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Cytoprotective Effect of Peptide Sedatin, an Agonist of μ/δ-Opioid Receptors, on Primary Culture of Pulmonary Fibroblasts of Albino Rats under Conditions of Oxidative Stress

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We studied the effects of a synthetic analogue of dermorphin peptide sedatin on DNA synthesis, nucleolar apparatus, and parameters of free radical oxidation in the primary culture of pulmonary fibroblasts under conditions of oxidative stress. Oxidative stress significantly enhanced production of superoxide anion radical in the culture, sufficiently inhibited DNA synthesis in fibroblasts, and reduced the size of cell nuclei and parameters of the nucleolar apparatus. Sedatin prevented accumulation of free radical oxidation products and changes in karyometry parameters induced by oxidative stress. The peptide completely eliminated changes in the parameters of fibroblast nucleolar apparatus and abolished the inhibitory effect of oxidative stress on the number of DNA-synthesizing cells. Pretreatment with non-selective opioid receptor antagonist naloxone hydrochloride partially abolished the effects of sedatin in the primary culture of pulmonary fibroblasts.

Key Words: *opioid peptides; DNA synthesis; oxidative stress; fibroblasts*

Opioid peptides exhibit pronounced neuro- [12], cardio- [8] and gastroprotective [2] effects under various pathological conditions associated with oxidative stress. *In vivo* protective effects of opioids can be implemented through various mechanisms, including both direct cytoprotective effects of opioids and effects mediated by changes in the regulatory continuum of the body.

Peptide sedatin (H-Arg-Tyr-D-Ala-Phe-Gly-OH) is a synthetic analogue of dermorphin and a nonselective μ/δ-opioid receptor agonist [5]. Sedatin is used in veterinary and is considered as a promising preparation for correction some human pathologies.

Here we analyzed *in vitro* cytoprotective effect of sedatin in the primary culture of pulmonary fibroblasts under conditions of oxidative stress.

MATERIALS AND METHODS

The study was carried out on the primary culture of pulmonary fibroblasts from newborn albino Wistar rats. Biopsy specimens from the right lung after mechanical disaggregation and treated with crab pancreas collagenase (500 U/ml; BioloT) at 37°C for 15 min. After double washing with Hanks' saline (BioloT), the material was plated in culture flasks. Culturing was carried out in DMEM medium (BioloT) supplemented with 10% fetal calf serum (Sigma) in an atmosphere

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with 5% of CO_2. Passage 5 cells were used in the experiments.

Incubation of pulmonary fibroblasts in the presence of sedatin (Peptos) was carried out for 6 h at peptide concentration in the culture medium of 0.1 μM. For the analysis of the involvement of opioid receptors in the effects of sedatin, nonselective opioid receptor antagonist naloxone hydrochloride (NH) (Sigma-Aldrich) was added to the culture medium in a concentration of 10 μM 30 min prior to sedatin.

Oxidative stress was induced by adding 0.001 ml 3% H_2O_2 (60 μM) to 15 ml incubation medium for 2 h [4]. The following groups were formed: intact control (group 1); sedatin (group 2); NH+sedatin (group 3); oxidative stress (group 4); sedatin+oxidative stress (group 5); NH+sedatin+oxidative stress (group 6). Each group consisted of 6 monolayer cultures.

Generation of superoxide-anion radicals by pulmonary fibroblasts was evaluated by the method of lucigenin-dependent chemiluminescence (LDCL) [1.11]. Fibroblasts were harvested with trypsin-versen (BioloT), washed three times with Hanks' solution, and counted in a Goryaev's chamber. For chemiluminescent analysis, 10^6 cells were used. Lucigenin (Sigma-Aldrich) was added to the cell suspension in a final concentration of 5 μM. LDCL was recorded on a Perkin Elmer LS 50B fluorescent spectrometer, the signal was standardized using applied Finlab software. Lucigenin-dependent light sum (Sluc) was recorded over 5 min and expressed in relative units.

DNA synthesis in pulmonary fibroblasts was analyzed by ^{3}H-thymidine autoradiography. Cell monolayers were incubated in Hanks' solution, supplemented with ^{3}H-thymidine in concentration of 1 μCi/ml (1 h at 37°C). After incubation, the monolayers were washed with Hanks' saline and fixed in 96% ethanol. The slides with fibroblast monolayer were coated with nuclear emulsion film (Ilford Photo) and incubated at 4°C for 28 days. Then, they were treated with D-19 developer, fixed in 33% sodium hyposulfite, and stained with hematoxylin. Index of labeled nuclei (ILN) was calculated based on examination of 10^4 fibroblasts in each monolayer and expressed in percents. Label intensity was analyzed semiquantitatively as low (silver grains occupying <50% nucleus area) and high (fused silver grains occupying >50% nucleus area). For analysis of nuclear label intensity distribution, 200 labeled fibroblast nuclei were examined in each monolayer.

Parameters of the nucleolar apparatus were evaluated in AgNOR-stained fibroblast monolayers by computer morphocytometry using the MEKOS-C complex image analyzer [3]. The area of fibroblast nucleus, number of nucleolar organizer regions (NOR), and total NOR area over the nucleus were assessed. Nucleus and NOR areas were calculated based on measurement of 50 cells. For analysis of NOR number, 200 fibroblast nuclei were examined.

The data were processed using Statistica 6.0 software. The differences were significant at $p<0.05$ (Student *t* test).

RESULTS

In group 2, sedatin inhibited production of superoxide radicals: LDCL intensity was significantly reduced by 33% (Fig. 1). After exposure to sedatin, ILN reflecting the proportion of S-phase fibroblasts and label intensity indirectly reflecting the rate of DNA synthesis did not differ from the control (Table 1). Morphometric analysis showed that sedatin had little effect on the area of fibroblast nuclei and NOR count. However, the total area of NOR incubation of fibroblasts with the peptide significantly decreased (Table 2).

Addition of NH to the culture medium (group 3) abolished the effect of sedatin on superoxide anion generation (Fig. 1) and total NOR area (Table 2). Parameters fibroblast monolayer in the third group did not differ from the control.

In group 4, oxidative stress induced a significant increase (by 80.9%) in LDCL intensity in the culture (Fig. 1). Pronounced activation of free radical oxidation in the culture was followed by significant inhibition of DNA synthesis in cultured cells. ILN significantly decreased by 30.2% (Table 1). The fraction of cells with high label intensity decreased by 9 times under the influence of oxidative stress (Table 1). According to K. J. Davies [7], oxidative stress is accompanied by DNA synthesis blockade in order to protect the cell from oxidative damage. In H_2O_2-treated fibroblasts, the area of cell nuclei and total NOR area significantly decreased (by 32.3% and 20.3%

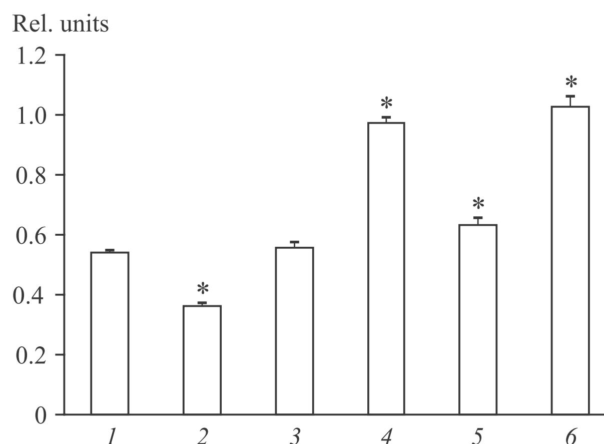

Fig. 1. LDCL intensity in the suspended culture of pulmonary fibroblasts of newborn white rats. *1*) Control, *2*) sedatin, *3*) NH+sedatin, *4*) stress, *5*) sedatin+oxidative stress, *6*) NH+sedatin+oxidative stress. *$p<0.05$ in comparison with the control.

TABLE 1. DNA Synthesis in the Primary Culture of Pulmonary Fibroblasts (*M*±*m*)

Group	ILN, %	Label intensity, %	
		low	high
Control	17.01±1.92	20.67±1.90	79.34±1.90
Sedatin	16.43±1.50	17.99±2.10	82.27±2.10
NH+sedatin	15.68±1.50	20.24±1.37	79.76±1.37
OS	11.87±1.17*	91.24±3.06*	8.77±3.06*
Sedatin+OS	13.37±2.48	94.20±2.53*	5.80±2.53*
NH+sedatin+OS	14.22±2.42	97.50±1.10*	2.50±1.10*

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

TABLE 2. Nucleolar Apparatus Parameters in the Primary Culture of Pulmonary Fibroblasts (*M*±*m*)

Group	Nucleus area, μ^2	Total NOR, μ2	NOR count
Control	217.48±6.03	16.43±0.47	3.53±0.05
Sedatin	205.29±5.51	14.09±0.43*	3.42±0.04
NH+sedatin	198.11±5.46	16.52±0.50	3.46±0.05
OS	147.14±4.71*	13.09±042*	3.37±0.05*
Sedatin+OS	168.08±7.55*	15.33±0.91	3.54±0.05
NH+sedatin+OS	165.49±3.70*	13.37±0.43*	3.27±0.04*

respectively) in comparison with the control, NOR count in fibroblast nuclei also decreased (Table. 2). According to previous reports [10,13], oxidative stress inhibits the synthesis of ribosomal RNA, which leads to shifts in the parameters of the nucleolar apparatus.

The intensity of H_2O_2-induced changes in the primary fibroblast culture was significantly lower in monolayers preincubated with sedatin (group 5). LDCL intensity in these cultures remained significantly higher than in the control (by 16.9%), but was significantly lower than in group 4 (by 35.4%; Fig. 1). After exposure to H_2O_2, the number of S-phase fibroblasts in cultures preincubated with sedatin did not significantly differ from the control. However, the proportion between nuclei with high and low label intensity remained the same as in group 4 and significantly differed from the control (Table 1). In group 5, the area of cell nuclei was significantly lower than in group 1 (by 22.7%), but higher than in group 4. The number and total area of nucleoli in group 5 did not differ from the control (Table 2). Hence, sedatin exhibited potent antioxidant and cytoprotective effects *in vitro*. Intracellular mechanisms of the observed cytoprotective action of mixed μ/δ-opioid receptor agonist can include activation of SOD and glutathione peroxidase [14], normalization of ionic composition [6], and a decrease in intracellular Ca^{2+} concentration in cells [9].

In group 6 fibroblasts, ILN did not significantly differ from the control. The decrease in label intensity decrease revealed in group 4 was maintained in this group. We observed a decrease in the nucleus area (by 23.9%), NOR count (7.4%), and total NOR area (by 18.6%) in comparison with the control. These morphological changes developed against the background of marked increase in LDCL intensity by 90.9%.

The results attest to direct antioxidant and cytoprotective properties of sedatin. The cytoprotective effect of sedatin is at least partially mediated by opioid receptors, because NH abolished many effects of sedatin in both intact cultures and cultures exposed to oxidative stress. Molecular mechanisms of the cytoprotective effect of sedatin require further investigation.

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