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COMPLEX SYSTEMS BIOPHYSICS

Studies of Nitric Oxide Production in Rat Tissues in Postnatal Development by Electron Paramagnetic Resonance Spectroscopy

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Abstract—Paramagnetic complexes containing nitric oxide (NO) have been assayed in rat heart and liver tissues by EPR spectroscopy to investigate the time variation of NO production during postnatal development. Nitric oxide levels have been assessed from the intensity of a characteristic EPR signal belonging to the $(DETC)_2–Fe^{2+}–NO$ complex. The results show that the content of NO in liver tissues increases within the age interval from 28 to 56 days but does not change significantly in postadolescence. In heart tissues, the nitric oxide level increases in adulthood compared to puberty. The NO level in the rat liver is significantly higher than in heart tissues in all studied age groups.

Keywords: nitric oxide, rat, development, heart, liver, electron paramagnetic resonance

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Nitric oxide, NO, is a gaseous compound, which is also a highly labile, short-lived, and reactive free radical. It serves as a chemical messenger in many physiological and pathophysiological processes [1–6]. Living tissues produce NO in two ways: enzymatically and nonenzymatically. The enzymatic NO synthesis is mediated by the enzyme NO synthase, which oxidizes the amino acid L-arginine with the presence of O_2 and NADP-H. A by-product of this reaction is another amino acid, L-citrulline [7]. Coronary and endocardial endothelium and cardiac muscle cells normally produce the bulk of NO and regulate heart functions via vessel-dependent and vessel-independent pathways [1, 8–11]. Nitric oxide controls the vasomotor tone, blood pressure, the proliferation of endothelial and smooth-muscle cells in the vessel wall, and myocardial contractility. It is also involved in the development of atherosclerosis and hypertension [9–14]. The toxic action of NO on cardiac muscle cells in health conditions was shown in [15].

Nitric oxide is widespread in the central and peripheral nervous systems [6, 16, 17]. As a signaling molecule, it modulates adrenergic and cholinergic actions on the heart [4, 10, 16–19]. The NO system is essential in the adaptation of the body to environmental perturbations, including changes in locomotion [20–22]. NO system activation is one of the mechanisms by which the body averts stress-related injuries. This system takes part in the activation of antioxidant enzymes, thereby limiting the stress response [23, 24]. Being highly reactive, NO can be deposited by interactions with various substances: thiols, proteins, sugars, metal ions, hemes, etc. These are kept in a wide range of tissues and organelles, which implies the presence of NO and its complexes in various tissues. The NO stock can be an additional nonenzymatic NO source in the case of a shortage. The nonenzymatic path is generally meant to be nitrite or nitrate reduction to NO [3]. This is one of the ways to prevent toxic effects of excessive NO [25, 26].

The considerable role of NO in many physiological and pathophysiological processes and the scarcity of information on NO synthesis rates in a growing body determine the significance of studies in this field. Therefore, the quantitation of NO as an intracellular, intercellular, histic, and interorganic mediator in various tissues is a topical issue.

MATERIALS AND METHODS

Nitric oxide was assayed in heart and liver tissues of rats of the ages 28, 56, 81, and 110 days ($n = 10$ in each of the four age groups) by the spin tracking method. All applicable regulations for the care and use of animals were followed. The spin trapping technique is based on the reaction of the NO radical with a spin trap molecule [27, 28]. The Fe^{2+} complex with diethyldithiocarbamate (DETC) was used for NO trapping to form the stable triple complex $(DETC)_2–Fe^{2+}–NO$

Abbreviations: NO—nitric oxide; DETC—diethyldithiocarbamate; EPR—electron paramagnetic resonance.

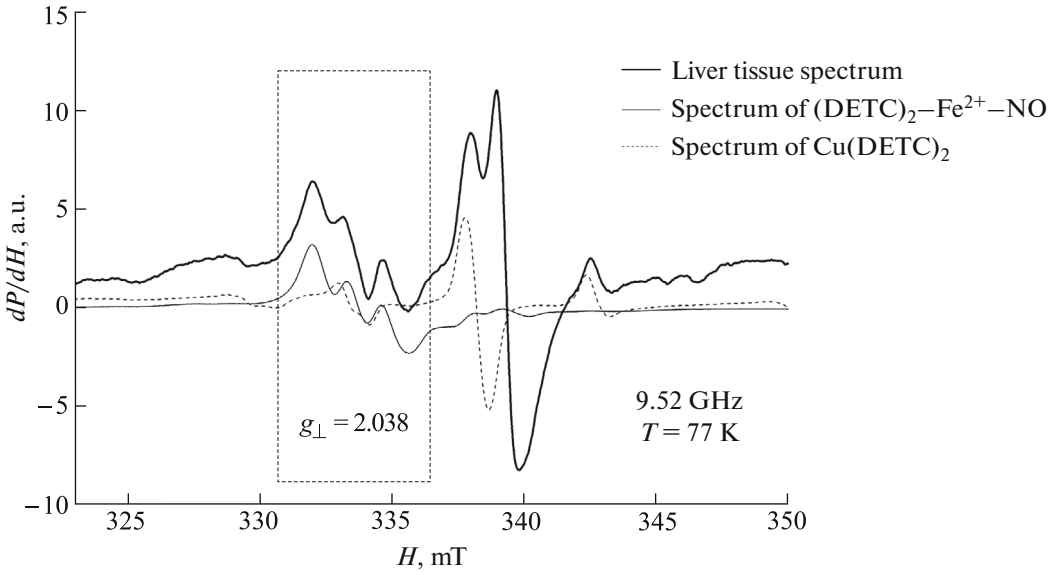

Fig. 1. The EPR spectrum of rat liver tissue. The dashed curve shows the spectrum of the $(DETC)_2-Fe^{2+}-NO$ complex, $p < 0.05$.

[27–29]. Aqueous DETC-Na (500 mg/kg) in 2.5 mL of water was injected intraperitoneally, and iron(II) citrate ($FeSO_4 \cdot 7H_2O$, Sigma, United States; 37.5 mg/kg) + sodium citrate, 187.5 mg/kg was injected intramuscularly, such that the triple complex formed inside the body (for details, see [30, 31]). The DETC–Fe(II) complex interacts with NO to yield the stable radical $(DETC)_2-Fe^{2+}-NO$. This complex is paramagnetic (SFe = 1/2, IN = 3/2); hence, it may be detected by electron paramagnetic resonance (EPR) [28]. The complex is characterized by its easily recognizable EPR spectrum with g = 2.08, and triplet hyperfine coupling (Fig. 1). Nitric oxide levels were assessed from the amplitude of the characteristic EPR signal belonging to the $(DETC)_2-Fe^{2+}-NO$ complex. The signals were compared according to their integrated intensities, as the integrated intensity of an EPR signal varies directly with the paramagnetic complex concentration [29]. Thirty minutes after the injections, the animal was narcotized with urethane, immobilized on a surgery table, and dissected. Organs were quickly dried and frozen in liquid nitrogen in EPR tubes. The EPR spectra were recorded at 77 K with an EPR spectrometer of the X range ER-200E-SRC or EMX/plus (Bruker, United States) with an ER-4112HV temperature control system. All experiments were conducted at the following parameters: microwave power, 30 mW; scan width, 5 G; enhancement, 4×10^4; time constant, 100 ms; scan time, 50 s; and number of scans, 8. The ordinary computer of the Aspect 3000 spectrometer (Bruker, United States) was used for spectrum accumulation and recording.

Statistical evaluation. The mean values of measured parameters and standard errors of the mean $M \pm SEM$ were calculated. The significance of the deviations of mean NO levels in different tissues of rats of different age groups was assessed with the Student's t-test and the Mann–Whitney U-test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

As reported in the literature, 28-day old rats are juvenile; this age corresponds to the maximum rate of cardiac sympathetic innervation development. The 56-day age corresponds to adolescence, which is accompanied by pronounced changes in the endocrine system that profoundly affect the regulation of the cardiac function. The age of 81 days corresponds to the transition from adolescence to sexual maturity; 110-day old rats are sexually mature [32–34]. We believe that our experimental design follows the main phases of rat development and traces the formation of cardiac regulation throughout the postnatal phases. The comparison of EPR spectra of atrial tissues from rats of different ages revealed no notable changes in the NO level from day 56 to day 81. By the age of 110 days, the level of $(DETC)_2-Fe^{2+}-NO$ increased by 19% compared to 56 days ($p < 0.05$, Fig. 2). In ventricular tissues, unlike atrial tissues, the mean NO level increased by 30% from day 56 to 81 ($p < 0.05$). Rats at 81 and 110 days did not differ significantly in this parameter (Fig. 3). In rat liver tissues, the integrated EPR signal intensity increased by 85% on the average from day 28 to 56 ($p < 0.05$) but did not change by day 110 (Fig. 4). Nitric oxide is involved in most metabolic processes in the liver; therefore, its variation points to developmental changes in the liver metabolism rate.

The highest NO levels were found in rat livers, lower levels occurred in atrial tissues, and still lower

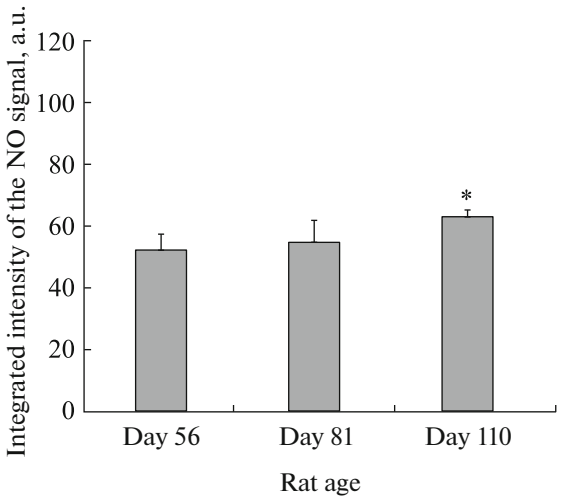

Fig. 2. The integrated intensities of the signal from the EPR trap $(DETC)_2$–Fe^{2+}–NO in rat atrium tissue. The *Y*-axis shows the integrated intensity of the signal. $*p < 0.05$.

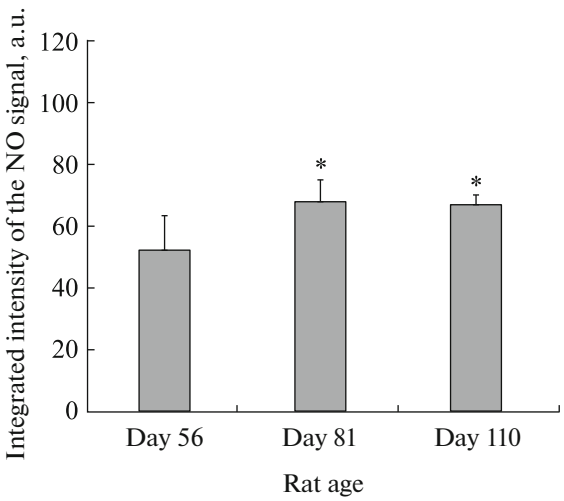

Fig. 3. The integrated intensities of the signal from the EPR trap $(DETC)_2$–Fe^{2+}–NO in rat ventricle tissue. The *Y*-axis shows the integrated intensity of the signal. $*p < 0.05$.

levels occurred in ventricular tissues of the heart. This observation may be related to the fact that the liver is a potent blood filter. The effect of NO on the liver is not confined to septic conditions. There is evidence of an important NO role in functional aberrations recorded in the liver in ischemia, reperfusion, neoplasms, cirrhosis, and several other conditions [35].

It has been shown that the NO concentration in the human body varies with age. Its maximum, 152.0 ± 16.2 μg/mL, is observed in the age interval of 5–12 years. NO production then decreases and reaches the minimum value at 19–30 years [36]. With aging, the vascular endothelium function is impaired; the decrease in the endothelial NO production rate is thought to be the main cause of the impairment [37].

The densities of cholinergic and adrenergic terminals in the cardiac muscle rapidly increase in the age interval from 7 to 10 years. Their maximum concentration is constantly recorded in the right atrial wall, followed by the left atrium, right ventricle, and left ventricle wall. The maximum density of neural plexuses in heart walls is reached in sexual maturity. Starting from days 35–40, sympathetic activity decreases. Immunohistochemical studies have revealed colocation of NO in pericellular endings, cardiac neurons, and cholinergic synapses of humans and animals. The influence of NO and its metabolites manifests itself in areas of the brain that control sympathetic activity and the vagus nerve function. NO also modulates the transmission of vegetative activity to target organs by acting in the spinal cord, ganglia, and neuromuscular junctions [38].

Our experiments demonstrate that the NO content in rat liver tissues increases from day 28 to day 56 but does not change significantly in postadolescence. The NO content in heart tissues increases from adolescence to sexual maturity. The NO level stabilizes in different tissues at different ages: in liver tissues this occurs at the beginning of adolescence (day 56); in ventricles, by day 81; and in atria, by day 110. Nitric oxide modulates or mediates nearly all signaling pathways of the cardiovascular system at every step from the central nervous system to cardiac muscle cells [39, 40]. It appears that NO, along with neurotransmitters and hormones, is a key molecule in the formation of cardiovascular system regulation in postnatal development.

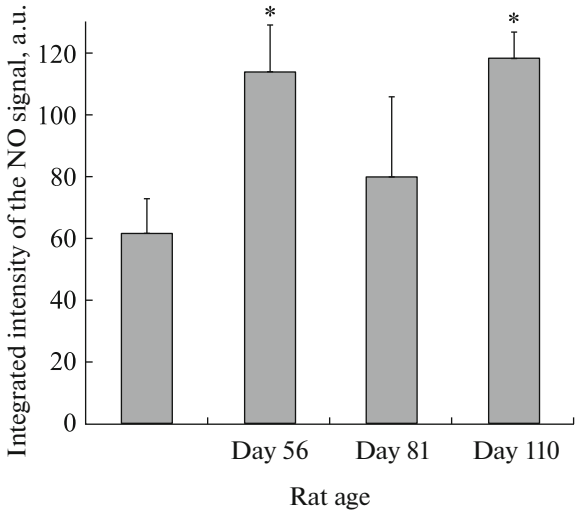

Fig. 4. The integrated intensities of the signal from the EPR trap $(DETC)_2$–Fe^{2+}–NO in rat liver tissue. The *Y*-axis shows the integrated intensity of the signal. $*p < 0.05$.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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