BIOGERONTOLOGY

Molecular Cellular Mechanisms of Peptide Regulation of Melatonin Synthesis in Pinealocyte Culture

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The effects of epithalone and vilone peptides on the synthesis of melatonin and factors involved in this process, arylalkylamine-N-acetyltransferase (AANAT) enzyme and pCREB transcription protein, were studied in rat pinealocyte culture. Epithalone stimulated AANAT and pCREB synthesis and increased melatonin level in culture medium. Simultaneous addition of norepinephrine and peptides into the culture potentiated the expression of AANAT and pCREB.

Key Words: *peptides; pinealocytes; melatonin; signal molecules*

Melatonin (MT) is the main hormone of the epiphysis (pineal gland), regulating the biological rhythms of the organism and involved in the maintenance of homeostasis by coordinating the immune and endocrine system activities [2,4,5,11]. The synthesis of MT depends on illumination and determines the endogenous circahoralian rhythms of many organs and systems work [6,13]. According to electron microscopic findings, there are three cell types in the epiphysis: type 1 pinealocytes, containing serotonin; type 2 pinealocytes, in which MT is synthesized from serotonin; and poorly differentiated transitional type 3 cells. Type 2 pinealocytes have the characteristics of endocrine cells and neurons [12,14].

Melatonin synthesis is controlled by arylalkylamine-N-acetyltransferase (AANAT). Norepinephrine (NE), the main neurotransmitter of the pineal sympathetic innervation, is the dominant inducer of MT production and AANAT stimulation [3,13]. Stimulation of AANAT synthesis is supported by a transcription factor pCREB (cyclic AMP-responsive element-binding protein), synthesized in the cells in response to triggering of mechanisms linked with the function of cyclic AMP (intracellular mediator of the hormonal signal).

During aging, the total pinealocyte count in humans reduces significantly in comparison with young age; in type 2 cells the changes manifest by modification of the nucleus shape, increase of heterochromatin share in the nucleus, clarification of the mitochondrial matrix, and formation of autophagosomes [7]. Changes in pinealocyte structure in subjects aged over 60 years lead to reduction of MT secretion and of its concentrations in the blood, plasma, and saliva at night [13,15]. Epiphyseal preparations normalize the low MT status in the blood of old monkeys and humans [8-10,12]. However, the molecular and cellular mechanisms of MT synthesis recovery under the effects of short peptides are unknown.

We studied the effects of peptides on the proteins involved in MT synthesis, AANAT and pCREB, and on MT concentration in the rat pinealocytes.

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MATERIALS AND METHODS

Pinealocytes were isolated from the pineal glands of adult male Wistar rats decapitated with guillotine at 10:00. Dissociated pinealocyte culture was grown in Petri dishes on a sublayer of slides in a CO_2 incubator at 36.7°C and 5% CO_2 for 3 days. Culture material was then divided into 3 groups: 1) control specimens without treatment of any kind; 2) specimens treated with NE in a concentration of 1 μg/ml (positive control); and 3) specimens treated with one of the studied peptides in a concentration of 100 ng/ml. The chosen concentration corresponded to the physiological concentration exhibiting biological activity. The cultures were treated with the studied bioactive substances for 3 h. Peptides were synthesized at Institute of Bioregulation and Gerontology: vilone (Lys-Glu) and epithalone (Ala-Glu-Asp-Gly) [13,15].

Pinealocyte cultures for immunocytochemical study were fixed in 95% ethanol. Immunocytochemical study was carried out with antibodies to pCREB (Upstate Biotechnology Inc., 1:500) and AANAT (Sigma, 1:1000). The reaction was visualized with peroxidase (Peroxidase VECTASTAIN Elite ABC Kit Standard, Alexis Biochemicals). Densitometric analysis of nuclear expression of pCREB and cytoplasmic expression of AANAT in pinealocyte culture was carried out using VIDAS image analyzer (Kontron) by optical density. Melatonin secretion from pinealocytes into cell medium supernatant was measured by ELISA with IHF GmbH kit (Camburg).

The data were statistically processed by Statistica 7.0 software. All experiments were carried out in accordance with regulation for studies on experimental animals, approved by Instruction of the EEC Scientific Council 86/609/EEC.

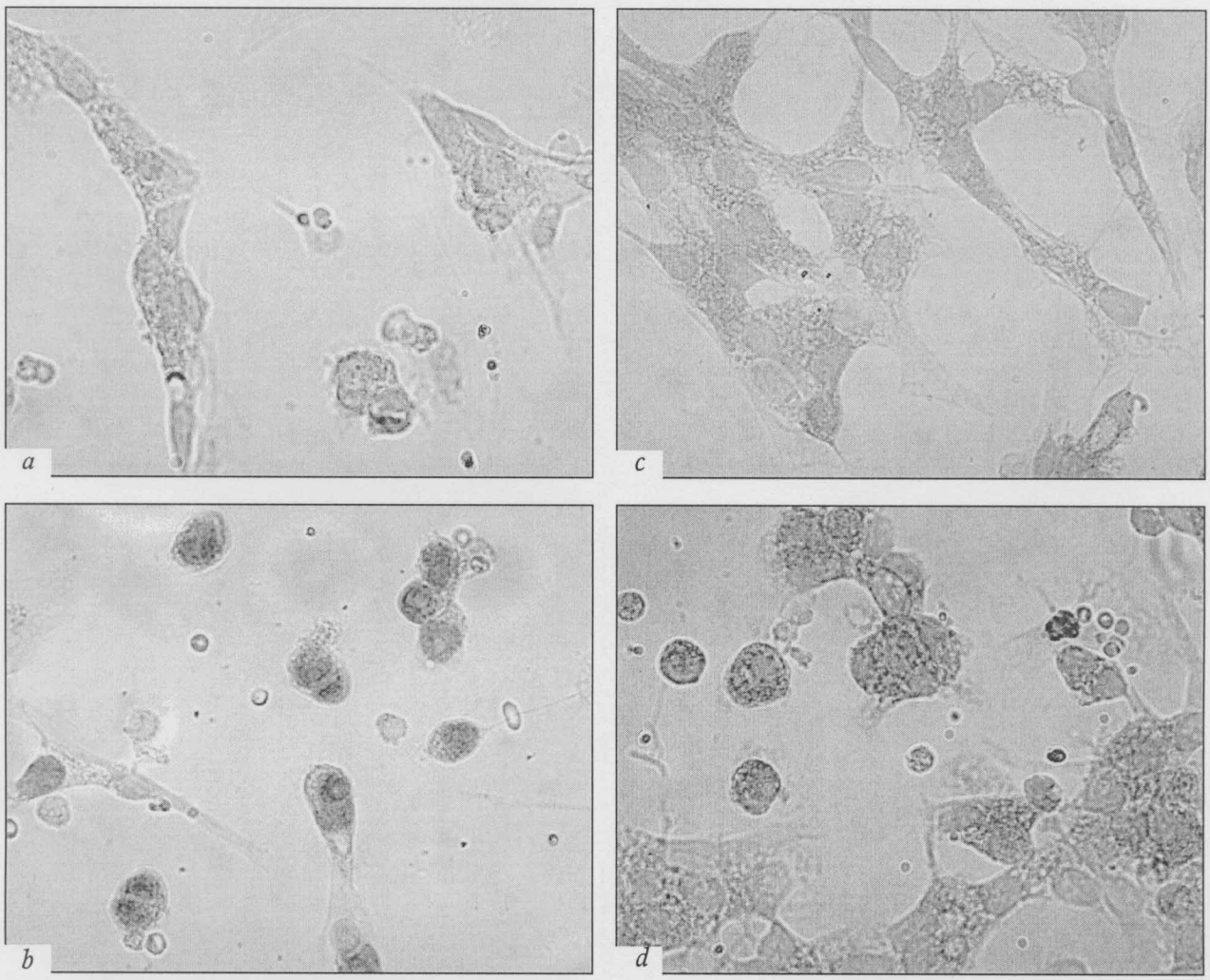

Fig. 1. Expression of signal molecules in pinealocyte culture (immunocytochemistry; ×400). *a*) expression of pCREB in control; *b*) expression of pCREB under the effect of epithalone (100 ng/ml); *c*) expression of AANAT in control; *d*) expression of AANAT under the effect of epithalone (100 ng/ml).

RESULTS

Studies by the immunocytochemical method showed a positive reaction to pCREB and AANAT for the majority of syncytium-forming cells in the control pinealocyte culture (Fig. 1, *a*, *b*). After peptide treatment only some cells remained immunopositive to these markers; these cells were located in clusters forming round cell chains (Fig. 1, *c*, *d*).

Studies of optical density of pCREB transcription protein expression in pinealocyte culture showed no changes in this parameter in the control over 3 h. Norepinephrine treatment (positive control) promoted a somewhat 16-fold increase of pCREB expression during the first 2 h and 4-fold after 3-h incubation (Fig. 2, *a*). Epithalone peptide promoted stable stimulation of pCREB expression (4-fold throughout the entire period of incubation; Fig. 2, *a*). Vilone increased this parameter depending on incubation duration. After 1 h pCREB expression increased 7-fold under the effect of vilone, while after 2- and 3-h incubation the expression of this transcription factor reduced to the control level (Fig. 2, *a*). These data indicated that addition of NE and vilone into pinealocyte culture induced a short-term stimulation of pCREB expression, while epithalone treatment caused a more lasting effect of this kind.

The data on potentiation of the effects of peptide and NE added into pinealocyte culture simultaneously are interesting; in our study the effects of epithalone and vilone on pCREB expression were much similar (Fig. 2, *b*).

Optical density of AANAT expression in the control somewhat increased over 2-3 h of observation. Vilone stimulated AANAT expression during 1-h incubation. However, prolongation of incubation showed that vilone effect on AANAT was short-term. Epithalone stimulated the expression of AANAT throughout the entire period of incubation and its effect was higher than that of NE (Fig. 2, *c*).

Hence, the findings of immunohistochemical studies indicate a significant effect of epithalone (pinealocyte tropic peptide) on the expression of pCREB transcription factor and AANAT enzyme in pinealocyte culture. Presumably, epithalone effect on AANAT and pCREB underlies the peptide regulation of pineal cell activity.

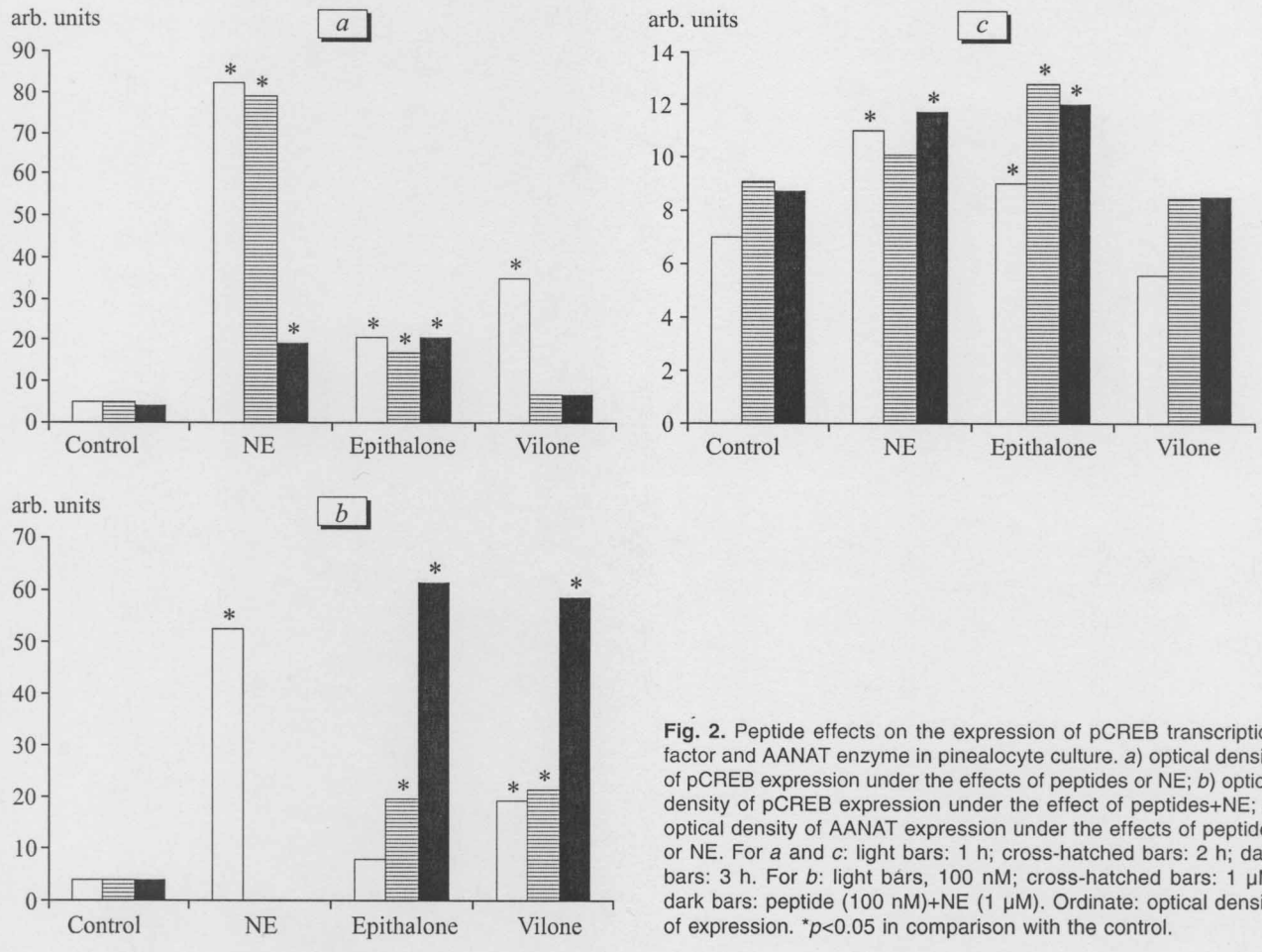

Fig. 2. Peptide effects on the expression of pCREB transcription factor and AANAT enzyme in pinealocyte culture. *a*) optical density of pCREB expression under the effects of peptides or NE; *b*) optical density of pCREB expression under the effect of peptides+NE; *c*) optical density of AANAT expression under the effects of peptides or NE. For *a* and *c*: light bars: 1 h; cross-hatched bars: 2 h; dark bars: 3 h. For *b*: light bars, 100 nM; cross-hatched bars: 1 μM; dark bars: peptide (100 nM)+NE (1 μM). Ordinate: optical density of expression. *$p<0.05$ in comparison with the control.

This result suggested study of the direct effect of epithalone on the level of MT released by pinealocytes into culture medium. Norepinephrine stimulated an increase of MT concentration in culture medium from 1897±273 to 7000±1350 pg/ml (3.5 times; $p<0.05$). Epithalone increased significantly ($p<0.05$) MT level, which reached 2451±121 pg/ml.

Epithalone obviously stimulated the expression of the key molecules involved in MT synthesis by pinealocytes. This fact confirmed the previous data on its effect on the pineal gland cells [1,2,4]. Epithalone stimulated the synthesis of AANAT present in the cell cytoplasm and the expression of pCREB located in the nucleus. Hence, intensification of MT synthesis under the effect of epithalone was explained by its modulation of the cellular cytoplasmic and nuclear structures. Potentiation of NE effect on the expression of AANAT and transcription factor pCREB trigger-related to cyclic AMP, and the formation of cell clusters under the effect of epithalone indicated that epithalone stimulated the MT synthesis by pinealocytes.

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