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THE ACTIVITY OF ANTIOXIDANT ENZYMES AND THE EXPRESSION OF THEIR GENES IN THE RAT BRAIN AFTER INJECTING OF VINDEBURNOL IN THE MULTIPLE SCLEROSIS MODEL

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АКТИВНОСТЬ АНТИОКСИДАНТНЫХ ФЕРМЕНТОВ И УРОВЕНЬ ЭКСПРЕССИИ ИХ ГЕНОВ В МОЗГЕ КРЫС ПРИ ВВЕДЕНИИ ВИНДЕБУРНОЛА В МОДЕЛИ РАССЕЯННОГО СКЛЕРОЗА

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ABSTARCT

The article presents the results of a study on the effectiveness of vindeburnol, a structural analogue of the cerebral vasodilator vincamine, in the model of multiple sclerosis. The drug was injected subcutaneously in rats after modeling experimental allergic encephalomyelitis at a dose of 20 μg/kg, from the first manifestation of a neurological deficit over the next 10 days. In the somatosensory cortex of animals, the activity of superoxide dismutase, glutathione peroxidase and glutathione reductase, as well as the expression level of the SOD1, GPX4, GPX6 and GSR genes using real-time polymerase chain reaction were studied. It was found that vindeburnol does not affect the expression of the SOD1 gene, as well as the activity of superoxide dismutase, while increasing the expression of the GPS4, GPS6 and GRS genes, which determines the increased activity of glutathione peroxidase and glutathione reductase in rats in the model of multiple sclerosis.

АННОТАЦИЯ

В статье представлены результаты исследования эффективности применения виндебурнола, структурного аналога церебрального вазодилататора винкамина, в модели рассеянного склероза. Препарат вводили подкожно крысам после моделирования экспериментального аллергического энцефаломиеллита в дозировке 20 мк/кг со дня первых проявлений неврологического дефицита в течение последующих 10 суток. В соматосенсорной коре животных исследовали активность супероксиддисмутазы, глутатионпероксидазы и глутатионредуктазы, а также уровень экспрессии генов SOD1, GPX4, GPX6 и GSR с использованием полимеразной цепной реакции в реальном времени. Установлено, что виндебурнол не оказывает влияния на экспрессию гена SOD1, как и на активность супероксиддисмутазы, при этом способствует повышению экспрессии генов GPS4, GPS6 и GRS, что определяет повышение активности глутатионпероксидазы и глутатионредуктазы у крыс в модели рассеянного склероза.

Keywords: multiple sclerosis, activity of antioxidative biocatalysts, gene expressions, vindeburnol.

Ключевые слова: рассеянный склероз, активность антиоксидантных ферментов, экспрессия генов, виндебурнол

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system that affects not only white, but also gray matter [1-2]. The histopathological feature of MS is the presence of foci of demyelination, as a result of which severe neurological disorders are observed in patients. In the process of development of demyelination, the progenitor cells of microglia and oligodendrocytes

penetrate into the inflammation zone, initiating a spontaneous remyelination process. A distinctive feature of the neuroinflammatory process is a local increase in the intensity of free radical processes, which leads to the release of certain cytokines and chemokines by glial cells. This creates an environment for enhancing cellular interactions, which leads to the ability of oligodendrocytes to synthesize new myelin [3]. In the acute phases of MS, the formation of an excessive amount of reactive oxygen species (ROS) is observed, which suppress the activity of antioxidant enzymes, which, as a result, leads to oxidative stress. The intensification of free radical processes also contributes to damage to biological macromolecules, such as polyunsaturated fatty acids in membrane lipids, as well as proteins and nucleic acids. This is confirmed by the results of a study of free radical processes in the blood and cerebrospinal fluid of patients with multiple sclerosis during the active phases of the disease. This indicates that an increase in ROS levels could lead to depletion of cellular antioxidants and the development of damage [4-7].

It was previously found that in the model of multiple sclerosis, vindeburnol, a structural analogue of the cerebral vasodilator vincamine, reduces the activity of oligodendorocytes and the volume of dimelinated regions in mice, and also increases the expression level of a number of genes involved in the survival of Locus coeruleus cells [8].

This study presents the results of a study of the effect of vindeburnol on gene expression of antioxidant enzymes, as well as on the activity of these antioxidants in rat brain in a model of multiple sclerosis.

Research methods. Animal experiments were carried out in compliance with European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (Strasbourg, 18 March, 1986).

The experiment was conducted on outbred white female rats weighing 200-250 g, which were divided into groups: 1 – control group (n = 8); 2 – experimental allergic encephalomyelitis (EAE) was simulated in animals (n = 8); 3 – after simulation of EAE, vindeburnol was administered subcutaneously at a dose of 20 μg / kg from the first manifestations of neurological deficiency over the next 10 days (to the peak of the clinical manifestations of the disease). The calculation of the dose of vindeburnol was carried out by calculating the LD50 for a given animal species with a confidence interval of 95%. The spinal cord of outbred white rats without neurological symptoms and modified Freund's complete adjuvant served as material for immunizing animals to reproduce EAE. The reaction of animals to the administration of the drug was evaluated by the level of manifestation of neurological deficit and pathomorphological changes in the brain of rats of experimental groups at the peak of the acute phase of the disease; in this formulation of an experiment on modeling EAE, signs of cerebral MS are manifested [9].

On the 30th day after the start of the experiment, the rats were decapitated; somatosensory cortex was isolated on ice, where free radical oxidation indices and gene expression levels (SOD1, GPX4, GPX6 and GSR) were studied.

Activity of glutathione peroxidase (GPx) in supernatant of hippocampus tissue was determined by speed of oxidation of reduced glutathione (GSH) in presence of tert-Butyl hydroperoxide according to the method [10]. Activity of glutathione reductase (GSR) was measured by speed of oxidation of NADPH oxidase according to the method [11]. The activities of GPx and GSR were expressed in μmol/min/mg protein. The activity of superoxide dismutase (SOD) in supernatants was determined by the intensity of inhibition of nitro blue tetrazolium reduction during superoxide radical generation during xanthine oxidation with xanthine oxidase at a wavelength of 560 nm, the enzyme activity was expressed in ΔE560 / mg protein [12].

The relative level of transcripts of the studied genes was investigated using the real-time polymerase chain reaction (Real-time PCR) method after reverse transcription. Total RNA from cortical and hippocampal tissues was isolated using the Aurum Total RNA fatty and fibrous tissue kit (BioRad). The amount and purity of the isolated RNA was evaluated spectrophotometrically using a NanoPhotometer (Implen). OD260/280 of RNA samples ranged from 1.80 to 2.00. Total RNA (0.5 μg) was reverse transcribed into cDNA, in a mixture volume of 25 μl. The reverse transcription reaction was carried out using reagents of the company Evrogen (Russia), in accordance with the manufacturer's instructions at 40°C for 40 minutes, and then inactivated at 92°C for 10 minutes. Design of forward and reverse primers for Real-time PCR was carried out in the Primer3 program with subsequent verification in IDT. The primers were synthesized by Synthol (Russia). The primer sequences are shown in table 1.

Real-time PCR was performed on a Bio-Rad CFX-96 amplifier using a ready-made iQ BioRad reaction mixture in a volume of 25 μl, including 50 pmol of forward and reverse primers and 1 μl of cDNA. Conditions for PCR: primary denaturation was carried out at 95°C for 3 minutes. 35 amplification cycles were carried out: 20s at 95°C, 10s at 60°C, 20s at 72°C. To verify the specificity after the end of the amplification cycles, a melting curve was constructed from 65°C to 95°C in increments of 0.5°C. SYBR Green I was used as a dye. The amplification reaction for each studied gene was carried out separately in duplicates. The HPRT1 gene encoding the hypoxanthine phosphoribosyltransferase 1 enzyme was used as the reference gene.

Table 1

Primer sequences

Gene	Forward Primer	Reverse Primer	melting point (°C)
SOD1	Gcg-gat-gaa-gag-agg-cat-gtt	Acg-gcc-aat-gat-gga-atg-ct	60
GPX4	Tga-gcc-gct-tat-tga-agc-ca	Cac-acg-caa-ccc-ctg-tac-tt	60
GPX6	Acg-tac-cct-gaa-ctg-aac-aca	Ccg-ttc-aca-tcc-ccc-ttc-tc	60
GSR	Gcc-ttc-acc-ccg-atg-tat-ca	Gcc-aac-cac-ctt-ctc-ctc-ttt	60
HPRT1	Tcc-tcc-tca-gac-cgc-ttt-tc	Atc-act-aat-cac-gac-gct-ggg	60

Relative expression was calculated by evaluating the Ct (Threshold cycle) values obtained during Real-time PCR. Next, the expression levels of the studied genes in the experimental and control samples were compared using the 2-ΔΔCt method [13].

Statistical processing of the research results was performed using the ANOVA method and the Statistica 10.0. A check for the normality of the distribution of experimental data was carried out using the Kolmagorov-Smirnov and Livin test. Data are presented as mean values and standard errors. The differences were considered statistically significant at $p<0.05$.

Results. Antioxidant enzymes (SOD, GPx, catalase) catalyze reactions with reactive oxygen species, leading to the formation of inactive products. There is a group of antioxidant enzymes that use reduced glutathione as a cofactor, electron and proton donor, and material for neutralizing electrophilic compounds. SOD neutralizes H2O2 and lipid peroxides, showing its activity in protecting, mainly, membranes. GPR reduces oxidized glutathione, thereby reactivating the system. An extensive group of glutathione-S-transferases, along with peroxidase activity, has the ability to alkylate toxic electrophilic compounds, conjugating them with HB-extra-erythrocyte hemoglobin. At the same time, the effect of detoxification is achieved, and GSH is irreversibly consumed [14]. Enzymes from the glutathione group, and also SOD, are predominantly present in the brain.

When studying the activity of antioxidant enzymes in the somatosensory cortex in the EAE model, a significant decrease in the activity was found: SOD by 59% ($p<0.05$), GPx by 37% ($p<0.05$) and GSR by 51% ($p<0.05$), relative to the control group. Under the conditions of the introduction of vindeburnol, the activity of these enzymes increased relative to the group of animals that were modeled by EAE. Nevertheless, the antioxidant defense link in these conditions did not correspond to the physiological norm: in animals in the EAE model and the introduction of vindeburnol, the activity of GSR, GPx and GSR was lower than the control level, respectively, by 32% ($p<0.05$), by 25% ($p<0.05$) and 35% ($p<0.05$) (Table 2).

Table 2

The effect of vindeburnol on the activity of superoxide dismutade (ΔE560/mg protein), glutathione peroxidase (μmol/min/mg protein) and glutathione reductase (μmol/min/mg protein) in rat somatosensory cortex in the model of multiple sclerosis

Groups	SOD	GPx	GSR
control	54,77±3,04	29,25±1,64	23,53±1,21
EAE model	22,31±1,19*	18,35±0,96*	11,62±0,07*
EAE model + vindeburnol	37,42±2,53*	21,84±1,29*	15,37±0,83*

* – significant differences in indicators relative to the values in the control group (at $p<0.05$)

Based on the results of the study of gene expression of antioxidant enzymes presented in Figure 1, the following conclusions can be made.

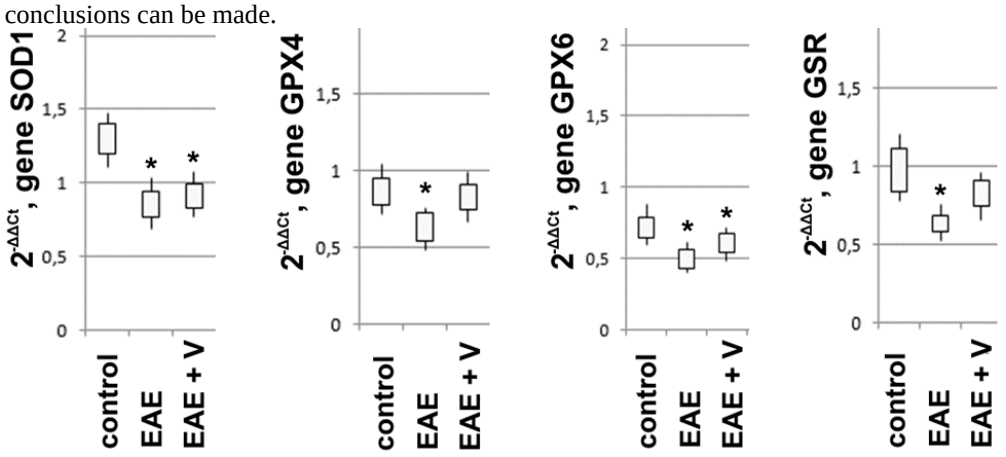

Fig. 1. The effect of vindeburnol on gene expression of antioxidant enzymes in rat somatosensory cortex in a model of multiple sclerosis. EAE - experimental allergic encephalomyelitis; V - vindeburnol
** – significant differences in indicators relative to the values in the control group (at $p<0.05$)*

First of all, under the conditions of modeling multiple sclerosis in the somatosensory cortex of rats, a decrease in the expression of these genes is observed. The most significant decrease in the expression level was found for the SOD1 and GSR genes. Moreover, the introduction of vindeburnol contributes to an increase in the expression level of the GPS4, GRS genes and, to a lesser extent, the GPS6 gene. Vindeburnol has no effect on SOD1 gene expression.

Conclusion. It has now been proven that the neuropathology of multiple sclerosis is associated with the development of oxidative stress, excitotoxicity and inflammation. Studies on multiple sclerosis report an important role for SOD, which has neuroprotective properties in this pathology [15-17]. So, over-expression of Klotho in the mouse ALS SOD1 model leads to delayed onset and progression of the disease, increased survival. Klotho reduced the expression of neuroinflammatory markers and prevented the loss of neurons in the motor cortex and, especially, in the spinal cord [18]. The results obtained in this work showed that the use of vindeburnol in the model of multiple sclerosis has a greater effect on the glutathione system through its effect on the expression of GPS4, GRS genes and, to a lesser extent, the GPS6 gene; this drug does not affect the expression of SOD1. As a result, the activity of SOD is not compensated by the introduction of vindeburnol (in contrast to the enzymes GPx and GSR).

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