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The potent vasodilating and guanylyl cyclase activating dinitrosyl-iron(II) complex is stored in a protein-bound form in vascular tissue and is released by thiols

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We studied the biological activity, stability and interaction of dinitrosyl-iron(II)-L-cysteine with vascular tissue. Dinitrosyl-iron(II)-L-cysteine was a potent activator of purified soluble guanylyl cyclase (EC_{50} 6 nM with and 100 nM without superoxide dismutase) and relaxed noradrenaline-precontracted segments of endothelium-denuded rabbit femoral artery (EC_{50} 10 nM superoxide dismutase). Pre-incubation (5 min; 310 K) of endothelium-denuded rabbit aortic segments with dinitrosyl-iron(II)-L-cysteine (0.036–3.6 mM) resulted in a concentration-dependent formation of a dinitrosyl-iron(II) complex with protein thiol groups, as detected by ESR spectroscopy. While the complex with proteins was stable for 2 h at 310 K, dinitrosyl-iron(II)-L-cysteine in aqueous solution (30–360 μM) decomposed completely within 15 min, as indicated by disappearance of its isotropic ESR signal at g_{av} = 2.03 (293 K). Aortic segments pre-incubated with dinitrosyl-iron(II)-L-cysteine released a labile vasodilating and guanylyl cyclase activating factor. Perfusion of these segments with *N*-acetyl-L-cysteine resulted in the generation of a low molecular weight dinitrosyl-iron(II)-dithiolate from the dinitrosyl-iron(II) complex with proteins, as revealed by the shape change of the ESR signal at 293 K. The low molecular weight dinitrosyl-iron(II)-dithiolate accounted for an enhanced guanylyl cyclase activation and vasodilation induced by the aortic effluent. We conclude that nitric oxide (NO) produced by, or acting on vascular cells can be stabilized and stored as a dinitrosyl-iron(II) complex with protein thiols, and can be released from cells in the form of a low molecular weight dinitrosyl-iron(II)-dithiolate by intra- and extracellular thiols.

Dinitrosyl-iron(II)-dithiolate; Endothelium-derived relaxing factor; Soluble guanylyl cyclase; *N*-Acetyl-L-cysteine; Nitric oxide; Electron spin resonance spectroscopy

1. INTRODUCTION

Nitric oxide is formed in organisms from endogeneous (L-arginine) and exogeneous (organic nitrates, sodium nitroprusside) sources and accounts for such biological responses as relaxation of smooth muscle, inhibition of platelet activation, modulation of neurotrasmission and non-specific cell-bound cytotoxicity (for review see [1]). Paracrine actions of NO require its intercellular transfer from a generator to a target cell. However as NO is extremely labile in oxygen-containing solution, a paracrine action of free NO is questionable. Recently, it was hypothesized that NO might be stabilized in organisms by binding to iron(II)-dithiolate complexes [2]. This assumption was based on the detection of paramagnetic dinitrosyl-iron(II) complexes with protein thiol groups in cells and tissues after exposure to endogeneous or exogeneous NO [3–6]. These so-called '2.03-complexes' [2] are in equilibrium with dinitrosyl-iron(II) complexes of low molecular weight thiols [7]. The latter possess biological activity similar to NO and endothelium-derived relaxing factor, i.e. they induce hypotension [8], relax vascular smooth muscle [9] and inhibit platelet aggregation [10].

The objective of the present investigation was to assess whether NO can be transiently stored as a dinitrosyl-iron(II) complex with protein thiols in the vascular wall, and whether a dinitrosyl-iron(II) complex with low molecular weight thiols could function as a diffusible NO carrier to facilitate paracrine NO actions.

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2. MATERIALS AND METHODS

2.1. *Materials*

Dinitrosyl-iron(II)-L-cysteine (3.6 mM in water) was prepared as described [11], with L-cysteine in 20-fold excess of iron. The complex was obtained in quantitative yield with respect to iron, as ascertained by double integration of its ESR signal under non-saturating conditions against an *N*-oxyl-2,2',6,6'-tetramethyl-4-piperidinol standard [7]. Dinitrosyl-iron(II)-L-cysteine was diluted in ice-cold Tyrode's solution (see below) immediately before use. NO gas (99.8%, Messer, Griesheim, Germany) was purified by high vacuum (0.01 mm Hg) distillation, Iron(II)-sulfate, L-cysteine, *N*-acetyl-L-cysteine and superoxide dismutase (bovine blood) were obtained from Sigma, Dreeich, Germany. Soluble guanylyl cyclase was purified to apparent homogeneity from bovine lung [12].

2.2. *Measurement of vasodilator activity*

The vasodilator response of precontracted (0.03–0.3 μM noradren-

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aline) endothelium-denuded segments of rabbit femoral artery to dinitrosyl-iron(II)-L-cysteine was measured as described previously [13]. The segments were perfused with 30 ml/h while the organ bath solution was continuously exchanged with 60 ml/h (held at 310 K). For both perfusates oxygenated Tyrode's solution (composition in mM: Na^+ 144.0, K^+ 4.0, Ca^{2+} 1.6, Mg^{2+} 2.0, Cl 140.4, HCO_3 11.9, H_2PO_4 0.36, glucose 11.2, calcium-disodium EDTA 0.025, pO_2 300 mm Hg) was used. Superoxide dismutase (0.1–1 μM) was added to the luminal perfusate, and pO_2 was reduced to 30 mm Hg. After dilution in cold (283 K) Tyrode's solution, dinitrosyl-iron(II)-L-cysteine was perfused through the segment within 2 min. To demonstrate the release of vasodilator activity from dinitrosyl-iron(II) complex with proteins, the perfusate from a donor segment (endothelium-denuded rabbit aorta) pre-incubated with dinitrosyl-iron(II)-L-cysteine (see below) was transferred into a precontracted detector segment (endothelium-denuded femoral artery; transmit time 2 s).

2.3. *Pre-incubation of aortic segments with dinitrosyl-iron(II)-L-cysteine*

The lumen of an aortic segment (200 mg wet weight, 4.8 cm length) placed in the organ bath was incubated (5 min) with a dinitrosyl-iron(II)-L-cysteine solution (0.3 ml). Thereafter, the bathing and the intraluminal solutions were quickly exchanged with fresh Tyrode's solution. The segments were then frozen in liquid nitrogen either immediately or after defined periods of perfusion.

2.4. *Measurement of guanylyl cyclase activation*

Activation of guanylyl cyclase was measured by formation of [^{32}P]cyclic GMP from [α-^{32}P]GTP as described [14]. Dinitrosyl-iron(II)-L-cysteine was incubated (10 min, 310 K, 100 μl final volume) wth guanylyl cyclase (1 μg/ml), glutathione (2 mM), superoxide dismutase (1 μM), gamma-globulin (0.1 mg/ml), $MgCl_2$ (4 mM), EGTA (1 mM), HEPES (15 mM), pH 7.4, and [α-^{32}P]GTP (0.1 mM). For measurement of guanylyl cyclase stimulation by the aortic perfusate, the effluent (130 μl) was collected over 15 s in a test tube containing guanylyl cyclase/[α-^{32}P]GTP solution (50 μl) and was incubated for 1min at 310 K [15]. Guanylyl cyclase activation was related to the activation induced by 0.1 mM sodium nitroprusside (100 %), which increased basal guanylyl cyclase activity 40-fold.

2.5. *Measurement of ESR spectra*

ESR spectra were recorded either at 77 K on frozen probes (0.7 ml aqueous solution or one aortic segment) contained in a quartz Dewar with 5 mm inner diameter chilled by liquid nitrogen, or at 293 K on probes (40 μl aqueous solution or a piece of aortic tissue) contained in a quartz capillary tube (1 mm i.d.) on a Bruker ESR 420 and on a Varian E4 spectrometer as described [11]. Instrument settings were (if not indicated otherwise): microwave frequency 9.33 GHz, microwave power 20 mW, modulation amplitude 0.5–10 Gauss, time constant 0.1–2 s.

3. RESULTS

3.1. *Biological activity of dinitrosyl-iron(II)-L-cysteine*

Dinitrosyl-iron(II)-L-cysteine potently activated purified soluble guanylyl cyclase (EC_{50} 100 nM; Fig. 1A) and relaxed precontracted endothelium-denuded arterial segments (EC_{50} 10 nM; Fig. 1B). Superoxide dismutase (1 μM) increased the guanylyl cyclase activating potency of dinitrosyl-iron(II)-L-cysteine by 10-fold (Fig. 1A). Dinitrosyl-iron(II)-L-cysteine (1 μM) in Tyrode's solution (pO_2 30 mmHg) retained biological activity at 273–283 K for more than 10 min, but at 310 K lost it with a half life of 30 s, as measured by a decrease in its guanylyl cyclase-stimulating activity. The instability of dinitrosyl-iron(II)-L-cysteine was paralleled by a loss of its absorbance maximum at 395 nm (ε_{395} = 3000 $M^{-1} \cdot cm^{-1}$) and by disappearance of its ESR signal (see below). Preliminary experiments revealed that the decomposition of the complex largely followed apparent zero order kinetics, the half life (2 min at 10 μM dinitrosyl-iron(II)-L-cysteine, 310 K, Tyrode's solution) being increased by low oxygen tension, low temperature, superoxide dismutase, L-cysteine, high dinitrosyl-iron(II)-L-cysteine concentrations, and decreased by the superoxide generating system xanthine/xanthine oxidase (data not shown).

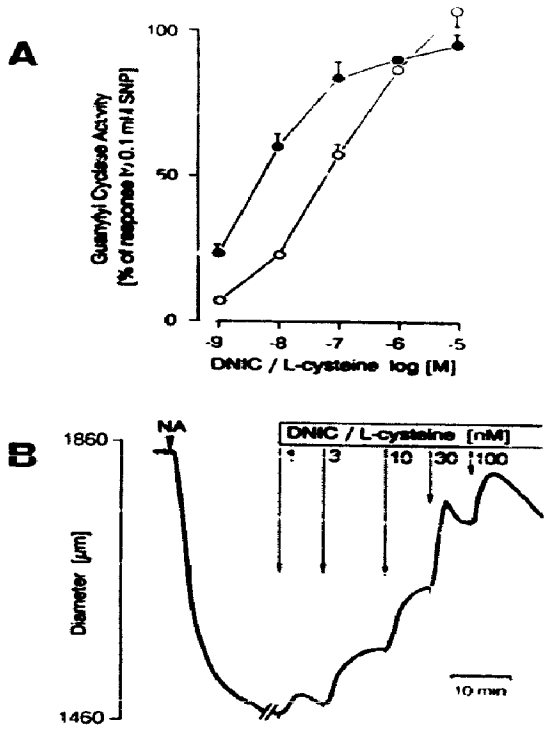

Fig. 1. Dinitrosyl-iron(II)-L-cysteine (DNIC/L-cysteine) activates purified soluble guanylyl cyclase (A) and relaxes endothelium-denuded rabbit femoral artery (B). (A) Mean values ± SEM of 3 experiments performed in triplicate in the presence (●) and in the absence (○) of superoxide dismutase (1 μM). (B) Representative tracing from one of 3 experiments. NA = noradrenaline (0.3 μM). Superoxide dismutase was present in the perfusate (0.1 μM).

3.2. *Formation of dinitrosyl-iron(II) complex with proteins in vascular tissue*

The anisotropic ESR signal of dinitrosyl-iron(II)-L-cysteine at $g_\perp$ = 2.037 and $g_{||}$ = 2.012 recorded at 77 K (Fig. 2A) converts to an isotropic ESR signal at g = 2.03 with a hyperfine structure (hfs) in liquid solution at 293 K (Fig. 2C). Exposure of a wet aortic segment to an oxygen-free NO atmosphere (15 min; pNO 300 mm Hg) induced the formation of a paramagnetic species within

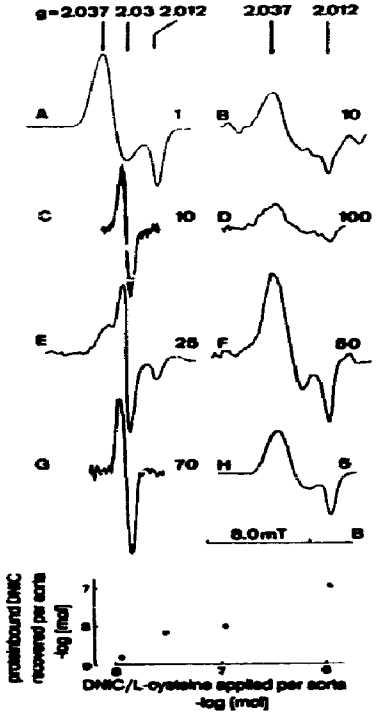

Fig. 2. ESR signals of dinitrosyl-iron(II)-L-cysteine (0.1 mM) at 77 K (A) and 293 K (C), of endothelium-denuded rabbit aorta after eqilibration with NO (B, 77 K; D, 293 K), of dinitrosyl-iron(II)-L-cysteine(0.36 mM)-preincubated rabbit aorta frozen (77 K) 5 min (E) and 2 h (F) after washing and perfusion with Tyrode's solution (containing superoxide dismutase, 0.1 μM), and of an *N*-acetyl-L-cysteine (0.1 M)-containing solution used for incubation of the segment from (F), and recorded at 293 K (G) and 77 K (H). Numbers beneath the spectra indicate the instrument gain (relative units). (Bottom) Dependency of the amount of dinitrosyl-iron(II) complex with proteins (protein-bound DNC) detected in a rabbit aortic segment (200 mg wet weight) at 77 K (15 min after washout) on the amount of dinitrosyl-iron(II)-L-cysteine applied (in 0.3 ml) intraluminally to the segment. Values show means from 3 experiments performed on each concentration. Error bars are smaller than symbols.

the tissue, exhibiting an ESR signal at 77 K with features similar to that of dinitrosyl-iron(II)-L-cysteine (Fig. 2B). However, the shape of the signal did not change on recording at 293 K (Fig. 2D), indicating the macromolecular nature of the paramagnetic species, i.e. dinitrosyl-iron(II) complex with protein thiols [3,7]. Incubation (5 min) of aortic segments with dinitrosyl-iron(II)-L-cysteine (0.036–3.6 mM) resulted in a concentration-dependent formation of paramagnetic complexes of dinitrosyl-iron(II) with protein thiol groups in the vascular wall, with about 10% recovery of paramagnetic centers at all concentrations of dinitrosyl-iron(II)-L-cysteine tested (Fig. 2, bottom). The ESR signal from dinitrosyl-iron(II)-L-cysteine-treated segments was not discernibly different from the signal exhibited by NO-treated segments (Fig. 2B). However, at room temperature, the broad signal from dinitrosyl-iron(II) complex with proteins was overlayed by a narrow (7-Gauss width) isotropic ESR signal at $g = 2.03$ (Fig. 2E), which is attributed to unreacted dinitrosyl-iron(II)-L-cysteine. This signal disappeared completely after washing of the segments (15 min), whereas the signal from dinitrosyl-iron(II) complex with proteins was largely maintained for up to 2 h at 310 K (Fig. 2F). As estimated from double integration [7], after washing (15 min) the concentration of dinitrosyl-iron(II)-L-cysteine was at least 100-fold lower than that of the dinitrosyl-iron(II) complex with proteins. A high concentration of *N*-acetyl-L-cysteine (0.1 M/0.1 ml/50 mg aorta/5 min, 310 K) converted the dinitrosyl-iron(II) complex with proteins into a dinitrosyl-iron(II) complex with low molecular weight thiols and generated a composite ESR signal in the tissue similar to that shown in Fig. 2E. In addition a dinitrosyl-iron(II) complex with *N*-acetyl-L-cysteine appeared in the solution, as detected by its ESR signal at 293 K (Fig. 2G) and 77 K(Fig. 2H). Furthermore, the content of dinitrosyl-iron(II) complex with proteins was reduced more rapidly by perfusion (60 ml/h for 2 h) with *N*-acetyl-L-cysteine (5 mM) (from 7.5 nmol/g tissue after 5 min incubation with dinitrosyl-iron(II)-L-cysteine (36 μM) to 4.2 ± 0.2 nmol/g tissue in the absence, and to 1.9 ± 0.2 nmol/g tissue in the presence, of *N*-acetyl-L-cysteine; $n = 3$).

3.3. *Release of vasodilating and guanylyl cyclase activating factor from dinitrosyl-iron(II) complex with proteins in rabbit aorta*

Effluent from aortic segments pre-incubated for 5 min with dinitrosyl-iron(II)-L-cysteine (36 μM) induced activation of soluble guanylyl cyclase (Fig. 3A) and relaxation of precontracted rabbit femoral artery segments (Fig. 3B). After an initial rapid decrease in the biological activity of the effluent due to the washout of unreacted dinitrosyl-iron(II)-L-cysteine, the activating factor was detected in the perfusate in a constant amount for at least 40 min. If *N*-acetyl-L-cysteine (1 mM) was perfused through the dinitrosyl-iron(II)-L-cysteine (36 μM)-preincubated aorta 20–40 min after washout, the guanylyl cyclase activating and relaxing effects of the effluent increased (Fig. 3A,B). This effect was not observed if *N*-acetyl-L-cysteine was added distally to the donor segment, thereby excluding the possibility that the effect was due to a direct interaction between *N*-acetyl-L-cysteine and the vasodilator/guanylyl cyclase activator.

The ESR signal of dinitrosyl-iron(II) complex with low molecular weight thiols could be detected in the superfusate, but only after pre-incubation with the highest concentration of dinitrosyl-iron(II)-L-cysteine (3.6 mM). In this condition, the paramagnetic complex was released at a constant rate (0.15 ± 0.05 nmol/ml/5 min for at least 20 min), and induced maximal guanylyl cyclase activation and vasodilation (data not shown).

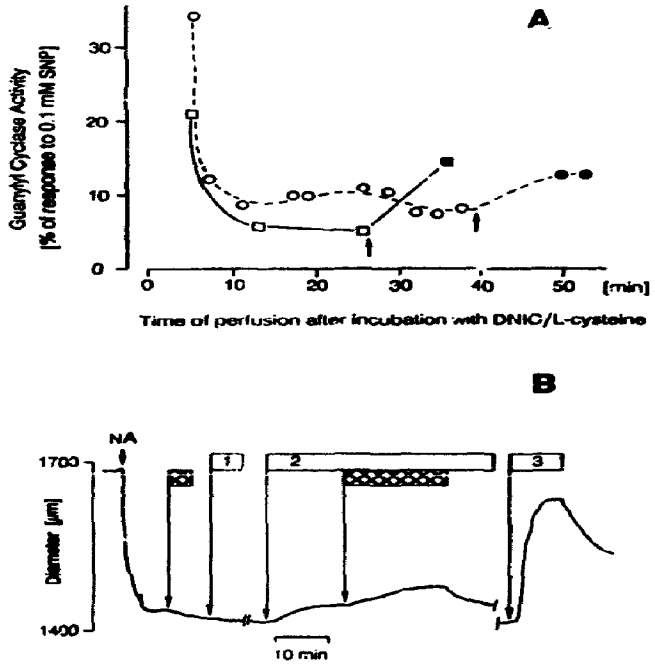

Fig. 3. Activation of soluble guanylyl cyclase (A) and relaxation of precontracted (0.3 μM noradrenaline (NA)) endothelium-denuded rabbit femoral artery (B) by the intraluminal perfusate from a dinitrosyl- iron(II)-L-cysteine(DNIC/L-cysteine)-preincubated rabbit aortic segment after different periods of perfusion. (A) Mean values from two representative experiments (○,□) performed in triplicate (SEM smaller than symbols) with 36 μM dinitrosyl-iron(II)-L-cysteine applied during pre-incubation. Intraluminal perfusion with *N*-acetyl-L-cysteine (1 mM; indicated by arrows) increases guanylyl cyclase activation by the perfusate (●,■). (B) The upper bars indicate perfusion of the femoral artery with 1, 2, and 3 aortic effluent 20 min after washout of pre- incubate (aorta pre-incubated without (1), with 36 μM (2) and with 360 μM (3) dinitrosyl-iron(II)-L-cysteine, respectively). The cross- hatched bars below indicate perfusion of *N*-acetyl-L-cysteine (1 mM) through both segments. Representative tracing from one of 3 experiments.

4. DISCUSSION

There is an ongoing discussion as to whether NO, which is an immediate product formed from L-arginine by endothelial NO synthase [16], is sufficiently stable to account for paracrine actions, such as endothelium-dependent relaxation. It has been proposed that NO generated from L-arginine, but also from nitrovasodilators, may be rapidly bound and stabilized by low- and high-molecular weight iron–sulfur complexes [2]. We have provided evidence that dinitrosyl-iron(II)-L-cysteine has pharmacological properties similar to those described for EDRF; it potently activates purified soluble guanylyl cyclase [15], relaxes endothelium-denuded precontracted rabbit femoral artery segments [17], decomposes quickly in aqueous solution, and its stability is affected by oxygen, superoxide dismutase, xanthine oxidase and temperature in a similar manner to EDRF [1]. Furthermore, we have shown that incubation of aortic tissue with NO gas and dinitrosyl-iron(II)-L-cysteine results in formation of quite stable dinitrosyl-iron(II) complex with protein thiols. According to former investigations, probably several different cytosolic and microsomal proteins with apparent molecular weights in the range 50–120 kDa are preferentially targeted by dinitrosyl-iron(II) complex in animal tissues [18]. These protein complexes act as NO stores in the tissue, and by reaction with intracellular low molecular weight thiols decompose and release low molecular weight dinitrosyl-iron(II)-dithiolate complexes into the extracellular space. This was evident from the finding that the effluent from a dinitrosyl-iron(II)-L-cysteine-pre-incubated aortic segment exhibited biological activity (relaxation of detector segment; guanylyl cyclase activation) for a much longer time than expected from the half life of dinitrosyl-iron(II)-L-cysteine in solution. We have also provided evidence that extracellular low molecular weight thiols probably promote the release of dinitrosyl-iron(II) complex from the macromolecular store, as in the presence of the membrane-permeable *N*-acetyl-L-cysteine [19], the dinitrosyl-iron(II) complex with proteins was partially converted into the low molecular dinitrosyl-iron(II) complex. The latter complex was released into the extracellular space, and was detected by both ESR spectroscopy and by the increase in biological activity of the perfusate. In conclusion, the dinitrosyl-iron(II)-dithiolate complex possesses biological properties similar to EDRF. Consequently, we propose that EDRF can be stored and released in a fashion similar to that demonstrated here for dinitrosyl-iron(II)-L-cysteine.

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