Peptides Stimulate Expression of Signal Molecules in Neuronal Cultures from Animals of Different Age

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Molecular mechanisms of the neuroprotective effect of peptide T-33 and cortexin were studied on organotypic cultures of the brain from young and old Wistar rats. The effective concentration of peptide T-33 stimulating proliferative activity of neurons considerably surpassed that of cortexin. Cortexin and peptide T-33 stimulated the expression of serotonin, Ki-67, and vimentin in cells of the brain cortex; peptide T-33 was most effective in this respect.

Key Words: *nervous system; peptides; signal molecules; cell cultures; aging*

The nervous system performs a regulatory function and participates in homeostasis maintenance at the organ, tissue, molecular, and cellular levels. Signal molecules, *e.g.* regulatory peptides are involved in the maintenance of functional activity of the nervous system. Aging of the nervous system is characterized by a decrease in the synthesis of regulatory peptides and reduced sensitivity of the target cells to these peptides. Disturbances in peptide regulation impairs the resistance of neurons to external and internal destabilizing factors and can be a mechanism of underlying accelerated aging and the development of some pathologies.

Peptide regulators developed at the St. Petersburg Institute of Bioregulation and Gerontology help to restore functional activity of the nervous system in age-related involution [8].

Treatment with the peptide bioregulator cortexin promoted recovery of CNS functions in the early posttraumatic period, which confirms its stimulatory effect on reparative processes in the brain [4]. Cortexin showed high efficiency in the treatment of GABA-deficient epilepsy, epilepsy with generalized convulsive and focal seizures of different etiology and duration of the disease. Moreover, cortexin demonstrated high efficiency as a component of complex therapy of encephalopathies of different genesis [7]. The molecular mechanism of activation of neuron renewal under the action cortexin is assumed to be "related to changes in the expression of genes regulating the synthesis of own neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF)" [1].

Synthetic peptide T-33 (Glu-Asp-Arg) also exhibits pronounced neuroprotective properties. In patients with brain injury after aftereffects, oral treatment with the tripeptide led to regression of focal symptoms and improvement of speech in motor and sensory aphasia. In patients with cerebral asthenia, oral administration of peptide T-33 reduced the number of errors during proofreading task and improved the integral performance index [3]. Peptide T-33 exhibited pronounced neuroprotective properties, which manifested in protection of nerve cells from oxidative damage caused by hypoxic exposure. Peptide T-33 increases activity of antioxidant enzymes in the brain and liver of pregnant female rats exposed to hypobaric hypoxia and protects the offspring from its effects; it also produces a protective effect on neurons of rat cerebellum and human neuroblastoma cells [12]. This peptide can enter cell nucleus and nucleolus and bind DNA and histones [10]. In patients with traumatic brain injury and stroke sequelae, the course of peptide T-33 treatment considerably changed bioelectric activity of the brain, which

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manifested as better modulation of electroencephalogram. Thus, the peptide bioregulators cortexin and T-33 improves the integral functions of the brain [5,9].

Here we compared the effects of neuroprotective peptide bioregulators on the expression of signal molecules in cultured cells of the brain cortex from animals of different age.

MATERIALS AND METHODS

Fragments (~1 mm^3) of the brain cortex from young (3 months) and old (24 months) Wistar rats were cultured in Petri dishes in 3 ml medium as described previously [13]. The medium contained 35% Eagle's medium, 35% Hanks solution, 25% fetal calf serum, 0.6% glucose, 0.5 U/ml insulin, and 100 U/ml gentamicin. The explants of the brain cortex were cultured for 3 days in an incubator at 36.7°C and 5% CO_2. The cultures were divided into 3 groups: control (addition of physiological saline) and two experimental groups (addition of cortexin and T-33). The peptides (cortexin and T-33) were added to the brain cell cultures in concentrations of 0.01, 0.05, 1.0, 10, 20, 50, and 100 ng/ml.

For evaluation of proliferation in cultures, area index (AI) was calculated as the ratio of the total explant area (including the zone of migrating cells to the area of the central zone (%). The explants were visualized using a microtelevision attachment (series 10, MTN-13, Alpha-Telecom). AI was calculated using PhotoM 1.2 software. For each substance, 20-25 experimental and 20-23 control explants were analyzed.

For evaluation of the expression of signal molecules, organotypic cultures were fixed with cold (-20°C) 96% ethanol and permeabilized with 0.5% Triton X-100. Immunocytochemical staining of the cortical cells was performed using primary monoclonal antibodies against Ki-67 (1:50; Novocastra), p53 (1:50; Novocastra), calmodulin (1:50; Dako), serotonin (1 50; Dako), and vimentin (1:50; Dako). The results of immunocytochemical analysis were evaluated morphometrically using a computer-assisted microscopic image analysis system consisting of Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, and Videotest Morphology 5.2 software. The relative area of marker expression was calculated as the ratio of the area occupied by immunopositive cells to the total area of cells in the field of view and expressed in %.

Statistical processing of the results was performed using the Statistica 7.0 software, the conformity of variables to normal distribution law was carried out using the Shapiro–Wilk test. Statistical homogeneity of samples was evaluated using nonparametric Kruskal–Wallis *H* test.

RESULTS

Concentration dependence of the neuroprotective effect of the peptides. T-33 peptide and cortexin changed AI in organotypic cultures of the cerebral cortex cells from young Wistar rats in a concentration-dependent manner. Peptide T-33 increased the area index in concentrations of 0.05 and 1.0 ng/ml and decreased it in concentrations of 20, 50, and 100 ng/ml (Table 1).

The decrease in AI induced by peptide T-33 in concentrations >20 ng/ml and reflecting suppression of culture growth can be explained as follows. It is known that in dissociated cultures, cell proliferation is stopped after attaining 80% confluence. This so-called contact inhibition phenomenon is related to spatial interaction of cells during migration and local expression of various signal molecules [6,14]. Since we assessed AI of the growth zone in organotypic cultures of cortical cells on day 3 of the experiment, it seems likely that peptide T-33 at concentrations >20 ng/ml initially stimulated cell growth, but before this term a phenomenon resembling the contact inhibition phenomenon in dissociated cultures was attained and apoptosis prevailed over cell proliferation. The rapid growth of cell cultures stimulated with peptide T-33 at high concentrations led to activation of cell metabolism and exhaustion of the culture medium, which resulted in a decrease in AI.

Cortexin produced a stimulating effect on the AI in higher concentrations that peptide T-33 (10 and 20 ng/ml; Table 1). Thus, the maximum increase on AI observed after addition of 0.05 ng/ml peptide T-33 and 20 ng/ml cortexin suggest that peptide T-33 produces stimulating effect on the growth of cortical cell cultures and this effect is comparable to the effect of

TABLE 1. Effect of Peptides on the Expression of Signal Molecules in Cell Cultures of the Brain Cortex from Young Rats (*M*±*m*)

Peptide concentration, ng/ml	Area index, %	
	peptide T-33	cortexin
0.05	27*	2
0.1	20*	2
1.0	15	3
10	11	19*
20	-15	25*
50	-14	18
100	-15	15

Note. *p<0.05 in comparison with the control (taken as 0).

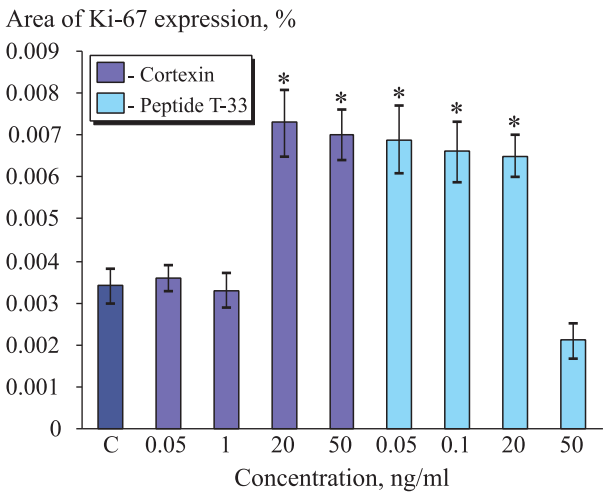

Fig. 1. Effect of peptide T-33 and cortexin in various concentrations of the expression of proliferation activating protein Ki-67 in organotypic cultures of the brain cortex from young rats. *$p<0.05$ in comparison with the control.

cortexin and is achieved at lower concentration. These findings were confirmed by the results of immunohistochemical analysis of Ki-67 expression. Peptide T-33 increased the area of transcription factor Ki-67 expression in organotypic cell cultures from rat cortex by more than 2 times at concentrations of 0.05, 1.0, and 20 ng/ml, while cortexin increased the area of Ki-67 protein expression in higher concentrations –20 and 50 ng/ml (Fig. 1). Thus, analysis of the effects of the test peptides on AI and Ki-67 expression showed that the effective concentration of peptide T-33 and cortexin were 0.05 and 20 ng/ml; therefore, these concentrations were used in further comparative studies of the neuroprotective properties of peptide bioregulators.

Effect of peptides on the expression of signal molecules in cell cultures of the brain cortex from young and old rats. After addition of peptide T-33 to the cell culture of the cerebral cortex from young rats, the expression of Ki-67, serotonin, and vimentin increased by 2, 1.9, and 3.5 times, respectively, in comparison with the control group, while expression of p53 and calmodulin remained at the control level (Table 2). After addition of cortexin, the area of expression of Ki-67 and serotonin increased by 2.2 times and vimentin by 3.6 times in comparison with the control, the area of p53 and calmodulin expression did not significantly change. Neuroprotective peptide bioregulators T-33 and cortexin produced similar stimulatory effect on the expression of signal molecules.

In cerebral cortex cell cultured from old rats, peptide T-33 increased Ki-67 and serotonin expression areas by 1.7 and 2.2 times, respectively, and had no effect on the expression of p53, calmodulin, and vimentin. In the presence of cortexin, the expression area of Ki-67 and serotonin increased by 1.6 and 1.7 times, respectively, in comparison with the control, whereas expression area of p53, calmodulin, and vimentin remained practically unchanged (Table 3). Thus, peptide T-33 produces more potent stimulatory effect on the expression of signal molecules in the cerebral cortex cell culture from old animals in comparison with cortexin.

Thus, the neuroprotective effect of peptide T-33 on organotypic cultures of the brain cortex from young animals is achieved at lower concentrations in comparison with cortexin. This suggests that peptide T-33 is a promising substance for further study as the neuroprotective agents of new generation. This conclusion agrees with the data of other investigators demonstrating that short peptide cortagen also exhibits its biological activity similar to that of cortexin, but in lower doses [2,11].

Peptide T-33 and cortexin produce a stimulating effect on the expression of markers of functional activity of cerebral cortex cells (Ki-67 serotonin, vimentin), while short peptide T-33 exhibits more pronounced stimulatory effect on the studied signaling molecules in the nervous tissue. Since synthesis of peptide T-33

TABLE 2. Effect of Peptides on the Expression of Signal Molecules in Cell Cultures of the Brain Cortex from Young Rats ($M\pm m$)

Immunocytochemical marker	Group		
	control	peptide T-33	cortexin
Ki-67	0.0034±0.0004	0.0069±0.0008*	0.0073±0.0008*
p53	0.0049±0.0006	0.0053±0.0004	0.0048±0.0003
Calmodulin	0.0065±0.0006	0.0072±0.0008	0.0028±0.0004*
Serotonin	0.0033±0.0004	0.0061±0.0006*	0.0073±0.0005*
Vimentin	0.0010±0.0002	0.0035±0.0004*	0.0036±0.0007*

Note. Here and in Table 3: *$p<0.05$ in comparison with the control.

TABLE 3. Effect of Peptides on the Expression of Signal Molecules in Cell Cultures of the Brain Cortex from Old Rats (*M*±*m*)

Immunocytochemical marker	Group		
	control	peptide T-33	cortexin
Ki-67	0.0081±0.0010	0.0140±0.0004*	0.0013±0.0003*
p53	0.0015±0.0003	0.0017±0.0004	0.0012±0.0003
Calmodulin	0.0010±0.0005	0.0110±0.0010	0.0013±0.0004
Serotonin	0.0011±0.0002	0.0024±0.0003*	0.0019±0.0003*
Vimentin	0.0020±0.0004	0.0140±0.0004	0.0010±0.0003*

is cheaper that the synthesis of cortexin, the above findings open new prospects for the creation of a more accessible and effective peptide bioregulator exhibiting neuron- and geroprotective activities.

Stimulation of the synthesis of serotonin, factor of proliferative activity of neurons in Ki-67 protein, and cytoskeleton protein vimentin under the effect of peptide T-33 can be a molecular mechanism of targeted therapy of neurodegenerative pathologies and depressive disorders, as it is known that these pathologies are associated with impaired signaling cascades regulated by biogenic amines [15].

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