity in Human Cerebellar Cortex in Chronic Alcoholic Intoxication (Opt. Dens. Units, $X \pm S_x$)

Cerebellar cortex cells	NADPH diaphorase activity (control)	NADPH diaphorase activity in decompensated alcoholemia (3-5‰)
Basket neurons	65.4±2.3	71.4±4.1
Purkinje cells	58.7±3.3	84.4±2.8
Granular cells	38.2±1.2	41.4±2.3
Microvascular endothelium	46.4±6.3	78.4±2.7

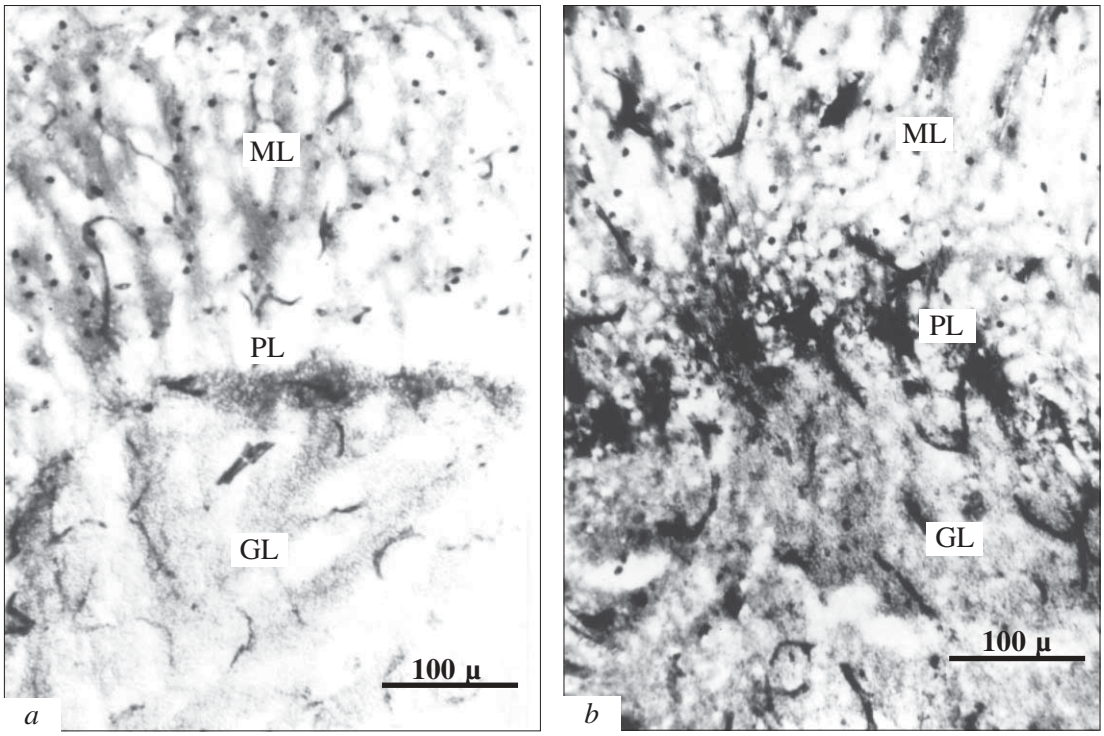

Fig. 2. Location of NADPH diaphorase in human cerebellar cortex. *a*) constitutive NADPH diaphorase in molecular layer neurons and granular cells; *b*) NADPH diaphorase induction in Purkinje cells and microvessels in chronic alcoholic intoxication. NADPH diaphorase reaction.

adaptation activation (induction) of the enzyme, which can develop in response to long-lasting and/or single excessive intake of ethanol. Enzyme induction in neurons and endotheliocytes is triggered by degenerative, metabolic, and ischemic changes, which inevitably result from toxic effect of ethanol and products of its oxidation on the brain. The consequences of enzyme induction, in turn, can be related to activation of protective mechanisms.

Multifactorial mechanism of the protective effect of NO can be explained by its different involvement in modification of glutamate (NMDA and non-NMDA type) receptors. This regulation is of priority significance, particularly during the formation of abstinence syndrome, when hyperstimulation of NMDA receptors potentiates the development of convulsive fits [8,12]. Passage of calcium ions through NMDA receptor channels is closely associated with activation of calmodulin (allosteric fragment of NOS). That is why induction of NADPH diaphorase observed in our experiments and NOS production in sites of ethanol accumulation associated with it can be regarded as obligatory components in the chain of neurotropic effects of ethanol. It is known that during prolonged alcoholization of animals NOS inhibitors provoke toxic effect of NMDA [5] and that apoptosis of cerebellar cortical neurons under conditions of antenatal exposure to ethanol is more pronounced in mice with genetically determined nNOS deficiency [6].

By contrast, during long lasting alcohol consumption under conditions of increased transmission of stimulatory amino acids and hyperstimulation of NMDA receptors the neurotropic effect of NO can become destructive. Single dose of ethanol stimulates NO production in the cerebral capillary endothelium, where NO functions as a neuroprotector [11]; however, after several hours the effect becomes opposite [14]. The increase in NO concentration in this situation leads to accumulation of free radicals, which potentiate the toxic effect of ethanol. Toxic oxidants impair the relationships between cell membrane components and cytoplasmatic proteins, triggering the mechanisms of necrosis and apoptosis, which augments energy imbalance in cells in the presence of massive delivery of NO [9]. One more possible variant was described by E. G. De Master *et al.* [7]: long experimental treatment with ethanol led to an appreciable increase in NO concentration, which blocked AlDH activity in neurons, while the neurodestructive effects of underoxidized acetaldehyde were enhanced manifold.

The behavior of NO in chronic alcohol intoxication can consist of two opposite effects: cytotoxic and neuroprotective. Induction of NOS and AlDH in neuronal cytoplasm and capillary endothelium detected in our experiments can be regarded as two sides of the same process associated with the formation of a mechanism protecting the cells from toxic effect of acetaldehyde and the modulatory NOergic component.

The shift of the physiological action of NO depends on the dose and duration of exposure to ethanol and acetaldehyde, degree of neuron hyperstimulation, and production of superoxides.

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