Nitric Oxide Production in Tissues of 7- and 16-Week-Old Rats under Mobility Restriction

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Abstract—Electron paramagnetic resonance is used to study the intensity of nitric-oxide production by analyzing the amount of NO-containing paramagnetic complexes in the heart, liver, and spinal-cord tissues of 7- and 16-week-old rats growing under conditions of restricted mobility. Nitric-oxide production is assessed from the intensity of the EPR signal belonging to the (DETC)₂—Fe²⁺—NO complex. The obtained results show that growth under conditions of restricted motility leads to an increase in the production of NO in all organs under study with the greatest increase observed in 7-week-old rats.

Keywords: nitric oxide, heart, liver, spinal cord, electron paramagnetic resonance

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INTRODUCTION

Motor activity is a main feature of animals and human beings, an integral part of the life and development of each organism. The normal functioning of the body has developed for thousands of years in combination with lively motor activity. In the conditions of the industrial and urban environment, the life of a modern person requires from the body very unusual forms of vital activity of its organs and systems, which are far from the necessary requirements laid down by evolution with respect to the level of motor activity and safety. Automation and computerization of work and life, passive recreation, and the development of transport and other conditions have resulted in a modern person having a sedentary lifestyle. The level of physical activity during life often changes in the direction of its increase or decrease under the influence of any requirements of the external environment. If a person changes his lifestyle so that his necessary physical activity becomes low, then his body must adapt to a new state. A specific adaptation develops in these cases, which is reduced to structural and metabolic dysfunctions of many organs and systems of the body. The growing restriction of motor activity is becoming a serious health threat, including entailing increased risk of cardiovascular disease.

A large amount of factual material on the effect of restriction of motor activity on the human and animal organisms has been accumulated, though most of these data were obtained in the course of solving specific problems of space medicine. Limitation of motor activity leads to morphological and functional shifts in the main life-supporting systems, such as nervous, cardiovascular, muscular, and endocrine systems, up to pathological conditions determined by the duration and degree of restriction of motor activity [1–6]. The main factor leading to this process is a decrease in the afferent influx, which causes a decrease in the tone of the central nervous system, a change in the structure and function of synapses, and muscle trophism [6].

The role of the free radical compound nitric oxide (NO) in the mechanisms of development of various pathological conditions of the body is of great interest. Nitric oxide is widely distributed in the nervous and cardiovascular systems and is known as one of the most important signaling molecules that regulate the physiological functions of the body and cell metabolism [7–14]. Excessive formation of NO can substantially reduce the tone of smooth muscle cells, impair endothelial function, and directly inhibit the myocardial contractility observed during septic and hemorrhagic shock and acute myocardial infarction [14–16]. On the one hand, NO has a toxic effect associated with the disruption of mitochondrial oxidative phosphorylation and the formation of free radical compound peroxynitrite anion, which blocks a number of neuronal receptors, inactivates the SOD enzyme, and causes a deepening of free radical oxidation that leads to cell death [16–19]. In addition to vasodilating, neurotransmitter, and stress-limiting properties, there is

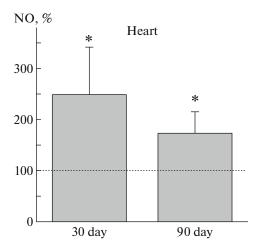


Fig. 1. Changes in the production of NO in rat heart tissues after 30 and 90 days of immobility with respect to that in the control group. The y axis reflects changes in the integral intensity of the signal from the (DETC)₂–Fe²⁺–NO complex (expressed in %) with respect to the control group. The significance compared to the control group is *p < 0.05.

no doubt that NO is involved in the reactions of oxidative stress, glutamate-calcium cascade, and inflammation [13]. Nitric oxide performs its physiological functions either by binding to iron (Fe) ions in the heme or through S-nitrosylation of proteins, as well as taking part in a number of biochemical reactions [13, 20-23]. The mechanism of NO toxicity includes covalent modification of proteins when interacting with their thiol groups, as well as direct DNA damage. Moreover, there is an opposite point of view, according to which an excess of NO serves as a compensatory factor. By activating soluble heme-containing guanylate cyclase and increasing the synthesis of cyclic guanosine monophosphate (cGMP), NO may also protect neurons from the toxic effects of glutamate. Thus, the intrinsic dual nature of NO is manifested in many natural modulators, and the protective and damaging properties of NO seem to be determined by its intracellular concentration [13, 21-26]. It is believed that the NO system is among the universal factors that regulate the stress and adaptive responses of the body. It is known that the NO system plays an important role in the adaptation of the body to various changes in the external environment and external conditions leading to stress (Manukhina, Malyshev, 2000; Manukhina, 2006; Sitdikov, Zaripova, Gainutdinov, 2017). To date, the impossibility of maintaining an optimal state of human health and the processes of adaptation of the body to various environmental factors, including physical activity, without normal cellular metabolism of NO has been proven [25–28].

Thus, the NO system is one of the most promising targets for therapeutic interventions under conditions of mobility restriction. The aim of this study was to carry out a comparative analysis of the NO content in the tissues of the spinal cord, heart, and liver of rats growing under conditions of unlimited and limited mobility. Mobility was restricted by placing the animals in special cages called "cage boxes" starting from the age of 3 weeks.

RESEARCH METHODS AND PROCEDURES

The study was conducted in accordance with the principles of the Basel Declaration and the recommendations of the local bioethical committee of Kazan Federal University. Various methods of partial and complete immobilization of animals are published. Simulation of motor-activity restriction by keeping animals in small cages has become widely used. Taking into account the characteristics of daily motor-activity restriction, the main requirement is met by the model of increasing limitation of mobility with a gradual increase in the time of immobilization of rats in cage boxes. By moving the partition, the volume of the box is changed in accordance with the size of the animal. In the first 2 days, the time of restriction of motor activity was 1 h, and then increased by 2 h every 2 days. The obtained experimental model made it possible to create the same degree of mobility restriction strictness for all animals, which is a necessary condition for obtaining comparable results. This model is characterized by the absence of additional damaging factors and the simplicity of technical design and makes it possible to regard the observed changes as a result of the adaptation of the body to the actual decrease in motor activity. It should be noted that the used mode of mobility restriction is not severe and undoubtedly reduces the stress response. Animals were released every day, and they had the opportunity to compensate for forced hypokinesia.

The studies were applied to laboratory outbred rats, which were divided into the following two groups: group I was the control group kept under standard vivarium conditions, and group II was the experimental group housed with limited mobility. Regarding age, the animals were divided into two groups: of 7- and 16-week-old rats. The infant rat grew up under conditions of restricted mobility when they reached the age of 3 weeks; therefore, the duration of mobility restriction was 30 and 90 days in the first and second groups, respectively. In each age group, n = 15. Electron paramagnetic spectroscopy with use of a spin trap is among the most efficient and direct methods for detecting and quantifying NO in biological samples. The spin trap method is based on the reaction of the NO radical with a spin trap. A complex of Fe²⁺ with diethyldithiocarbamate (DETC) was used to capture NO and form the stable ternary complex (DETC)₂-Fe²⁺-NO [29, 30]. To form this complex in the body, the animals were injected with an aqueous solution of DETC-Na at a dose of 500 mg/kg in 2.5 mL of water intraperitoneally and with a solution containing iron citrate

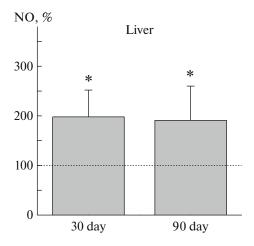


Fig. 2. Changes in the production of NO in rat liver tissues after 30 and 90 days of immobility relative to the control group of animals. The *y* axis reflects changes in the integral intensity of the signal from the $(DETC)_2$ — Fe^{2+} —NO complex (expressed in %) with respect to the control group. The significance compared to the control group is *p < 0.05.

(iron(II)) sulfate FeSO₄·7H₂O (Sigma, United States) at a dose of 37.5 mg/kg and sodium citrate at a dose of 187.5 mg/kg intramuscularly (the details of the method have been published earlier in [31, 32]). The nitric oxide trap was introduced 30 min before dissection. The DETC-Fe(II) complex interacts with NO and gives rise to the formation of stable radical (DETC)₂-Fe²⁺-NO. This complex is paramagnetic $(S_{\text{Fe}} = 1/2 \text{ and } I_{\text{N}} = 3/2)$ and can be detected by the EPR method [30]. The complexes are characterized by an easily recognizable EPR spectrum with a g-factor of g = 2.035 and a triplet hyperfine structure. The amount of NO was estimated from the intensity of the characteristic EPR signal belonging to the (DETC)₂-Fe²⁺–NO complex. The signals were compared for the integral intensity values, since the integral intensity of the EPR signal is directly proportional to the concentration of paramagnetic complexes [30]. Thirty minutes after drug administration, the rat anesthetized with urethane was fixed on the operating table and cut, and the removed organs were quickly dried and frozen in liquid nitrogen in capillaries for measurements. The heart, liver, and spinal cord tissues of the animals were taken for the study. The EPR spectra of the prepared samples were recorded on an ER-200E-SRC Bruker EMX/plus X-band EPR spectrometer with an ER 4112HV temperature attachment at 77 K. The following parameters were kept constant in all experiments: microwave power of 30 mW, modulation of 5 G, amplification of 4×10^4 , time constant of 100 ms, spectrum recording time of 50 s, and number of accumulations of 8. The computer of an Aspect 3000 spectrometer from Bruker was used to accumulate and record the spectra. Immediately before the measurement, the finished sample truncated according to the

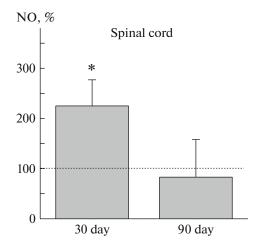


Fig. 3. Changes in the production of NO in the tissues of the spinal cord of rats after 30 and 90 days of immobility relative to the control group of animals. The y axis reflects changes in the integral intensity of the signal from the $(DETC)_2$ - Fe^{2+} -NO complex (expressed in %) with respect to the control group. The significance compared to the control group is p < 0.05.

shape of the measurement cuvette is weighed. The weight of the samples needed to be about 100 mg. The amplitude of the EPR spectra was always normalized to the weight of the sample and to the amplitude of the EPR signal of the reference sample (the details of the technique for measuring the EPR signals have been published earlier in [31]).

For statistical processing, mean value M of the measured parameter and standard error $M \pm \text{SEM}$ of the mean were obtained. Using Student's t-test and the Mann–Whitney U-test, we tested the significance of differences in the mean values of NO levels in different tissues of rats of different ages. The differences were considered significant at p < 0.05.

RESULTS AND DISCUSSION

The EPR method was used to study the heart, spinal cord, and liver tissues of 7- and 16-week-old rats growing under conditions of restricted mobility, and those of control rats of the corresponding ages. In all measured EPR spectra, a characteristic triplet signal from a complex based on a spin trap (DETC)₂—Fe²⁺— NO was recorded, the integral intensity of which is directly proportional to the NO content in the sample.

The comparison of the EPR spectra of heart tissues of 7- and 16-week-old rats growing under conditions of limited mobility revealed an increase in the content of NO in heart tissues by 148.7% after staying under 30-day hypokinesia and by 73.2% after 90-day hypokinesia with respect to the parameters of rats in the control group (p < 0.05). Growth under conditions of 30- and 90-day restriction of mobility caused an increase in the content of NO in the liver tissues of rats

with respect to the control group (p < 0.05) by 98 and 91%, respectively. In the tissues of the spinal cord of rat pups growing under conditions of limited mobility for 30 days, an increase in the intensity of NO production by 125% relative to the level of this parameter in control rats of the corresponding age is found (p < 0.05). With restriction of mobility for 90 days, the production of NO in the tissues of the spinal cord does not substantially differ from the level observed in rats in the control group.

Thus, growth under conditions of limited mobility leads to an increase in the production of NO in all the studied samples of tissues, except for the tissues of the spinal cord of rats growing under 90-day hypokinesia. The reaction of the rat organism to hypokinesia depends on the duration of motor-activity restriction. The most pronounced increase in the content of NO in the tissues of rats was found during 30-day mobility restriction. Most likely, this is associated with characteristic peculiarities of this age, since 7 weeks of age corresponds to the onset of puberty. In addition, local stress-limiting systems are activated at the organ level during 1 month of action of mobility restriction, and the nitric-oxide generation system is one of them. It is possible that growth under conditions of a 30-day mobility restriction causes the mobilization of all reserve adaptation mechanisms. An increase in the production of nitric oxide was also revealed when mobility was limited for 90 days, but it was less pronounced than in the case of 30-day hypokinesia. This is probably associated with the development of the adaptive effect of an adequate stress response by this time. Activation of the NO system is one of the mechanisms by which the body prevents stress damage.

CONCLUSIONS

The observed increase in the intensity of NO formation under conditions of restricted mobility in rats allows us to conclude that there are close relationships between the level of NO in the body and the motoractivity regime, especially for a growing organism. Given that our model includes two componentsnamely, direct hypokinesia and stress from the applied procedures—there are NO-dependent mechanisms of the response of the body to immobilization stress. This is important because any experimental technique for motor-activity restriction includes a stress component that cannot be isolated in its pure form. It is interesting that an increase in the level of NO in cells effectively prevents, regardless of the source, a substantial increase in its amount during stress and associated tissue damage by inhibiting the inducible form of the enzyme (iNOS) or through the formation of protective antioxidant or other proteins [33]; i.e., there is a complex feedback mechanism. The data obtained broaden our understanding of the role of NO and NO synthases in the activity of the internal organs of rats growing under stress conditions in early postnatal

ontogenesis. Under conditions of limited mobility, the NO system can act as a stabilizing system that prevents the destruction of skeletal muscles and other tissues, and triggers the molecular mechanisms of body adaptation. In view of the well-known fact that hypokinesia causes significant changes in the cardiovascular system, in internal organs, and in the system of blood flow and oxygen supply to the body, it can be assumed that some of these changes are caused by a stationary increase in the production of nitric oxide in tissues that are most important for the body's activity.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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