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ESR Study of the Nitric Oxide Production in Tissues of Animals under an External Influence on the Functioning of the Cardiovascular and Nervous Systems

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Abstract. Electron spin resonance (ESR) of the ternary $(DETC)_2$–Fe^{2+}–NO complex has been applied to determine the nitric oxide production in tissues of rats and snails. A preliminary ESR study of the NO content in tissues of rats before and after artificially induced acute myocardial infarct was performed. The analysis of the obtained results shows that the nitric oxide production during the first hour after the moment of inducing myocardial infarct decreases. It is also demonstrated that ESR may be useful in the study of the influence of the long-term sensitization of snails on the nitric oxide production in their body. The changes in the NO production after the external influences in both cases are discussed.

1 Introduction

Nitric oxide (NO) is one of the most important transmitters which participates in the functioning of various systems of an organism. For vertebrates, its role in the functioning of the cardiovascular system is of particular importance. Here NO controls the vascular tonus, arterial pressure, proliferation of endothelial and smooth-muscular cells of the vascular walls [1–3], participates in the occurrence of atherosclerosis and hypertension, controls contractility of the myocardium [4–6]. In these processes, NO plays the role of the molecule modulating adrenergic and cholinergic influences on the heart [7, 8]. The endothelial cells of the coronary vessels are the source of NO [9, 10]. The formation of the excessive amount of NO can promote the damage of cells inducing even their death. A superfluous formation of NO can noticeably decrease the tonus of the smooth-muscular cells, deteriorate the function of the endothelial tissue and directly inhibit the retractive function of the myocardium [11]. The latter is observed at the septic

and hemorrhagic collapse [12], and acute infarct [13]. According to the opposite opinion, an excessive amount of NO serves as the compensating factor and favors the tissue perfusion and prevents arrhythmia at reperfusion [11]. NO is able to actuate the apoptosis of the smooth-muscular cells [14] and cardiomyocytes [15]. The NO effects are coupled with the influence of NO on the ion channels, the transmitter release, the calcium ions exchange, the cell metabolism and its genome [16]. Therefore pronounced effects of the endogenic and exogenic NO on the functioning of the heart can be expected. These effects are coupled with the change of the regulation of the heart through the cholinergic receptors and adrenoreceptors. Thus two opposite kinds of the NO influence are possible. First, it is a stimulating positive influence. Second, it is a toxic and damaging action leading to the cell death. Most probably only the amount of NO in the body matters. On this basis it is not clear what quantity of NO in tissues of cardiovascular system can be considered as small or large.

The role of NO in the functioning of the nervous system is also very important. It is well known [17–19] that NO participates in the development, maturation and aging of brain, forms learning and memory processes. At the moment it is considered that NO plays the role of a signal molecule in different parts of the nervous system influencing the function of synaptic formations. NO-synthesizing neurons have been also found in the nervous system of invertebrates including mollusks [20]. Invertebrates are very suitable for the study of their behavior because of the simplicity of their nervous system consisting of a small amount of giant nervous cells. On the scale of single cells it is possible to deduce not only the sensor signals entering the nervous system and its output but also a set of intermediate events motivating one or another behavior reaction [21, 22]. The molecular mechanisms of memory retention are extensively studied in the context of the interrelation of associative learning with sensitization as the elementary form of the nonassociative training. At this training an animal learns to increase the response on the stimulus earlier noneffective which follows after the application of strong stimulus in another area. One of such forms of nonassociative training of mollusks is the long-term sensitization which influences the defensive and food reflexes in different directions [23, 24]. So far the relationship between the formation of the long-term sensitization and the quantity of the NO synthesized by neurons has not been studied.

Summarizing the above observations, one can conclude that the study of the NO production in tissues of animals before and after the experimental myocardial infarct and in tissues of mollusks before and after the formation of the long-term sensitization is of special interest.

Recently, electron spin resonance (ESR) became the most effective method to detect and to quantitatively determine the NO content in biological tissues (see, e.g., refs. 25–27). This occurred due to the procedure suggested by Vanin et al. [28], who used the spin-trapping method. This method is based on the reaction of a radical (incidentally of NO) with a spin trap. As a result of this reaction, an adduct with the typical ESR spectrum is formed. Vanin et al. [28] used the Fe^{2+} complex with diethylthiocarbamate (DETC) to trap NO and to form the

stable ternary $(DETC)_2$–Fe^{2+}–NO complex in different tissues of animals. The spectral feature of the ternary $(DETC)_2$–Fe^{2+}–NO complex is the easily recognizable ESR spectrum with a g-value of 2.035–2.040 and with the a triplet hyperfine structure. Later on [25, 29], it has been shown that with an appropriate organic solvent of the $(DETC)_2$–Fe^{2+} complex nonsoluble in water it is possible to reach a good linear relationship between the intensity of the ESR signal of the $(DETC)_2$–Fe^{2+}–NO complex and the concentration of NO and to improve the detection threshold of this method down to 50 nM.

In this article we present preliminary results of the ESR study of the NO production in different tissues of rats in good health and in the ischemia state in the course of the artificially caused acute myocardial infarct. The comparative analysis of the NO content in different tissues of rats in good health and in the ischemia state shows that the NO production is decreased in the body of animals with heart ischemia. In addition, we demonstrate the possibility to apply this procedure to study the relationship between the formation of the long-term sensitization and the quantity of NO synthesized by neurons of mollusks.

2 Experimental

2.1 Reference Samples

To identify the ESR spectrum of the $(DETC)_2$–Fe^{2+}–NO complex in the studied tissues of the animals, synthetic reference samples containing a certain amount of NO trapped by the $(DETC)_2$–Fe^{2+} complex have been prepared. For the preparation of these reference samples the saturated solution of the DETC–Na and iron citrate (iron sulfate ($FeSO_4$) plus sodium citrate) were used. We added the known concentration of the sodium nitroprusside as an NO donor to this solution instead of the endogenic NO from biologic samples. We diluted all substances in bidistilled water. The $(DETC)_2$–Fe^{2+} complex nonsoluble in water which was formed as a result of this reaction was dissolved in the organic solvent (we used ethyl acetate). For ESR measurements the organic samples with equal weights were put into the quartz tubes with a diameter of 3 mm. ESR experiments were performed on a Bruker ER-200 spectrometer at X-band at the liquid nitrogen temperature. The typical ESR spectrum of a reference sample is shown in Fig. 1.

The analysis of the observed spectrum showed that the signal with $g = 2.04$ completely originates from the $(DETC)_2$–Fe^{2+}–NO complex. The amplitude of the ESR signal was compared with the amplitude of the ESR signal of diphenylpicrylhydrazyl (DPPH) sample which was always placed into the microwave cavity of the spectrometer and kept at room temperature. The ESR signal intensity of the $(DETC)_2$–Fe^{2+}–NO complex in reference samples had a good linear relationship with the concentration of sodium nitroprusside (or NO).

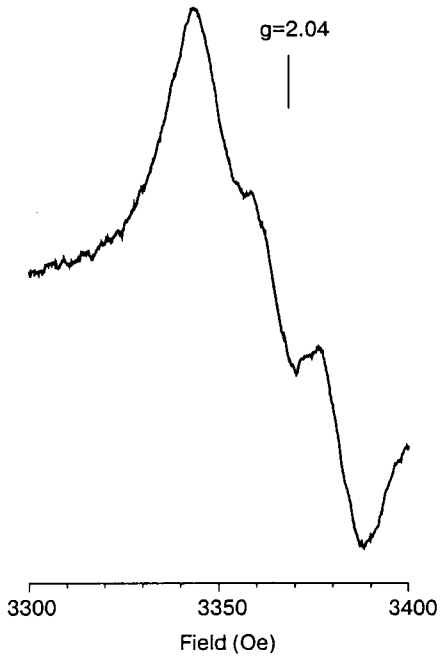

Fig. 1. ESR spectrum of the $(DETC)_2$–Fe^{2+}–NO complex in the reference sample. The known concentration of the sodium nitroprusside as an NO donor was added into this sample instead of the endogenic nitric oxide from biologic samples.

2.2 Formation of the Ternary $(DETC)_2$–Fe^{2+}–NO Complex in Tissues of Rats and Snails

The experiments were performed with male white rats with a weight of 180–260 g. In accordance with the Vanin's procedure [27, 28] in order to form the $(DETC)_2$–Fe^{2+}–NO complex, the water solution of the DETC–Na complex was injected into the bodies of rats intraperitoneally at a dose of 500 mg/kg in 2.5 ml of water and solution of iron citrate (II) (iron sulfate (II) at a dose of 37.5 mg/kg plus sodium citrate at a dose of 187.5 mg/kg) subcutaneously. The DETC–Na complex interacting with iron citrate (iron sulfate ($FeSO_4$) plus sodium citrate) forms a DETC–Fe (II) complex nonsoluble in water which in its turn traps NO. As a result, the stable $(DETC)_2$–Fe^{2+}–NO complex which can be detected by ESR is formed. Forty minutes after the injection of chemicals the rats were decapitated. We took the heart, liver and pancreas for the sample preparation. The extracted organs were quickly dried, weighed and cooled in the liquid nitrogen in special disposable syringes for the following measurements.

For our experiments with mollusks, a grape snail *Helix lucorum* of the Crimea population has been chosen as a subject of study. In these experiments sexually mature individuals homogeneous in size and weight (30 g) have been used. In order to form the ternary $(DETC)_2$–Fe^{2+}–NO complex, we injected into the body

of the snails the same solution as we used for rats recalculating the amount of the animal's weight. We used the nervous system and heart of the snails as the samples for our study. Due to small sizes of their organs, we used two snails for the preparation of each sample.

3 Results

3.1 ESR Spectra of Tissues of Rats in Good Health

Most of measurements we performed on tissues of the heart, liver and pancreas of rats in good health because ESR spectra of the $(DETC)_2$–Fe^{2+}–NO complex for these organs were most intensive. The weight of the samples was in the range between 150 and 300 mg, but most of the samples were of 200 mg. The amplitude of the ESR spectrum was normalized to the weight of the sample and to the amplitude of the ESR signal of DPPH. As it was mentioned above, the DPPH reference was always kept in the microwave cavity of the spectrometer at room temperature.

The typical ESR spectrum of the frozen right-hand side of the heart of a rat in good health 40 min after the injection of DETC plus Fe^{2+}-citrate is shown in Fig.. 2 (spectrum a). For other studied organs we obtained similar spectra. The comparison of the obtained spectra with the ESR spectrum of the reference sample (Fig. 1) allows us to refer univocally the observed signals to the ESR signal of the ternary $(DETC)_2$–Fe^{2+}–NO complex. It is necessary to note that unlike the ESR spectrum of the reference samples, for tissues we observed

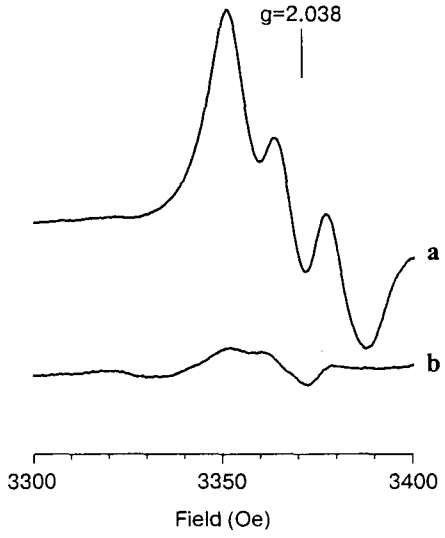

Fig. 2. ESR signal of the heart of rats in good health (**a**) and in the ischemia state (**b**).

the ESR signals with $g = 2.00$ and $g = 1.94$ in addition the ESR signal of the $(DETC)_2$–Fe^{2+}–NO complex with $g = 2.038$ and triplet hyperfine structure (these additional signals do not fall into the magnetic field range shown in figures). Most probably the latter are due to free radicals and the Fe^{2+} and Cu^{2+} complexes. However, here we will not discuss their nature and will concentrate our attention on the ESR signal of the $(DETC)_2$–Fe^{2+}–NO complex.

3.2 ESR Spectra of Tissues of Rats in the Ischemia State

In experiment, the acute myocardial infarct was induced following the Selye et al. procedure [30] by banding the right-hand coronary artery. Ten to fifteen minutes after inducing the experimental myocardial infarct, the $(DETC)_2$–Fe^{2+} complex was injected into the body of animals (the experiments have been performed with 20 animals), and after 40 min the rats were decapitated. 23 rats in good health were used as reference.

The ESR measurements were performed on tissues of the heart, liver and pancreas. Since the accumulation of NO by the spin trap took place during the first hour after inducing the experimental myocardial infarct, the state of the animals under study was closer to the ischemia state than to the infarct. The typical ESR spectrum of the frozen part of the right-hand side of the heart of a rat 40 min after the injection of the $(DETC)_2$–Fe^{2+} trap is presented in Fig. 2 (spectrum b).

An essential difference between ESR spectra of the heart of the rat in good health (Fig. 2, spectrum a) and the heart of a rat in the ischemia state (Fig. 2 spectrum b) is clearly seen. The same result was obtained for all other studied organs. Figure 3 demonstrates the integral intensities of the resonance lines of the $(DETC)_2$–Fe^{2+}–NO complex for the different tissues of rats in good health

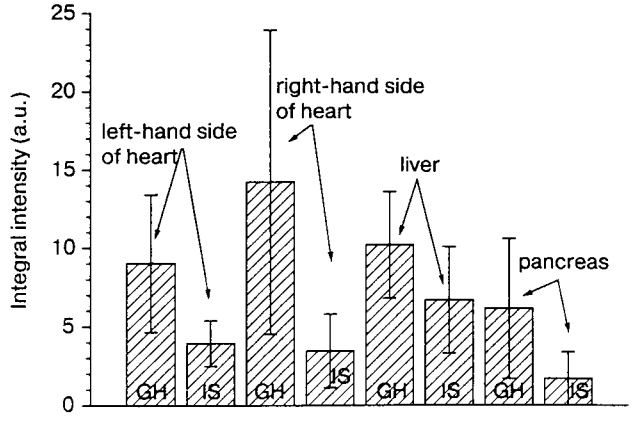

Fig. 3. Integral intensities of the ESR lines of the $(DETC)_2$–Fe^{2+}–NO complex for the different tissues of rats in good health (GH) and in the ischemia state (IS).

and in the ischemia state averaged over all measurements. The integral intensity of the ESR lines of the $(DETC)_2$–Fe^{2+}–NO complex for the animals in the ischemia state is noticeably smaller than that of the animals in good health.

3.3 ESR Spectra of Tissues of the Unschooled Snails

ESR measurements have been performed with the tissues of nervous system and heart of snails. Well-resolved ESR spectra of the $(DETC)_2$–Fe^{2+}–NO complex were observed for both organs of snails 40 min after the injection of the $(DETC)_2$–Fe^{2+} spin trap. The ESR spectrum of the nervous system of these reference unschooled snails are shown in Fig. 4 (spectrum a). For tissues of the heart we obtained similar spectra.

3.4 ESR Spectra of Tissues of the Snails after the Formation of the Long-Term Sensitization

The long-term sensitization of the defensive reflex of mollusks was produced by previously developed procedure [24]. The animals were influenced by the electrical pulse packet on the head area 4 times per day during four days with the interval of 1.5–2 h. The duration of each packet was 0.5 s. The amplitude of the square-wave pulses within a packet was 6–8 mA, duration was 10 ms, and the frequency of the repetition was 50 Hz. The real amplitude of the current during the pacing was controlled with an oscilloscope. During the electrical provocation the animals were placed on a copper electrode plate covered by a

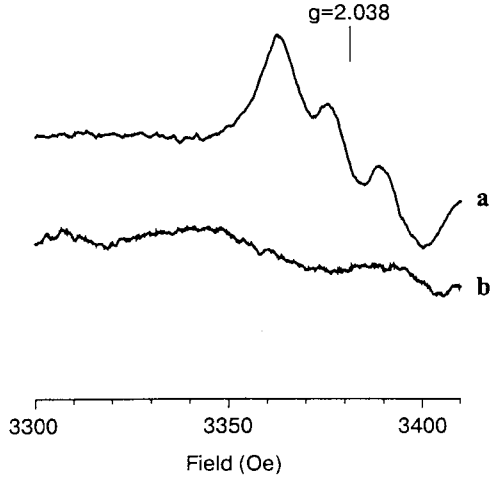

Fig. 4. ESR signal of the nervous system of the unschooled snails (**a**) and of the snails after the formation of the long-term sensitization (**b**).

layer of a wet paper. A metallic rode attached to the head area of the snail represented the second electrode. The criteria for the long-term sensitization was a considerable increase of the pneumostome closing time as a reply to the testing irritation in comparison with initial reaction. The full closing of the pneumostome was determined as the positive reaction on the stimulus.

After establishing the fact that the long-term sensitization is formed, the spin-trap DETC plus Fe^{2+}-citrate was injected into the body of snails. The measurements were performed similar to those for the reference snails. The ESR spectrum of the $(DETC)_2$–Fe^{2+}–NO complex in the nervous system of snails after the formation of the long-term sensitization is shown in Fig. 4 (spectrum b).

The comparison of the corresponding ESR spectra for nervous system of the body of snails shows that after the formation of the long-term sensitization of the defensive reflex the NO production in the body of snails is noticeably decreased. The same result was obtained for the heart tissues of snails. We tested 10 unschooled snails and 10 snails after the formation of the sensitization of the defensive reflex.

4 Discussion

The comparison of the ESR spectra of the ternary $(DETC)_2$–Fe^{2+}–NO complex in tissues of rats in good health and rats in the heart ischemia state shows that the intensity of the ESR spectrum for animals with the heart ischemia is noticeably smaller than that for healthy animals. These results are in contradiction with the data obtained by Vanin et al. [15] that after the myocardial infarct the NO production by organism increases. We suppose that this contradiction is caused by the time difference in taking the probe on the NO content. As it was mentioned above, in our study the accumulation of NO by the spin trap took place during the first hour after inducing the myocardial infarct. In ref. 15, the probes were taken 24 h after inducing the infarct and later. In order to eliminate the above contradiction, we suppose that the NO production abruptly decreases at the moment of inducing the infarct (or in the ischemia state). This is what we observe in our experiment. We may also suppose that with time after inducing the infarct the NO production in the body of animals starts to increase abruptly reaching the values which are higher than the normal level. Just this increase of the NO production was probably observed by Vanin et al. [15]. Of course, in order to confirm or to neglect these suppositions, a systematic study of the NO production at different moments after inducing the infarct is necessary. Nevertheless, it is possible to conclude that the expected effects of the endogenic and exogenic nitric oxide on the functioning of the heart [16] are really observed experimentally. They are due to the change of the regulation of the heart through the cholinergic receptors and adrenoceptors. This means that the quantitative determination of the NO content in tissues of rats at different stages of behavior of the experimental infarct may give an opportunity to develop a method for the diagnostics of the dynamics of the myocardial infarct.

Our study shows that ESR of the ternary $(DETC)_2$–Fe^{2+}–NO complex formed by the corresponding spin trap injected into the body of animal gives a possibility to study the influence of the long-term sensitization on the NO production in different tissues of mollusks. Our results for snails are very similar to those for rats. Here we also observed a decrease of the NO production after the external influence.

5 Summary

A preliminary ESR study of the NO content in different tissues of rats before and immediately after artificially induced acute myocardial infarct shows that the NO production during the first hour of the ischemia state decreases.

The possibility to use this method when studying the influence of the long-term sensitization on the NO content in tissues of mollusks is demonstrated. It is found that the NO production decreases after the formation of the long-term sensitization of the defensive reflex.

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