14

Application of EPR spectroscopy to study the content of NO and copper in the frontal lobes, hippocampus and liver of rats after brain ischemia

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Electron paramagnetic resonance (EPR) spectroscopy was used to record the content of nitric oxide (NO) and copper in brain tissues (frontal lobes and hippocampus) and liver of healthy rats and rats after ischemia modeling. Ischemia was simulated by ligation of the carotid arteries, followed by taking 3 ml of blood from the common carotid artery. Signals from triple complexes (DETC) were recorded by EPR spectroscopy of complexes $(DETC)_2$-$Fe^{2+}-(NO)$ and $Cu(DETC)_2$. Based on direct measurements by EPR spectroscopy, it was shown that a day after the modeling of ischemia, NO production in the hippocampus decreases by an average of 30% and there is a tendency to decrease NO in the frontal lobes and liver. The copper content decreased by an average of 3 times in the frontal lobes and the hippocampus by an average of 20% a day after ischemia modeling, and a tendency to decrease was noted in the liver. Thus, brain hypoxia is accompanied not only by a decrease in NO production, but also by signs of weakening of the antioxidant system in the hippocampus and frontal lobes, which further worsens the functional state of the homeostasis system.

Keywords: electron paramagnetic resonance, spin trap, nitric oxide, cerebral ischemia, frontal lobes, hippocampus.

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Introduction

Nitrogen oxide (NO) is one of the key signal molecules which control physiological functions of the body, including nervous system [1,2]. NO is produced from *L*-arginine amino acid by enzymes from NO-synthase (NOS) family and participates in control of a set of cell functions, including blood vessel tone control, neurotransmission, learning, immune response and other functions [3]. In life of animals and humans, the role of NO in functioning of cardiovascular [4,5] and nervous systems [1,6]. Participation of NO in the development mechanisms of various pathological states of body is of great interest [4,7,8]. Fact have been accumulated which demonstrate that NO biosynthesis is one of the key factors in pathophysiological response of brain to hypoxia–ischemia [6,9,10]. NO performs its physiological functions by bonding with ferrous ions (Fe) as part of haem or through S-nitrosylation of proteins and is involved in a range of biochemical reactions [11].

Long-term oxygen deficiency leading to brain hypoxia is one of the reasons why NO is involved in a pathological process. hypoxia is accompanied by the development tissue ischemia which always occurs when oxygen supply tissues does not meet the actual demand of tissues [6,9]. In case of reduced oxygen content in inspired air, cerebral blood flow disorders leading to lack of oxygen supply of brain regions, brain ischemia occurs which can finally cause ischemic stroke accompanied with brain tissue and function damage [12,13]. Therefore, study of pathogenesis, prevention methods, correction and stroke mechanisms is important both in terms of theory and practice.

Currently, development of brain ischemia followed by stroke are associated with brain blood flow disorders and with brain control disorders by NO system [14,15]. Unfortunately, there is no single opinion regarding the role of endogenous NO in the processes flowing during nervous system damage [16]. And this can be explained. There is a large amount of methods for NO production measurement in biological systems. Recently, electron paramagnetic resonance method (EPR) has become one of the most effective methods of NO detection and quantitative determination in biological tissues [11,17,18]. This was ensured by a technique developed by Vanin et al. who used a spin trapping based on radical (NO in this case) reaction with a spin trap [19]. As a result of the reaction, an adduct with typical EPR spectrum is formed. It has been found that Fe^{2+} and diethyldithiocarbamate (DETC) forms stable ternary complex $(DETC)_2$-$Fe^{2+}-NO$ in case of NO trapping. These complexes are characterized by an easily recognized EPR spectrum with g-factor $g = 2.035-2.040$ and triplet extra-thin structure [11,18]. The method's sensitivity is $0.04-0.4$ nM, the method allows to carry out direct measurements, is high sensitive due to the use of spin traps.

Thus, NO content dynamics in brain tissues during occurrence and progress of brain ischemia is still understudied, despite the fact that the main contribution is made by NO produced by nNOS and iNOS [1,14,20].

The purpose of the research was to study the consequences of experimental ischemic brain damage on NO production intensity in rat frontal lobes, hippocampus and liver using EPR spectroscopy with spin traps,

1. Experimental procedure

1.1. ischemic stroke simulation in rats

Ischemic brain damage was simulated in accordance with the approved protocol of the Ethics Committee of the Institute of Physiology, National Academy of Sciences of Belarus (NAS Belarus), Minsk. Tests were carried out in day time on 4-week male white rats (initial weight 139–145 g).

Animals were kept in standard vivarium conditions (with maintenance of 12/12-hour lighting and darkness rate, air temperature at $23 \pm 1^{\circ}$C and stable supply- exhaust ventilation) with free access to water and food (*ad libitum*) and food ration in accordance with laboratory animal care rules.

The animals were subdivided into two groups: 1-st group ($n = 10$) — intact (control) rats, control group of animals was not subjected to surgery interventions and was tested in the same conditions as other groups of rats; 2-nd group ($n = 10$) — rats subjected to hypotoxic exposure (10 min blood flow interruption by means of carotid artery ligation at vocal chord level and collection of 3 ml blood from the total carotid artery).

1.2. Compliance with ethical standards

For the purpose of the research, animals (rats) were used, animal handling standards were complied with. Stroke was simulated in accordance with the approved protocol of the Ethics Committee (protocol № 1 dated 31.01.2019) of the Institute of Physiology, National Academy of Sciences of Belarus (NAS Belarus), Minsk.

All operations and tissue extractions were carried out in anaesthetized animals (55.6 mg/kg ketamine, 5.5 mg/kg xylazine, 1.1 mg/kg acepromazine, abdominally) [10,21].

1.3. Generation of ternary $(DETC)_2$-Fe^{2+}–NO complex in rat tissues

For sample preparation for EPR spectra measurement, spin trap technique was used [17]. As earlier [22,23], spin trap components for nitrogen oxide (DETC–Na, $FeSO_4$, sodium citrate) were introduced 30 min before extraction of the test tissues. As spin trap, Fe^{2+} and diethyldithiocarbamate complex was used— $(DETC)_2$-Fe^{2+}. Spin trap and NO ($(DETC)_2$-Fe^{2+}–NO) complex is characterized by an easily recognized EPR spectrum with g-factor $g = 2.038$ and triplet extra-thin structure [11, 17, 24] DETC-Na was introduced abdominally in a dose of 500 mg/kg per 2.5 ml of water. Solution mixture: iron sulfate ($FeSO_4 \cdot 7H_2O$, Sigma, USA) in a dose of 37.5 mg/kg and sodium citrate in a dose of 187.5 mg/kg (1 ml of water per 300 g of animal weight), prepared directly before introduction was introduced subcutaneously in three points — right and left hip and rostral part of interscapular region. The experiment and methods are detailed in [23,24]. Tissue samples were frozen immediately in liquid nitrogen and were transferred in frozen condition from Minsk to Kazan in plastic containers with dry ice. Spin trap and NO complex ($(DETC)_2$-Fe^{2+}–NO) is kept well in such condition and the signal from the complex is not changed within a month [18]. In addition, spin trap interacts with Cu and forms $Cu(DETC)_2$ complex which can be also recorded by EPR spectroscopy method [25].

1.4. Measurements by EPR spectroscopy method

Spectra of $(DETC)_2$-Fe^{2+}–NO and $Cu(DETC)_2$ complex were measured using Bruker X (9.50 GHz) EMX/plus spectrometers with ER 4112HV and ER 200 SRC temperature accessory with magnetic field modulation 100 kHz, $2 \cdot 10^{-4}$ T modulation amplitude, microwave emission power 30 mW, time constant 200 ms and temperature 77 K in Bruker finger-type Dewar flask. Modulation amplitude, amplification and microwave power in all experiments were selected with the assumption of the absence of remodulation and EPR signal saturation, and these parameters were kept unchanged throughout the measurements. Directly before the measurement, the prepared sample cut to the shape of the measurement cell was weighed. Sample weight was about 100 mg. EPR spectra amplitude was always rated to the sample weight and EPR signal amplitude of the reference sample (EPR signal measurement techniques were detailed before in [26]).

1.5. Statistical processing of the result

The result is presented as $M \pm m$ (average $\pm$ standard error of mean). Statistical data processing was carried out using Student's t-criterium. The differences were considered relevant at $p < 0.05$.

2. Study results and their discussion

EPR spectroscopy method was used to study NO production intensity and copper content (as indicator of 1st and 3rd superoxide dismutase subunits) in frontal lobes, hippocampus and liver in brain hypoxia simulation caused by carotid artery ligation during 10 min on both sides and collection of 3 ml of blood from the total carotid artery.

Figure 1 shows EPR spectra of frontal lobes of the control rat (Figure 1, *a*) and the rat after hypoxia caused by carotid artery ligation followed by collection of 3 ml of blood from the common carotid artery (Figure 1*b*) 24 h after

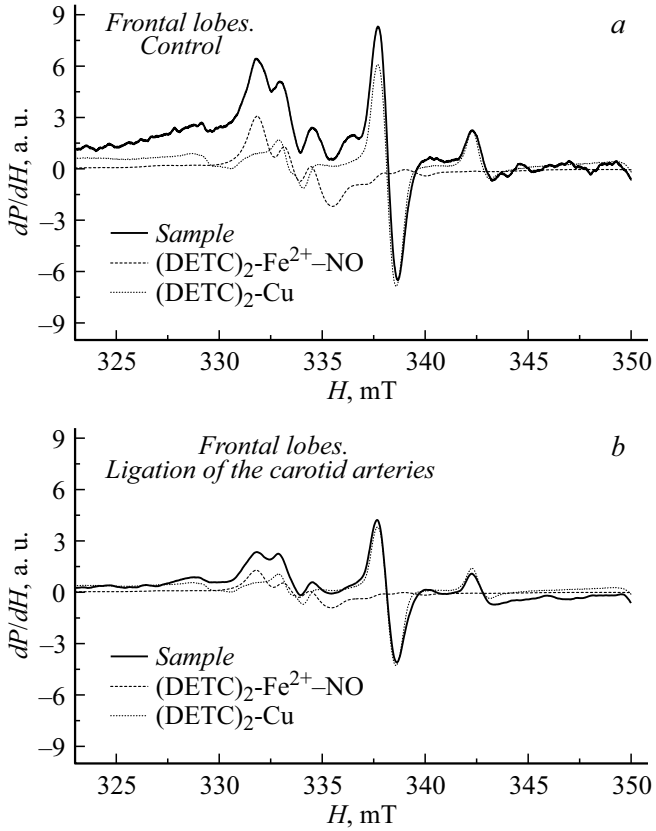

Figure 1. Examples of EPR spectra of frontal lobes of a control rat (a) and a rat after hypoxia caused by carotid artery ligation followed by collection of 3 ml of blood from the common carotid artery (b) 24 h after brain ischemia. The animals were injected with spin trap components $(DETC)_2$-Fe^{2+} — citrate. $g_{cp} = 2.038$.

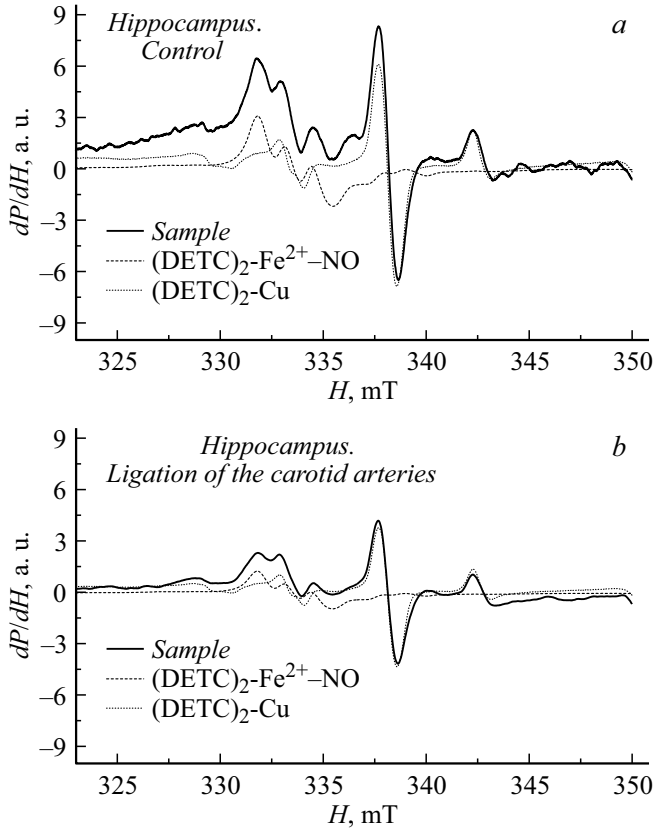

Figure 2. Examples of EPR spectra of hippocampus of a control rat (a) and a rat after hypoxia caused by carotid artery ligation followed by collection of 3 ml of blood from the common carotid artery (b) 24 h after brain ischemia. The animals were injected with spin trap components $(DETC)_2$-Fe^{2+} — citrate. $g_{cp} = 2.038$.

brain ischemia. This spectrum shows typical triplet signal from $(DETC)_2$-Fe^{2+}–NO complex with g-factor equal to 2.038 [17]. Moreover, signal from $(DETC)_2$-Cu complex is also present in this region. Figure 2 shows EPR spectra of hippocampus tissues in normal rats (Figure 2, a), and rats on day 1 after ischemic stroke simulation (caused by carotid artery ligation followed by collection of 3 ml of blood from the common carotid artery) (Figure. 2, b). Solid line shows the spectrum of the sample, dashed line shows the signal from nitrogen oxide associated with spin trap as part of the complex spectrum $((DETC)_2$-Fe^{2+}–NO$)$. Relative change of the amount of NO-containing complex and $Cu(DETC)_2$ complex was assessed by the integral intensity of signal from these complexes.

Figure 3 shows statistical data by integral signal intensities $(DETC)_2$-Fe^{2+}–NO in spectra of test samples of biological tissues which express spectra features in case of ischemic stroke caused by carotid artery ligation followed by collection of 3 ml of blood from the total carotid artery for NO production assessment in brain tissues (frontal lobes and hippocampus) and liver 24 h after ischemia simulation. Analysis results demonstrate reduction of NO production after ischaemic stroke simulation in hippocampus by 30% on average ($p < 0.05$) and downward trend of NO production in frontal lobes and liver. Figure 4 shows statistical data by integral signal intensities $(DETC)_2$-Cu. The results show that copper content 24 h after the ischemia simulation is definitely reduced in frontal lobes on average by a factor of 3 ($p < 0.05$) and in hippocampus on average by 20% ($p < 0.05$), and in only a downward trend is noted in liver. Thus, hypoxia is followed not only by reduced NO production, but also by hippocampus antioxidant system degradation and in frontal lobes which additionally deteriorates the functional condition of nervous system.

Brain stroke is a key cause of death and the most often cause of disability worldwide [12]. It is known that hypoxia is followed by the brain region oxygen supply failures therefore brain ischemia occurs which often finally causes ischemic stroke [27]. Earlier, we carried out experiments where we simulated ischemic stroke by various methods: 5 min hypobaric hypoxia, which was achieved by conditional lifting of animals at 4500 m above sea level [24], by carotid artery ligation [8,28]. A more complicated version was used herein — carotid artery ligation was combined with collection of 3 ml of blood from the common carotid artery.

This research clearly shows that brain ischemia development is followed by reduced NO production intensity.

On the one hand, development of brain ischemia followed by stroke are associated with cerebral blood flow disorder and with brain tissue blood flow control disorders by NO system [6,9,14,26,29]. On the other hand, hypoxia itself caused by ischemic stroke is followed by early death of cells in various brain regions and then programmed late death of other brain cells occurs by apoptosis [30]. In this hypoxia- ischemia processes, NO role is questionable: NO is capable of performing neurotoxic and neuroprotective functions [9,16]. Reasons of contradictory functions of NO include synthesis by different NO-synthases as a main source of NO [6,14], presence of nitroreductase component of NO cycle [4,9], and significant number of depot for NO which interact with iron-containing complexes (e.g. haem structures) with thiols and other compounds [10,31,32].

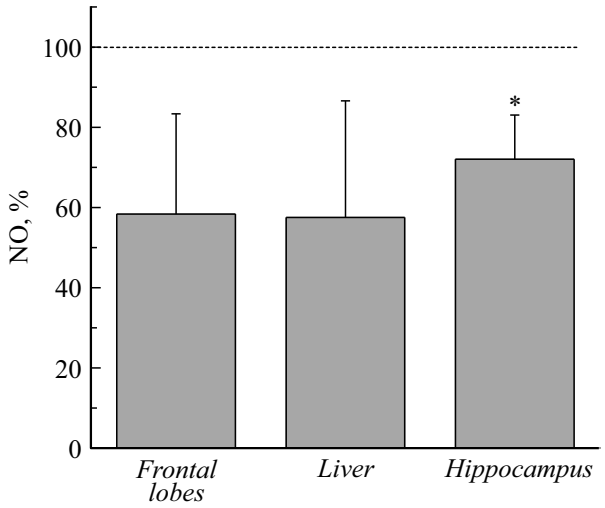

Figure 3. NO content in brain frontal lobes, liver and hippocampus in hypoxia simulation caused by carotid artery ligation 24 h after ischemia. Y-axis — specific intensity of signal of $(DETC)_2$-Fe^{2+}−NO complexes in tissue samples of animals after ischemia simulation, in percentage of signal intensity of $(DETC)_2$-Fe^{2+}−NO complexes in a control group. * — difference from control (t-test, $p < 0.05$).

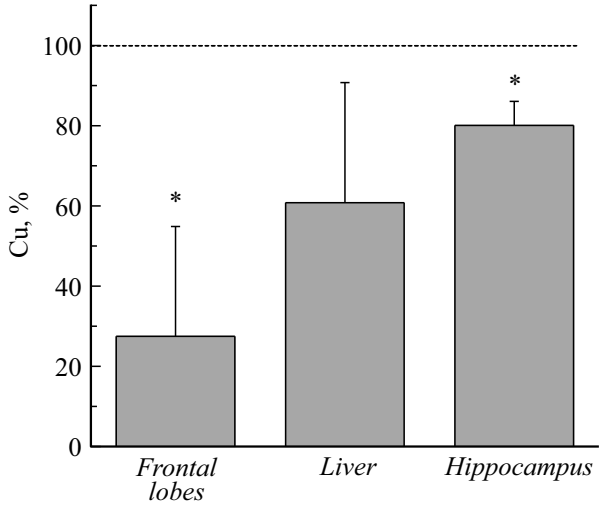

Figure 4. Copper content in brain frontal lobes, liver and hippocampus in hypoxia simulation caused by carotid artery ligation 24 h after ischemia. Y-axis — specific intensity of signal of $Cu(DETC)_2$ complexes in tissue samples of animals after ischemia simulation, in percentage of signal intensity of $Cu(DETC)_2$ complexes in a control group. * — difference from control (t-test, $p < 0.05$).

Conclusion

EPR spectroscopy method is used to study intensity of NO production and copper content (as superoxide dismutase indicator) in rat frontal lobes, hippocampus and liver after stimulation of ischemic damage of brain. These molecular components constantly draw attention of researchers who study brain functioning mechanisms in normal and pathological conditions. It has been shown that 24 h after ischemia simulation NO production is reduced in hippocampus and NO reduction trend is observed in frontal lobes and liver, and copper content is definitely reduced in frontal lobes and hippocampus and in liver ($p < 0.05$) to a small extent. Thus, it may be supposed that hypoxia is followed not only by reduced NO production, but also by hippocampus antioxidant system degradation and in frontal lobes which additionally deteriorates the functional condition of nervous system.

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Conflict of interest

The authors declare that they have no conflict of interest.

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