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Role of Amino Acid Arginine and Nitric Oxide in Mechanisms of Cytoprotective Effect of Non-Opiate Leu-Enkephalin Analogue *In Vitro*

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Incubation of primary culture of pulmonary fibroblasts with non-opiate analogue of leu-enkephalin (NALE; Phe-D-Ala-Gly-Phe-Leu-Arg, 0.1 μM) reduced generation of superoxide anion-radical (by 20.7%) and decreased the number of $p53^+$ cells (by 40.2%) induced by exposure to H_2O_2 (60 μM). The cytoprotective effect of NALE was potentiated by NO synthase inhibitor L-NAME (1 mM): the number of $p53^+$ cells decreased by 65.3% and morphometric parameters of the cell nuclei and nucleoli were improved. Incubation of pulmonary fibroblasts culture with peptide G (Phe-D-Ala-Gly-Phe-Leu-Gly, 0.1 μM) also significantly reduced the damaging effect of H_2O_2: the number of $p53^+$ cells decreased by 73.5%, the area of cell nuclei returned to normal, and generation of superoxide anion-radical decreased by 18.4%. These results indicate that C-terminal amino acid Arg and activation of NO synthase are not involved in the direct cytoprotective effect of NALE.

Key Words: *peptide analogue of leu-enkephalin; cytoprotective effect; oxidative stress*

Synthetic enzyme-resistant analogue of leu-enkephalin dalargin (Tyr-D-Ala-Gly-Phe-Leu-Arg) exhibits potent cytoprotective properties [13]. Potential efficacy of dalargin as a cytoprotective factor that can reduce the systemic inflammatory response in the treatment of new coronavirus infection is discussed [4]. However, wide clinical use of dalargin is limited by its affinity to μ- and δ-opioid receptors. The presence of amino acid Arg in the dalargin molecule reduces its passage through the blood—brain barrier, due to which dalargin produces primarily peripheral effects [2]. However, the effect of dalargin on the CNS cannot be excluded [1,10], especially under conditions of pathologically increased permeability of the blood—brain barrier and during the neonatal period.

In our previous studies, we have demonstrated the protective effect of peptide Phe-D-Ala-Gly-Phe-Leu-Arg, a structural analogue of dalargin, on cells of the brain [9], heart [17], and liver [6] under conditions of oxidative stress and its direct cytoprotective effect *in vitro* [7]. Peptide Phe-D-Ala-Gly-Phe-Leu-Arg has no affinity to opioid receptors due to the presence of N-terminal Phe instead of Tyr [11] and, therefore, it was called non-opiate analogue of leu-enkephalin (NALE). The mechanisms of the cytoprotective effect of NALE remain unclear. Probably, its effect is determined by the C-terminal Arg and stimulation of NO synthesis by NO synthase [21].

Our aim was to analyze the involvement of C-terminal amino acid Arg and NO-synthase activation into the mechanisms of the direct *in vitro* cytoprotective effect of NALE in cell culture.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of the Far Eastern State Medical University.

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Primary culture of pulmonary fibroblasts was obtained from left lung of 5-day-old albino Wistar rats. The lung fragments were kept in DMEM with high concentration of antibiotics (0.5 mg/ml gentamicin and 25 μg/ml amphotericin B, BioloT) for 4 h at room temperature. Then, the tissue was minced and incubated with crab pancreas collagenase (750 U/ml; BioloT) for 10 min at 37°C. Collagenase was inactivated by double washing with Hanks solution (BioloT); centrifugation was carried out in a cooling centrifuge (Eppendorf 5427R). The cell suspension was placed in culture flasks (CellATTACH) in DMEM (BioloT) containing 10% fetal calf serum (BioloT). The cells were incubated in a CO_2 incubator (Sanyo) at 5% CO_2.

Passage 6 pulmonary fibroblasts were used in the experiments. Peptides NALE (Almabion) and original peptide G (Phe-D-Ala-Gly-Phe-Leu-Gly (Almabion) were added to the culture medium in a concentration of 0.1 μM. In a special experimental series, non-selective inhibitor of NO synthase N-nitro-L-arginine methyl ester (L-NAME; Sigma-Aldrich) was added to the culture medium in a final concentration of 1 mM. This concentration of L-NAME provides effective suppression of NO generation *in vitro* [12]. Oxidative stress was induced by 2-h incubation of fibroblasts with 60 μM H_2O_2 [8].

The following experimental groups were formed: 1) control (intact cell culture); 2) NALE (incubation with NALE for 6 h); 3) NALE+L-NAME (incubation with NALE and L-NAME for 6 h); 4) peptide G (incubation with peptide G for 6 h); 5) H_2O_2 (incubation with H_2O_2 for 2 h); 6) NALE+H_2O_2 (incubation with NALE for 6 h with addition of H_2O_2 during the last 2 h of incubation); 7) NALE+L-NAME+H_2O_2 (incubation with NALE and L-NAME for 6 h with addition of H_2O_2 during the last 2 h of incubation); 8) peptide G+H_2O_2 (incubation with peptide G for 6 h with addition of H_2O_2 during the last 2 h of incubation).

Final passaging, incubation with peptides, and exposure to H_2O_2 were carried out in Petri dishes (Nunc) with glass slides for morphological analysis of cell monolayers. To study other characteristics of cells, they were removed from the dishes with trypsin—Versene (BioloT), washed 3 times with Hanks solution (BioloT), and resuspended in 1 ml Hanks solution.

The total number of cells in the suspension was counted using Fuchs—Rosenthal chamber; the production of superoxide anion radical was determined by the lucigenin-dependent chemiluminescence [3]. Chemiluminescence was recorded on a LS 50B luminescence spectrometer (Perkin Elmer) with signal standardization using the Finlab software. Lucigenin (Sigma-Aldrich) was added in a final concentration of 5 μM. The luminescence light sum over 5 min was measured, activity of superoxide anion radical generation was expressed in relative units.

Fibroblast monolayers were stained with silver nitrate (AgNOR staining) to assess morphometric parameters of the nucleoli and nuclei [5]. Image analysis was carried out on computerized morphometric apparatus MECOS-C (MECOS). Parameters of the nucleoli are integral characteristic of the cell response to stress [15]. We measured the area of fibroblast nuclei and the number and total area of nucleoli in the nucleus. In each group, at least 250 cells were examined.

For complex assessment of the functional state of fibroblasts, expression of p53 protein, a transcription factor accumulating in the cell under conditions of cell stress and DNA damage [19], was also measured. Immunohistochemical staining for p53 (clone DO-7, Leica Biosystems) was performed using the Novolink Polymer Detection System kit (Leica Biosystems) according to the manufacturer's protocol. Labeled cells were counted under a light microscope (×1000): proportion of cells with brown nuclei was analyzed by viewing 2500 fibroblasts in each experimental group.

Statistica 6.0 software (StatSoft, Inc.) was used for statistical analysis of the results. After verification of the normality of distribution, the arithmetic mean and standard error of the mean (M±SEM) were calculated. The experimental and control groups were compared using the Student's t test for independent samples; the differences were significant at $p<0.05$.

RESULTS

Cell number. As in our experiments we used the integral parameter, total number of cells harvested from the substrate, the data were not subjected to statistical processing and are descriptive.

Incubation with NALE and peptide G induced a decrease in the number of cells harvested from the substrate by 35.5 and 22.5%, respectively, in comparison with the control (Fig. 1). After incubation with NALE against the background NO synthase blockade with L-NAME, the number of cells surpassed the control value by 19.4%.

Addition of H_2O_2 to the culture medium was followed by a decrease in cells number by 36.9%. Pre-incubation of cells with peptides neutralized the negative effect of H_2O_2. Moreover, the number of fibroblasts in the groups NALE+H_2O_2, NALE+L-NAME+H_2O_2, and peptide G+H_2O_2 slightly surpassed the control values (by 10.5, 2.8, and 7.2%, respectively; Fig. 1).

Relative number p53$^+$ fibroblasts. Incubation of fibroblasts with NALE and peptide G was followed by a significant increase in the number of p53$^+$ cells in comparison with the control (by 4.36 and 4.8 times, respectively; Fig. 2). The number of p53$^+$ cells in

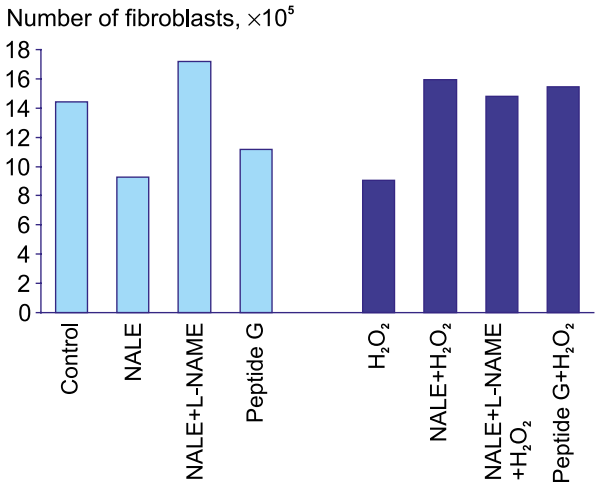

Fig. 1. Number of fibroblasts harvested from the substrate after incubation with NALE and peptide G under conditions of oxidative stress and NO synthase blockade.

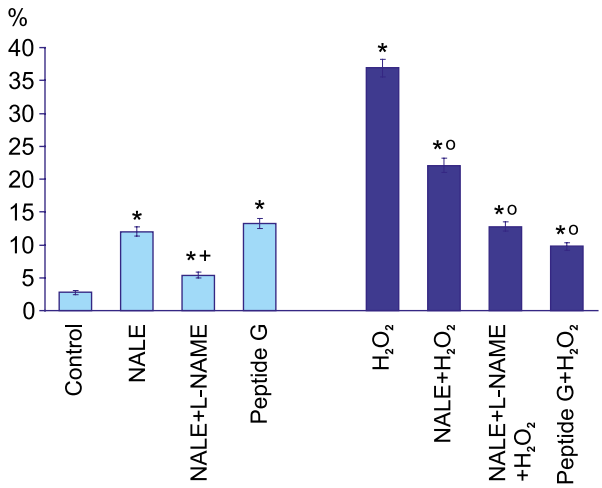

Fig. 2. Percentage of $p53^+$ fibroblasts after incubation with NALE and peptide G under conditions of oxidative stress and NO synthase blockade. $p<0.05$ in comparison with *control, $^+$NALE, $^oH_2O_2$.

cultures treated with NALE+L-NAME was higher by 1.96 times than in the control, but lower (by 2.2 times) than in cultures treated with NALE without inhibition of NO synthase.

Addition of H_2O_2 to the culture medium 13.37-fold increased the fraction of $p53^+$ fibroblasts. Pre-incubation of cells with NALE, NALE+L-NAME, and peptide G significantly decreased the number of $p53^+$ cells by 1.67, 2.88, and 3.78 times, respectively, in comparison with cultures treated with H_2O_2 alone (Fig. 2). However, the proportion of $p53^+$ fibroblasts in all cultures exposed to H_2O_2 significantly surpassed the control.

Morphometric parameters of fibroblast nucleoli and nuclei. In cell cultures exposed to peptide G, the area of cell nuclei and the number and total area of nucleoli did not differ from the control (Fig. 3). In cells incubated with NALE, a significant increase (by 29.1%) in the total area of nucleoli was revealed. In cultures exposed to NALE and NO synthase inhibitor L-NAME, the effect was less pronounced: the total area of nucleoli was lower than after incubation with NALE alone by 11.2%, but still surpassed the control (by 14.6%; Fig. 3).

Exposure to H_2O_2 induced a significant decrease in the area of fibroblast nuclei and total area of nucleoli (by 23.16 and 26.15%, respectively). Pre-incubation with NALE did not correct the negative changes caused by H_2O_2. Combined exposure to NALE and NO synthase inhibitor L-NAME before addition of H_2O_2 improved the state of cultured cells: the total area of nucleoli was significantly greater (by 12.16%) than after exposure to H_2O_2 and the area of fibroblast nuclei did not differ from the control. In cell cultures pre-incubated with peptide G before exposure to H_2O_2, the area of nuclei did not differ from the control, but the total area of nucleoli was below the control by 31.97%.

Generation of superoxide anion radical by fibroblasts. Incubation with NALE alone and in combination with L-NAME as well as incubation with peptide G did not change the intensity of superoxide anion radical generation in the cell culture in comparison with the control (Fig. 4).

Incubation of fibroblasts with H_2O_2 significantly intensified generation of superoxide anion radicals (by 67.2%). Pre-incubation with NALE and peptide G significantly reduced the intensity of superoxide anion radical generation by 20.65 and 18.41%, respectively. However, blockade of NO synthase with L-NAME abolished the effect of NALE.

Thus, exposure to H_2O_2 induced oxidative stress and stimulated generation of superoxide anion radicals in the primary pulmonary fibroblast culture. The effect was accompanied by a decrease in the number of cells, mean area of nuclei, and total area of nucleoli as well as significant increase in the number of $p53^+$ fibroblasts. The nucleoli are stress sensor of cells. Activation of free radical oxidation leads to damage of the nucleolar structure due to loss of nucleolar proteins. Some nucleolar proteins are released into the nucleoplasm, bind to MDM2 protein, and inhibit its activity, which leads to stabilization of p53 protein [14]. It is well known that p53 plays the key role in induction of cell apoptosis. Nucleus dehydration and a decrease nucleus size are most important changes in apoptosis [20]. Activation of apoptotic death leads to a decrease in cells number. Thus, the effects observed in fibroblast culture under influence of H_2O_2 corresponded our previous data [7] and indicated significant damaging effect of H_2O_2 on the cell culture.

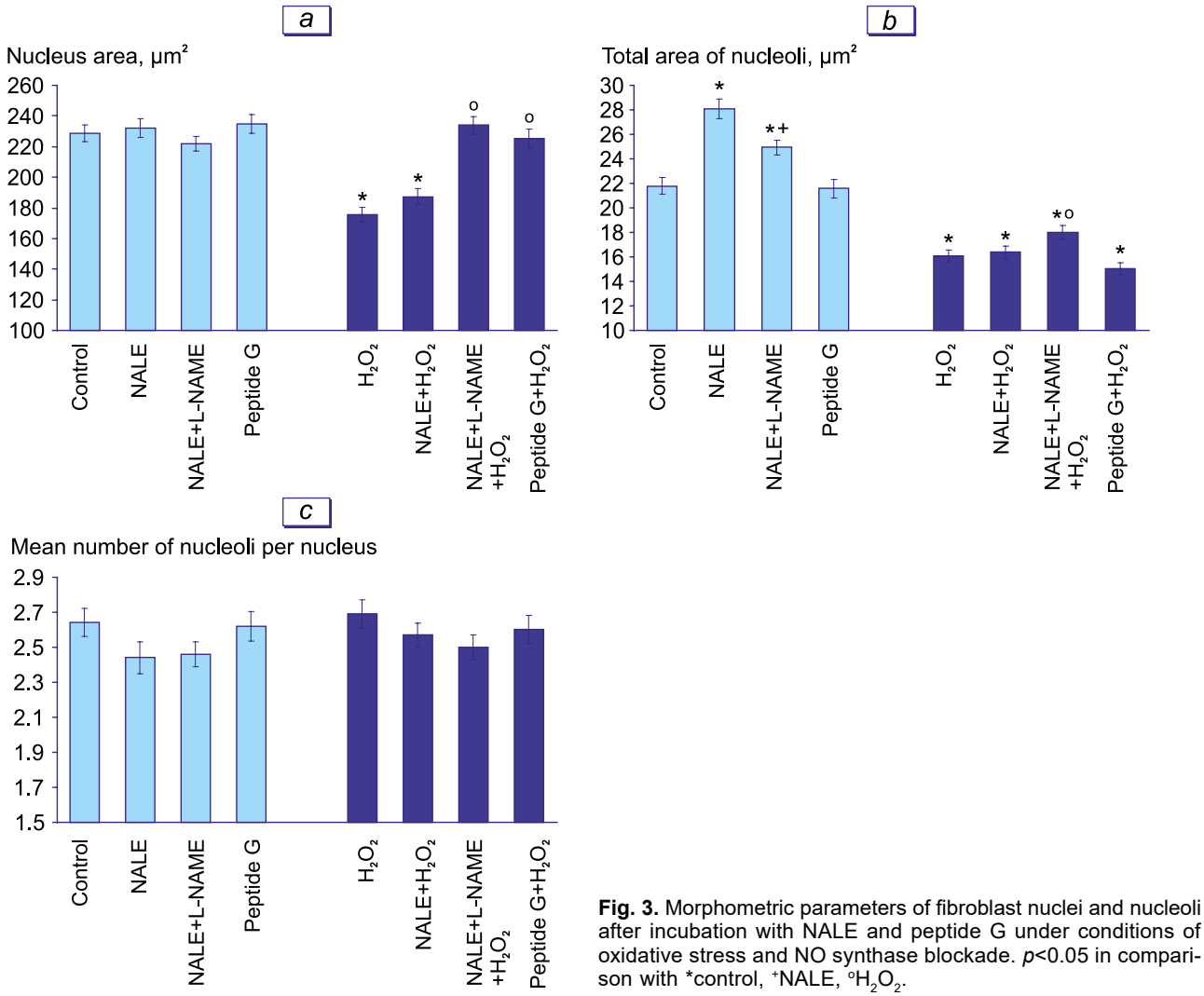

Fig. 3. Morphometric parameters of fibroblast nuclei and nucleoli after incubation with NALE and peptide G under conditions of oxidative stress and NO synthase blockade. $p<0.05$ in comparison with *control, $^{+}$NALE, $^{o}H_2O_2$.

Changes induced by H_2O_2 persisted after pre-incubation with NALE: the mean area of fibroblast nuclei and the total area of nucleoli were significantly lower than in the control; the number of $p53^{+}$ cells and intensity of superoxide radical anion generation surpassed the control levels, but were significantly lower than after exposure to H_2O_2 alone. Hence, pre-incubation of fibroblasts with NALE reduced the damaging effects of H_2O_2 on the cell culture. In previous studies, we also observed pronounced corrective effect of NALE on pulmonary fibroblast culture exposed to H_2O_2 [7].

Incubation with NALE against the background of NO synthase blockade (NALE+L-NAME+H_2O_2) did not reduce generation of superoxide anion radicals by cells under the action of H_2O_2, which attested to a role of the NO/NO synthase system in the implementation of the antioxidant effect of NALE. However, morphometry showed that the area of fibroblast nuclei returned to normal and the total area of nucleoli was

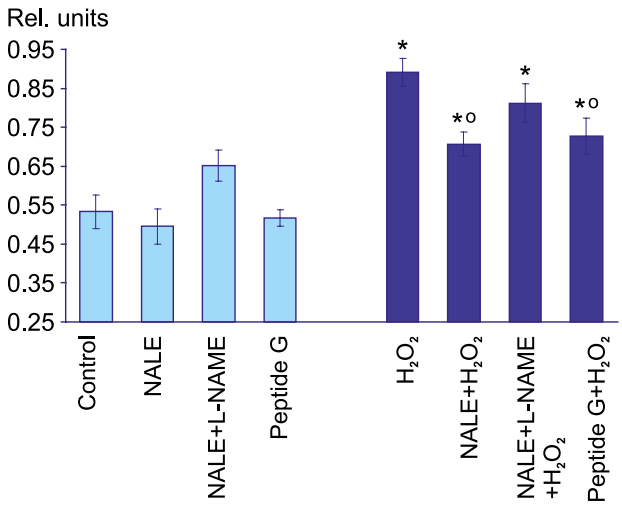

Fig. 4. Intensity of lucigenin-dependent chemiluminescence in fibroblast suspensions after incubation with NALE and peptide G under conditions of oxidative stress and NO synthase blockade. $p<0.05$ in comparison with *control, $^{o}H_2O_2$.

significantly higher than in cells exposed to H_2O_2 alone (though still remained below the control). The fraction of $p53^+$ fibroblasts decreased by almost 3 times. The total number of cells was close to the control. Thus, NO synthase blockade potentiated the ameliorative effect of NALE on morphological parameters of cells under conditions of oxidative stress.

Opposite changes in the intensity of superoxide anion radical generation and expression of p53 were also observed after incubation with NALE and L-NAME without oxidative stress modeling (in the absence of H_2O_2). Incubation of fibroblasts with NALE for 4 h did not affect ROS generation and led to a significant increase in the total area of nucleoli, which can be interpreted as an increase of anabolic protein-synthetic activity of cells; the number of $p53^+$ fibroblasts also significantly increased in these cultures, which can indicate a damaging effect of NALE. However, the role of p53 protein in cells cannot be interpreted univocally: this protein is known as a factor activating DNA repair and reducing chromosomal instability. In addition, some metabolic effects of p53 were reported [18].

Stimulation of the nucleolar apparatus after exposure to NALE against the background of NO synthase blockade (NALE+L-NAME) was less pronounced. The increase in the number of $p53^+$ cells under the effect of NALE was also less pronounced under these conditions.

Thus, blockade of NO synthase modulated the effects of NALE not only during oxidative stress, but also under normal conditions. This can be due to direct influence of L-NAME on cultured cells. The use of L-NAME for inhibitory analysis of the involvement of the Arg/NO-synthase/NO signaling pathway in the cytoprotective effects of NALE is difficult due to the presence of own effects of NO synthase blocker [16]. To assess the contribution of the Arg/NO-synthase/NO signaling pathway into the realization of the cytoprotective effects of NALE we replaced the C-terminal amino acid Arg for Gly in the molecule structure of original peptide G.

Incubation of fibroblasts with peptide G did not change the parameters of the nucleo-nucleolar apparatus and generation of superoxide anion radical. Peptide G, similar to peptide NALE, increased the number of $p53^+$ cells. Incubation of fibroblasts with peptide G reduced adverse effect of H_2O_2 on the cell culture. We also observed a significant increase in the area of cell nuclei, a decrease in the production of superoxide anion radicals, and a decrease in the number of $p53^+$ cells number, which attested to a significant cytoprotective effect of peptide G under conditions of oxidative stress. Moreover, the positive effect of the peptide G by some parameters (area of cell nuclei, expression of cell damage marker protein p53) exceeded the effect of NALE.

The results of this study indicate pronounced cytoprotective properties of non-opiate analogs of leu-enkephalin that did not depend on the presence of amino acid Arg in the peptide molecule and with the NO system.

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