GENERAL PATHOLOGY AND PATHOLOGICAL PHYSIOLOGY

In Vivo Accumulation of Nitric Oxide in Blood Vessels

B. V. Smirin, A. F. Vanin,* I. Yu. Malyshev, D. A. Pokidyshev, and E. B. Manukhina

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 127, No. 6, pp. 629-632, June, 1999
Original article submitted August 17, 1998

Against the background of NO-synthase blockade, diethyldithiocarbamate had no effect on the tone of isolated rat aorta, but induced relaxation of aorta preparations isolated after *in vivo* NO accumulation and isolated aorta incubated with dinitrosyl iron complex. Guanylate cyclase inhibitor methylene blue prevented the relaxation induced by diethyldithiocarbamate. These data suggest that accumulation of NO in the organism can result in its accumulation in the vessel wall.

Key Words: *nitric oxide; nitric oxide stores; dinitrosyl iron complex; adaptation to stress*

Nitric oxide (NO) is a labile short-lived molecule that acts as a transmitter in various biological processes. In a stabilized form this molecule can execute not only autocrine, but also paracrine functions. Stabilization of NO can be achieved via the formation of dinitrosyl iron complexes (DNIC) with thiol ligands or S-nitrosothiols, which then release NO. These NO-containing complexes form physiologically active NO stores in tissues [11,14].

Accumulation of NO in the form of DNIC can be detected by electron paramagnetic resonance [10,11], histochemical reaction for Fe(II) [6], and photorelaxation [9]. The release of NO from tissue stores can be induced by N-acetylcysteine [10,11] and diethyldithiocarbamate (DETC), which react with DNIC yielding vasoactive products. However, in all studies NO stores were induced by long-term incubation with NO donors or potent NO-synthase (NOS) inductors, for instance lipopolysaccharides, and detected in cell cultures or isolated vessels.

In the present study we revealed and evaluated physiologically active NO stores formed *in vivo* in blood vessels after elevation of NO content in the organism.

Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences; *Institute of Chemical Physics, Russian Academy of Sciences, Moscow

MATERIALS AND METHODS

Experiments were carried out on male Wistar rats weighing 230-280 g. The increased concentration of NO in the organism was created by treatment with the NO donor DNIC, heat shock [2], and adaptation to short-term stress [8]. DNIC were injected into the caudal vein in a dose of 170 μg/kg. The animals were decapitated 5 h postinjection. Heat shock was reproduced by heating in a thermostat for 15 min after the core temperature attained 41°C. The rats were decapitated 1 h after heating. Adaptation to stress was performed by immobilization in the supine position for 15 min on day 1, 30 min on day 2, 45 min on day 3, and then for 60 min every other day. A total of 8 immobilization sessions were carried out. The rats were decapitated after 1 or 8 sessions.

After decapitation, the thoracic aorta was isolated, cut free form adipose and connective tissues, and a 3-4-mm segment was prepared. The preparation was equilibrated in continuously oxygenated Krebs solution containing (in mM): 130 NaCl, 11 glucose, 14.9 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.18

0007-4888/99/0006-0571$22.00

KH_2PO_4, pH 7.4 (37°C) at an initial load of 1.2 g. The tone was recorded on a DY-1 isometric strength transducer with a Gemini dual-channel recorder (Ugo Basile).

After equilibration, the aorta rings were precontracted with norepinephrine (5×10^{-7} M) and when the reaction reached the plateau, the NOS inhibitor Nω-nitro-L-arginine (L-NNA, 10^{-3} M) was added for 20 min. For detection of NO stores at the plateau of L-NNA-induced reaction, 3×10^{-4} M DETC was added and the relaxation was recorded.

For *in vitro* formation of NO stores in the aorta wall, DNIC (10^{-6} M) were added to the norepinephrine-precontracted segment after inhibition of NOS with L-NNA. After complete degradation of DNIC in the chamber and restoration of the initial tone, NO accumulation was assessed by DETC-induced relaxation after or without washout. In some experiments, the preparation was incubated for 20 min with guanylate cyclase inhibitor methylene blue (3×10^{-5} M) before addition of DETC.

For evaluation of NO accumulation in the endothelium and smooth muscles, in some experimental series DETC-induced relaxation was studied on denuded aorta rings preincubated with DNIC or isolated from DNIC-treated rats. Endothelium was removed with a special catheter. Denuded preparations precontracted with 5×10^{-7} M norepinephrine did not respond by relaxation to acetylcholine (10^{-5} M).

The data were processed statistically using Student's test.

RESULTS

In the control, DETC had no effect on the tone of isolated aorta segment, but induced relaxation of preparation isolated from rats subjected to heat shock, or injected with NO donor DNIC, or adapted to stress (Fig. 1, Table 1). This indicates accumulation of NO complexes in the vascular wall capable of releasing free NO after both treatment with exogenous NO donors or stimulation of endogenous NO synthesis. Adaptation increased NO stores in the vascular wall: after completion of the adaptation course the stores were larger than after single immobilization session (Table 1).

DNIC releasing NO *in vitro* induced a transient relaxation of the aorta (Fig. 2). After the tone returned to baseline values (which indicated complete degradation of DNIC in the medium), NO stores were detected in the vascular wall. Moreover, reactions to DETC were similar in washed and non-washed preparations (Fig. 2, *a*, *b*). No repeated relaxation to DETC was observed, hence the product mediating this reaction can not be synthesized or replenished. In other words, NO stores are limited and can be exhausted. Methylene blue completely prevented DETC-induced relaxation of the aorta (Fig. 2, *b*). Hence, DETC-induced relaxation is mediated through NO release and activation of soluble guanylate cyclase [7]. Since this relaxation developed against the background of L-NNA treatment, it was associated with the release of accumulated NO, rather than with its *de novo* production.

After denudation DETC produced only a minor relaxation in some aorta preparation from rats injected with NO donor (Fig. 1, *c*). However, when the preparations were incubated with DNIC after denudation, DETC induced a more potent reaction than in preparations with intact endothelium (Fig. 1, *c*). These findings suggest that *in vivo* NO stores are primarily formed in the endothelium. However, smooth muscles in denuded vessels also can accumulate NO. NO stores formed in the vascular wall after incubation with NO donors were visualized by histochemical Fe^{2+}-specific reaction: positive staining was observed primarily in the endothelium, while in smooth muscles only weak and unstable staining was observed in endothelium-adjacent layers [6]. Our findings are consistent with these data.

NO stores can be presented by DNIC with thiol ligands (*e.g.*, cysteine or glutathione), protein thiol groups, or S-nitrosothiols. Detection of NO stores with DETC is based on the reaction of DETC with intracellular DNIC proceeding through the formation of nitrosonium ion (NO^+):

$$(RS^-)_2Fe^+(NO^+)_2+[(C_2H_5)_2=N\text{-}CS_2]^-\rightarrow$$
$$\rightarrow[(C_2H_5)_2=N\text{-}CS_2]^-{}_2Fe^+NO^++NO^++2RS^-.$$

TABLE 1. Measurement of NO Stores in Isolated Aorta by the Reaction of DETC-Induced Relaxation (*M±m*, *n*>5)

Series	DETC-induced relaxation, % of contractile response to norepinephrine and L-NNA
Control	0
Incubation with DNIC	
in vivo	10.48±2.76
in vitro (without endothelium)	51.00±18.31
Injection of DNIC	10.88±1.67
Injection of DNIC (without endothelium)	0
Heat shock	7.63±3.33
Adaptation to stress	
1 session	9.7±2.96
8 sessions	23.7±4.17*

Note. *$p<0.05$ compared with single adaptation session.

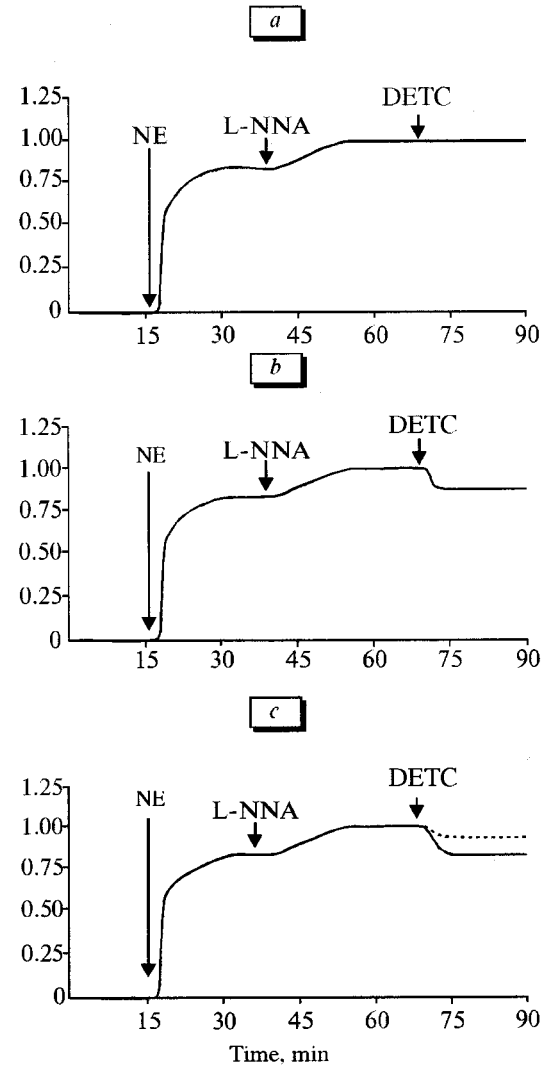

Fig. 1. Detection of NO stores formed after *in vivo* elevation of NO content in the organism. *a*) control; *b*) after procedures elevating NO content in the organism: injection of dinitrosyl iron complex (DNIC), adaptation to stress, and heat shock; *c*) denuded aorta: after injection of DNIC to rat (dotted line) and after *in vitro* incubation of denuded aorta with DNIC (solid line). Here and in Fig. 2: ordinate: contraction strength, g. NE: norepinephrine; L-NNA: Nω-nitro-L arginine; DETC: diethyldithiocarbamate; arrow indicates addition of the drug to the incubation medium.

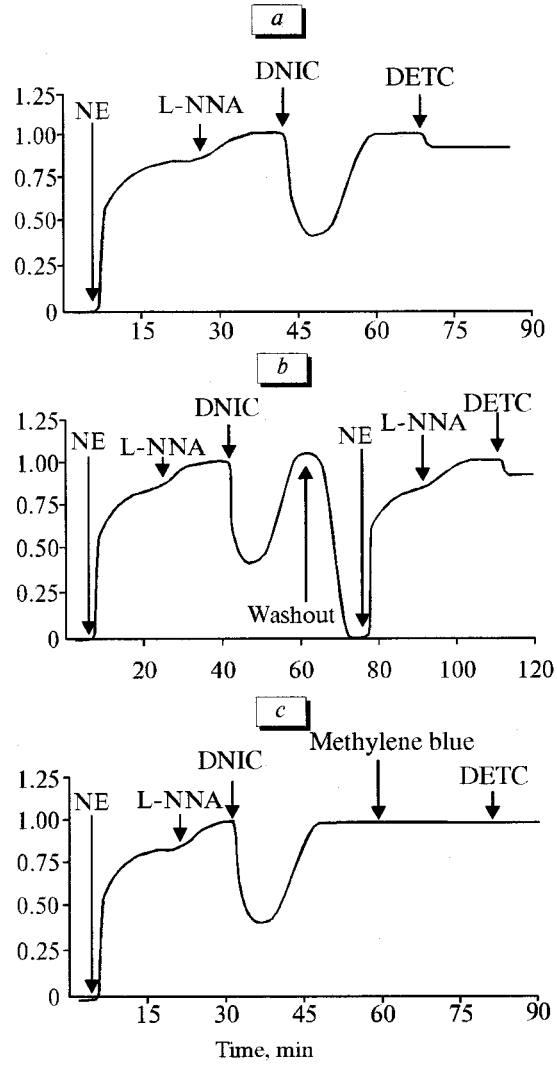

Fig. 2. Detection of NO stores formed after elevation of NO concentration *in vitro*. *a*) incubation of aorta preparation with dinitrosyl iron complex (DNIC) and addition of DETC without washout; *b*) incubation of aorta preparation with DNIC and addition of DETC after washout; *c*) effect of methylene blue on reaction of isolated aorta to DETC.

NO^+ binds with thiol with the formation of S-nitrosothiols possessing vasorelaxation activity [14].

Accumulation of NO is probably an adaptive reaction, which protects the organism against excessive NO concentrations under conditions of NO overproduction. Apart from elimination of NO excess from the blood, its accumulation in the vascular wall creates a reserve, which limits NO overproduction by inhibiting NOS through the feedback mechanism [5]. For instance, elevation of NO content induced by adaptation to stress or injection of NO donors effectively prevents NO overproduction and related cardiovascular disturbances in heat shock [3,4], while heat shock limits the damaging effects of lipopolysaccharides mediated through NO overproduction [12].

It can be hypothesized that the release of NO from tissue stores can ameliorate disturbances caused by insufficient NO production. This assumption is indirectly confirmed by the fact that the duration of the hypotensive effect of DNIC considerably surpassed its lifetime in the organism [1]. Moreover, adaptive preconditioning inducing a transient increase in NO production, in particular physical exercises, produces a sustained hypotensive effect [13]. Accumulation of NO in smooth muscles of denuded vessels observed by us also represents a compensatory reaction regulating vascular tone after damage to endothelium associated with atherosclerosis, heart catheterization, or vascular surgery.

Our findings suggest that enhanced NO production leads to its accumulation in the vascular wall in the form of physiologically active stores. These stores interact with DETC and release vasorelaxation agents, which allows to detect and evaluate quantitatively these NO stores by the magnitude of vascular response to DETC.

The study was supported by the Russian Foundation for Basic Research (grants Nos. 97-04-48370, 97-04-48371, and 96-04-48066), President Council and State Program for Supporting the Leading Scientific Schools (grand No. 96-15-970), and INTAS/OPEN CALL Program (grant No. 524).

REFERENCES

1. M. E. Galagan, E. V. Oranovskaya, P. I. Mordvintsev, *et al.*, *Byull. Vsesoyuz. Kardiol. Nauch. Tsentra*, No. 2, 75-80 (1988).
2. E. B. Manukhina, I. Yu. Malyshev, V. D. Mikoyan, *et al.*, *Byull. Eksp. Biol. Med.*, **121**, No. 5, 520-523 (1996).
3. E. B. Manukhina, D. A. Pokidyshev, E. B. Malenyuk, *et al.*, *Izv. Ross. Akad. Nauk, Ser. Biologiya*, No. 1, 54-58 (1997).
4. E. B. Manukhina, D. A. Pokidyshev, I. Yu. Malyshev, *et al.*, *Ibid.*, No. 2, 300-304 (1998).
5. J. Assreuy, F. Q. Cunha, F. Y. Liew, and S. Moncada, *Br. J. Pharmacol.*, **108**, 833-837 (1993).
6. F. W. Flitney, I. L. Megson, D. E. Flitney, and A. R. Butler, *Ibid.*, **102**, 842-828 (1992).
7. L. J. Ignarro, *Biochem. Pharmacol.*, **41**, 485-490 (1991).
8. F. Z. Meerson, P. I. Mordvintcev, E. B. Manukhina, *et al.*, *The Biology of Nitric Oxide*. Pt. 3, *Clinical and Physiological Aspects*, Eds. S. Moncada *et al.*, London (1994), pp. 182-185.
9. I. L. Megson, F. W. Flitney, J. Bates, and R. Webster, *Endothelium*, **3**, 39-46 (1995).
10. B. Muller, A. L. Kleschyov, and J. C. Stoclet, *Br. J. Pharmacol.*, **119**, 1281-1285 (1996).
11. A. Мьlsch, P. I. Mordvintcev, A. F. Vanin, and R. Busse, *FEBS Lett.*, **294**, 252-256 (1991).
12. A. J. Ryan, S. W. Flanagan, P. L. Moseley, and C. V. Gisolfi, *J. Appl. Physiol.*, **73**, 1517-1522 (1992).
13. H. Tanaka, D. R. Bassett, E. T. Howley, *et al.*, *J. Hypertens.*, **15**, 651-657 (1997).
14. A. F. Vanin, I. V. Malenkova, V. A. Serezhenkov, *Nitric Oxide*, **1**, No. 3, 191-203 (1997).