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Detection and Description of Various Stores of Nitric Oxide Store in Vascular Wall

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We analyzed the possibility of the existence of various NO pools in the vascular wall. Incubation of isolated rat aorta with dinitrosyl iron complex (NO donor) led to the formation of NO stores in the vascular wall detected by vascular relaxation response induced by diethyldithiocarbamate and N-acetylcysteine. Comparison of the effects of successive application of diethyldithiocarbamate and N-acetylcysteine revealed two NO pools (one pool responded to both agents, while other responded only to N-acetylcysteine). Inhibition of guanylate cyclase with methylene blue abolished the response to diethyldithiocarbamate, while the reaction to N-acetylcysteine decreased by the value, corresponding to diethyldithiocarbamate-dependent relaxation. It is hypothesized that in the vascular wall NO is stored in the form protein-bound dinitrosyl iron complexes and S-nitrosothiols in hydrophilic and hydrophobic cell compartments.

Key Words: *nitric oxide; NO store; dinitrosyl iron complexes; S-nitrosothiols*

Modern studies of the role NO in the regulation of vascular relaxation are focused on the mechanisms of its storing and transport. NO lifetime in the organism is only few seconds, but this free radical can be stabilized in NO-containing complexes (NO stores). Dinitrosyl iron complexes (DNIC) and S-nitrosothiols (RS-NO) are now considered as the main forms of NO stores [2].

In biological systems, DNIC and RS-NO exist in low-molecular and protein-bound forms bound via thiol groups. The protein forms are more stable due to higher affinity to $Fe^+(NO^+)_2$ groups in DNIC or NO^+ nitrosonium ion in RS-NO complex. As a result, the dynamic equilibrium between the low-molecular and protein-bound forms of DNIC and RS-NO is shifted towards the protein forms. Therefore, these protein forms of DNIC and RS-NO constitute the intracellular NO store. However, the protein DNIC and RS-NO are little effective as NO donors due to inefficient release of NO from these compounds and their low lability in cells. NO transfer to intracellular targets is effected by low-molecular DNIC and RS-NO, which is determined by their high mobility and low affinity to NO. The formation of these DNIC and RS-NO forms is promoted by high concentrations of low-molecular thiols and other compounds accepting $Fe^+(NO^+)_2$ and NO^+ groups from protein-bound DNIC and RS-NO [2]. For evaluation of NO stores, N-acetylcysteine (N-AC) [11] and diethyldithiocarbamate (DETC) [4,10] were used. These compounds penetrate into cells and form low-molecular RS-NO and DNIC forms or mononitrosyl-iron complexes (MNIC) with N-AC and DETC, respectively. These complexes can donate NO or NO^+ affecting various intracellular targets.

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The increase in NO concentration in tissues and cells irrespective of its mechanisms always leads to the formation of NO stores, and usually this store is revealed in the wall of isolated vessels [3,4,10]. Erythrocytes also can store and transport NO in the form of S-nitrosylhemoglobin [9]. NO store previously formed in the vascular wall *in vivo* or *in vitro* can be detected by electron paramagnetic resonance [14] or by relaxation of isolated aorta in the presence of agents inducing NO release from the store. For detection of NO store in isolated vessels photorelaxation reaction [12] and relaxation induced by DETC [4] or N-AC [10,11] are currently used.

Both DETC and N-AC are water-soluble substances. However, binding of DETC to ions of the transition metals such as iron or copper leads to the formation of water-insoluble complexes, which are predominantly located in cell membranes. Thus, comparison of activity of N-AC and DETC as the substances accepting NO from its protein stores can shed light upon two NO stores located in hydrophilic and hydrophobic cell compartments. Here we compared these pools by analyzing the formation of NO store in the wall of rat thoracic aorta after its incubation with DNIC-glutathione complex.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats weighing 280-320 g. After decapitation, thoracic aorta was isolated and cleaned from adipose and connective tissues. Aorta rings (length 3.5 mm) were placed into a thermostabilized chamber (37°C) filled with 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 KH_2PO_4, pH 7.4. The initial load was 1.2 g. The contraction force was recorded with an DY-1 transducer in a two-channel Gemini recorder (Ugo Basile).

After 1 h stabilizing period, contraction of the preparation was induced with norepinephrine (5×10^{-7} M). When the plateau was achieved, the preparation was incubated with DNIC (10^{-5} M) for 30 min in order to form NO store in the vascular wall [4]. After washout, the tone was restored with norepinephrine (5×10^{-7} M) and the vessel was incubated with N^{ω}-nitro-L-arginine (L-NNA, a blocker of NO-synthase, Sigma, 10^{-4} M) for 20 min. NO store was detected by relaxation of aorta rings in response to N-AC (Zambon France, 10^{-4}-10^{-3} M) or DETC (Sigma, 3×10^{-4} M). The degree of relaxation was assessed in percents of contraction induced by combined action of norepinephrine and L-NNA.

For evaluation of the role of guanylate cyclase (GC) in the vasodilator effect of N-AC and DETC, the preparation was incubated with GC inhibitor methylene blue (3×10^{-5} M) for 20 min after preincubation with NO donor. Then relaxation was induced by N-AC or DETC, and this reaction was compared with the response of the preparations not incubated with methylene blue.

To reveal individual peculiarities in the effects of N-AC and DETC on NO store, two experimental schemes were used. In the first scheme, after incubation of the preparation with DNIC (formation of NO store), norepinephrine (precontraction), and L-NNA relaxation responses to N-AC and then to DETC were recorded. In the second scheme, DETC was applied before N-AC.

The degree of relaxation was measured in percents of contraction produced by combined action of norepinephrine and L-NNA.

Dimeric DNIC-glutathione complex used to form NO store in the vascular wall were synthesized as described elsewhere [1].

The results of 5 or more experiments are presented as $M\pm m$. The results were analyzed statistically using Student's t test at $p<0.05$.

RESULTS

The paper presents original records of contraction and relaxation of isolated aortal preparations in the presence of various agents.

Control preparations not incubated with DNIC did not relax in response to N-AC or DETC. Probably, such preparations had no NO stores, or their NO stores were too small to be detected. This agrees with previous data on the absence of N-AC- or DETC-induced relaxation in preparations without previously formed NO stores [4]. Relaxation induced by these agents was observed in vessels isolated from rats 5 h after DNIC administration.

N-AC-induced relaxation of the preparation expressed in percents of contraction peaked at 5×10^{-4} M (30.5±5.6%), and further increase in N-AC concentration produced only negligible increase in relaxation response to 33.3%±3.9% (10^{-3} M, Fig. 1, *a*). The response to DETC was observed at 3×10^{-4} M (13.9±0.8%, Fig. 1, *b*). Further increase in DETC concentration was accompanied by toxic action on vessels, while the decrease in DETC concentration to 10^{-4} M drastically diminished the relaxation response.

Methylene blue, an inhibitor of soluble GC, completely abolished relaxation of aortal preparation in response to 3×10^{-4} M DETC (Fig. 2, *a*). By contrast, the effect of N-AC was inhibited only partially: relaxation induced by N-AC decreased to 15.8±2.0% (Fig. 2, *b*). When aorta preparation was relaxed in response to N-AC (10^{-3} M), addition of DETC (3×10^{-4} M) did

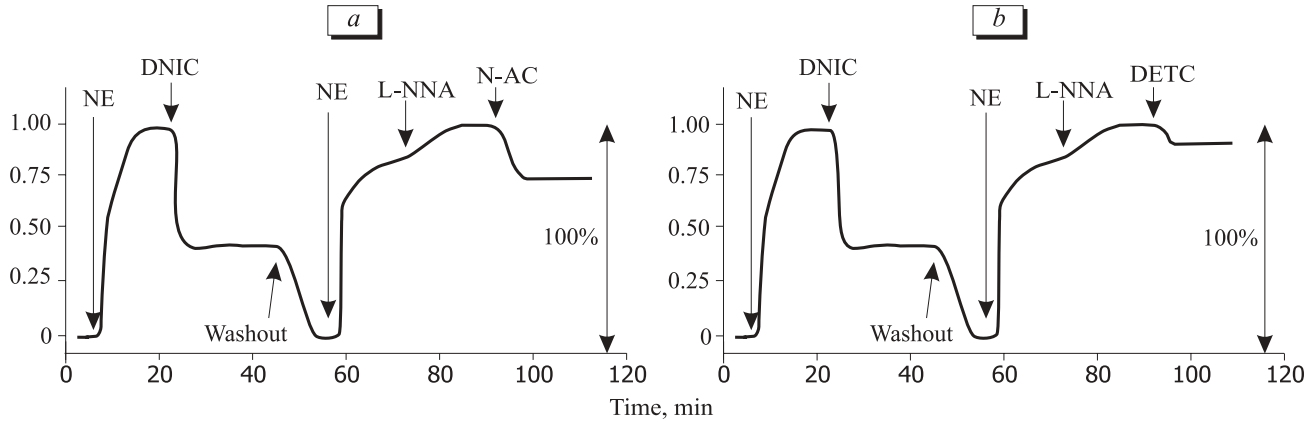


Fig. 1. Detection of NO store in the wall of rat aortal preparation by relaxation reaction to N-acetylcysteine (N-AC; *a*) and diethyldithiocarbamate (DETC; *b*): experimental protocol. Here and in Figs. 2 and 3: ordinate: contraction force (g). NE: norepinephrine, L-NNA: N^{ω}-nitro-L-arginine, DNIC: dinitrosyl iron complexes. Arrows show the moments of application.

not potentiated this response (Fig. 3, *a*). However, reversal application of these agents produced an additive relaxation effect. When the vessel was relaxed by DETC (13.9±0.8%), addition of N-AC induced further relaxation by 10.4±1.8% (Fig. 3, *b*). The total relaxation was 24.3±1.8%, which significantly differed from relaxation induced by N-AC alone (33.3%±3.9%).

Relaxation induced by N-AC or DETC was observed in the presence of NO-synthase inhibitor L-NNA. However, in this case the repetitive response to DETC or N-AC could not be elicited. This fact can indicate depletion of NO store caused by DETC or N-AC. Thus, NO store is not replenished without synthesis of endogenous NO, so vascular relaxation induced by both examined agents results namely from the release of NO from its store and not by *de novo* synthesis of NO.

Evidently, both examined substances (N-AC and DETC) relax the vessels by affecting NO store, which can be formed by RS-NO or DNIC. The degree of this relaxation is determined by the nature of metabolites formed during this interaction. These data show that the products formed in the reaction of DETC with NO store are less efficient in this respect than those formed in the reaction with N-AC. The interaction of DETC with RS-NO or DNIC produces S-nitrosylated DETC **(1)** alone or MNIC-DETC **(2)**, respectively:

$$\mathrm{RS\text{-}NO+(C_2H_5)_2}\!\begin{matrix}=\mathrm{S}\\ \mathrm{S}\end{matrix} \longrightarrow \mathrm{RS+C_2H_5C}\!\begin{matrix}=\mathrm{S}\\ \mathrm{SNO}\end{matrix}, \qquad (1)$$

$$\mathrm{(RS)_2Fe+(NO^{+})_2+(C_2H_5)_2}\!\begin{matrix}=\mathrm{S}\\ \mathrm{S}\end{matrix} \longrightarrow$$

$$\longrightarrow \left[\mathrm{(C_2H_5)_2C}\!\begin{matrix}=\mathrm{S}\\ \mathrm{S}\end{matrix}\right]_2 \mathrm{FeNO^{+}+C_2H_5C}\!\begin{matrix}=\mathrm{S}\\ \mathrm{SNO}\end{matrix}. \qquad (2)$$

According to [6], recombination of DETC-SNO molecules releases neutral NO molecule. In addition, neutral NO can be released during MNIC-DETC disintegration **(3)**:

$$\left[\mathrm{(C_2H_5)_2C}\!\begin{matrix}=\mathrm{S}\\ \mathrm{S}\end{matrix}\right]_2 \mathrm{FeNO^{+}} \longrightarrow \mathrm{Fe^{2+}+NO+2C_2H_5C}\!\begin{matrix}=\mathrm{S}\\ \mathrm{S}\end{matrix} \qquad (3)$$

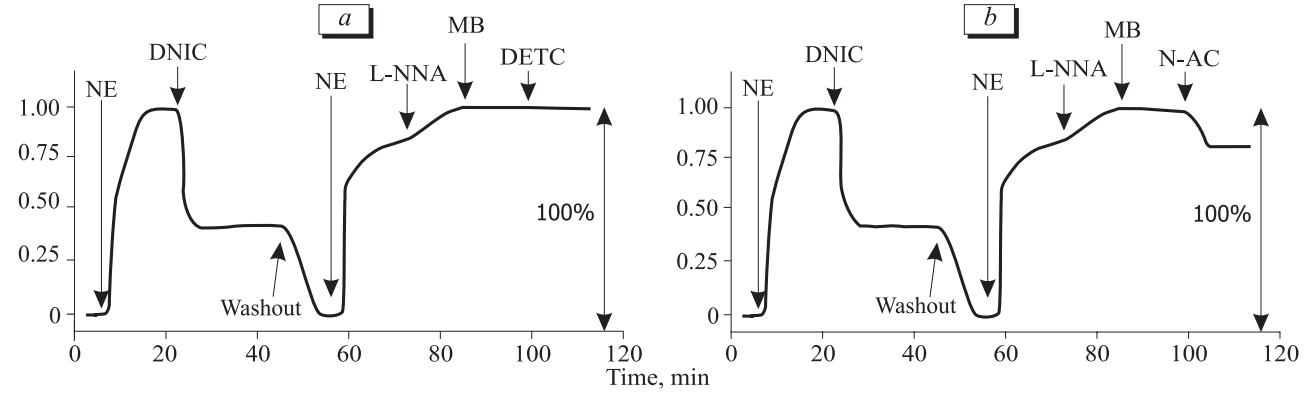


Fig. 2. Effect methylene blue (guanylate cyclase inhibitor) on relaxation of rat aortal preparation induced by DETC (*a*) and N-AC (*b*): experimental protocol. MB: methylene blue.

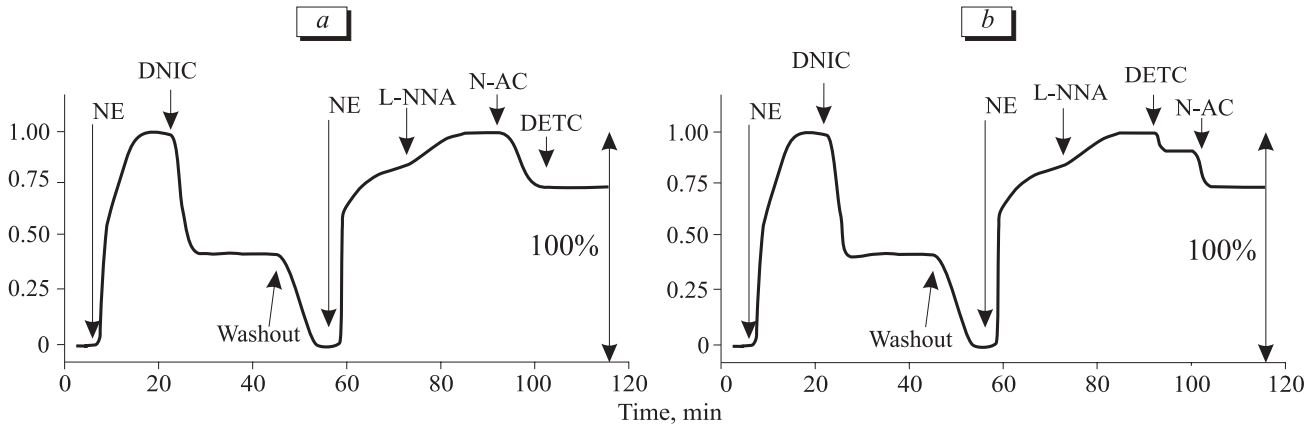
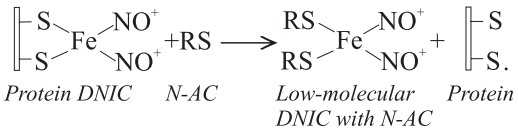


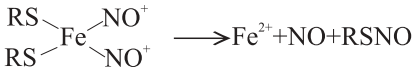
Fig. 3. Detection of DETC-sensitive (*a*) and N-AC-sensitive (*b*) stores of NO-storing complexes in the wall of rat aortal preparation: experimental protocol.

Neutral NO molecule initiates vascular relaxation by activation of soluble GC. It was observed in our experiments, where DETC-induced relaxation was determined entirely by this process and was suppressed by methylene blue, an GC inhibitor. By contrast, experiments with N-AC revealed both GC-dependent and GC-independent vasorelaxation. This result is explained by specificity of metabolites formed in the reaction of N-AC with NO stores, *i.e*. with RS-NO and DNIC proteins. Interaction with protein DNIC results in transfer of $Fe^{+}(NO^{+})_2$ groups to N-AC accompanied by the formation of low-molecular DNIC with N-AC **(4)**.



(4)

Low-molecular DNIC can efficiently S-nitrosylate the SH-groups in proteins [13]. For example, such a reaction with thiol groups in Ca^{2+} and K^{+} channels can produce GC-independent vascular relaxation [8], which we observed here. Disintegration of low-molecular DNIC activated GC by NO released from DNIC **(5)**:



(5)

This reaction was manifested in partial inhibition of relaxation by methylene blue. S-nitrosylation of ionic channel proteins can also occur under the action of S-nitrosoacetylcysteine (S-NO-N-AC), which is produced during interaction with RS-NO and N-AC stores of NO (reaction of renitrosylation).

Less pronounced vasodilation induced by DETC can be explained by predominant localization of this agent in the hydrophobic phase. The degree of DETC participation in vascular relaxation is determined by hydrophobic character of DETC-iron complexes, which pronouncedly decreases water solubility of DETC in biological tissues.

Since not only free DETC, but also iron-carbamate complexes can destroy both DNIC and RS-NO producing only MNIC-DETC, it cannot be excluded that DETC interacts predominantly with lipid-soluble membrane-bound NO store, while N-AC interacts with both water and lipid-soluble NO stores.

It should be noted that DETC produced a more pronounced toxic effect on vessels compared to N-AC. Toxicity of DETC can be manifested in weakening of subsequent vascular responses, so successively applied DETC and N-AC produce less pronounced relaxation (by 28%) compared to their individual effects. It is also possible that this toxicity is manifested in smaller vasodilator activity of DETC in comparison with N-AC, since 10 mM N-AC produces the same relaxation as 300 mM DETC.

Thus, exogenous DNIC can initiate the formation of NO stores in blood vessels in the forms of protein-bound DNIC and RS-NO. These stores can be located both in hydrophilic and hydrophobic cell compartments. The degree of vasorelaxation induced by DETC and N-AC is determined by the nature of interactions of these agents with NO stores.

It is widely thought that in biological organism NO stores play a role of NO buffer. On the one hand, binding of NO surplus in a store protects the organism from its cytotoxic and excessive vasodilatatory action. On the other hand, NO store can serve as a reserve, which can be used when needed. Probably, the processes of formation and disintegration of NO store can participate in prevention of disturbances caused by excess or deficiency of NO, so the directed modulation of these processes can have clinical implications [3]. Among the two examined agents, clinical use of N-AC seems to be more desirable, since this substance

more efficiently releases NO from its stores than DETC [5,7].

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