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PHYSIOLOGY

Regulatory Peptides Protect Brain Neurons from Hypoxia in Vivo

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Short peptides that have been characterized in previous studies performed in the St. Petersburg Institute of Bioregulation and Gerontology of the Northeastern Division of the Russian Academy of Medical Sciences [1–3] protect neurons from oxidative stress [4]; however, under in vitro conditions, the peptide action has been observed at high (millimolar) concentrations. The anti-oxidant activity of these peptides is not high; therefore, their biological effect could be mediated by a natural system of antioxidant defense [5]. If this is the case, the peptide protective effect could be observed at almost physiological concentrations under the in vivo conditions. This was aimed at verifying this assumption. The protective effect of short regulatory peptides (vilon, epitalon, pinealon, and vesugen) was studied under the conditions of oxidative stress caused in animals by hypobaric hypoxia.

Male Wistar rats weighing 185–200 g were used in our experiments. Oxidative stress was induced under the conditions of hypobaric hypoxia in an altitude chamber with adjustable airflow, which prevented hypercapnia [6]. By means of a vacuum pump, the pressure in the altitude chamber was reduced to 0.125 atm for 1 min. Under these conditions, the animals were kept until respiration arrest; after this, the animals were returned to the normal-pressure conditions by supplying air into the chamber for 1 min.

The following parameters were recorded: the time (s) until the respiration arrest "at the height"; the time (s) of posture recovery, i.e., the period from respiration arrest to the moment when the experimental animal assumed normal posture after "descending from the height" and respiration recovery under normobaric conditions; and the restitution time (s), the total duration of the recovery of physiological activity after hypoxia. To determine the protective effect of the peptides studied, the death rate and coefficient of restitution (the ratio of the restitution time to the time before respiration arrest in the altitude chamber) were estimated.

The results obtained were processed statistically using the Mann–Whitney and Kruskal–Wallis tests; the differences were assumed to be significant at $p < 0.05$.

The peptides vilon (Lys-Glu), vesugen (Lys-Glu-Asp), pinealon (Glu-Asp-Arg), and epitalon (Ala-Glu-Asp-Arg) were injected daily at a dose of 10 μg/kg body weight intraperitoneously for five days before hypoxia. The rats of the control group were injected with saline according to the same scheme. Each group contained 10 animals.

All the regulatory peptides that were injected intraperitoneously before hypobaric hypoxia increased significantly the animal resistance to hypoxia (Table 1): the time before respiration arrest increased from 72 s (control) to 149–188 s (vilon, pinealon, epitalon); vesugen was the least efficient (88 s). At the same time, vesugen (like epitalon) promoted a rapid posture recovery: after cessation of hypoxia, this parameter returned to the control level. After injection of vilon or pinealon, the time of posture recovery was somewhat higher, which corresponded to a longer time for which the animals respired under hypoxia. The total restitution time increased as compared to the control in the cases of vilon and pinealon administration and remained at the control level in the cases of vesugen and epitalon administration. Thus, after the administration of pinealon and epitalon, the best coefficients of restitution were detected, though the effect of pinealon was accompanied by a higher death rate of the animals (Table 1). In total, pinealon seems to be the most efficient protective preparation ensuring the best rate of adaptation to hypoxia. Therefore, in the next experi-

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Table 1. The effect of regulatory peptides on the physiological parameters of rats exposed to acute hypobaric hypoxia

Compound studied	Time until respiration arrest, s	Time of posture recovery, s	Restitution period, s	Coefficient of restitution (3/1)	Death rate, %
Control	72 ± 10	126 ± 21	202 ± 32	2.8	10
Vilon	149 ± 47 ($p < 0.08$)	152 ± 31	302 ± 50 ($p < 0.08$)	2.0	20
Vesugen	88 ± 9	111 ± 16	194 ± 24	2.2	10
Pinealon	184 ± 30 ($p < 0.014$)	152 ± 16	291 ± 35 ($p < 0.1$)	1.6	10
Epitalon	150 ± 38 ($p < 0.08$)	103 ± 28	185 ± 33	1.2	20

Note: The significance of difference from the control group of animals injected with saline is shown.

mental series, the effect of pinealon was studied under the conditions of prenatal hypobaric hypoxia.

In these experiments, female rats were exposed to hypoxia on the 10th day of pregnancy, when the nervous system orimordia are developed [7]. After the start of an acute hypobaric hypoxia, pinealon was administered to half of the animals (five intraperitoneous injections every other day at a dose of 10 μg/kg body weight), whereas the animals of another group, which served as a control, were intraperitoneously injected with saline according to the same protocol. The number of offspring was estimated, as well as their physiological behavior and resistance of isolated cerebellar neurons to oxidative stress.

The number of offspring per female exposed to hypoxia was 9 ± 3, whereas in the group that received pinealon, this number was 14 ± 2 ($p < 0.05$), which was close to that typical of intact animals. On the 12th day, a portion of the grown up youngs was sacrificed, and their cerebellum was subjected to cytometric analysis. The remaining four-week-old animals were used to evaluate their behavior in the open field test.

A suspension of granulated cerebellar cells was obtained as described previously [8, 9]; to induce oxidative stress, they were incubated in the presence of either hydrogen peroxide or N-methyl-aspartate (NMDA), the ligand for NMDA glutamate receptors. The amount of reactive oxygen species (ROSs) and the neuronal death rate were determined using intracellular probes carboxymetoxi-2',7'-dichlordihydrofluorescein diacetate (CDCF-DA, 100 μM) and propidium iodine (PI, 5 μM) by means of an EPICS XL flow cytometer (Beckman Coulter, United States).

Neuron incubation in the presence of either hydrogen peroxide (20 mM for 20 min) or NMDA (1 mM for 40 min) strongly increased the death rate of these cells (Figs. 1a, 1b). In a suspension of neurons from animals born to females exposed to hypoxia and not treated with pinealon, the cell death rate was doubled after treatment with hydrogen peroxide, whereas in a cell suspension obtained from the offspring of pinealon-treated females, the neuronal death rate was about 40% under the same conditions. The initial number of dead cells in a primary culture was also nearly two times lower than in the suspension of cerebellar neurons isolated from pinealon-treated rats.

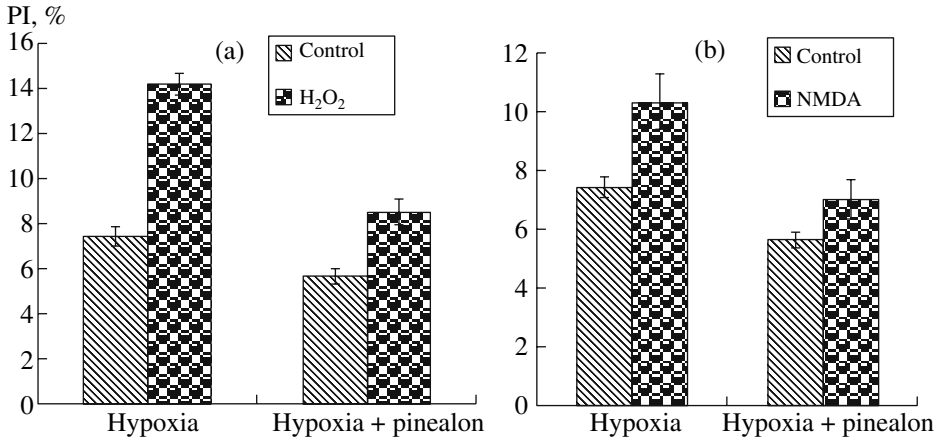

Fig. 1. The proportion of dead cells in the primary culture of rat cerebellar neurons incubated in the presence of either (a) hydrogen peroxide or (b) NMDA. Incubations in the presence of 20 mM hydrogen peroxide and 1 mM NMDA continued for 20 and 40 min, respectively.

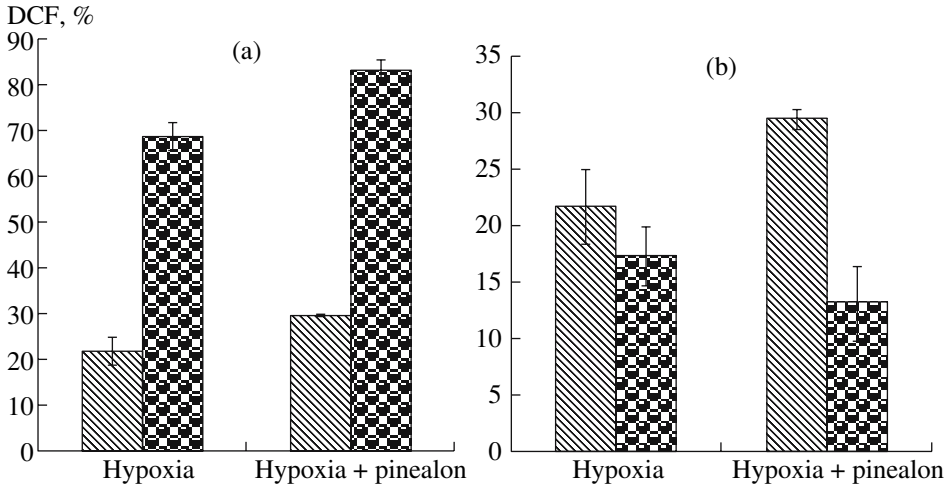

Fig. 2. The amount of ROSs in a primary neuron cultures incubated in the presence of either (a) hydrogen peroxide or (b) NMDA. The conditions are the same as in Fig. 1.

Thus, the cells isolated from cerebellum of animals born to pinealon-treated rats displayed a higher resistance to oxidative stress. Hypoxia proved to lead to a strong (almost threefold) increase in the initial steady-state level of ROSs. Against this background, the oxidative neuronal stress caused by a nonspecific factor (hydrogen peroxide) increased the amount of free radicals to the level almost similar in the hypoxic animals treated and not treated with pinealon. At the same time, the response to NMDA was quite different in the cells of the compared animals. Against the background of a high steady-state ROS level, NMDA caused no additional increase in the amount of free radicals in the offspring of hypoxic rats, which resembled the situation in the case of intact neurons [7]. In the neurons of the offspring of pinealon-treated hypoxic rats, the amount of free radicals was even decreased (Fig. 2). This evidence suggests that pinealon protects neuronal cells from the excitotoxic effect of NMDA.

Since there was no correlation between the neuronal death rate and the increase in the level of ROSs under the conditions of oxidative stress, pinealon seems to prevent cell death by increasing membrane resistance, as it was observed in our previous model experiments with erythrocyte osmotic hemolysis in the presence of pinealon, rather than by directly limiting ROS production. Probably, the effect of pinealon is mediated by an increase in the resistance of the internal system of antioxidant enzymes, as it is typical of other bioregulators [4, 5].

Four-week-old experimental animals were tested in the open field. The following parameters were recorded: the horizontal activity (HA), the number of squares crossed during a set period of time (3 min); the vertical activity (the number of raisings to an upright position); the number of explored burrows (a parameter characterizing the exploratory activity); the number of grooming episodes; the number of entries to the field center and the number of boles, which characterized

Table 2. Physiological parameters of animal behavior in the open field test

Parameter	Sensitive to hypoxia ($n = 6$)	Sensitive to hypoxia + pinealon ($n = 11$)	Resistant to hypoxia ($n = 9$)	Resistant to hypoxia + pinealon ($n = 12$)
HA	36 ± 6.9	53 ± 5.1 $p < 0.05$	45 ± 3.3	57 ± 3.3 $p < 0.05$
VA	2 ± 0.8	5 ± 0.8 $p < 0.05$	2 ± 0.6	6 ± 1.1 $p < 0.05$
Burrow-exploring activity	1.5 ± 0.5	1.5 ± 0.6	2.7 ± 0.7	3 ± 0.5
Number of grooming episodes	1.5 ± 0.8	3.5 ± 1.2	8.7 ± 2.9	10 ± 2.4
Number of boles	2.7 ± 0.4	1.8 ± 0.6	2.0 ± 0.7	4.1 ± 0.8
Number of entries to the field center	0.01 ± 0.1	0.27 ± 0.1	0.17 ± 0.1	0.13 ± 0.1

Note: The significance of difference from the group of hypoxic animals that received no pinealon is shown.

anxiety and emotional activity. The offspring was divided into two groups according to the response of their mothers to hypobaric hypoxia during pregnancy—the sensitive and resistant groups—which were studied separately. The results are shown in Table 2. The offspring of pinealon-treated rats, both sensitive and resistant to hypoxia, displayed significantly higher HA and VA than the offspring of pinealon-untreated rats exposed to hypoxia. The HA of the rats resistant to hypoxia was somewhat higher than the HA of hypoxia-sensitive rats; therefore, the most pronounced pinealon effect was observed in the group of hypoxia-sensitive animals. Pinealon proved to have no significant effect on the exploratory activity (burrow exploring).

In the rats sensitive to hypoxia, pinealon noticeably increased the frequency of grooming episodes and the number of entries into the open field center, though the increase was nonsignificant because of the wide individual variation of the responses. In contrast, pinealon had no effect on the offspring of the animals resistant to hypoxia.

Our results suggest that pinealon has a pronounced antihypoxic effect. The pinealon capability of increasing the neuronal resistance to hypoxic stress is of a complex nature; it is based not so much on the inhibition of ROS increase in cells in response to stress as on limiting the excitotoxic effect of NMDA. The pinealon effect on brain metabolism in the stress-sensitive animals is expected to be the most pronounced.

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