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COMPARATIVE AND ONTOGENIC BIOCHEMISTRY

State of Antioxidative Protection in Central Nervous Ganglia of the Mollusc *Lymnaea stagnalis* at Modulation of Activity of the NO-Ergic System

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Abstract—In experiments on mollusc *Lymnaea stagnalis,* the state of antioxidative protection is studied in central nervous ganglia during a long-term activation (inhibition) of synthesis of nitrogen monoxide (NO) in the body. The effect of the blocker of NO-synthase N^G-nitro-L-arginine (L-NNA) at the background of enhancement of pulmonary respiration has been found to be associated with a rise of levels of reduced glutathione and TBK-active products in the nervous tissue at preservation of a relatively high superoxide dismutase activity and a low glutathione peroxidase activity compared with the control group and the animals treated with the metabolic precursor of NO synthesis L-arginine. In spite of the revealed disturbances of balance of the body pro- and antioxidative system, DNA electrophoresis detected no products of its degradation, which can indicate the absence of massive programmed death of the nervous tissue cells in *Lymnaea stagnalis* during modulation of activity of the NO-ergic system.

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Key words: antioxidants, nervous system, nitrogen monoxide, apoptosis, mollusc.

INTRODUCTION

Nitrogen monoxide (NO) is a signaling molecule and is widely used by quite different representatives of animal kingdom from Coelenterata to the higher vertebrates [1, 2]. At present there are a lot of experimental data indicating the involvement of the NO-ergic system in the regulation of the activity of various physiological processes including the neuronal ones [3]. The presence of one unpaired electron in structure of an NO molecule allows ascribing it to the bioradicals [4] with all their characteristic properties.

It is reasonable to suggest that under conditions of activation (inhibition) of NO production the intracellular system of antioxidative protection will be also modified. As a result, the level of free-radical forms in the cell can be changed, which, in turn, can activate some range of functions. In particular, superoxide anion appears as the intracellular second messenger by modifying activity of various proteins including the gene-regulatory ones [5]. Also the subject of full-scale discussion is the possibility of induction of processes of cell death at changes of level of the antioxidative cell protection [6].

In the mid-1990s, the NO-synthase activity was revealed in fresh-water pulmonate molluscs [7]. The subsequent experiments have shown the involvement of NO in regulation of the food-procuring [8], respiratory [9], and passive-defensive behavior [10]. It is to be noted that it is the amount of

oxygen in tissues which is responsible for generation of its active forms. Nevertheless, no investigation of the effects associated with prolonged modulation of the NO-ergic system activity has been carried out. The main attention of the authors studying the molluscan antioxidative system was focused on its role in elimination of consequences of the increased level of free-radicals in the body as a rule in response to contamination of environment with heavy metals and organic pollutants [11, 12].

The goal of the present work was to study the state of antioxidative protection in central nervous ganglia of *Lymnaea stagnalis* at prolonged stable activation (inhibition) of nitrogen monoxide synthesis as well as a possibility of the accompanying induction of programmed death of the nervous system cells.

MATERIALS AND METHODS

The work was carried out on a representative of fresh-water pulmonary molluscs—the common pond snail *Lymnaea stagnalis* L. The snails were kept at the laboratory in 5-liter aquaria (5 individuals in each) at the water temperature of 24–26°C. The food was the leaves of dandelion located in the lower part of the aquaria. Adult animals weighing 2–4 g were used in the experiments. The water was changed every 3 days.

Modulation of activity of the NO-ergic system. The molluscs were incubated for 2.5 h in solutions of NO-synthase blocker prepared in the sedimented tap-water: N^G-nitro-L-arginine (L-NNA) or of metabolic precursor of NO synthesis—L-arginine, at a concentration of 1×10^{-4} or 1×10^{-3} M, respectively (the used preparations were from Sigma, USA). Then the animals were returned back to the aquaria and kept there under the constant action of L-NNA (1×10^{-5} M) or L-arginine (1×10^{-4} M). The procedure of incubation was repeated at the 4th and 8th days of experiment. The control group of molluscs did not undergo any action of the preparations from the nitrogen monoxide group.

Respiratory behavior. The number of respiratory cycles (opening-closing of respiratory orifice) for 0.5 h of observation as well as duration of cycle in each mollusc individually were recorded. There was calculated the mean time of the respiratory act and the total duration of respiration for 0.5 h of observation.

Preparation of homogenate of the nervous tissue. The ring of central and buccal ganglia was isolated in molluscs at the 10th day of their incubation in solutions of L-NNA and L-arginine. Three nervous systems of molluscs were taken for one sample and weighed on a VLP-200 analytical balance with precision of 0.05 mg. Using a glass homogenizer, the ganglia were ground in 1.2 ml of cold (4°C) Ringer's solution for Lymnaea (mM): 44 NaCl, 1.7 KCl, 4 $CaCl_2$, 1.5 $MgCl_2 \times 6\ H_2O$, 10 HEPES, pH 7.5. A half of each sample was frozen and kept at –70°C for not longer than 2 months. The other half was used for determination of the level of reduced glutathione.

Biochemical analysis of the system of antioxidative protection in the mollusc nervous tissue. Activity of superoxide dismutase (SOD, EC 1.15.1.1) was determined by the spectrophotometric method based on estimation of the rate of autooxidation of flavonoid quercetine in its presence or its absence (according to [13]). Activity of Se-dependent glutathione peroxidase (Se-GP, EC 1.11.1.9) was determined from its ability to detoxicate tret-butylperoxide in the presence of reduced glutathione (G-SH) [14]. The level of G-SH was determined spectrophotometrically (at 412 nm) from coefficient of molar extinction (12 600 $M^{-1} \times cm^{-1}$) [15]. Intensity of processes of lipid peroxidation was determined from formation in the nervous tissue homogenates of TBK-active products [16]. The protein content was determined by Bradford's method [17].

Isolation and electrophoresis of DNA. Isolated nervous systems (5 in each sample) were minced in twenty volumes of SDS-buffer for cell lysis (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1% SDS, pH 7.5) and incubated at 42°C for 3 h. One half of preparation in each series was treated with RNase (25 μg/ml) for the last hour of incubation. Then 5 M solution of sodium perchlorate was added to the tubes to a final concentration of 0.2 M and gently stirred for 5 min, after which the sample was added with equal amount of the chloroform : isoamyl alcohol mixture in ratio of 24 : 1 and centrifuged for 20 min at 10000 rev/min. The water fraction was aspirated and additionally

Effect of NO-synthase inhibitor (L-NNA) and of metabolic precursor of NO-synthesis L-arginine on characteristics of pulmonary respiration of *Lymnaea stagnalis*

Experimental series	Parameters of pulmonary respiration					
	frequency of respiration (act/30 min)		duration of one respiratory act (s)		the total duration of pulmonary respiration (s/30 min)	
	after 1 day	after 9 days	after 1 day	after 9 days	after 1 day	after 9 days
Control	1.2 ± 0.09	1.1 ± 0.15	110.9 ± 5.83	94.1 ± 5.10	139.1 ± 12.54	123.0 ± 14.38
L-arginine	1.4 ± 0.16	1.0 ± 0.09	100.3 ± 6.96	84.3 ± 7.07	138.2 ± 15.27	103.0 ± 14.34
L-NNA	1.4 ± 0.21	1.3 ± 0.12$^{\#\#}$	127.6 ± 8.11$^{\#}$	95.2 ± 8.21	205.5 ± 27.96$^{*,\#}$	142.4 ± 17.16$^{\#\#}$

Note: The number of animals in all series of experiments—36. Asterisk indicates statistically significant differences as compared with control group in the same time diapason ($p < 0.05$); # and ## indicate statistically significant differences as compared with animals exposed to action of L-arginine in the same time diapason ($p < 0.05$ and $p < 0.02$, respectively).

cleared from protein by the chloroform : isoamyl alcohol mixture. The DNA from water fraction was sedimented with two volumes of 96% ethyl alcohol cooled to –10°C, the pellet was rinsed by 70% ethanol, dried, and dissolved in 50 μl TE-buffer (10 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The obtained DNA preparation was stored at –70°C until use. Electrophoresis was performed in 1.5% agarose gel in 40 mM Tris-acetate buffer containing 0.5 μg/ml ethidium bromide or acridine orange. After DNA separation the gels were scanned in ultraviolet light using an Image-Master VDS0CL system (Amersham, USA).

Statistical analysis. Experimental data ($\bar{x} \pm S_{\bar{x}}$) were processed by the commonly accepted methods of variation statistics. The number of molluscs used in behavioral experiments and of the studied samples is specified for each series. The statistical significance of obtained results was estimated applying the Student's criterion or the criterion of correspondence χ^2.

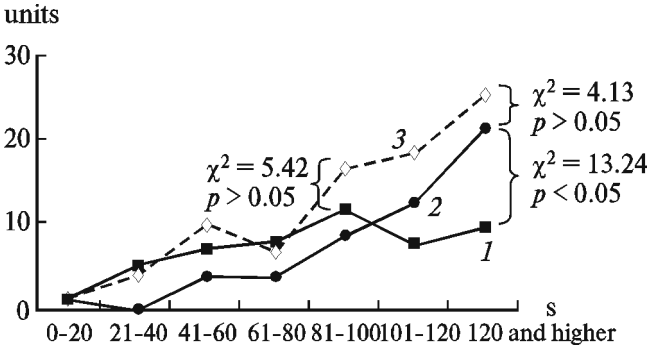

Fig. 1. Distribution of respiratory acts of the mollusc *Lymnaea stagnalis* for duration. *Abscissa*: duration of the open state of pneumostome (c); *ordinate*: frequency of the open state with a given duration (units). (*1*) L-arginine, (*2*) L-NNA, (*3*) control group. Indicated are the values χ^2 and level of significance *p*.

RESULTS

Respiratory behavior. Next day after incubation of molluscs in L-NNA and L-arginine solutions, there were revealed changes in parameters of pulmonary respiration in the indicated experimental series as compared with each other and with control (see table). Specifically, in animals under action of NO-synthase inhibitors, duration of the single respiratory act increased (1.3 times) as compared with molluscs under effect of L-arginine. A pronounced 15-fold rise in the total duration of pulmonary respiration was noticed as compared with control group and with the snails placed into solution of metabolic precursor of NO synthesis. No statistically significant changes in the frequency of respiration were revealed. Analysis of the curve of distribution of respiratory acts for duration (Fig. 1) has shown its shift to the side of the shorter cycles under effect of L-arginine as compared with animals treated with L-NNA. The observed character of the effect of drugs from the group of nitrogen monoxide is preserved on the

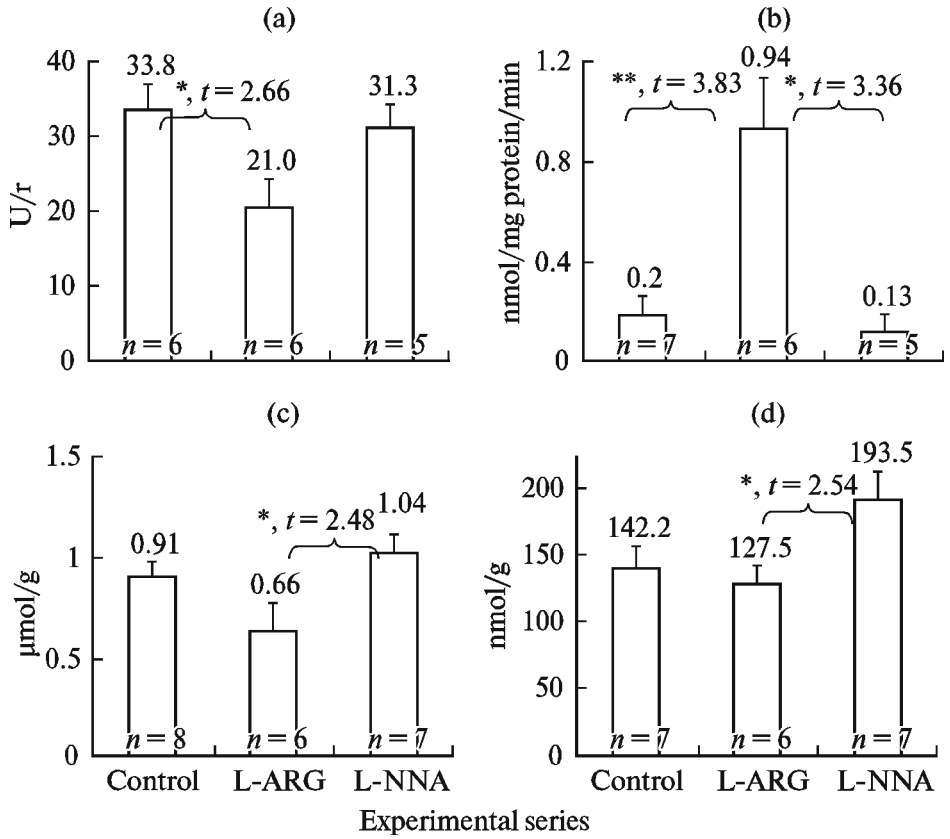

Fig. 2. Effect of NO-synthase inhibitor (L-NNA) and of metabolic precursor of NO synthesis (L-arginine) on the state of the antioxidative system in central nervous ganglia of *Lymnaea stagnalis*. *Vertical axis*: (a) activity of superoxide dismutase (U/g), (b) activity of glutathione-peroxidase (nmol/mg of protein/min), (c) concentration of reduced glutathione (μmol/g), (d) level of TBK products (nmol/g); *horizontal axis*: experimental series. Presented values are the studied parameters (numbers above bars) and the number of samples (*n*) for each experimental series. Asterisks indicate statistically significant differences: * $p < 0.05$, ** $p < 0.02$. The value of Student's *t*-criterion is indicated for statistically significant differences.

whole for 9 days: an increased total duration (1.4 times) and frequency (1.3 times) of pulmonary respiration under action of L-NNA as compared with action of L-arginine. No statistically significant differences were found in other respiratory parameters between molluscs of different experimental groups.

The system of antioxidant protection. In 10 days after the beginning of experiment there was observed a change in activity of the key antioxidative enzymes in the mollusc nervous tissue (Fig. 2). Under conditions of the NO synthesis stimulation, there was noted a cardinal (5–7 times) rise of the activity of Se-GP as compared with control group and the group undergone action of L-NNA. At the same time, activity of SOD decreased 1.5 times. The blockade of NO synthesis resulted in a 1.5-fold increase of the G-SH level as well as the amount of TBK-active products in the nervous tissue as compared with conditions of action of L-arginine. No differences in the total protein content were observed for any experimental groups of molluscs: 29.1 ± 2.27 mg/ml (L-NNA), 26.1 ± 2.73 mg/ml (L-arginine), and 28.2 ± 3.78 mg/ml (control).

DNA electrophoresis. Analysis of electrophoregrams (Fig. 3) performed at the 12th day after the beginning of action of NO drugs did not reveal the presence of the products of DNA degradation (low-molecular or corresponding by the size to nucleosomal DNA, about 180 b.p.) in any of experi-

mental series (in 3 repeats for each). The observed fragments of the size of about 550 b.p. (series with L-NNA) disappeared after treatment of the preparation with RNase in the course of isolation. Staining of the gel with acridine orange also permitted identifying the indicated fragments as the single-chain ones, i.e., most probably as mRNA.

DISCUSSION

The main physiological process leading to formation of free-radical forms is a «respiratory explosion», as a rule in the cells of immune system, at which practically the entire consumed oxygen is spent for formation of the oxygen anion-radical by the NADPH-oxidase complex. Hence, the amount of consumed oxygen is the key factor for characterization of the state of anti-oxidative system of the body protection. Specifically, there are observed pronounced changes in activities of several enzymes (SOD, Se-GP, catalase) in the liver and muscle cells in the molluscs *Otala lacteal* and *Helix aspersa* during transition from the torpid state (hibernation) into the active one, when the oxygen consumption is elevated many times [18, 19], and activity of anti-oxidative enzymes of *Mytilus galloprovincialis* directly depends on pO_2 in water [20].

The revealed enhancement of pulmonary respiration preserved for a long time under action of inhibitor of NO-synthase should facilitate development of oxidative stress in the Lymnaea nervous tissue. This is indirectly indicated by an increase of the level of TBK-active products and by preservation of the sufficiently high SOD activity found in molluscs kept in L-NNA solution. The paradox in action of this substance consists in that in parallel with a rise of respiratory activity it suppresses production of NO whose interaction with superoxide anion leads to formation of an extremely toxic intermediate—peroxinitrite ($ONOO^-$); as a result, the total «oxidative» background in the tissue can decrease.

The fact of the almost complete absence of action of L-arginine on parameters of the Lymnaea pulmonary respiration as compared with control group can be explained as follows. In the body of freely moving, feeding molluscs, production of nitrite monoxide is at the constantly high level [21].

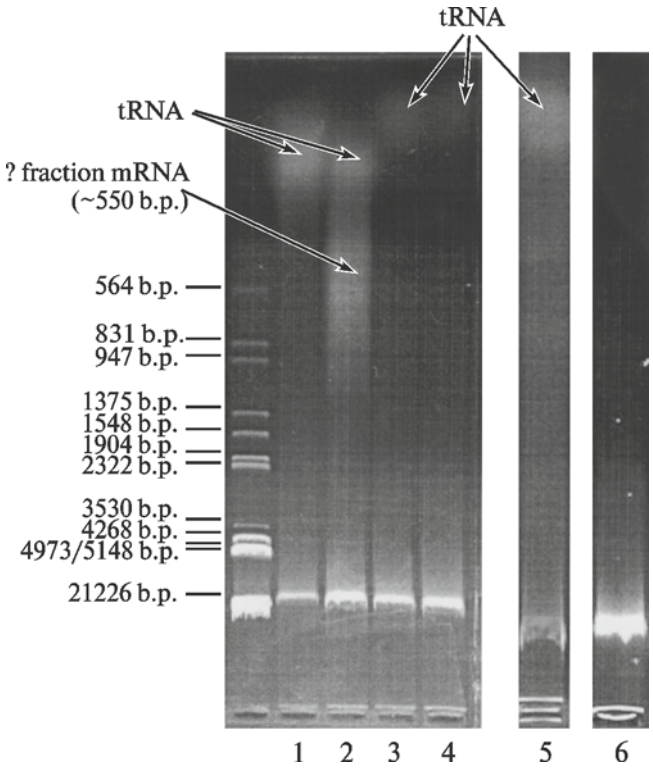

Fig. 3. Electrophoregram of DNA isolated from the nervous tissue at modulation of activity of the NO-ergic system in *Lymnaea stagnalis*. (1) L-ARG, (2) L-NNA, (3) L-ARG (treatment with RNase), (4) L-NNA (treatment with RNase), (5) control, (6) control (treatment with RNase).

As a result, the nervous system is under tonic effect of this signaling molecule, including the effect of accumulation of a great number of NO-ergic cells in buccal ganglia [7]. In this connection the observed by some authors [9] inhibitory action of NO donors on the functional activity of central generator of the respiratory rhythm can also produce a compensatory effect by preventing development of oxidative stress. Also remarkable is the fact of a sharp elevation of the Se-GP activity under these conditions on the background of a decrease of the G-SH level and of SOD activity, especially in comparison with the animals undergone the action of L-NNA. It is obvious that an enhancement of NO production still leads the system of cell anti-oxidative protection to an additional stress.

The oxidative action is considered to be one of the key factors of aging process [22]. In other

words, during aging, the cells lose the ability to counteract the oxidative damage of the key molecules increasing with age. Specifically, an increase of the SOD and Se-GP activities with age is observed in the tissue of gills and mantle musculature in some cephalopoda, for example, in *Sepia officinalis* and *Lolliguncula brevis*, on the background of a decreased level of catalase and of unchanged activity of glutathione reductase [23]. These species are characterized by the short lifespan, which can be explained by the low total activity of antioxidative enzymes.

The cell death due to intracellular signals belongs to the programmed cell death, unlike apoptosis, at which destruction results from the cell stimulation with external signals. An increase of intracellular Ca^{2+}concentration is a most important intracellular event during development of the programmed cell death phenomenon. In spite of the well-known ability of free-radical forms and especially of nitrite monoxide to affect the Ca^{2+}-channels of the cytoplasmic membrane and intracellular calcium depots, and, consequently, the content of free calcium in the cell cytoplasm [3], we failed to obtain evidence for massive neuronal death at modulation of NO production in the Lymnaea body. In this case the presence in several series of experiments (incubation in L-NNA) of mRNA fragments can indicate activation of certain genes whose protein products are able to resist disturbance of balance of the body pro- and antioxidative systems. Thus, tissues of the sea scallop *Chlamys farreri* contain the PCF peptide that has the pronounced protective action against oxidative stress, first of all due to activation of SOD and catalase and to a decrease in the level of lipid peroxidation. In the model investigations the protective PCF action is associated with a decreased level of Ca^{2+} inside the cells, enhancement of their proliferative activity, and a decrease of percent of the apoptotically dying cells [24]. The peptide isolated from *Meretrix meretrix* was successfully used for suppression of proliferation of carcinoma cells from the gastric digestive glands, which can be due to its produced increase of the SOD activity on the background of decreased activity of tyrosine kinase [25].

Thus, the prolonged modulation of activity of the NO-ergic system in body of the mollusc *Lymnaea stagnalis* alters anti-oxidative status of the nervous tissue. The observed effects are realized at the cellular level, probably due to pronounced readjustments of respiratory activity, which is indicated by a change in the oxygen amount consumed by the animals. In spite of the revealed disturbance in the balance of the body pro- and antioxidative systems, the massive programmed death of cells of the Lymnaea nervous tissue is not observed.

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