Effect of Tripeptide Lys-Glu-Asp on Physiological Activity of Neuroimmunoendocrine System Cells

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Tripeptide Lys-Glu-Asp stimulates proliferation and inhibits apoptosis in organotypic cultures of neuroimmunoendocrine system cells. Lys-Glu-Asp accelerates cell renewal processes (decrease of apoptosis marker p53 and increase of proliferation marker Ki-67) in the pineal gland; this effect is more pronounced in cultures derived from old animals than in young cultures. The tripeptide induces the expression of low-differentiated lymphocyte marker CD5 and macrophage marker CD68, but in "old" cultures this effect is less pronounced than in "young" ones. Thus, in tissue culture Lys-Glu-Asp primarily affects the nervous and endocrine tissues during aging and produces a less pronounced effect on the nervous tissue. Physiological activity of the tripeptide consists in modulation of associative learning of honey bee in the model of short-term and the long-term memory.

Key Words: *tripeptide; organotypic culture; neuroimmunoendocrine system; memory*

Processes of cell proliferation and differentiation involve a set of signaling mechanisms, including cytokines and cytomedins. Peptides entering the cell evolutionally represent a system of signaling molecules epigenetically regulating gene expression and cells differentiation [1,4,7]. Tripeptide Lys-Glu-Asp [5] synthesized at the St.-Petersburg Institute of Bioregulation and Gerontology consists of amino acids with charged residues: glutamate and aspartate (acidic) and lysine (basic), the most frequent constituents of proteins [9]. It was shown that tripeptide Lys-Glu-Asp exhibits a pronounced immunoprotective effect and restores the structure of rat thymus and spleen in the model of radiation-induced aging [6]. Moreover, this tripeptide produces opposite effects on mesenchymal SC of KF-1 rats and human K-562 erythromyelopoiesis precursor cells [8]. Thus, Lys-Glu-Asp stimulates immune and mesenchymal cells, but physiological aspects of its effect on the neuroimmunoendocrine system are poorly understood. Organotypic culture derived from rats of different age allowing studies of the expression of signaling molecules in different organs during aging is a convenient model for molecular and physiological studies. Insects are often used as a model for studying common biochemical and physiological mechanisms of memory trace formation in the neuroimmunoendocrine system of vertebrates and invertebrates [10,11]. The combination of these models in the same study allows tracing physiological effects of the tripeptide at the molecular, cellular, and organism level.

We studied the effects of Lys-Glu-Asp on cell proliferation and apoptosis in organotypic cultures of the neuroimmunoendocrine system tissues and on physiological mechanisms of short- and long-term memory storage.

MATERIALS AND METHODS

The study consisted of two parts: evaluation of the effect of the test peptide on tissue cultures and its neu-

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roprotective effect in the model of conditioned reflex in bee. Such a combination of experimental models allows comprehensive characterization of physiological effects of the test peptide on the neuroimmunoendocrine system.

First, we studied the effect of the tripeptide on organs of the neuroimmunoendocrine system in organotypic culture. The tissues for organotypic culturing were taken from young (3-month-old, *n*=10) and old (24-month-old, *n*=10) male Wistar rats after decapitation with a guillotine. The tissues were placed in a sterile Petri dish and cut into fragments (~1 mm^3, explants). We studied 100 explants of 7 tissues: spleen (immune tissue), subcortical structures of the brain, including the pineal gland possessing neuroimmune functions (neural tissue), testes and prostate gland (endocrine glands). The explants were placed in Petri dishes with collagen coating (3.5×2.5 mm, Jet Biofil; 10-12 explants per dish) and were cultured in 3 ml of nutrient medium consisting of Hanks solution (45%), Eagle's medium (45%), fetal bovine serum (10%), glucose (10 mg/ml), and gentamicin (0.5 mg/ml). Lys-Glu-Asp in an effective concentration of 0.05 ng/ml was added to experimental explants (control explants were incubated in pure medium). The explants were cultured in CO_2-incubator at 36.7°C and 5% CO_2 for 3 days (this time period is required for the formation of the growth zone consisting of proliferating and migrating cells) [2,3].

The area index (AI) was calculated as the ratio of the total explant area (including the zone of migrating cells) to central zone area and expressed in arb. units. For visualization of the explants, a microteleattachment for microscope was used (series 10, MTN-13, Alfa-Telekom). AI of the explants was calculated using PhotoM 1.2 software. For each tissue, 20-25 control and experimental explants were analyzed. Changes in AI caused by the tripeptide were expressed in percents of the control value. AI was evaluated for samples from the spleen, testis, prostate gland, and subcortical structures.

For immunocytochemical study of the growth zones of the pineal gland and spleen explants, they were fixed in 96% ethanol, permeabilized with 0.5% triton X-100, and immunocytochemical reaction was performed using primary antibodies to low-differentiated lymphocyte marker CD5 (1:30, Novocastra), macrophage marker CD58 (1:30, Novocastra), proliferation marker Ki-67 (1:50, Novocastra), and apoptosis marker p53 (1:50, Novocastra) and standard single-stage protocol with high-temperature antigen unmasking in citrate buffer (pH 6.0); standard biotinilated antimouse Ig were used as secondary antibodies. The reaction products were visualized using avidin-biotin-horseradish peroxidase complex with diaminobenzidine (ABC-kit, Dako). Morphometry was performed using a computer-assisted image-analysis system consisting of Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, Intel Pentium 4 computer, and VidiotestMorphology 5.0 software. In each case, 5 fields of view were analyzed at ×100. The marker expression area (a common morphometric parameter characterizing the number of cells expressing the marker) was calculated as the ratio of immunopositive cell area to whole cell area in the field of view and expressed in percents.

The second part of the study was carried out on the model of conditioned reflex storage in short-term and long-term memory in insects. This experiment allows evaluation of the effect of the peptide on conditioned behavior and extends the data obtained on cell cultures. The experiments were carried out on 15-30-day-old bees *Apis mellifera carnika Pollm*. The bees were deprived of food and isolated from the family for 3 h for creating standard unconditioned and conditioned background and then they were presented a combination of conditioned (carnation odor) and unconditioned (reinforcement, 50% sucrose) stimuli. The percentage of bees responding to odor by proboscis extension (from the total number of bees) was determined 1 and 180 minutes after learning. Before learning, the level of food motivation was determined by the percent of bees extending proboscis in response to direct stimulation of antenna and front leg chemoreceptors with sucrose solution (unconditioned response). Bees of the experimental group received systemic injection of 2 μl test tripeptide in concentrations of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M (into posterior part of the thorax). Controls received 2 μl NaCl (0.9%).

Statistical analysis of the data on tripeptide effect on AI, expression of markers in organotypic culture, and memory characteristics of bees was performed using two-sample Wilcoxon rank-sum test. This test was chosen for evaluation of data reliability because it is more accurate than Student's test and has no data distribution limitations. Statistica 7.0 was used for statistical processing.

RESULTS

The tripeptide stimulated cell proliferation in organotypic culture of all tissue explants in comparison with the control (Fig. 1).

Immunocytochemical study showed that the thripeptide stimulated CD68 expression in culture of the immune spleen cells of young and old rats (Table 1). The increase in the expression of CD5 (marker of low-differentiated lymphocytes) under the effect of the tripeptide was noted only in cultures derived from young animals (Table 1). The absence of the stimulating effect of the tripeptide in splenic culture from old ani-

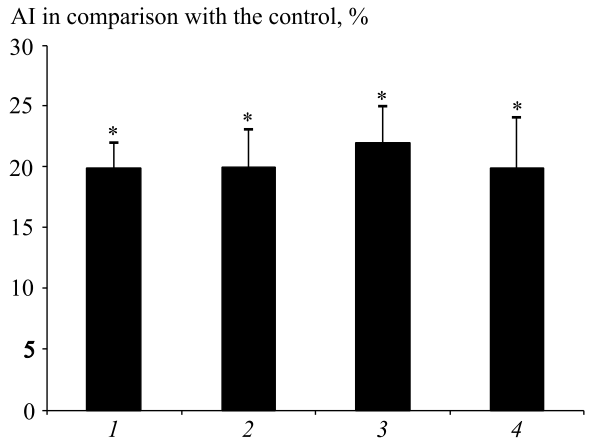

Fig. 1. Effect of Lys-Glu-Asp on AI of explant growth zones in organotypic cultures of rat neuroimmunoendocrine system. *1*) spleen, *2*) testes, *3*) prostate gland, *4*) subcortical structures. Control: zero line. *$p<0.05$ in comparison with the control.

mals can be explained by initially high expression of this marker. Thus, the tripeptide enhanced proliferative activity of undifferentiated T cells and macrophages (Fig. 1). These findings agree with the previous data on stimulating effect of Lys-Glu-Asp on proliferation of splenic cells after radiation damage [5].

Immunocytochemical assay of proliferation and apoptosis markers in organotypic cell cultures of the pineal gland from rats of different ages showed positive effect of the tripeptide on cell renewal (Table 2). We showed that the stimulating effect of Lys-Glu-Asp on proliferation and its inhibiting effect on apoptosis were more potent in cultures from old animals than in cultures from young animals by several times. We previously showed that Ki-67 expression in human pineal gland decreases and expression of proapoptotic marker p53 increases with age. Thus, the tripeptide has an apparent restorative effect on cells of pineal gland during its age-related involution.

Experiments on bees showed that tripeptide effect on memory formation depends on the background conditioning level. The tripeptide in concentrations of 10^{-7} and 10^{-4} M impaired short/long-term memory storage of conditioned response by ~30% in bees with initially high background conditioning (85-95% control bees reproduced the response), but did not affect it in bees with low initial level of conditioning (51-69% control bees reproduced the response). The tripeptide also affected alimentary excitability: it increased by more than 20% in comparison with the control only in case of initially low food motivation (only 75% of the bees initially had food motivation). These findings suggest that Lys-Glu-Asp has differently directed modulating effects on food motivation and associative activity of honey bee.

Thus, we showed that the tripeptide stimulates proliferation and inhibits apoptosis in cells of the pineal gland, the central organ of the neuroimmunoendocrine system; in "old" cultures this effect is more pronounced than in "young" ones. The tripeptide stimulates the proliferation of T cell precursors and macrophages in the spleen, but this effect decreases with aging. Thus, this tripeptide promotes activation of the expression of signal molecules in different organs of the neuroimmunoendocrine system. These data agree with the results obtained in studying conditioning and memory formation in bees [10].

TABLE 1. Effect of Lys-Glu-Asp on Expression Area of Low-Differentiated Lymphocyte Marker CD5 and Macrophage Marker CD68 in the Growth Zone of Organotypic Spleen Cultures from Young and Old Rats

Marker	Control		Tripeptide	
	young rats	old rats	young rats	old rats
CD5	0.42±0.05	8.43±0.80	3.30±0.90*	9.62±2.07
CD68	1.20±0.31	1.35±0.38	3.45±1.10*	6.68±1.38*

Note. Here and in Table 2: *$p<0.05$ in comparison with the control.

TABLE 2. Effect of Lys-Glu-Asp on Expression Area of Proliferation Marker Ki-67 and Apoptosis Marker p53 in Organotypic Pineal Gland Culture from Young and Old Rats

Marker	Control		Tripeptide	
	young rats	old rats	young rats	old rats
Ki-67	0.83±0.09	0.12±0.02	1.43±0.31*	2.19±0.41*
p53	0.59±0.11	4.16±1.15	0.07±0.02*	0.68±0.20*

Thus, peptide Lys-Glu-Asp produces a pronounced stimulating and modulating effect on cell renewal processes in the tissues of the diffusive neuroimmunoendocrine system in rats during aging and on conditioning in insects.

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