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COMPLEX SYSTEMS BIOPHYSICS

EPR Study of Nitric Oxide Production in Rat Tissues under Hypokinesia*

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Abstract—EPR spectroscopy was used to study the intensity of nitric oxide (NO) production upon modeling 60-day progressive hypokinesia (restriction of motor activity) in rats and estimating the content of $(DETC)_2$-Fe^{2+}-NO complexes in heart and liver tissues. In 30 days of hypokinesia, there was a 2–3-fold increase in tissue NO. Administration of a nonspecific inhibitor of NO synthases, L-NAME, to hypokinetic rats prior to measurement decreased their NO level even below the untreated control. Our results show that the intensified NO production in hypokinesia is mainly due to NO synthases, rather than to the nitrite reductase pathway.

Keywords: nitric oxide, hypokinesia, rat, heart, liver, electron paramagnetic resonance
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INTRODUCTION

Nitric oxide (NO) is known to be a paramount signaling molecule modulating the physiological functions of the organism and the cell metabolism [1, 2]. Its role is documented for the central and autonomous nervous systems, for cardiovascular function [3–5] and blood supply to the brain and the heart, where deviations in NO level may incur risks of stroke and infarction [6, 7]. The NO system is also essential in adaptation to environmental changes and external conditions such as physical load [8, 9].

The literature provides evidence of two opposing modes of NO influence on the physiology of various tissues: (i) positive, stimulatory, versus (ii) toxic, damaging action that may lead to cell death [10–12]. Hence it can be asserted that the "sign" of effect depends on the amount of NO, yet it is not clear what amounts should be regarded as low, normal, or elevated.

A very topical medico-social problem is hypokinesia/hypodynamia, associated with the way of life, occupation, prolonged inactivity caused by illness, etc. Hypokinesia incurs reduced load on the musculature, leading to alteration of tissue function and morphology, up to pathological states depending on its duration and degree [13, 14]. In this context, here we assessed the changes in NO production in the tissues of rats grown under restrained motility.

* The experimental data contained herein fully correspond to the original publication but the presentation had to be substantially revised for the English version. *A.G.*

EXPERIMENTAL

Model of hypokinesia. Mongrel albino infant (3-week) rats were divided into two groups (20 animals each): (I) controls kept in standard vivarium conditions; (II) animals under progressive hypokinesia, which was imposed by confinement in a small box for 1 h in the first two days, further adding 2h every other day, so that by day 25 of treatment the animals stayed in boxes for 23 h a day, and were kept that way till day 60 [15]. Prior to measuring the tissue NO levels, half of the animals taken from each group received L-NAME.

EPR spectroscopy. In view of the short lifetime of NO in tissues and its low concentrations, the most expedient method of detecting and quantitating NO in biological samples is EPR [3, 16] with spin traps [17]. Here we used diethyl thiocarbamate (DETC) combined with iron, which trap the available NO in a mononitrosyl complex $(DETC)_2$-Fe^{2+}-NO [18] producing a characteristic triplet about $g = 2.035$. The details of our protocol have been described previously [6, 19]. Tissue samples of 100 mg were examined using a Bruker EMX/plus spectrometer with a low-temperature attachment ER 4131VT, at 140 K, operation frequency 9.445 GHz, modulation amplitude 5 G at 100 kHz, microwave power 2 mW, time constant 81 ms. The settings were selected so as to avoid overmodulation and signal saturation, and were the same for all measurements.

Statistical treatment. Data were presented as $M \pm SEM$. Differences between groups were assessed with

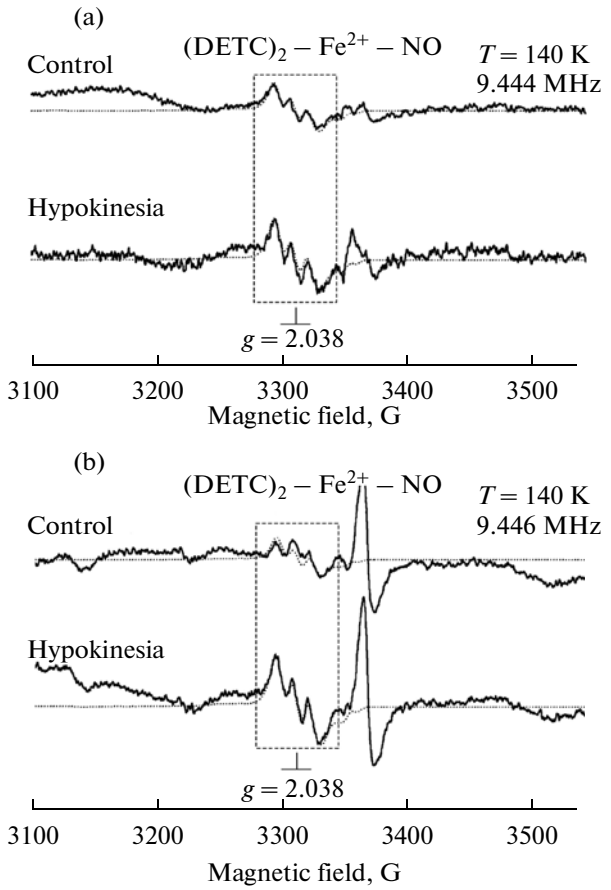

Fig. 1. EPR spectra of (a) heart and (b) liver samples from control rats and those after 60-day hypokinesia. The $(DETC)_2$-Fe^{2+}-NO signals are framed.

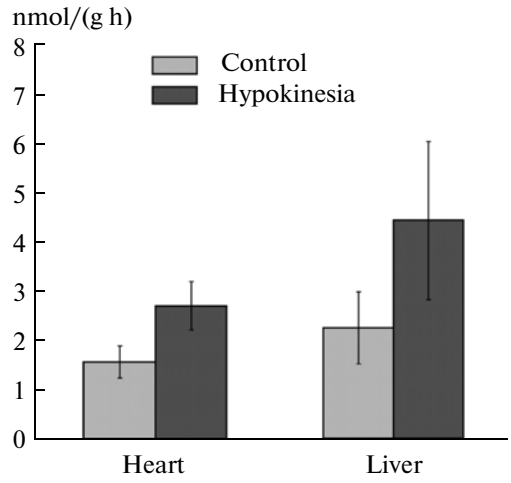

Fig. 2. Content of spin-trapped NO in heart and liver samples from control rats and those after 60-day hypokinesia ($M \pm SEM$, calculated from spectra exemplified in Fig. 1).

the Student's *t*-test and Mann–Whitney *U*-test, and deemed significant at $p < 0.05$.

RESULTS AND DISCUSSION

Quantitative processing of EPR spectra. EPR is widely used to determine the content of paramagnetic particles [17, 20–22], often by comparison with a reference sample or standard [23, 24]. Here we used a specimen of charcoal containing $0.75 \cdot 10^{13}$ spins.

To determine the quantity of NO produced in tissues, we first synthesized the $(DETC)_2$-Fe^{2+}-NO complex (from DETC, iron sulfate + sodium citrate, and sodium nitroprusside as the NO donor); this aqueous solution was frozen in liquid nitrogen.

Its EPR spectrum at the assay temperature (140 K) contained the characteristic triplet in the g = 2.03 region, and was further taken as the reference signal of trapped NO. This signal (calibrated with respect to the quantity of NO molecules) was entered into the program for analyzing the experimental spectra. [It should be noted that $(DETC)_2$-Fe^{2+}-NO is stable enough, so the shape of the EPR spectrum did not vary from sample to sample.] The NO content in tissues was deduced from least-squares fitting of the experimental records with simulated reference signals of varied intensity.

Changes in tissue NO content upon hypokinesia. Figure 1 exemplifies the EPR spectra of heart and liver samples from control rats and those subjected to 60-day hypokinesia. Frames demarcate the typical $(DETC)_2$-Fe^{2+}-NO triplets; signal intensity is proportional to the NO content. In Fig. 2 these data are expressed in conventional units of NO production; it is obvious that in both organs of hypokinetic rats the NO level is nearly doubled over the control.

It should be admitted that the model used here does not allow explicitly distinguishing the NO-related effects of hypokinesia proper and those of the attending immobilization stress (though the latter was unlikely to be dramatic in gradual adaptation at young age).

As mentioned in Introduction, the literature presents disparate views on the influence of surplus NO in (patho)physiological conditions—beneficial or aggravating, specifically as regards the cardiac function. Anyway, the changes in tissue NO levels observed here are not inconsistent with the general changes in the organ and body functions observed in hypokinesia [13, 14].

Sources of NO elevation in hypokinetic rat tissues. As shown in the brain, NO is continuously produced from arginine by constitutive NO synthases (NOS)—endothelial, neuronal, and mitochondrial [1, 5]—to ensure adequate blood supply, to modulate cell activity and metabolism [2, 11]. However, NO can be produced in the body via another pathway involving nitrite reductase [4]. In order to discern the origin of surplus NO in hypokinesia, we used a nonspecific NOS inhibitor L-NAME. As evident from Fig. 3,

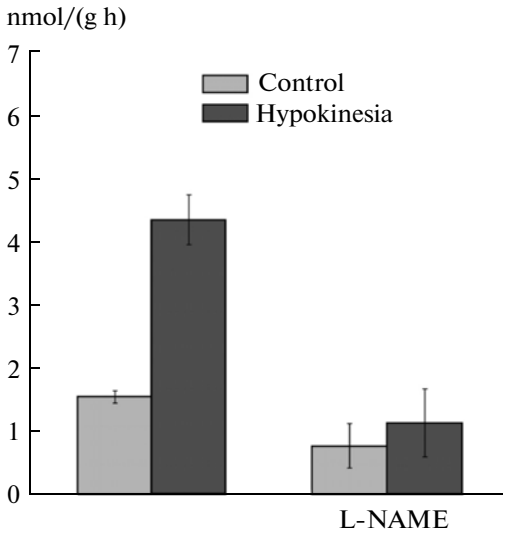

Fig. 3. Effect of NOS inhibitor L-NAME on the NO content in rat heart tissue, control and after 30-day hypokinesia.

administration of L-NAME to rats upon 30-day hypokinesia and not long before measurements decreased the amount of NO trapped in heart tissue to a level even lower than that in untreated controls but not significantly different from L-NAME-treated controls. Hence it can be concluded that enhanced NO production under hypokinesia is mainly due to NOS activity, while other sources are not markedly influenced by hypokinesia.

CONCLUSIONS

Considering the involvement of NO in many processes including cardiovascular function, and on the other hand, the shortage of information on the NO levels and effects in hypokinesia of a growing organism, studies in this direction appear quite topical. Here we have shown that the NO content rises significantly in vital organs of hypokinetic animals. Earlier we found that NO production in various tissues decreased in rats subjected to intense training (hyperkinesia) [8]. These observations strongly suggest close connection between NO levels and motor activity. Inasmuch as hypokinesia is known to cause substantial alterations in most of the body systems and organs, it may be supposed that such alterations are partly caused by a persistent excess of NO in key tissues.

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