Investigation of Changes in NO Content during Long-Term Sensitization in Edible Snail Using EPR-Spectroscopy: Effects of Antibodies to Calcium-Binding Protein S-100

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EPR-spectroscopy experiments (electron paramagnetic resonance) demonstrated a decrease in NO production in the nervous system and heart of edible snail *Helix lucorum* after formation of long-term sensitization, a neurobiological model of anxiety and depression. The protective effect of antibodies to Ca^{2+}-binding protein S-100 in dilution of 10^{-12} on the formation of long-term sensitization was accompanied by partial recovery of NO synthesis in the nervous system and heart. These findings indicate that the imbalance in Ca^{2+}-binding protein S-100 can lead to inhibition or modulation of some processes during plastic reorganization in the body and especially during pathological processes.

Key Words: *nitric oxide; long-term sensitization; neurobiological model of anxiety; Ca-binding protein S-100; electron paramagnetic resonance*

Numerous studies during the recent decade showed that simple chemical compound NO is an intra- and intercellular transmitter with diverse signal functions [2,9]. The effects of NO are associated with its influence on ionic channels, transmitter secretion, calcium ion exchange, and cell metabolism and genome [10,13]. Activity of NO synthase responsible for NO formation was identified in the nervous system of invertebrates, including mollusks [15]. NO regulates neuron plasticity properties in edible snail: NO synthase blockers promote habituation, while NO donors facilitate sensitization [4]. It was also demonstrated that serotonin and NO donors mutually potentiate the effect of activation of serotonergic system in edible snail [5].

Long-term sensitization (LS) of the defense reflex, which can be defined as potentiation of the reflex response under the influence of a strong or damaging irrelevant stimulus appear to be the neurobiological model of anxiety and depression [12]. This model can be used for the studies of membrane mechanisms of the formation of persistent excitation focuses in animal nervous system [3,7]. It is known that serotonin is necessary for LS formation; bearing in mind mutual interaction of serotonin and NO donor in activation of the serotonergic system in edible snail [5], it was interesting to study changes in NO content during LS formation. We found a protective effect of antibodies to Ca^{2+}-binding

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protein S-100 on the formation of LS as a neurobiological model of anxiety and depression [11]: injection of antibodies against Ca^{2+}-binding protein S-100 in dilution 10^{-12} before the start of LS formation (10 minutes before first electrical stimulus) inhibits potentiation of defense reactions of pneumostome closing and ommatophore (ocular horn) withdrawal.

The aim of this study was quantitative evaluation of NO content in edible snail tissues during LS; possible influence of low doses of antibodies against S-100 on NO content during LS was also studied.

MATERIALS AND METHODS

Experiments were carried out on terrestrial pulmonate gastropod *Helix lucorum* (edible snail) from Crimean population. LS of defense response was formed [3] by stimulation of animal head with electric pulse bursts (4 times per day for four days with 1.5-2 h intervals). Parameters of stimulation: 0.5 sec burst duration, 6-8 mA amplitude of rectangular pulses, 10 msec pulse duration, 50 Hz pulse frequency. Significant lengthening of the closed state of peumostome in response to presentation of a testing tactile stimulus compared to baseline was a criterion of changed defense response and LS formation.

During LS formation, the defense reaction of pneumostome closing and ommatophore withdrawal in response to the testing stimulus was evaluated and the locomotion rate was measured. The time of closed pneumostome state after tactile stimulation of the pallial cushion area with a brush hair was measured. The reaction of pneumostome closing was chosen because it appears to be the ini-

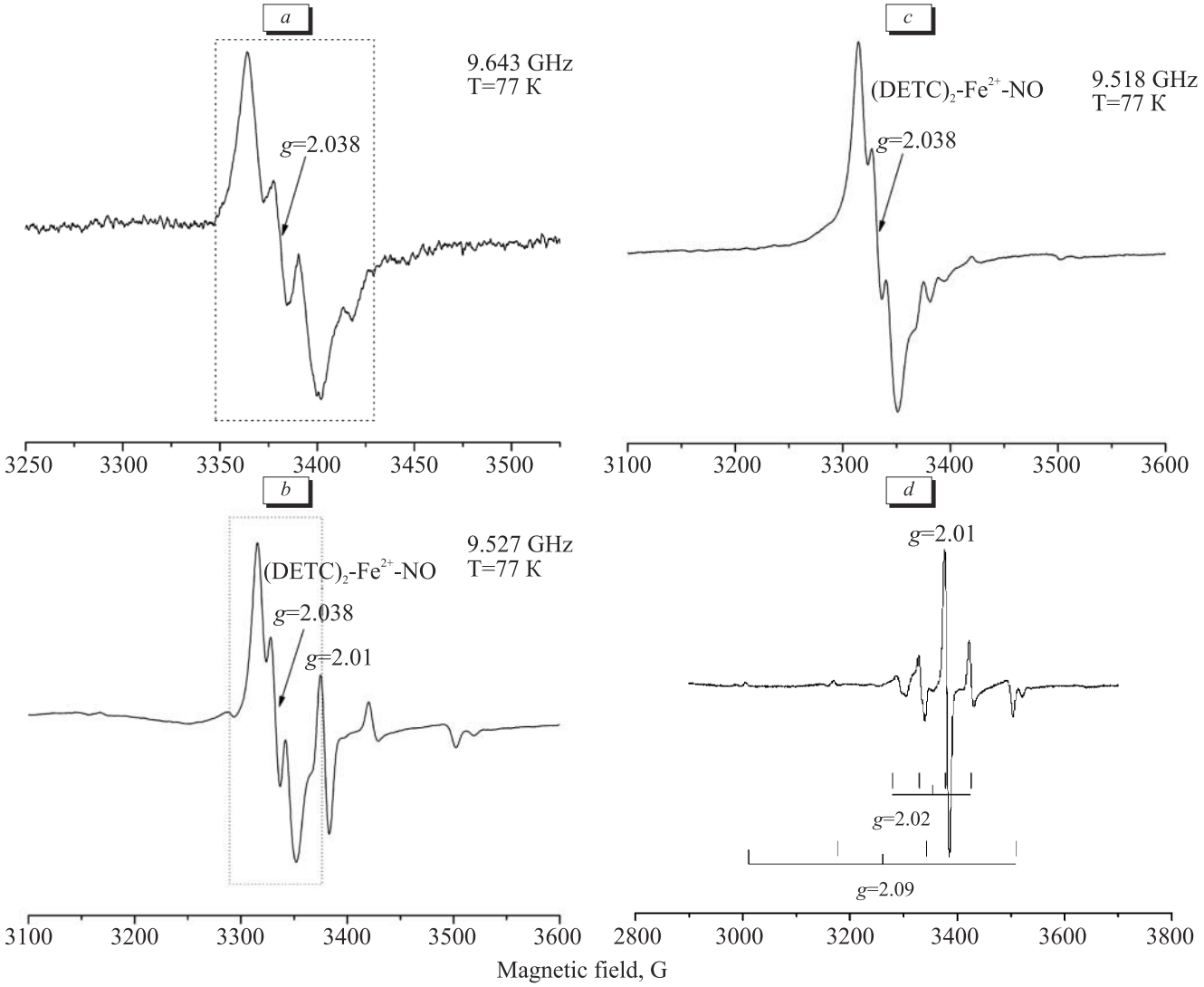

Fig. 1. EPR spectra. *a*) EPR spectrum of $(DETC)_2$-Fe^{2+}-NO spin trap in snail tissues; *b,c*) standard EPR spectra of mononitrazol complexes of non-hem iron DETC in snail nervous system and heart, respectively; *d*) EPR spectrum of Cu-$(DETC)_2$ in snail tissues.

tial component of defense reactions [11]. Moreover, the amplitude of ommatophore withdrawal was measured in response to both tactile stimulation with Frey hair of the middle and front parts of the foot and ommatophore contraction was visually assessed (in percents). To this end, the maximum ommatophore length was taken as 100% and the degree of withdrawal was documented (as 0, 25, 50, 75 or 100%) [14]. Furthermore, video recording with subsequent computer analysis was performed. The minute run speed on vertical wall of rectangle glass terrarium cage was measured [8]. The details of testing procedures were described by us previously [11].

The effects of antibodies against Ca^{2+}-binding protein S-100 on LS formation were assessed. To this end, low doses of AB-S100 (LAT-S100, dilution 10^{-12}, Materia Medica) were administered in a volume of 0.1 ml to 50% snails every day before electric stimulation, another group (control) was simultaneously injected with 0.1 ml saline. Apart from behavioral and electrophysiological tests, NO content was measured by EPR-spectroscopy.

EPR is one of the most effective methods for NO detection and measurement in biological tissues due to spin trapping approach proposed by A. F. Vanin *et al.* This method was used in our experiments for evaluation of changes in NO content during LS formation [6]. Two groups of snails (control and with developed LS) received injections of 500 mg/kg sodium diethyldithiocarbamate (DETC, DETC—Na, chemically pure), 187.5 mg/kg sodium citrate and 37.5 mg/kg iron sulfate ($FeSO_4 \times 7\ H_2O$, Sigma) into sinus node for the formation of mononitrosyl iron complexes. The interaction between DETC—Na and iron citrate leads to the formation of DETC—Fe^{2+}complexes, which in turn interacts with NO [2] yielding stable radical $(DETC)_2$-Fe^{2+}-NO detectable by EPR-spectroscopy. Since DETC—Fe^{2+} complex is not water-soluble, all components forming the spine trap were injected separately and in different time into the sinus node area of snail (lacking pain receptors) for prevention complex formation before interaction with NO. The EPR spectrum was recorded on a EPR ER-200 spectrometer (Brucker) at 77 K. EPR spectra in the nervous system and heart of snail were analyzed. Two snails were used for preparing each sample because of small sizes of snail organs.

Thus, four groups of snails were used in this study: intact, sensitisized after preliminary injection of saline (active control), sensitisized after preliminary injection of LAT-S100, and snails injected with LAT-S100 (second active control).

The results were presented as *M*±*SEM*. Statistical significance of differences between the mean neuron parameters in different experimental series was evaluated using Student's *t* test and Mann—Whitney *U* test.

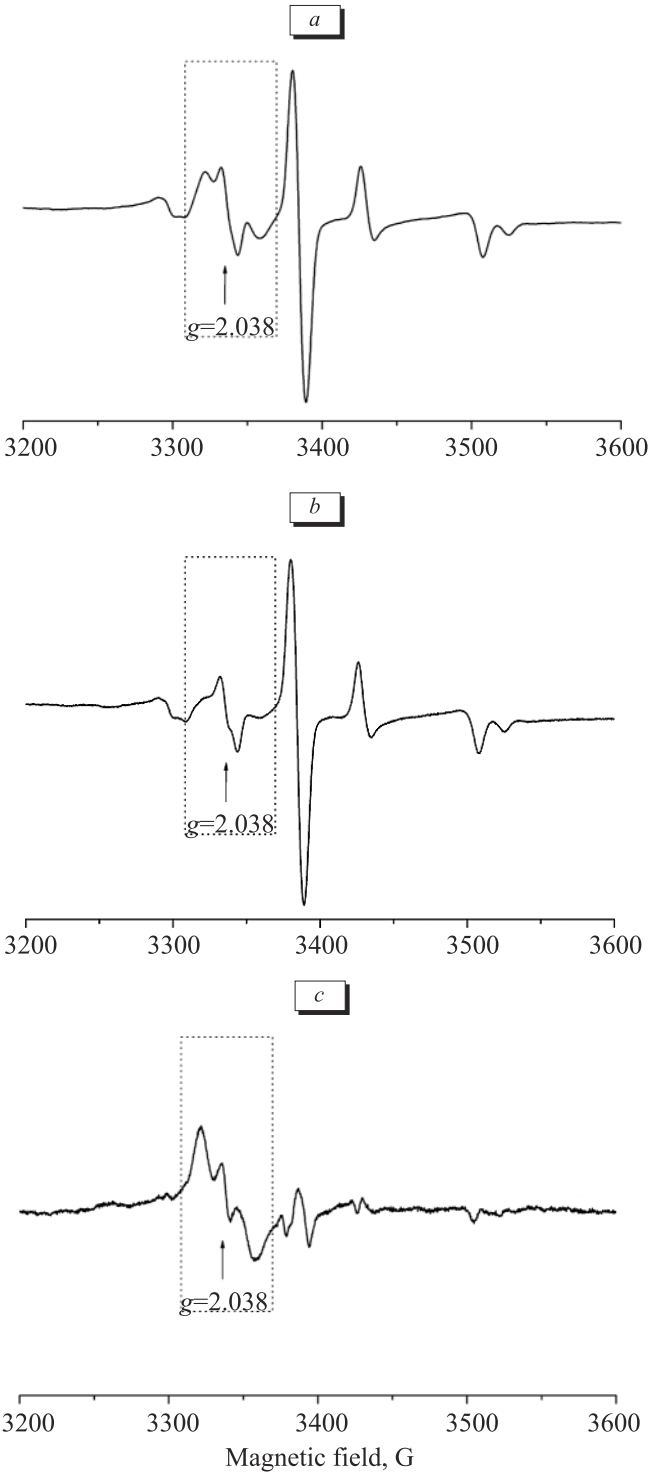

Fig. 2. The procedure of $(DETC)_2$-Fe^{2+}-NO spin trap signal extraction in snail tissues. *a*) original experimental EPR spectrum of snail nervous system tissue; *b*) perpendicular structure of EPR spectrum of Cu-$(DETC)_2$ complex; *c*) spectrum of the spin trap obtained after subtraction of spectrum *b* from spectrum *a* (for descriptive reasons the signal amplitude was amplified by 3 times).

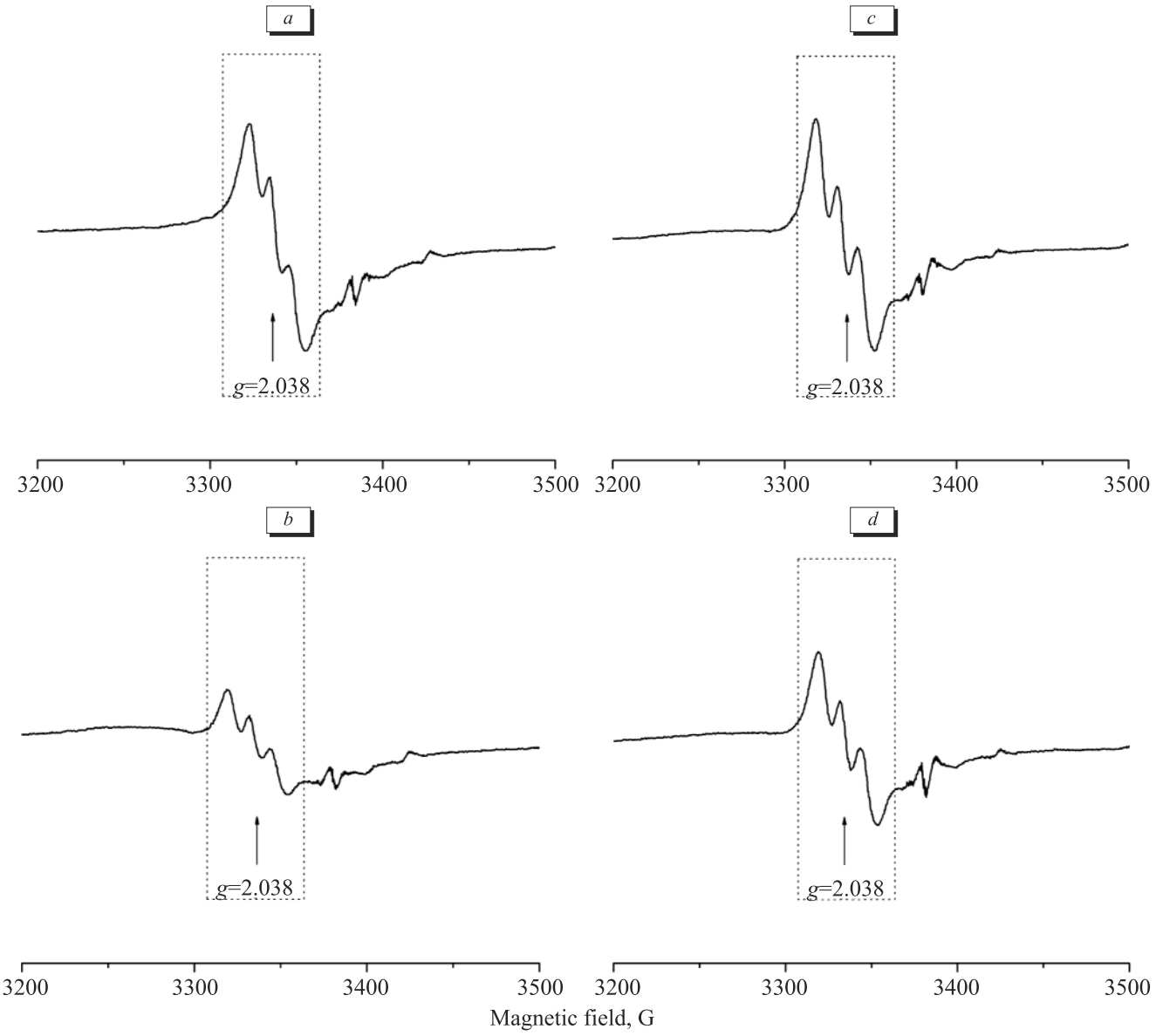

Fig. 3. EPR signals from nervous system (after subtraction of complex Cu-$(DETC)_2$ spectrum) of experimental snails. *a*) intact snails; *b*) snails with developed LS; *c*) snails receiving LAT-S100; *d*) snails receiving LAT-S100 before LS formation.

RESULTS

The results of LS formation were described in our previous reports [11]. Presentation of electrical stimuli for 4 days increased the closed pneumostome time from 15.2±0.6 to 61.7±2.8 sec ($p<0.01$), which confirmed LS formation. Preliminary saline injection before electric stimulation had no effect on LS formation and snail behavior. However, preliminary injection of AB-S100 in dilution of 10^{-12} before LS formation (10 minutes before the first electric stimulus) prevented potentiation of ommatophore withdrawal defense reaction and significantly reduced lengthening of the closed pneumostome period in comparison with snails receiving saline [11]. Changes in locomotor activity in both groups were similar.

NO production edible snail tissues (nervous system and heart) after the same influences was measured by EPR (Fig. 1, *a-c*). After injection of spin trap components, well-resolved EPR signals from $(DETC)_2$-Fe^{2+}-NO complex with g-factor 2.038 and triplet ultrafine structure were observed in all studied tissues. The integral intensity of these signals is the measure of NO amount produced during the presence of the spin trap in the body. Since copper is the binding ion in the hemolymph of edible snail, paramagnetic complex Cu-$(DETC)_2$ is formed

apart from the paramagnetic complex $(DETC)_2$-Fe^{2+}-NO (Fig 1, *d*). This complex has a multicomponent spectrum and one its line overlaps with the analyzed triplet of the spin trap. The spin trap signal can be isolated after subtracting of the Cu-$(DETC)_2$ spectrum from the total EPR spectrum (Fig. 2).

A total of 60 snails were used: 15 snails comprised the control group, 20 snails daily received LAT-S100 and 10 snails received saline before electric stimulation, and 15 snails daily received LAT-S100 solution without electric stimulation. After formation of LS of the defense reflex, the intensity of NO production in snails markedly decreased (Fig. 3). The analysis of NO content in snails receiving antibodies to Ca^{2+}-binding protein S-100 and sensitisized snails receiving and not receiving AB-S100 showed that injections of LAT-S100 insignificantly increased NO concentration in the nervous system, while NO production reduced after LS formation recovered in case of preliminary AB-S100 injection. Integral intensity of the triplet signal from the spin trap is proportional to NO amount (Fig. 3). Integral intensity was assessed by the double integration of the triplet signal.

Thus, we showed that formation of LS, the neurobiological model of anxiety and depression, is accompanied by a decrease in NO formation rate in snails. Comparative analysis of EPR measurements of NO concentration in the nervous system and heart of edible snails indicates that AB-S100 disturb the formation of LS, *i.e*. can eliminate the phenomena and signs of anxiety and this effect is accompanied by recovery of NO concentration. These results suggest that Ca^{2+}-binding protein S-100 imbalance can inhibit or modulate some processes developing during plastic changes in the organism, especially during pathological processes.

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