PROTECTION OF HIPPOCAMPAL CELL CULTURES AGAINST HYPOXIA AND SUBSEQUENT REOXYGENATION BY AN ANTIOXIDANT OF THE STERICALLY HINDERED PHENOL CLASS

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The study of the mechanisms of neurodestructive processes and the search for ways of preventing them in hypoxia, hypoglycemia, and cerebral ischemia is an urgent problem in experimental neurology. Results of numerous recent investigations [5] have convincingly demonstrated the leading role of glutamate in hypoxic damage to neurons in different brain structures. A considerable rise of the extracellular glutamate concentration in hypoxia/ischemia as a result of its increased rate of release from presynaptic terminals and disturbance of its reuptake leads to hyperactivation of the glutamate receptors of the postsynaptic membrane and of the ionic channels under their control. A result of this is rapid inflow of Ca^{2+} into the neurons [4, 10], followed by activation of Ca^{2+}-dependent lipolytic and proteolytic enzymes and the formation of free radicals, which leads to delayed death of nerve cells [8, 11-13]. In addition, there is evidence that elevation of the intracellular calcium level in the posthypoxic period, leading to delayed death of neurons, may be the result of activation of free-radical reactions and, in particular, of lipid peroxidation, disturbing the barrier function of neuron membranes [1, 6].

The possibility of preventing destruction of neurons during hypoxia and subsequent reoxygenation with the aid of a new lipophilic antioxidant of the sterically hindered phenols class (U-18), which has justified itself well in giving protection against free-radical injury [3], was studied in the present investigation.

EXPERIMENTAL METHOD

Hippocampal cell cultures from 17-18-day mouse embryos were obtained by enzymic dissociation and grown on coverslips (22 × 22 mm) in plastic Petri dishes 40 mm in diameter, by the method described by the writers previously [7]. During the experiments a Plexiglas chamber of our own design was used, in which the dishes with the cultures in nutrient medium with and without the addition of U-18 in a concentration of 25 μM were placed, was used. After the chamber had been ventilated for 30 min with a hypoxic gas mixture (95% N_2 + 5% CO_2) it was hermetically sealed and left in an incubator at 35.5°C for 6-7 h. The cultures were then taken from the chamber and placed for 12 h in a CO_2 incubator (95% air + 5% CO_2) at the same temperature. In another series of experiments U-18 was added to the nutrient medium only after removal of the cultures from the hypoxic chamber. Damage to the neurons in culture was estimated quantitatively by morphological and biochemical methods. For morphological assessment of the degree of damage, the cell cultures were fixed with a mixture of 96% ethanol, 40% formalin, and glacial acetic acid in the ratio of 7:2:1 and stained by Nissl's method or with vanadium-hematoxylin [2]. The degree of damage was expressed as the percentage of cells which died relative to their total number in each culture in 15 fields of vision, each with an area of 0.1 mm^2. Quantitative biochemical assessment of death of the neurons was carried out by recording spectrophotometrically the lactate dehydrogenase (LDH) concentration in the nutrient

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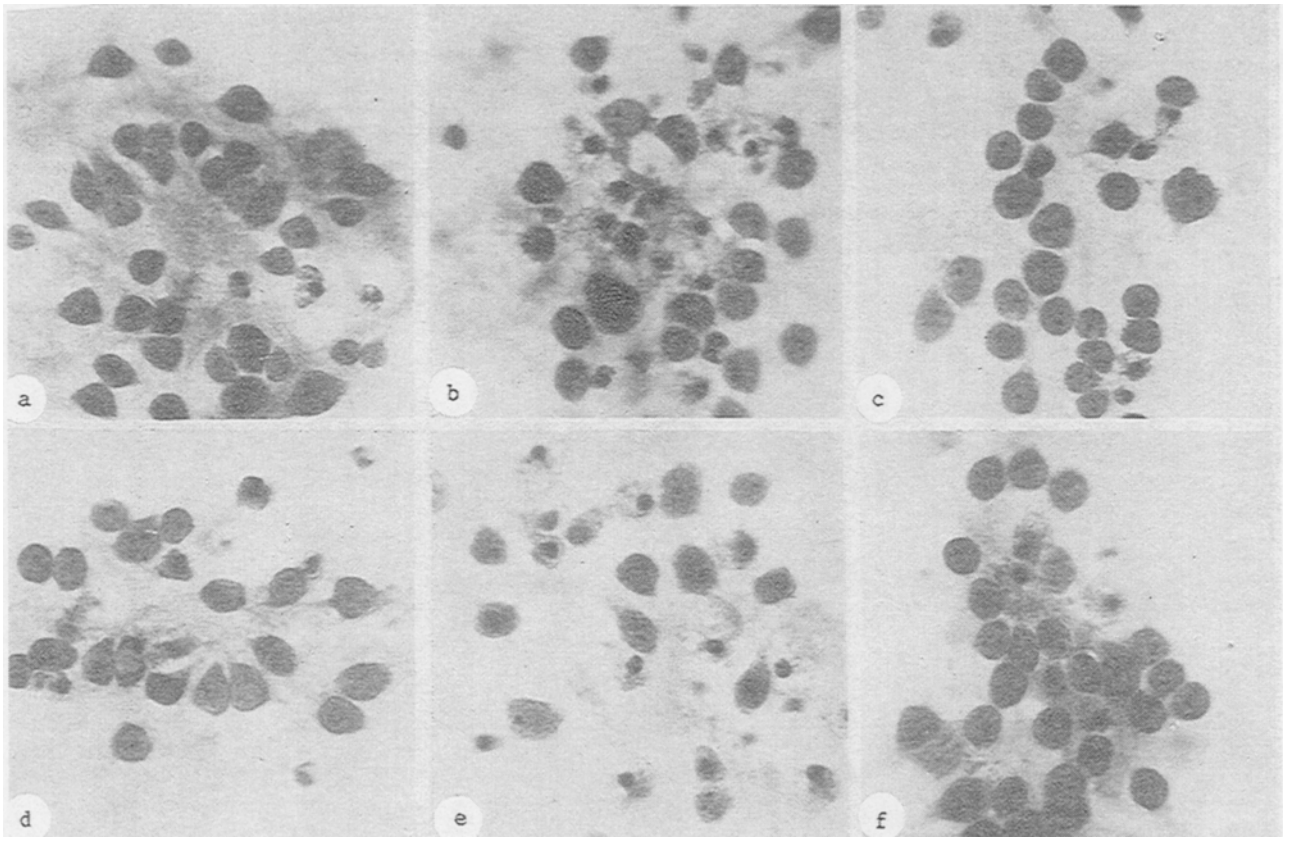

Fig. 1. Action of hypoxia and subsequent reoxygenation on hippocampal neuronal cultures and effect of the antioxidant U-18 on their injury: a) control culture; b-d) cultures after exposure to hypoxia for 7 h and reoxygenation for 12 h: b) in medium without U-18, c) after addition of U-18 to medium before exposure to hypoxia, d) addition of U-18 to medium immediately after exposure to hypoxia; e) after exposure to hypoxia for 7 h and reoxygenation for 3 h; f) after exposure to hypoxia (without reoxygenation) for 10 h. Stained with vanadium-hematoxylin, 20 days in vitro. Scale: 25 μ. Objective 40, ocular 10.

medium [9], using a standard kit of reagents from "Beckman." The quantity of enzyme escaping from the neurons was expressed as a percentage of the highest possible LDH activity measured in a parallel sample of nutrient medium, in which the control and experimental cultures were subjected to freezing and thawing. The significance of the numerical results and their differences was determined by Student's t test.

EXPERIMENTAL RESULTS

Cultures with a high degree of neuronal differentiation and with properly formed synaptic connections were used in the experiments [7]. Incubation in a hypoxic chamber for 6-7 h and subsequent culture in a CO_2 incubator for 12 h led to destruction of a high proportion of the neuron (Fig. 1a, b). During intravital study of these cultures in a phase-contrast microscope the destructive changes in the neurons appeared as a rule 2-3 h after exposure to hypoxia, after which the number of damaged neurons rose rapidly to reach a maximum after 7-8 h. Glial cells (astrocytes), as an immunocytochemical investigation for acid gliofibrillar protein showed, remained intact (this part of the experiment was performed by Senior Scientific Assistant T. M. Solomatina). Subsequent culture for 1-2 days

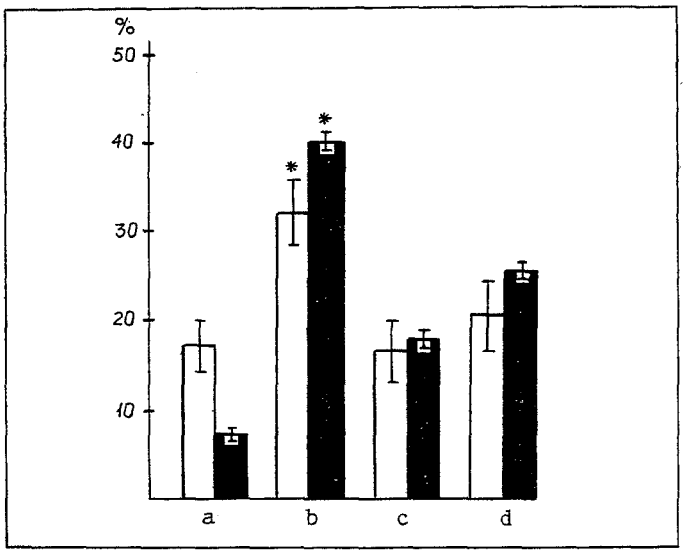

Fig. 2. Quantitative parameters of neuron destruction following exposure to hypoxia and to antioxidant U-18: a) control values (n = 11); b-d) after hypoxia for 7 h and reoxygenation for 12 h: b) in medium without U-18 (n = 17), c) after addition of U-18 to medium before exposure to hypoxia (n = 9), d) after addition of U-18 to medium immediately after exposure to hypoxia (n = 5). Asterisk indicates significant difference from a ($p < 0.005$), c ($p < 0.001$), and d ($p < 0.05$); n) number of cultures studied. Unshaded columns – LDH release, black columns – dying cells.

led to no appreciable increase in the number of damaged neurons. LDH activity in medium obtained from cultures subjected to hypoxia and subsequent reoxygenation amounted to 32.2 ± 3.8%, compared with 18.0 ± 2.9% in the control cultures, whereas the corresponding numbers of dying neurons were 40.2 ± 1.0 and 7.2 ± 0.6% (Fig. 2a, b). The high LDH activity in the control can evidently be explained by gradual accumulation of the enzyme in the nutrient medium due to the fact that spontaneous death of a certain proportion of nerve and glial cells takes place during long-term culture. It must be emphasized that measurements of LDH activity in the medium after freezing of the control cultures and of cultures subjected to hypoxia did not reveal any significant differences, evidence that exposure to hypoxia has no effect on this enzyme.

Addition of the antioxidant to the nutrient medium significantly weakened the neurodestructive processes induced by hypoxia. If the antioxidant was present in the medium throughout the experiment, i.e., before and after exposure to hypoxia, LDH activity was comparable with that in the control, namely 16.5 ± 3.3%, and the number of dying neurons was reduced by 17.7 ± 0.9% (Figs. 1c and 2c). A similar effect – LDH activity 20.5 ± 4.0%, number of dying neurons 25.3 ± 0.9% – also was observed on the addition of antioxidant to the nutrient medium immediately after exposure to hypoxia, i.e., only for the reoxygenation period (Figs. 1d and 2d). Incidentally, this action of U-18 is unconnected with blockade of glutamate receptors, for testing isolated neurons with this compound by the patch-clamp method did not reveal any properties of a glutamate receptor antagonist in it (I. N. Sharonova and V. S. Vorob'ev, personal communication).

The results suggest that injury to neurons during hypoxia and subsequent reoxygenation takes place mainly during the reoxygenation period. This hypothesis was confirmed by comparative statistical analysis of results obtained in parallel series of experiments, in which some cultures were subjected to hypoxia for 7 h, followed by reoxygenation for 3 h (Fig. 1e), whereas others were exposed to hypoxia for 10 h only (Fig. 1f). The number of dying neurons in the

first series was significantly greater (twice as many) and LDH activity was almost 25% higher than in the second group, where these indicators of the degree of destruction of neurons were comparable with the control values.

The results as a whole thus indicate that free-radical reactions (most probably lipid peroxidation) play an important role in neuronal destruction during hypoxia and subsequent reoxygenation; these reactions, moreover, are involved in the injury process mainly during the reoxygenation stage.

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COMPARATIVE ELECTROPHORETIC CHARACTERISTICS OF ALKALINE PHOSPHATASE ISOZYMES IN MATERNAL BLOOD PLASMA AND EXTRACTS OF CHORION FRONDOSUM AT DIFFERENT STAGES OF PREGNANCY

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In recent years the number of enzymic methods used in clinical practice for the early detection of pathological changes has increased considerably [1]. One of the most important conditions in this connection is the use of methods of investigation of enzymes or their molecular forms in blood, urine, saliva, and seminal fluid which correlates most closely with tissue enzymes, for this not only determines the interpretation of clinical data, but also makes it possible to estimate the quality and efficacy of treatment and the prognosis for development of pathological processes. One of the best ways of tackling this problem is through a parallel study of the activity of an enzyme in

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