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# Lipophilic Antioxidant U-18 and Superoxide Dismutase Prevent Cultured Hippocampal Neurons from Destruction during Hypoxia and in the Posthypoxic Period

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The lipophilic antioxidant U-18 from the class of hindered phenols prevents the destruction of cultured hippocampal neurons during hypoxia and also in the posthypoxic reoxygenation period, apparently by being stably incorporated into their phospholipid membranes and by safeguarding these from free-radical damage in the course of reoxygenation. On the other hand, the protection afforded to the cultured hippocampal neurons by superoxide dismutase is probably due to its ability to interfere with the posthypoxic neuron-degrading processes mediated through hyperproduction of superoxide radicals in the neuronal cytoplasm.

**Key Words**: *nerve cell culture; hippocampus; hypoxia; antioxidants*

Results of numerous *in vivo* and *in vitro* experimental studies attest to important roles of free radicals and lipid peroxidation (LPO) in causing damage to brain neurons during hypoxia/ischemia [5,6,8,15]. One of the factors initiating these conditions is activation of several intracellular enzymes (phospholipase $A_2$, xanthine oxidase, NO synthase) by calcium ions. The hyperstimulation of glutamate receptors resulting from increased presynaptic release of glutamate and impairment of the mechanisms of its reuptake under conditions of energy deficiency causes calcium to accumulate in the cytosol. As a consequence, the level of low-molecular substrates and the activity of enzymes involved in the generation of reactive oxygen species become greatly elevated during hypoxia.

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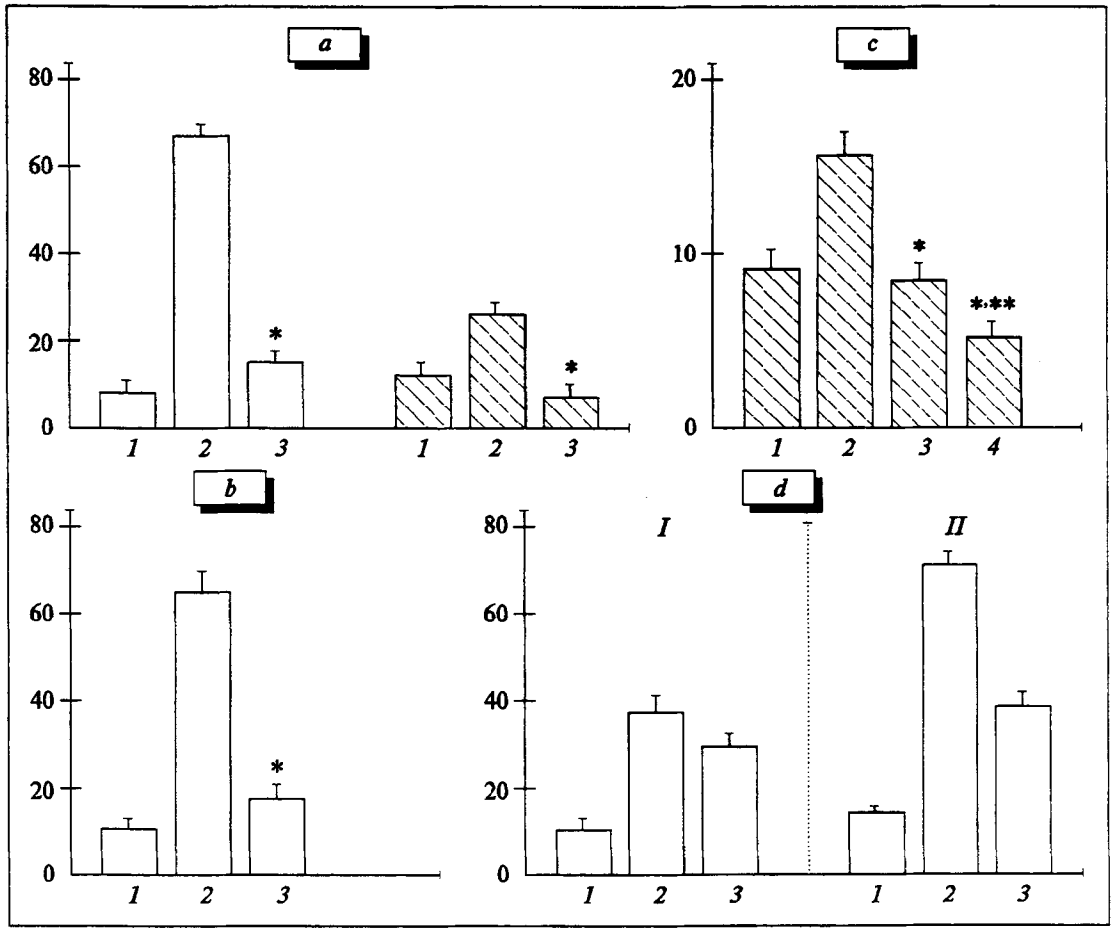


**Fig. 1.** Quantitative characteristics of the destruction of cultured hippocampal neurons during hypoxia and after the addition of antioxidants U−18 (25 μM) or SOD (300 U/ml). White bars: percentage of dead cells; dark bars: released LDH (units). *a*: *1*) control; *2*) hypoxia for 8 h + reoxygenation for 18 h without U−18; *3*) hypoxia for 8 h with U−18 + reoxygenation for 18 h with U−18. *b*: *1*) control; *2*) hypoxia for 15 h without U−18; *3*) hypoxia for 15 h with U−18. *c*: *1*) control; *2*) hypoxia for 8 h + reoxygenation for 16 h without SOD; *3*) hypoxia for 8 h + reoxygenation for 16 h with SOD; *4*) control in the presence of SOD. *d*: *1* (*I*, *II*) control; *2*) incubation with glutamate (100 μM) for 7−8 h (*I*) or 18−24 h (*II*) without U−18; *3*) incubation with glutamate (100 μM) for 7−8 h (*I*) or 18−24 h (*II*) with U−18.*$p<0.05$ in comparison with *2* in each test series; **$p<0.05$ in comparison with *3* in Fig. 1, *d*. Number of cultures tested: 6−14 in *a*; 5−6 in *b*; 15−25 in *c*; 6−14 in *d*.

Neurons are believed to sustain the greatest damage in the posthypoxic reoxygenation period, i.e., under circumstances particularly favorable for the formation of free radicals [14]. The LPO activated during this period greatly aggravates the destructive processes initiated in the hypoxic period so as to make them irreversible and cause delayed neuronal death.

In *in vivo* experiments, separation of the hypoxic period from the posthypoxic one is necessarily rather arbitrary. In nerve cell cultures, the process of neuronal destruction and the impact of neuroprotectors on the latter can be examined during both hypoxia and reoxygenation. In this work, we examined the synthetic lipophilic antioxidant U-18 from the class of hindered phenols [2] and the naturally occurring antioxidative metalloenzyme superoxide dismutase (SOD) for their effects in hippocampal cell cultures subjected to hypoxia with or without subsequent reoxygenation.

## MATERIALS AND METHODS

Dissociated hippocampal cells from 17- to 19-day embryos of C57Bl mice were cultured, as we described earlier [9], on cover glasses in plastic dishes 40 mm in diameter placed in a $CO_2$ incubator (95% air + 5% $CO_2$) at 35°C. The tests were conducted with cultures containing differentiated neurons and mature synaptic junctions (16-20 days *in vitro*) [9]. Before the cultures were exposed to hypoxia, the nutrient medium was removed from the dishes and stored in an incubator, while the cultures were washed three times in a balanced salt solution containing (in mM) 113.0 NaCl, 5.0 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 1.0 $Na_2HPO_4$, 24.0 $NaHCO_3$, and 5.0 glucose. Thereafter the antioxi-

dant (either U-18 or SOD) was added to this solution in the appropriate concentration and the cultures were incubated there for 30 min before being placed (in dishes without lids) in a hermetically sealed chamber filled with a gaseous mixture of 95% nitrogen and 5% carbon dioxide to be exposed to hypoxia for periods required by the experimental conditions. Oxygen concentrations in the chamber, measured polarographically, never exceeded 1%. For reoxygenation, the cultures were removed from the chamber, washed in the balanced salt solution, placed in the same nutrient medium as before, and returned to the $CO_2$ incubator for 16-20 h. Neuronal destruction and the protective action of the antioxidants were evaluated, as described by us previously, by counting the number of dead neurons (relative to the total number of neurons) in the cultures stained with vanadium hematoxylin [1] and by measuring lactate dehydrogenase (LDH) activity in absolute units/ml in the incubation medium [11]. Cultures incubated for appropriate periods in the balanced salt solution under normoxic conditions served as controls. The results were treated statistically using Student's *t* test.

## RESULTS

After the exposure to hypoxia (for 6-8 h) and subsequent reoxygenation (for 16-20 h) 67±1.8% of the neurons were dead. The presence of the U-18 antioxidant (25 μM) in the incubation medium during hypoxia reduced more than fourfold (to 15.5±1.9%) the proportion of neurons found to be dead after the termination of the reoxygenation period, during which the cultures were incubated without the antioxidant (Fig. 1, *a*). These findings correlated with the time course of LDH activity (6.5±2.3 units with U-18 *vs.* 26.6±2.3 units without it). Without reoxygenation, a similar destructive effect (62.3±5.1% of dead neurons) was achieved with cultures exposed to hypoxia for 15 h. It is important to note that in the presence of U-18 the proportion of nerve cells dying during this period was also significantly reduced (to 17.5±3.9%) (Fig. 1, *b*).

In cultures subjected to hypoxia and subsequent reoxygenation, a marked protective effect was exerted by SOD (300 units/ml). In the incubation media obtained from these cultures, LDH activity was 7.7±1 units as compared to 15.8±2.1 units in the absence of SOD (Fig. 1, *c*). SOD also exerted a protective effect in the control cultures incubated in the balanced salt solution without being exposed to hypoxia: LDH activity was 4.1±0.9 units *vs.* 9.3±1.4 units in the control cultures incubated without SOD.

As suggested above, one cause of excessive production of free radicals and activation of LPO in hypoxia/ischemia may be hyperstimulation of postsynaptic glutamate receptors. This suggestion is supported by the results of a study where protective effects of LPO inhibitors (21-aminosteroids) were demonstrated both against the cytotoxic activity of excitatory amino acids and during hypoxia and glucose deprivation [13]. A similar effect was produced by U-18 in our experiments. In cultures to which this antioxidant was added during their treatment with glutamate (100 μM) for 7-8 h or 18-24 h, the proportion of dead neurons was 26.5±1.8% and 38.1±2.7%, respectively, *vs.* 38.1±1.9% and 70.1±2.5% in cultures that did not contain U-18 (Fig. 1, *d*).

In our previous study, U-18 produced an antidestructive effect when added to cultures during the posthypoxic reoxygenation period [3]. The finding in the present study that this antioxidant is capable of preventing nerve cell death when present in the culture only during hypoxia may be taken as evidence of its prolonged effect, apparently as a result of its stable incorporation into the phospholipid membranes and the prevention of LPO through binding of free radicals that are actively produced during reoxygenation. On the other hand, the reduction in the number of dead neurons observed for cultures where U-18 was present during prolonged hypoxia (without subsequent reoxygenation) makes it likely that free radicals can also be produced to excess when the access of oxygen to nerve cells is limited, which, however, does not exclude the possibility of a membranotropic stabilizing action of U-18 not associated with its neutralization of free radicals.

The protective action of SOD seen in the present study confirms that this cytoplasmic antioxidant enzyme possesses neuroprotective properties observed previously in *in vivo* and *in vitro* studies where central neurons were subjected to cytotoxic or hypoxic damage [4,7,10,12], and appears to reflect the ability of SOD to prevent damage associated with the generation of superoxide radicals [7]. Since the lipophilic antioxidant U-18 and the hydrophilic antioxidant SOD can accumulate, respectively, in phospholipid membranes and cytoplasm, their antidestructive effects may be an indication of excessive production of reactive oxygen species in various morphofunctional compartments of the neuron.

Our results, in conjunction with those of other authors, attest to the ability of antioxidants

to protect neurons effectively from hypoxic death and widen prospects for the use of these compounds in disorders of cerebral circulation leading to ischemic disease.

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# Effect of Carnosine on the Activity of Lipoxygenase Isolated from Rabbit Reticulocytes

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The effects of the histidine-containing dipeptide carnosine and of histidine on the activity of 15-lipoxygenase from rabbit reticulocytes are studied. It is shown that low concentrations of carnosine (0.5-4 mM) activate oxidation of arachidonic acid by lipoxygenase, while high concentrations (>4 mM) inhibit the enzyme activity. Histidine elicits no activatory effect, although its inhibitory activity is comparable to that of carnosine.

**Key Words**: *15-lipoxygenase; reticulocytes; arachidonic acid; carnosine*

The processing of arachidonic acid (AA) results in the formation of prostaglandins and thromboxanes (the cyclooxygenase pathway) and leukotrienes and lipoxins (the lipoxygenase pathway) [6]. 15-Lipoxygenase of reticulocytes was originally described in 1979 [12]. This enzyme oxidizes AA to leukotrienes and actively produces lipoxin $B_4$ without the involvement of other lipoxygenases [10]. The lipoxygenase reaction is characterized by activation with lipoperoxides [11]. No other naturally occurring activators of these enzymes have been found. The enzymes are inhibited by various compounds, including antioxidants [1], since oxidation of unsaturated fatty acids is a free-radical process.

We examined the effect of the naturally occurring histidine-containing dipeptide carnosine, which elicits a potent antioxidant effect *in vivo* and *in vitro* [7], on the lipoxygenase pathway of AA oxidation. In addition to antioxidant activity, this dipeptide exhibits antistressor, immunomodulating,

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