Effect of Short Peptides on Expression of Signaling Molecules in Organotypic Pineal Cell Culture

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We demonstrated the influence of short peptides on the expression of signaling molecules in organotypic culture of the pineal gland from 3-month-old rats. Peptides Ala-Glu-Asp-Gly and Lys-Glu-Asp stimulate the expression of proliferative protein Ki-67 in pineal gland culture. These peptides as well as Glu-Asp-Arg and Lys-Glu do not affect the expression of apoptosis marker AIF. The synthesis of transcription factor CGRP by pinealocytes was stimulated only by Ala-Glu-Asp-Gly. Thus, peptide Ala-Glu-Asp-Gly tissue-specifically stimulates proliferative and secretory activities of pinealocytes, which can be used for recovery of pineal gland functions at the molecular level.

Key Words: *organotypic pineal cell culture; short peptides; signaling molecules*

The pineal gland is the major element coordinating activity of the neuroimmunoendocrine system. Pinealocyte dysfunction and, first of all, insufficient production of melatonin and neuropeptide CGRP can be the causes of accelerated aging of the organism [1,2,9]. Moreover, low functional activity of the pineal gland observed in humans over 60 leads to the development of pathologies of the neuroimmunoendocrine system manifesting in circadian rhythm disorders, neurodegenerative diseases, immunodeficient states, enhanced carcinogenesis, endocrinopathies, and cardiovascular diseases [2,4,8]. Numerous regulatory peptides affecting tissue development are released by different cells and tissues of the organism as endocrine and autocrine carriers of information about local state of the organ or tissue. Biological integrity of organisms at the cellular level is maintained by signals regulating the balance between the two basic physiological processes: proliferation and programmed cell death (apoptosis). The function of intercellular signals at the para- and autocrine levels is performed by secretory proteins (cytokines) and peptide bioregulators (cytomedins) produced in animal tissues and maintaining structural and functional homeostasis of cell populations containing and producing this factor [8,12]. Short peptides, analogs of cytomedins and containing amino acids predominating in the corresponding tissues, were synthesized in St. Petersburg Institute of Bioregulation and Gerontology [3,9,11,13].

The efficiency of peptide Ala-Glu-Asp-Gly in recovery of the pineal gland functions was demonstrated, but molecular mechanisms of this activity are poorly understood [3,11].

Here we studied the effect of some short peptides on the synthesis of signaling molecules reflecting the processes of cell renewal and synthetic capacity of pinealocytes in organotypic culture of rat pineal gland.

MATERIALS AND METHODS

Organotypic culturing preserving normal interaction between cell subpopulations makes it possible to explore different influences on the totality of the pineal gland cells, which would have been impossible in dissociated pinealocyte culture [10].

Experiments were carried out on 2-3-month-old Wistar rats from Rappolovo breeding center. The ani-

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mals were decapitated with a guillotine. The pineal gland was isolated with eye-surgery tools, placed in a sterile Petri dish, and divided into explants (~1 mm^3). The explants were transferred to Petri dishes (10 per dish) with collagen coating (35×2.5 mm, Jet Biofil) and cultured in 3 ml nutrient medium consisting of Hanks solution (45%), Eagle medium (45%), fetal bovine serum (10%) and supplemented with glucose (10 mg/ml) and gentamicin (0.5 mg/ml).

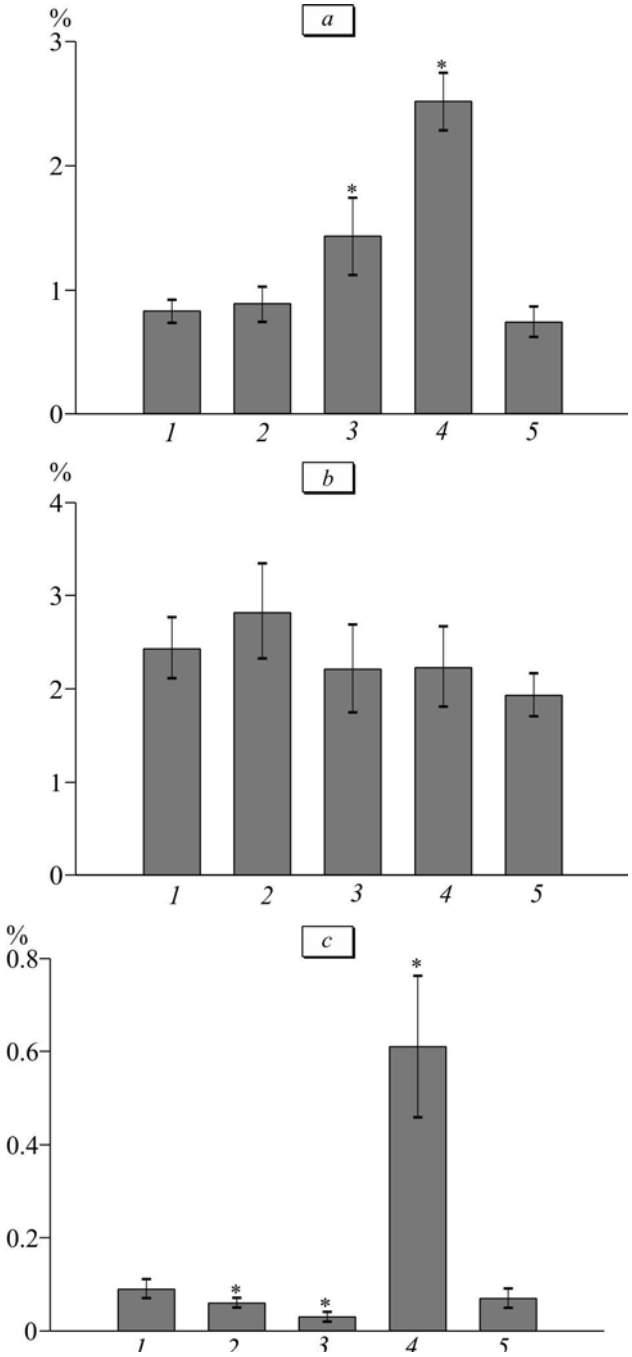

Fig. 1. The area of expression of signaling molecules in organotypic pineal culture. *a*) Ki-67, *b*) AIF, *c*) CGRP. *1*) control, *2*) AB-0, *3*) T-38, *4*) AE-0, *5*) T-33. *$p<0.05$ in comparison with the control group.

The explants (n=50) were incubated with saline (control) or one of the test peptides AE-0 (Ala-Glu-Asp-Gly), T-33 (Glu-Asp-Arg), T-38 (Lys-Glu-Asp), or AB-0 (Lys-Glu) in a concentration of 10 ng/ml. The incubation was performed in a CO_2 incubator (5% CO_2) at 36.7°C. The duration of culturing was 3 days (the period necessary for the formation of the growth zone consisting of proliferating and migrating pinealocytes with admixture of fibroblasts and macrophages) [8]. The pineal explants were fixed in 95% ethanol for immunocytochemical analysis in the explant growth zone.

Immunocytochemical reaction with antibodies to neuropeptide marker associated with calcitonin CGRP gene (1:500, Abcam), proliferative protein Ki-67 (1:60, Novocastra), and mitochondrial apoptosis factor AIF (1:500, Abcam) was carried out using a standard one-stage protocol with high-temperature antigen demasking in citrate buffer (pH 6.0). Biotinylated antimouse immunoglobulins (a universal set) were used as secondary antibodies. The reaction was visualized using a complex of avidin with biotinylated horseradish peroxidase and diaminobenzidine (ABC-kit, Dako).

Morphometry was performed using computer-assisted microscopic image analysis system consisting of a Nikon Eclipse E400 microscope, Nikon DXM1200 digital camera, Intel Pentium 4 computer, and VideotestMorphology 5.0 software. In each case, 5 fields of view were analyzed at ×100. The area of marker expression was calculated as the ratio of the area occupied by immunopositive cells to the whole cells area in a field of view and expressed in percents. Optical density of the expression was measured in arbitrary units. The area of expression characterizes the number of cells expressing the test marker and optical density characterizes the amount of marker protein released by one cell.

Statistical processing of the data included calculation of the mean, standard deviation, and confidence intervals.

RESULTS

Evaluation of the effect of short peptides on the expression of proliferation marker Ki-67 in organotypic pineal culture showed that peptides T-38 and AE-0 significantly increased the area of Ki-67 expression by 1.7 and 3 times, respectively, while peptides AB-0 and T-33 did not change this parameter (Fig. 1, *a*). Optical density of Ki-67 expression did not change after incubation with peptides AE-0, AB-0, and T-38 and significantly decreased by 1.6 times in the presence of T-33 peptide (Table 1). Thus, peptides AE-0 and T-38 enhance proliferative activity of pinealocytes from young rats. We previously showed that proliferative

activity of pinealocytes in long-living people sharply decreased in comparison with that in middle-aged individuals [7]; this suggests that T-38 and especially AE-0 can promote the recovery of proliferative activity of pinealocytes during aging.

Since proliferation and apoptosis are closely related processes, we studied the expression of mitochondrial apoptosis marker AIF. The choice of AIF protein instead of p53, the most prevalent marker of caspase-dependent apoptosis, is determined by the fact that, according to previous reports the expression of p53 in the pineal gland of humans over 60 does not change with age, whereas mitochondrial apoptosis increased [7]. However, in organotypic culture of the pineal gland none of the studied peptides affected the expression area and optical density of AIF (Fig. 1, *b*; Table 1). The absence of changes in AIF expression in pineal gland culture can be explained by the fact that the material was collected from young animals, in which mitochondrial apoptosis is at the physiological level and does not increase as it was noticed during aging.

Neuropeptide CGRP associated with calcitonin gene is a specific transcriptional protein produced in the brain, pineal gland, and thymus. The test peptides produced different effects on CGRP expression. AE-0 peptide increased the area of CGRP expression by 6.8 times in comparison with the control and simultaneously decreased optical density of CGRP expression by 1.8 times (Fig. 1, *c*; Fig. 2; Table 1). Thus, AE-0 peptide increased the number of pinealocytes expressing this transcriptional factor and simultaneously decreased the intensity of expression in cells. T-38 peptide decreased the area and optical density of CGRP expression by 3 and 1.6 times, respectively (Fig. 1, *c*; Table 1). Peptides AB-0 and T-33 had no effect on the expression of CGRP marker in organotypic culture of the pineal gland (Fig. 1, *c*; Table 1).

TABLE 1. Mean Optical Density of Expression of Signaling Molecules Ki-67, AIF, and CGRP in Organotypic Pineal Culture (arb. units; *M±m*)

Group	Ki-67	AIF	CGRP
Control	0.23±0.05	0.20±0.04	0.20±0.03
AB-0	0.17±0.03	0.23±0.05	0.28±0.08
T-38	0.15±0.04	0.21±0.04	0.12±0.03*
AE-0	0.21±0.06	0.16±0.03	0.11±0.02*
T-33	0.15±0.02*	0.19±0.03	0.15±0.04

Note. *$p<0.05$ in comparison with the control group.

These findings suggest that AE-0 peptide stimulates proliferative activity of pinealocytes and secretion of transcriptional protein CGRP, a regulatory factor participating in neuroimmunoendocrine interactions, while T-33 and AB-0 produce no such effects. T-38 stimulates proliferation of pinealocytes, which supplements the data about activation of proliferation of different cell types under the influence of this tripeptide [5]. However, T-38 does not modulate the expression of other proteins, *e.g.* CGRP, in the studied culture.

Since AE-0 was synthesized on the basis of the analysis of amino acids composition of the extract from animal pineal gland [6], its stimulatory effect on the expression of signaling molecules by pinealocytes attests to tissue-specific influence of this tetrapeptide demonstrated at the molecular level.

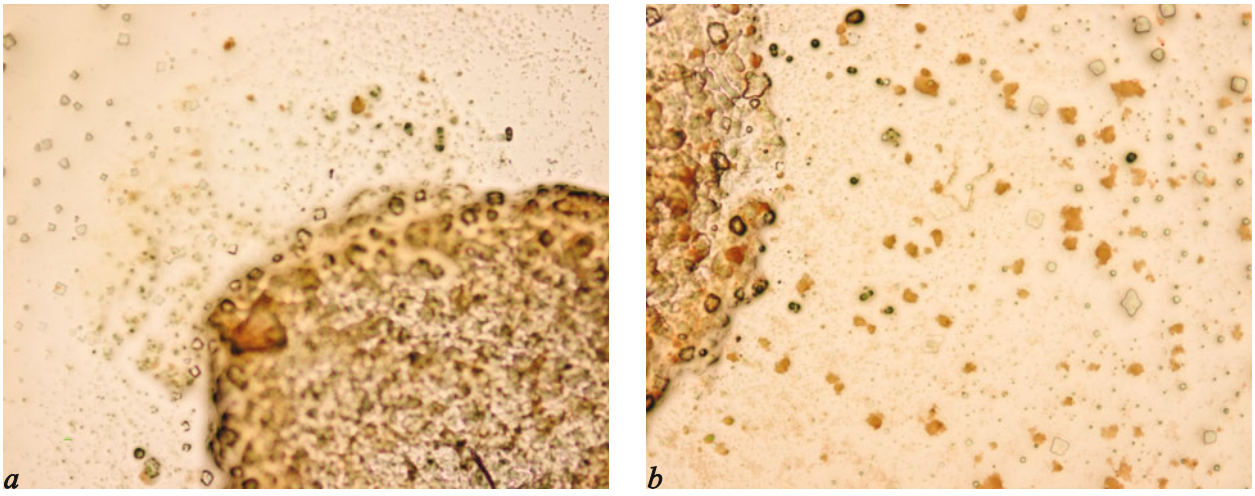

Fig. 2. Expression of neuropeptide CGRP in organotypic pineal culture: immunocytochemistry data (×100). *a*) control, *b*) after addition of 10 ng/ml AE-0 peptide. Continuous brown staining: explant zone, diffuse brown fragments around the explant zone: expression of the studied marker in pinealocyte monolayer.

It can be hypothetized that the stimulation of the expression transcriptional factor CGRP by AE-0 is determined by binding of this tetrapeptide to the gene encoding CGRP protein, because binding of AE-0 peptide to different genes was previously demonstrated [12,13].

Our experiments showed that tetrapeptide AE-0 produces a tissue-specific stimulatory effect on proliferative and secretory activities of rat pinealocytes in organotypic culture, which can be used for recovery of functional activity of the pineal gland, reduced during aging.

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