«STUDY OF THE ERYTHROPOIETIN AND CRYOMELT MN EFFECT ON THE BDNF AND NGF NEUROTROPHIC FACTORS' EXPRESSION IN VITRO»

Abstract

The regulation of expression of neurotrophins and their receptors in the brain is currently considered as one of the most promising ways to affect some pathological CNS conditions which result in neurons' degeneration and death.

At the same time, some of the known to-date compounds that are capable of regulating the neurotrophins' expression in the brain, such as anti-depressants, possess certain negative adverse effects. Such undesirable effects restrict clinical use of the above compounds.

The study investigated the activity of drugs, named respectively Erythropoietin N1 and Erythropoietin N2, in regulating the expression of the neurotrophic factors BDNF and NGF in vitro.

Erythropoietin N1 is a modified form of human recombinant erythropoietin with the addition (1: 1) of the drug Cryomelt MN (5 mg / ml).

The Erythropoietin N2 is medicinal form of human recombinant erythropoietin with a brand name "Erythrostim".

It has been shown that in cultured rat neuroglial cortex cells the drug at a 3 nM concentration caused statistically significant (2 – 5-fold) stimulation of both BDNF and NGF expression after 1 and 4 hours from drug injection, as compared to control non-stimulated cells. The highest stimulation of the BDNF neurotrophic factor expression was found 4 hours after Erythropoirtin N1 injection; it reached 340% compared to the control. Since BDNF possesses high neuroprotective and neurotrophic activities, it would be possible to create a pharmaceutical drug, taking Erythropoietin N1 as a base that could be actived against demyelinating diseases.

Introduction

The neutrophins are a family of the neural tissue regulatory proteins that are synthesized by neurons and neuroglial cells. They promote differentiation and maintain viability and functioning of peripheral and central neurons by using autocrine and paracrine regulatory mechanisms. The neurotrophins regulate neuronal differentiation, induce dendrite ramification (arborization) and axone growth (sprouting) towards target cells. In the mature nervous system the neurotrophins regulate both short-term synaptic transmission and long-term potential transmission, thus partaking in execution of nervous system plasticity that is required for its normal functioning.

The mature active BDNF forms are represented by stable homodimers with molecular mass of about 28 kD. They support growth of spinal sensor neurons as well as survival and growth of motor neurons and sensor, ganglion, dophaminergic, cholinergic and GAMK-ergic neurons. The BDNF is primarily produced by the neuroglial cells of cortex and medulla as well as by the Schwann cells associated with peripheral motor neurons.

Materials and Methods

Materials and Reagents

We used the following reagents: L-glutamin, MEM, F12, DMEM, fetal bovine serum (ICN), sucrose, BSA, SDS, EDTA, NaOH, Na2CO3, Na2HPO4, NaCl, PPO, POPOP, benzamidine, PMSF, BDNF, Tris, insulin, transferrin, progesteron, putrescine, Na2SeO3, trifluoroacetic acid, heptafluorbutanoic acid, trichloroacetic acid, acetonitryl, polyethylenimine, D-glucose, L-glutamine, aprotinine, leupeptine (Sigma, Sigma-Aldrich), CuSO4, paraformaldehyde, Ca,Na-tartrate, hydrochloric acid, toluene (Reachim), CaCl2, Folin reagent (Merck), triton X-100 ("Ferak, Berlin"). The tissue culture plasticware was from Nunc and Costar companies. The manufacturers of other used materials and reagents are indicated in the relevant sections.

Primary neuroglia cell culture

Primary neuroglia cell culture was obtained by a standard method (Cole, 1989). The Sprague-Dowly rats aged 1-3 days were asphyxiated by a 15 minute exposure to CO2 and submerged in 80% ethanol for one minute. All subsequent operations were carried out in aseptic conditions at 4-70C. Isolated brains were used for membranes-free cortex tissue isolation in Hanks salts buffer. The isolated tissues were washed once with Hanks salts buffer and transferred to the MEM/F12 (1:1) containing 20% fetal bovine serum and 2 мМ L-glutamine. The tissue was mechanically dissociated into single cells. The cell suspension obtained was washed once with cell culture medium by centrifugation at 200 x g. Cells were plated at 200 000 cells/cm2 in poly-L-lysin-treated 75 cm2 tissue culture flasks and cultured in 5% CO2-incubator (5% CO2 and 95% air) at 370C in MEM/F12 supplemented with 15% fetal bovine serum, 6 g/l D-glucose, 2 мМ

L-glutamine, 25 mg/l insuline, 100 mg/l transferrine, 20 nM progesteron, 100 nM putrescine and 30 nM sodium selenite, 100 µg/ml gentamycin. Fresh cell culture medium was added every 3-4 days. Cells were split at a ratio of 1:3 after they had reached the confluent state (the confluency time was 1.5 – 2 weeks).

Investigation of the Erythropoietin N1 and Erythropoietin N2 effect on the BDNF mRNA and NGF mRNA levels of expression in neuroglia cell culture

Total RNA isolation

To this end we used cells after the 3rd passage. Cells were seeded at a density of 100 000/cm2 in poly-lysine-treated 6-well clusters using the above cell culture medium. After cells have reached the confluent state the growth medium was changed to a serum-free medium (the one without fetal bovine serum). After 48 h incubation cells were fed with sterile solutions of tested compounds (0.04 ml per well, 3 nM final concentration, 3 parallel measurements per one experimental point). As a control, an equal volume of 0.9 % NaCl in water was used. After indicated time intervals the culture medium was aspirated, cells washed with cold phosphate-buffered saline and total RNA was isolated by the phenol-chlorophorm technique using YellowSolve Kit (Clonogen, Russia) according to manufacturer's instructions. The RNA purity and concentration was determined spectrophotometrically, and the RNA samples with ratio of A260/A280 >1.6 were used in the following experiments.

Reverse transcription and PCR

For reverse transcription 1 μg of total RNA was taken in the reaction mixture containing 8 U/ml of Moloney Murine Leukemia Virus (M-MLV)-reverse transcriptase, 10 мМ dithiothreitol, 800 μM dNTPs, random hexamer primers (20 μg/ml) and the first-strand buffer (50 мМ Трис-HCl, 75 мМ KCl, 3 мМ MgCl2) in 25 μl volume. The reaction was for 1 hr at 37oC followed by 10 minutes at 700C. The cDNA samples were stored at -200C.

The BDNF mRNA expression level was estimated using quantitative real-time PCR (Mx3000P system, Stratagene) using highly specific ds-DNA-binding dye SYBR green I. The reaction was carried out in 25 μl reaction volume containing either 2 μl cDNA of the sample, or the standard, or water (negative control), 250 μM dNTP mix (deoxynucleoside triphosphates), 2.5 мМ MgCl2, 15 мМ Трис-HCl (pH 8.8), 50 мМ KCl, 0,5% glycerol, 0.1% Tween 20, the intercalating dye SYBR Green I, 1 U of Taq DNA polymerase with enzyme activity-inhibited antibodies ("Syntol", Russia), and 10 pmols each of sense- and anti-sense primers ("Syntol", Russia, Table 1). The PCR conditions were as follows: start - 5 minutes at 950C, followed by 40 cycles including melting for 30 seconds at 950C, annealing for 30 seconds at 680C and elongation for 30 seconds at 720C, with fluorescence detection at the end of every elongation step.

Table 1

Target gene	Primers sequence (direct, reverse)
□-actin	5'-CTACAATGAGCTGCGTGTGGC-3' 5'-CAGGTCCAGACGCAGGATGGC-3'
BDNF	5'-AGCCTCCTCTGCTCTTTCTGCTGGA-3' 5'-CTTTTGTCTATGCCCCTGCAGCCTT-3'
NGF	5'-TCAGTGTGTGGGTTGGAGAT-3' 5'-AGCCTGTTTGTCGTCTGTTG-3'

For PCR product specificity validation, at the end of amplification the reactions were cooled to 60ºC, and 20 minutes later the melting curves were obtained by heating the reaction to 950ºC at a rate of 0.030C/sec with continuous fluorescence detection. For calibration curves generation, the cDNA sample mix was sequentially diluted with DNAse-free water to obtain a series of standard solutions with known relative concentration of the corresponding product. By using the manufacturer's software we detected the cycle number that corresponded to the maximal amplification rate, produced the calibrating curve that plotted the number of cycles against relative product concentration, and calculated the relative concentration in unknown sample with its subsequent normalization to the concentration of □-actin.

The significance of differences between grouped averages was estimated using the dispersion analysis (one-way ANOVA). The curve represents average grouped values corrected for standard mean deviations (Mean + SEM). The designation of confidence intervals are: * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$

Results and Discussion

We have carried out the evaluation of Erythropoirtin N1 and Erythropoietin N2 ability to regulate the expression of the most studied neurotrophins BDNF (brain-derived neurotrophic factor) and NGF (nerve growth factor) in cultured rat cortex astrocytes. To this end we used the method of quantitative real-time PCR (the PCR of reverse-transcribed products) in order to test the ability of Erythropoirtin N1 and Erythropoietin N2 to affect the BNDF and NGF gene expression at the mRNA level in cultured rat cortex astrocytes.

It was shown that the introduction into culture medium of Erythropoirtin N1 and Erythropoietin N2 at the concentration of 3 nM caused prominent upregulation in the BDNF and NGF expression, up to 2 – 5 –fold as compared to control cultures. Such an increase in expression is continuous and is observed both 1 h and 4 hrs after the introduction of compounds (Figures 1 and 2). The most prominent and long-lasting upregulating effect on BDNF expression was due to Erythropoietin N1. Compared to control culture, the BDNF expression rise was 340% (Figure 2).

The CNS myelin is formed as a result of axons spiral entrapment by the glial cells processes. The resulting myelin sheets in some cases could be destroyed by viruses and autoimmune reactions. As a result, the transmission capacity of nerves may be impaired leading, for instance, to motor disfunctions. The maintaining of neurons viability is a BDNF-dependent process that is executed by the neuroglial cells. We can suggest that the Erythropoietin N1-mediated upregulation of BDNF and NGF expression is one of the main molecular mechanisms of its therapeutic action. Since BDNF possesses high neuroprotective and neurotrophic activities, it could be possible to create a pharmacological drug on the basis of Erythropoietin N1 which would show activity in treatment of the demyelinating diseases.

Moreover, the detected biological activity of Erythropoietin N1 allows us to suggest its anti-depressant activity. It is known that the direct injection of BDNF into the hippocampus of laboratory animals leads to the anti-depressant effect.

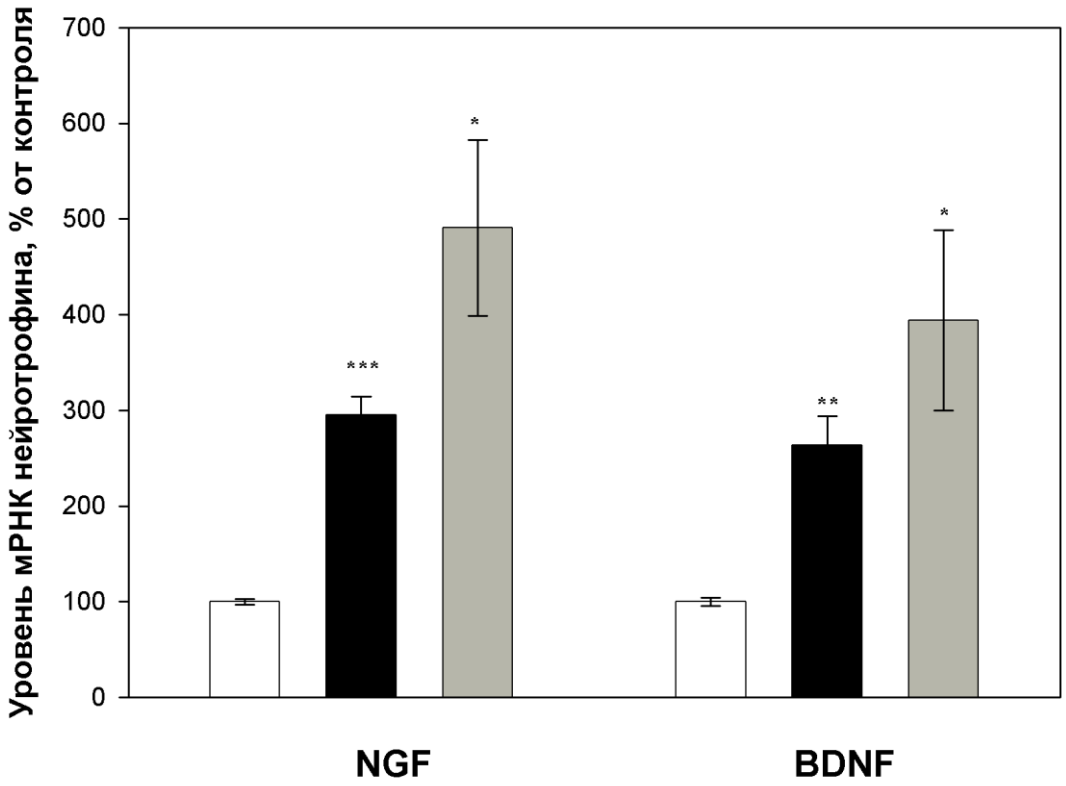

Figure 1. The effect of Erythropoietin N1 and Erythropoietin N2 preparations at a 3 nM concentration on the expression of the BDNF and NGF neurotrophic factors in cultured rat cortex astrocites during a one hour incubation (white bar – control, black bar – Erythropoietin N1, grey bar – Erythropoetin N2; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$). Ordinate – the mRNA neurotrophin levels, % of control.

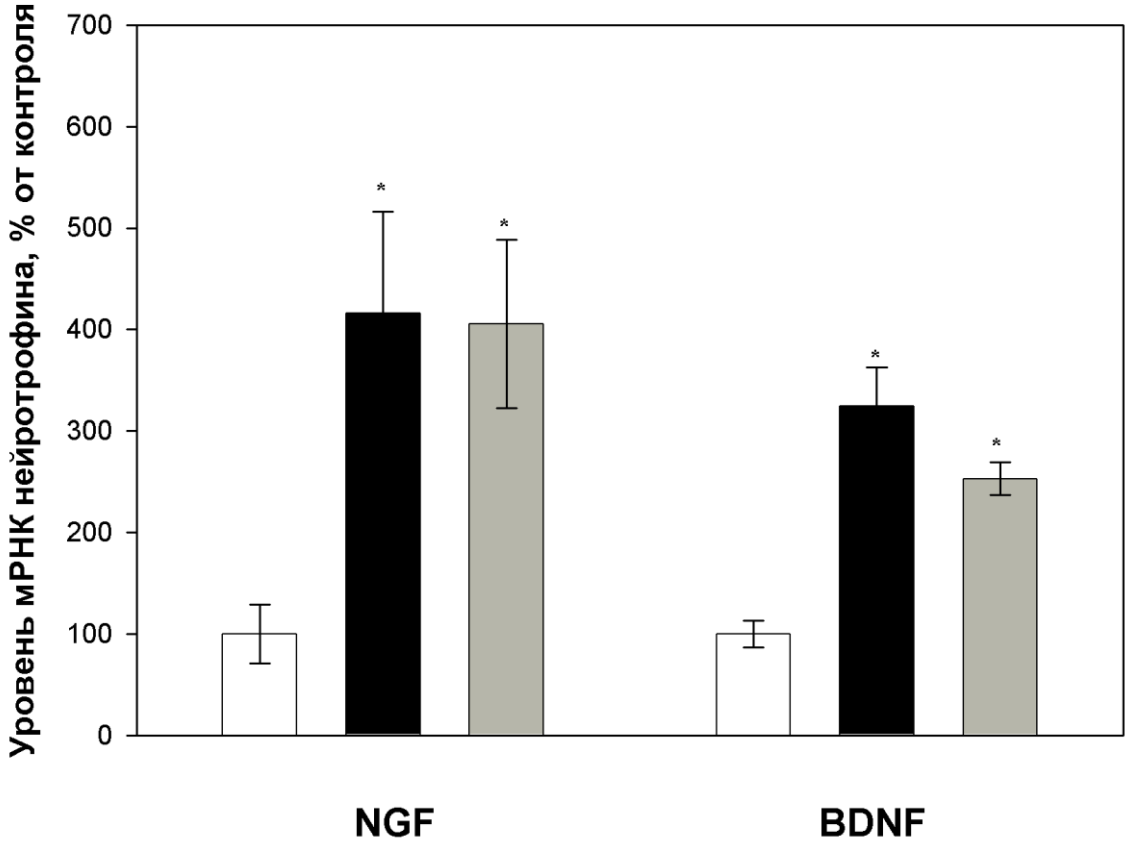

Figure 2.

The effect of Erythropoietin N1 and Erythropoietin N2 preparations at a 3 nM concentration on the expression of the BDNF and NGF neurotrophic factors in cultured rat cortex astrocites during a four hour incubation (white bar – control, black bar – Erythropoietin N1, grey bar – Erythropoetin N2; * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$). Ordinate – the mRNA neurotrophin levels, % of control.