Detection and Evaluation of NO Stores in Awake Rats

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We demonstrated and substantiated the possibility of detection and evaluation of NO stores in freely moving awake rats. NO stores were created by administering NO donor or by heat shock and were then detected by hypotensive reaction to diethyldithiocarbamate (blood pressure monitoring) under conditions of NO synthase inhibition. Electron paramagnetic resonance revealed NO release from its stores by incorporation into paramagnetic mononitrosyl-iron complexes with diethyldithiocarbamate. Five hours after administration of NO donor or heat shock diethyldithiocarbamate induced a blood pressure drop and the appearance of electron paramagnetic resonance signals from the mononitrosyl-iron-diethyldithiocarbamate complex in rat heart, liver, kidneys, and brain. The hypotensive reaction to diethyldithiocarbamate and electron paramagnetic resonance signals were absent in control rats.

Key Words: *nitric oxide; NO stores; diethyldithiocarbamate; dinitrosyl-iron complexes*

It is well established that NO, a universal regulator of physiological functions and potent endogenous vasodilator, is an unstable and highly reactive compound. At the same time, NO produces both autocrine and paracrine effects, *i.e.* NO modulates physiological and biochemical processes not only in cells where it was synthesized, but also in adjacent cells. Inhaled NO enters lung alveoli and induces dilation of distant vessels. It should be emphasized that NO produces a long-lasting vasodilating effect [13]. *In vivo* NO can form relatively stable compounds and can be stored in cells or transported for a long distances. Most authors believe that dinitrosyl-iron complexes (DNIC) with thiol-containing ligands and S-nitrosothiols (RS-NO) serve as NO stores [2].

Bioactive store of NO is a dynamic system including free and bound NO forms characterized by different stability [2]. Stationary level of these forms is insufficient for physicochemical assays, which makes difficult detection of NO stores in the organism. The content of DNIC in animals was evaluated only after addition of sodium nitrate (exogenous NO source) to drinking water for several days or 1 week after onset of bacterial infection [2].

Our previous studies showed that NO stores formed *in vivo* after treatment of animals with the NO donor or stimulation of NO synthesis can be detected in isolated rat aorta [8]. The method is based on mobilization of NO from stores in the vascular wall under the influence of diethyldithiocarbamate (DETC) inducing vasorelaxation. Until now there were no methods for detection and measurements of NO stores in the vascular wall of the whole organisms. In this work a modified method was used to demonstrate and substantiate the possibility of detecting and measuring NO stores in freely moving awake rats.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 230-250 g. The animals were narcotized with nembutal (40 mg/kg intraperitoneally). A polypropylene catheter was introduced into the aortic arch via the carotid artery to measure blood pressure (BP). Another catheter for administration of preparations was inserted into the jugular vein. The experiment was

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started 24 h after treatment. BP was measured in freely moving awake rats.

NO stores were created by pharmacological (administration of the NO donor) and non-pharmacological methods (heat shock, HS). Previous experiments with isolated vessels showed that these treatments increased NO level in the organism and led to the formation of NO stores [8]. NO stores were measured 5 h after administration of the NO donor or HS.

Diamagnetic (dimeric) DNIC with glutathione (200 μg/kg intraperitoneally) served as the NO donor. DNIC were synthesized as described elsewhere [1]. HS was induced by heating of rats in a thermostat at 65°C for 15 min.

NO synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA) in a dose of 50 mg/kg was administered 1 h before evaluation of NO stores. Similarly to the study of NO stores in isolated vessels [8], this treatment was performed to exclude the contribution of *de novo* synthesized NO into BP changes. BP was monitored in awake animals 5 h after administration of DNIC or HS. DETC in a dose of 20 mg/kg was injected intravenously during continuous monitoring of BP. Since DETC can induce the release of NO, it produces generalized NO-dependent vasodilation and BP drop, if the vascular walls contain a store of NO. The BP shifts in response to DETC administration reflected the content of stored NO.

The release of NO from its stores was assayed by electron paramagnetic resonance (EPR). We studied incorporation of NO into DETC complexes with the formation of paramagnetic mononitrosyl-iron complexes (MNIC) with DETC. The rats received DETC, $FeSO_4$ (20 mg/kg intraperitoneally), and sodium citrate (95 mg/kg subcutaneously) to induce the formation of MNIC. The animals were decapitated 30 min after treatment. The aorta, liver, kidneys, heart, and brain were removed, put in a press mold, frozen, and stored in liquid nitrogen. The content of NO in complexes was determined by the amplitude of EPR signals at *g*-factor of 2.041 and 2.014 (DNIC) [14,15]. EPR signals were recorded on a Bruker ECS-106 radiospectrometer (77K, microwave power 20 mW, modulation amplitude 0.5 mT).

The results were analyzed by Student's *t* test. The data are expressed as $M \pm m$.

RESULTS

DETC did not change BP in non-narcotized controls rats and in rats receiving L-NNA alone (Fig. 1, *a*). This attests to the absence of NO stores in the vascular wall of control animals. Otherwise, the content of stored NO was below the sensitivity limit for the applied method. After HS or administration of NO donor DETC produced generalized vasodilation and decreased BP (Fig. 1, *a*). The hypotensive reaction to DETC developed 5 min after treatment and progressed over 15-20 min. BP returned to normal by the 40th minute after DETC administration. DETC decreased BP by 13.0±3.4 and 17.5±4.1% 5 h after DNIC administration and HS, respectively (Fig. 1, *b*). At this term the NO donor cannot be detected in the tissue and blood [4].

The hypotensive effect was accompanied by the appearance of considerable contents of MNIC with DETC in tissues of experimental animals (Fig. 2). These complexes were found practically in all animals receiving DNIC. In these rats the content of complexes in the heart, liver, kidneys, and brain was 1.4-14.0, 19-

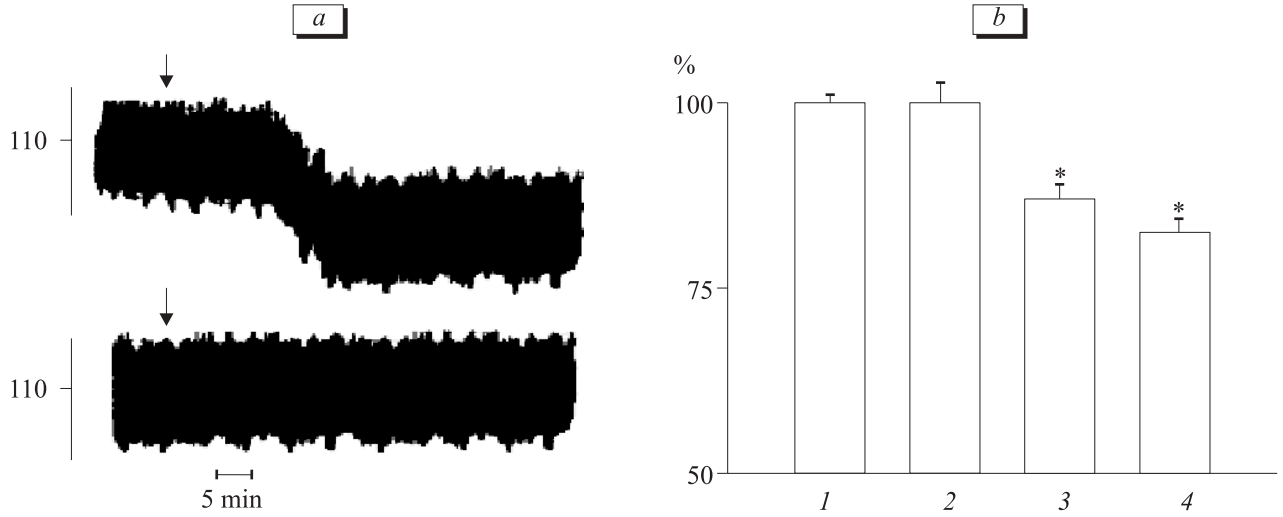


Fig. 1. *In vivo* assessment of NO stores formed after administration of NO donor dinitrosyl-iron complexes (DNIC) or heat shock (HS) in rats. Blood pressure (BP) in awake rats (*a*); arrow, administration of diethyldithiocarbamate (DETC). BP (mm Hg) after administration of DETC under conditions of NO synthase blockade (*b*). BP in the control before administration of DETC (*1*); BP in the control after administration of DETC (*2*); administration of DETC to animals receiving the NO donor DNIC (*3*); administration of DETC to animals after HS (*4*). **p*<0.05 compared to control animals receiving DETC.

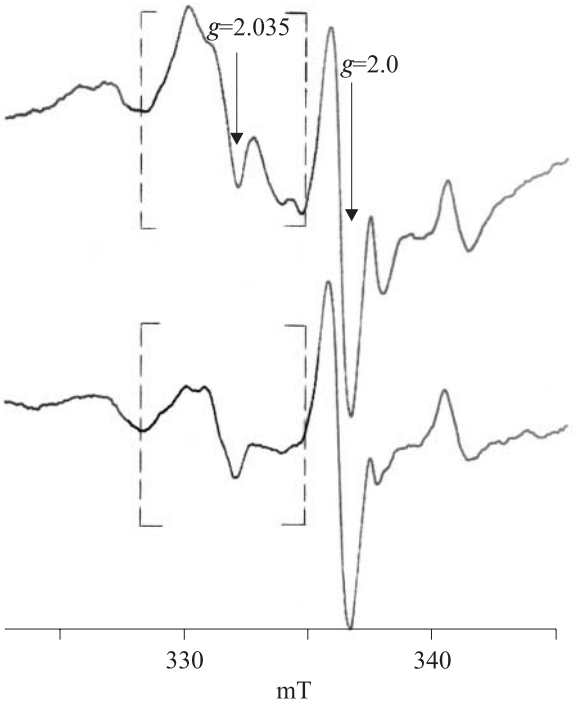


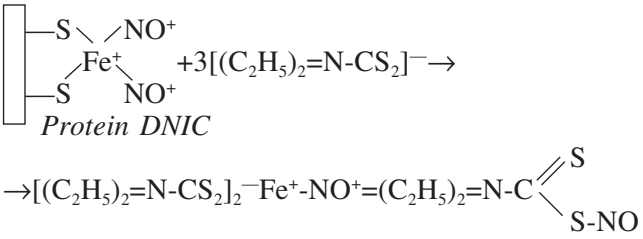
Fig. 2. Electron paramagnetic resonance (EPR) signals of mononitrosyl-iron-diethyldithiocarbamate complexes in rat liver after the formation of NO stores produced by the NO donor or HS (upper curve); control (lower curve). Recordings were performed under conditions of NO synthase blockade. Dotted frame: component of the hyperfine triplet structure of EPR signals reflecting NO content.

103, 1.8-5.2, and 2.3-4.0 ng/g tissue, respectively. MNIC with DETC were detected in 40% animals subjected to HS. In these rats the content of complexes in the heart, liver, kidneys, and brain was 3.0-24.0, 7.2-168.0, 12.0-20.4, and 1.2-7.2 ng/g tissue, respectively. MNIC with DETC were not found in control rats receiving the inhibitor of endogenous NO synthesis L-NNA. Even though these complexes appeared, their content was below the detection limit of the applied method (<1 ng/g tissue). These data suggest that the appearance of MNIC with DETC in tissues of experimental animals was related to the formation of NO stores induced by administration of exogenous NO (DNIC) or activation of endogenous NO synthesis (HS).

Experimental conditions did not allow us to evaluate the spontaneous release of stored NO that would affect BP. In our experiments the hypotensive reaction was observed only after administration of DETC. We did not analyze the mechanisms of these changes. Previous experiments with isolated vessels containing preformed NO stores showed that DETC-induced vasodilation is associated with activation of soluble guanylate cyclase [8], *i.e.* is caused by free NO molecules.

The study of the interaction between DETC and RS-NO or DNIC [3,10] showed that the formation of free NO in the biological system can be mediated by the following mechanisms. DETC attacks NO stores (protein RS-NO), which via the reaction of transnitrosation leads to the formation of DETC S-nitrose derivatives. Spontaneous homolytic disintegration of these derivatives results in the release of neutral NO molecules. DETC S-nitrose derivatives can undergo disintegration in the reaction with iron-DETC complexes, which leads to the formation of MNIC with DETC [14]. Further disintegration of these complexes is followed by the appearance of free NO molecules. Previous experiments revealed the ability of similar water-soluble MNIC with methyl-D-glucamine dithiocarbamate to induce the spontaneous release of NO [15].

The interaction of DETC with protein DNIC can lead to the formation of MNIC-DETC and S-nitrosated DETC:



Our previous results and published data indicate that NO stores in the vascular wall are formed under conditions of increased NO level in the blood, tissues, or incubation medium of the isolated vessel irrespective of the mechanisms of this increase. A direct correlation was found between the content of stored NO and concentration of NO metabolites in the plasma and vascular wall [5]. In our experiments HS induced overproduction of NO due to the appearance of inducible NO synthase and activation of endothelial NO synthase [12]. NO stores were formed in the vascular wall 5 h after HS. Similar results were obtained in our previous experiments with isolated rat aorta [8].

Exogenous low-molecular-weight DNIC rapidly spread in tissues and produce a hypotensive effect [3,4]. In animals this long-lasting effect (several hours) is related to the formation of protein DNIC due to transfer of $Fe^{+}(NO^{+})^{2}$ groups from low-molecular-weight DNIC to thiol groups in proteins. In narcotized and awake animals the degree of hypotension decreases with the reduction of EPR signals 1.5 h and 30 min after treatment, respectively. Our experiments showed that action of DETC on NO stores is followed by the development of hypotension.

Experiments with HS demonstrated that accumulation of NO excess in stores plays an important role in the prevention of adverse effects resulting from NO overproduction [9]. NO stores serve as an additional source of NO, which is important for the prevention of NO deficiency (*e.g.*, during hypertension) [6]. NO

released from stores produces a long-lasting cardioprotective effect [11]. However, excess accumulation of NO under certain conditions can be followed by relative NO deficiency and reduces the resistance to stress [7].

Physiological mechanism underlying the formation and disintegration of NO stores are poorly studied. Until now NO stores could be detected only in isolated vessels. The proposed method for studying NO stores in the organism can be used to control accumulation and transport of NO and consumption of stored NO.

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