Current Topics in Biophysics 2002, 26(1), 101-113

INFLUENCE OF TRANSITION METALS ON STABILITY OF VARIOUS S-NITROSOTHIOLS

ANATOLY F. VANIN[1,2], BERNARD MULLER[2], JACICARLOS L. ALENCAR[2], IRINA I. LOBYSHEVA[1,3], FRANCOISE NEPVEU[3], JEAN-CLAUDE STOCLET[2]

[1]Institute of Chemical Physics, Russian Academy of Sciences, 119991 Moscow, Kosygin Str.4, Russian Federation;
[2]Pharmacologie et Physico-Chimie des Interactions Cellulaires et Moleculaires, UMR CNRS 7034, Universite Louis Pasteur de Strasbourg, Faculte de Pharmacie, BP, F-67401 Illkirch Cedex, France;
[3]Laboratoire Pharmacophores Redox Phytochimie et Radiobiologie (EA -3030), Universite Paul Sabatier, Faculte des Sciences Pharmaceutiques, 35 chemin des Maraichers, F-31062 Toulouse Cedex 4, France.

The analysis of the destroying action of copper and iron ions on S-nitrosocysteine (cys-NO) demonstrated that the amount of intrinsic copper in the solutions was too low (≤0.2 μM) to produce a destabilizing effect on cys-NO. Only admixtured iron present in the solutions at the concentrations of about 1-2 μM was responsible for the process of cys-NO degradation, which occured through the formation of dinitrosyl iron complexes (DNIC) resulting in free NO liberation. The conclusion was drawn from demonstration of the capacity of copper/iron chelator, bathophenantroline disulfonic acid to selectively block the destructive effect of iron, but not of copper ions on cys-NO. Similar results were obtained in relation with the influence of intrinsic copper/iron on vasorelaxant properties of cys-NO on isolated rat aorta. By contrast, the concentrations of both intrinsic copper and iron ions in the solutions were proved to be not large enough to initiate the decomposition of S-nitrosoglutathione. The process was initiated when copper/iron ions were added to the solution at the concentration of 100 μM in the presence of reducing agents, ascorbate or GSH. S-dinitrosodithiotreitol (DTT-2NO) was decomposed spontaneously without any effect of intrinsic copper/iron ions in the solution. Nevertheless, the addition of copper/iron (20 μM) in the presence of ascorbate (1 mM) also accelerated the process of DTT-2NO decomposition. Such type of enhancing effect of copper/iron ions was suggested to be characteristics of enzymatic manner of RS-NO decomposition. The process of GS-NO or DTT-2NO decomposition catalyzed by iron ions (100μM) in the presence of GSH or DTT was associated with formation of DNIC with GSH or DNIC with DTT, respectively. These results allow to propose that the process of GS-NO and DTT-2NO decomposition catalyzed by iron ions proceeded, as with cys-NO, through the formation of DNIC. The subsequent decomposition of DNIC ensures appearence of free NO molecules in the solution.

INTRODUCTION

It is reasonable to suggest two opportunities for S-nitrosothiols (RS-NOs) to produce NO in the experiments on biological systems in vitro. First is the decomposition of RS-NOs catalyzed by intrinsic copper or iron ions in the medium (McAninly, Williams, Askew, Butler & Russel, 1993; Gorge, Meyer, Hothersall, Neild, Payne & Noronha-Dutra, 1995; Williams, 1996; Singh, Hogg, Joseph & Kalyanaraman, 1996; Dicks, Swift, Williams, Butler, Al-Sa`doni & Cox, 1996; Gorren, Schrammel, Schmidt & Mayer, 1996; Gorge, Hothersall, Neild & Noronha-Dutra, 1996; Butler & Rhodes, 1997; Al-Sa`doni, Megson, Bisland, Butler & Flitney, 1997; Dicks, Li, Munro, Swift & Williams, 1997; Dicks, Beloso & Williams, 1997; Vanin, Malenkova & Serezhenkov, 1997; Vanin, 1998; Gaston, 1999; Sorenson, Skiles, Xu, Aleryani & Kostka, 2000; Sheu, Zhu & Fung, 2000). Second is enzymatic NO release from RS-NOs for instance on cell surface (Zai, Rudd, Scriber & Loscalzo, 1999; Ramachandran, Root, Jiang, Hogg & Mutus, 2001). One-electron reduction of RS-NOs, resulting in the release of a neutral NO molecule ($RS^-NO^+ + e^- \rightarrow RS^- \ldots NO \rightarrow RS^- + NO$ (Williams, 1985; Scorza, Pietraforte & Minetti, 1985; Rubanyi, Johns, Wilcox, Bates & Harrison, 1991; Feelish & Stamler, 1996; Kashibo-Iwatsuki, Yamaguchi & Inoue, 1996; Xu, Vita & Keaney, 2000) is a more efficient process of RS-NO decomposition.

At the present time, most investigators (McAninly *et al.*, 1993; Gorge *et al.*, 1995; Williams, 1996; Singh *et al.*, 1996; Dicks *et al.*, 1996; Gorge *et al.*, 1996; Al`Sadoni *et al.*, 1997; Butler & Rhodes, 1997) have suggested that RS-NO decomposition in solution is catalyzed mainly by copper ions in accordance with Scheme 1:

$$Cu^+ + RS^-NO^+ \rightarrow Cu^{2+} + RS^- + NO$$
$$Cu^{2+} + RS^- \rightarrow Cu^+ + RS^\bullet$$

$$RS\text{-}NO \rightarrow NO + 1/2(RS\text{-}SR)$$

(net reaction) (Scheme 1)

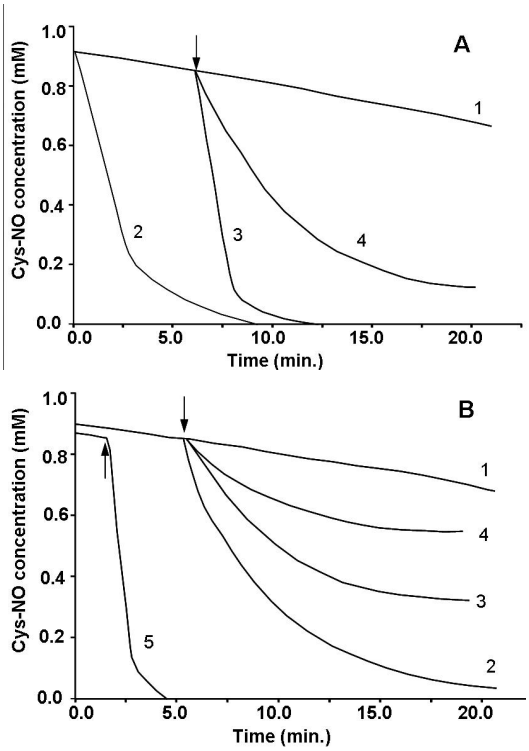


Fig.1. Kinetics of the decomposition of cys-NO preparations synthesized at the ratio of $[HNO_2]$: [cysteine] = 1.5 : 1 followed by dilution in Hepes buffer, 150 mM, pH 7.4 (preparation **A**) - curves **1**, top and bottom panels or by treating with a strong basic solution (preparation **B**) - curve **2**, top panel). The preparations **A** were added with 1mM or 50 μM of ascorbate (curves **3** or **4**, top panel, respectively), 20 μM Cu^{2+}, Fe^{2+} , Fe^{3+} or 50μM ascorbate + 20μM Cu^{2+}/Fe^{2+} (curves **2-5**, respectively, bottom panel).

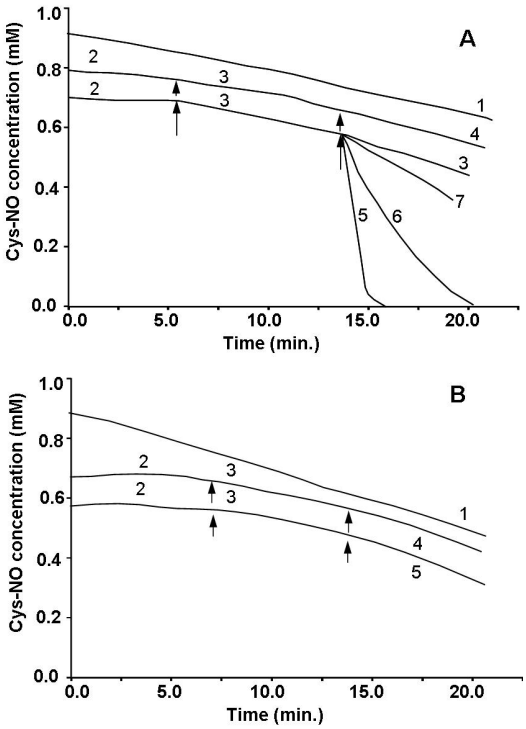
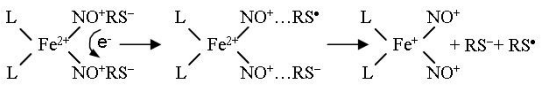


Fig.2. Influence of subsequent additions (shown by arrows) of ascorbate (1mM) (curves **3**, top and bottom panels), Fe^{3+} (20μM) (curves **4**, top and bottom panels) and Cu^{2+} (20, 2 or 0.2μM) (curves **5-7**, respectively, top and bottom panel) on the stability of cys-NO (preparation **A**) in the presence of BPDS (1mM) (top panel) or BCS (1mM) (bottom panel).Curves **2** (top and bottom panels): BPDS or BCS were added alone. Curves **1** (top and bottom panels): initial preparation of cys-NO.

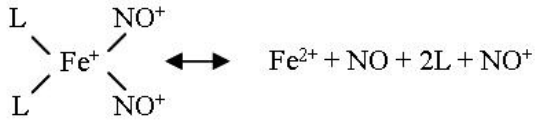
The main argument in favour of this mechanism is the blocking action of the so called "selective" Cu^{+} chelator, neocuproine on RS-NO degradation (Williams, 1996; Dicks *et al.*, 1996; Gorge *et al.*, 1996; Al`Sadoni *et al.*, 1997). However recent data demonstrate that NO initiates the binding of iron ions (initially Fe^{2+}) to neocuproine, resulting in the formation of EPR-detectable stable dinitrosyl iron complexes (DNIC) with neocuproine (Vanin, Serezhenkov & Malenkova, 2001). Predominant localization of unpaired electron on iron atom in DNIC ensures the transition of electron configuration of iron from d^6 (Fe^{2+}) to d^7 (Fe^{+}) Vanin, Stukan & Manukhina, 1996). These data imply that the hypothesis on the main role of copper irons on RS-NO degradation in physiological medium warrants a critical reevaluation.

As regard to Fe^{2+}-catalyzed decomposition of RS-NOs, iron is suggested not serve as a redox agent (Vanin *et al.*, 1997; Vanin, 1998). Each ion Fe^{2+} ion binds two RS-NO molecules and provides migration of an electron between these molecules (i.e., their mutual one-electron oxidation reduction) and thereby decomposition (Scheme 2). The NO molecule and nitrosonium ion (NO^{+}) emerging from this decomposition maintain their connection with iron, which results in formation of dinitrosyl iron complexes (DNICs). Diverse anionic compounds including thiols can serve as a ligands (L) in these complexes (Vanin *et al.*, 1997; Vanin, 1998).



(Scheme 2)

In accordance with the chemical equilibrium between DNIC and its constituents (Scheme 3; Vanin *et al.*, 1997; Vanin, 1998), appearance of DNICs in the solution gives free NO molecules.



(Scheme 3)

Thus, according to this proposed mechanism, NO is generated by the DNICs formed during RS-NOs decomposition, rather than directly by RS-NOs.

As to enzymatic NO release from RS-NOs catalyzed by protein disulfide isomerase (PDI), a proposed plausible mechanism is:

$$2RS\text{-}NO + PDI\text{-}2SH \rightarrow 2NO + 2RSH + PDI(S\text{-}S)$$

(Scheme 4)

followed by reducing of the oxidized form of protein, for example by the plasma membrane NADH-oxidoreductase. The role of enzymatic systems for NO release on cell surface as well as inside cells is demonstrated (Kowaluk & Fung, 1990; Gorge *et al.*, 1996; Gaston, 1999; Zai *et al.*, 1999; Sorenson *et al.*, 2000; Ramachandran *et al.*, 2001).

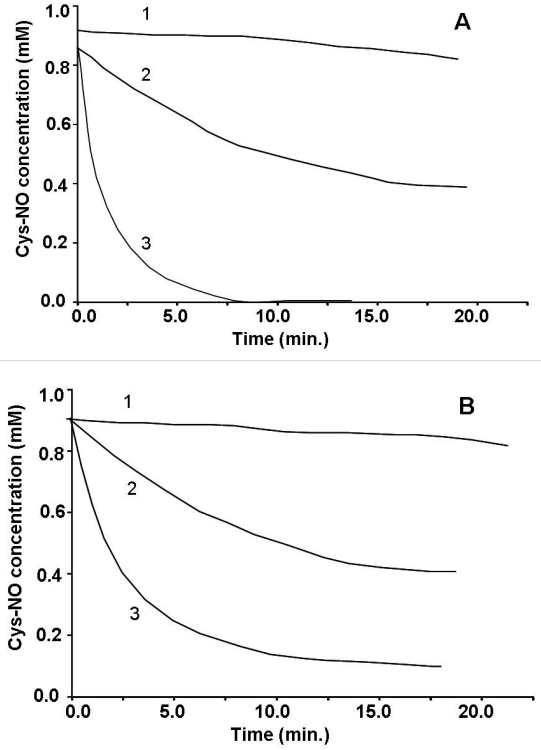


Fig.3. Influence of addition of L-cysteine (1mM or 50μM) (curves **1** or **2**, respectively, top and bottom panels), L-cysteine (1 mM) + 20μM Cu^{2+} (curve **3**, top panel) or L-cysteine (1 mM) + 100μM Fe^{3+} (curve **3**, bottom panel) on the stability of cys-NO (preparation **A**).

The main purpose of the present study was to analyse the influence of copper and iron on the process of decomposition of RS-NOs: S-nitrosocysteine (cys-NO), S-nitrosoglutathione (GS-NO) and S-dinitrosodithiotreitol (DTT-2NO).

MATERIALS AND METHODS

Materials

Na-ascorbate, disodium salt of basthocuproine disulfonic acid (BCS), disodium salt of bathophenantroline disulfonic acid (BPDS), 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolydine-NO-oxyl (3-carboxy-proxyl), sodium citrate, L-cysteine, reduced glutathione (GSH), dithiotreitole (DTT), all from Sigma (St.Louis, MO,USA), ferrous sulfate, ferric chloride, cupric sulfate, all from Fluka (Buchs, Switzerland) were used in experiments. Gaseous NO was obtained as described elsewhere (Vanin *et al.*, 1997).

Synthesis of RS-NOs and dinitrosyl iron complexes.

RS-NOs were synthesized by addition of 0.125 ml of 1N HCl to a mixture of 200 mM of L-cysteine and 300 mM $NaNO_2$, 200 mM of GSH and 270 or 300 mM $NaNO_2$ or 100 mM DTT and 250 or 300 mM $NaNO_2$ in 15 mM Hepes buffer (pH 7.4) followed by snap freezing of aliquots (10 to 20 μl) in liquid nitrogen. Two ml of 150 mM Hepes buffer (pH 7.6) were added to frozen aliquots, resulting in the preparation of RS-NO solutions at pH 7.3-7.4. The solutions were used for optical assays and contraction experiments. In some experiments a strong basic solution was added to acid cys-NO solution to increase pH value to 7.3-7.4. The concentrations of RS-NOs were estimated by optical absorbance at 340 nm. Paramagnetic DNICs with cysteine or GSH were obtained by treating the $FeSO_4$ and thiol solutions with gaseous NO in a Thunberg tube in 10 mM Hepes buffer (pH 7.4) at a molar ratio 1: 30 as described by Vanin *et al.* (Vanin, Malenkova & Serezhenkov, 1997). Similar approach was used for synthesis of DNIC with phosphate. DNIC with BCS was synthesized by addition of 10 mM of BCS to 3 mM solution of DNIC with phosphate in 100 mM phosphate buffer as described elsewhere (Vanin, Stukan & Manukhina, 1996). DNIC with DTT was synthesized by mixing 0.5 ml of $FeSO_4$ in distilled water (pH 5.5) and 4.5 ml of DTT solution in Hepes buffer (pH 11) in NO gas followed by evacuation of NO from the tube. The

concentrations of DNICs were determined by EPR method.

Optical and EPR assays

Optical spectra from the solutions of RS-NOs in 150 mM Hepes buffer were recorded using Beckman (model 25) or Varian Cary 300 Bio spectrometers in 2 ml open cuvettee ast ambient temperature. The initial concentrations of RS-NOs was 0.9 mM in most assays. The decomposition of RS-NOs was monitored spectrophotometrically by decrease of absorbance at 340 nm with time.

EPR spectra were recorded from the solutions of DNICs, cys-NO or GS-NO at ambient temperature in 100 μl pipettes or at 77K in quartz dewar filled by liquid nitrogen using a Bruker-IBM ER 300 (Bruker, Germany) or Miniscope (Magnettech, Germany) EPR spectrometers at X-band, respectively. To estimate the concentration of a paramagnetic centers using standard samples, the nitroxyl radical sample (3-carboxy-proxyl) or paramagnetic DNIC with cysteine solution of known concentration were used.

Contraction experiments

Male Wistar rats (280-350 g) were killed by cervical dislocation. The thoracic aorta was removed, cleaned of connective and fat tissues and cut into rings (2-3 mM length). The endothelium was removed by gently rubbing the intimal surface of the rings with curved forceps. Rings were then mounted under passive tension of 2 g in 10 ml organ bathes filled with a Krebs solution (composition in mM: NaCl 119; KCl 4.7; $MgSO_4$ 1.17; $CaCl_2$ 1.25; KH_2PO_4 1.18; $NaHCO_3$ 25; glucose 11) continuously kept at 37°C and bubbled with 95% O_2 / 5% CO_2. Tension was measured with an isomeric force transducer. After equilibration period of 60 min, aortic rings were precontracted with noradrenaline (NA, 1 μM). Acetylcholine (1 μM) was subsequently added. Rings were considered as endothelium-denuded when the addition of acetylcholine failed to induce a relaxing effect. After a washing period of 60 min, the rings were precontracted by NA (0.1 μM, a contraction that produced around 80% of maximal response to NA in endothelium-denuded rings). When the contraction reached a steady-state level, RS-NOs or DNIC were added to the bath (either as single concentration or in a cumulative manner). In some experiments, when the concentration induced by 0.1 μM NA reached a steady level, L-cysteine (10 μM), BPDS (100 μM), BCS (100 μM), ascorbate (500 μM), $FeSO_4$ or $CuSO_4$ were added to the bath, before the addition of cys-NO or DNIC.

Relaxing effect was expressed in percentage of contraction, 100% contraction being the tone induced by 0.1 μM NA. The EC_{50} values (concentration that produced 50% relaxation of precontracted vessels, relaxation being measured at its maximum) were determined by log-logit regression.

Statistical analysis

Statistical comparisons were performed using

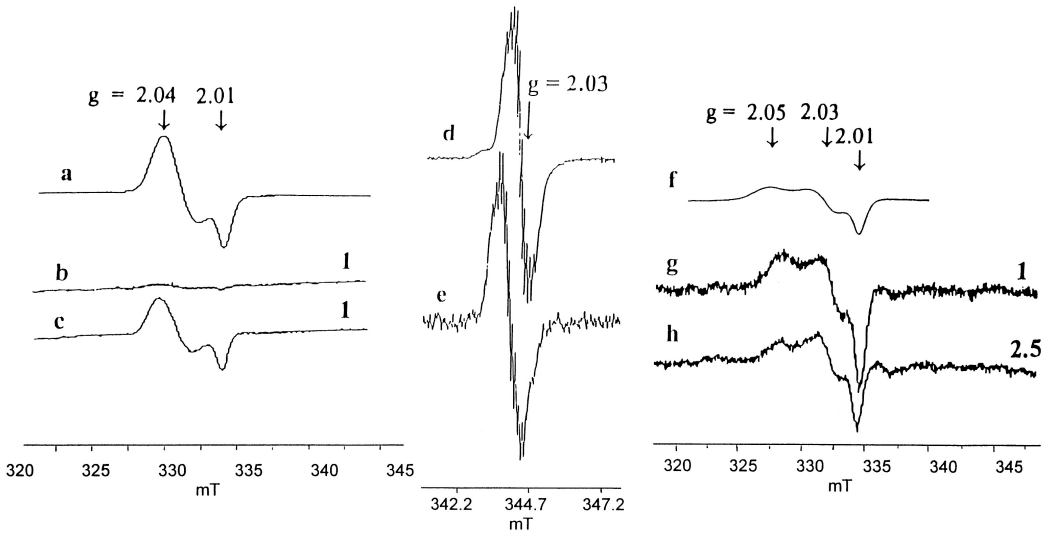


Fig.4. EPR spectra from the solution of cys-NO (0.9 mM, preparation **A**) + L-cysteine (1mM) recorded immediately or 10 min after addition of 100μM $FeCl_3$ (spectra **b** or **c,e**,respectively) or from anaerobic phosphate buffer solution (150 mM, pH 7.4) containing 100μM citrate complex of Fe^{2+} (ratio of Fe : citrate = 1 : 5) 1 or 3 min. after addition of 0.45 mM cys-NO (preparation **A**) (spectra **g** or **h**, respectively). The spectra **a,d** or **f** are from DNIC with cysteine or DNIC with phosphate, respectively. Spectra were recorded at 77K (**a-c, f-h**) or ambient temperature (**d,e**). The intensity of the EPR signals corresponded to 67μM or 17μM and 5μM of DNIC with cysteine (the signal **c**) or DNIC with phosphate (the signals **g** and **h**), respectively.

the unpaired Student`s t-test. *P* values less than 0.005 were considered to be statistically significant.

RESULTS

Cys-NO and GS-NO decomposition in aqueous solutions at neutral pH

The stability of cys-NO increased in the solutions at pH 7.3-7.4 when the ratio of nitrite to cysteine used for synthesis was increased (data not shown). That was in accordance with the data demonstrating the reversible character of the nitrosation process (Beloso & Williams, 1997):

$$RSH + HNO_2 \leftrightarrow RSNO + H_2O$$

Free thiols remaining in the solution can reduce copper or iron ions to Cu^+ or Fe^{2+}, which are capable to destroy RS-NOs. It was found that relatively stable cys-NO preparation was obtained when the ratio of the concentration of HNO_2 (formed from nitrite under acid conditions) to that of cysteine was equal to 1.5: 1. Less than 25% of the initial amount of cys-NO was decomposed during the first 20 min in solution. (Fig. 1, curve 1). This degree of cys-NO stability ensured investigations of the effects of various agents on the stability of the preparation (see below).

Cys-NO preparations which were stable in the solutions at neutral pH values were obtained if pH value characteristics of initial acid cys-NO solutions was increased by dilution in strong Hepes buffer (150 mM, pH 7.4) (preparation **A** of cys-NO). However, rather unstable cys-NO preparation was obtained (Fig. 1, curve 2, top panel) when the pH value was increased by addition of small amount of strong basic solution (preparation **B** of cys-NO). Evidently, rapid decomposition of the preparation was due to hydrolysis of cys-NO resulting in the accumulation of free cysteine (Williams, 1996). The process could be ensured by high local transitory increasing pH value in the solution during the procedure of its mixing with strong basic solution.

In comparison to cys-NO, GS-NO was characterized by a greater stability in buffer solution at neutral pH: it remained stable for at least 1 hour even at the ratio of nitrite to GSH equal to 1.35: 1 (data not shown).

Effect of ascorbate, copper, iron and chelating agents on cys-NO or GS-NO stability

When added at final concentrations ≥ 0.25 mM, ascorbate completely destroyed within 2 min cys-NO prepared at the ratio of nitrite to cysteine 1.5: 1 (preparation A) (Fig. 1, curve 3, top panel). At lower concentrations of ascorbate (50 μM), partial decomposition of cys-NO occured (Fig. 1, curve 4, top panel). By contrast, when added even at final concentration of 1 mM, ascorbate did not influence the stability of GS-NO at least within 20 min (data not shown). Addition of copper or iron (20 μM) also accelerated the degradation of cys-NO in the following range in the activity of ions: Cu^{2+} > Fe^{2+} > Fe^{3+} (Fig. 1, curves 2-4, bottom panel). Simultaneous addition of 50 μM ascorbate led to complete degradation of cys-NO within 10 min

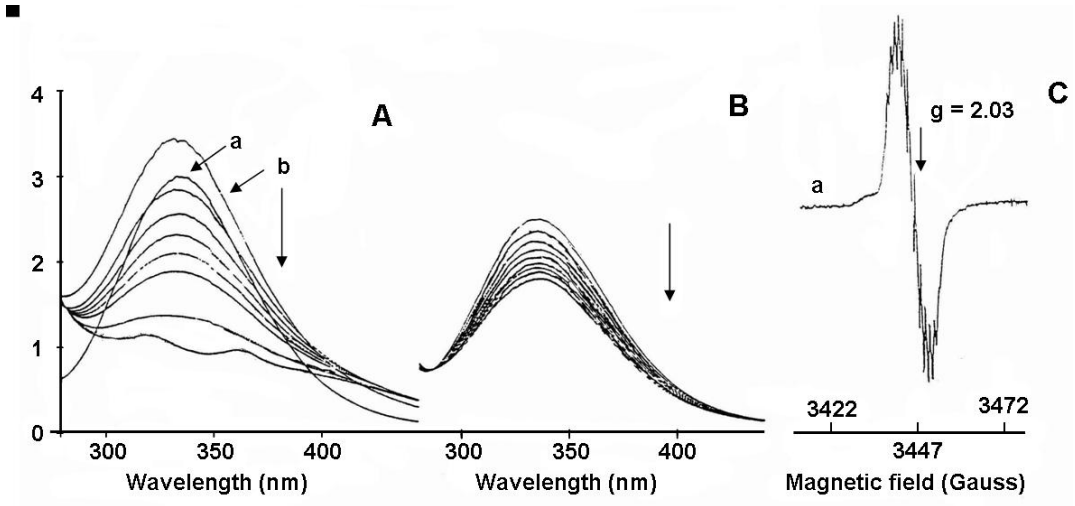


Fig.5. Influence of GSH and Fe^{2+} on stability of GS-NO in 150 mM Hepes buffer, pH 7.2. **A**:curve **a**, initial GS-NO solution (3 mM) + GSH (10 mM); curves **b**, after addition of 0.1 mM $FeSO_4$ to the solution with subsequent recording of spectra in 5 minutes. **B**: GS-NO (2mM) + GSH (10 mM) solution with subsequent recording of spectra in 5 min;**C**: the EPR signal from the solution of GS-NO (3 mM) + GSH (10 mM) + $FeSO_4$ (0.1 mM) incubated within 30 min followed with increasing pH value from 7.2 to 11. Recordings were made at ambient temperature.

(Fig. 1, curves 5, bottom panel).

In contrast to destroying effect on cys-NO, Cu^{2+} or Fe^{2+} even of 100 μM did not notably influence the stability of GS-NO at least within 20 min. The decomposition of the GS-NO was initiated by the metal ions when they were added to the solution in the presence of ascorbate (data not shown).

Iron and copper chelators, BPDS or BCS (1 mM) (Fig. 2, curves 3, top or bottom panels) abolished the destroying effect of 1 mM ascorbate on cys-NO, thereby stabilizing this RS-NO. This result clearly demonstrates that the destroying activity of ascorbate on cys-NO was mainly due to its capacity to reduce intrinsic copper or iron to Cu^{+} or Fe^{2+}, which catalyzed cys-NO decomposition. Both BPDS and BCS completely eliminated also the destroying action of iron ions (20 μM) even in the presence of 1 mM ascorbate (Fig. 2, curves 4, top and bottom panels). When iron concentration was increased to 100 μM, BCS still eliminated the action of iron (data not shown). The protection of cys-NO by BPDS was not complete in this case (data not shown). However in contrast to BCS, BPDS failed to protect cys-NO against the destroying effect of copper ions in the presence of 1 mM ascorbate, even when 0.2 μM of copper ions was added (compare Fig. 2, curves 5-7, top panel or curve 5, bottom panel). Thus, BPDS displayed a selective inhibitory effect towards iron ions, while BCS completely inhibited the effect of both copper and iron.

Similar protective effects of BPDS (0.25 mM) and BCS (0.25 mM) on GS-NO stability were observed when they were added to the solutions of GS-NO (0.9 mM), containing 1 mM ascorbate and 100 μM copper or iron (data not shown).

Thus, because BPDS was able to efficiently block the destroying effect of ascorbate on cys-NO, the following conclusions may be drawn: (i) the amount of intrinsic copper in cys-NO solution was not large enough to produce any effect on cys-NO; (ii) only admixtured (intrinsic) iron was responsible for the process of cys-NO decomposition. Experiments with BPDS and copper addition allow to indirectly evaluating the higher level of intrinsic copper in the solution. Indeed, notable decomposition of cys-NO was detected at the concentration of 0.2 μM in the presence of 1 mM BPDS and ascorbate (Fig. 2, curve 7, top panel). This demonstrates that the higher level of intrinsic copper was not more than 0.2 μM. As regard to

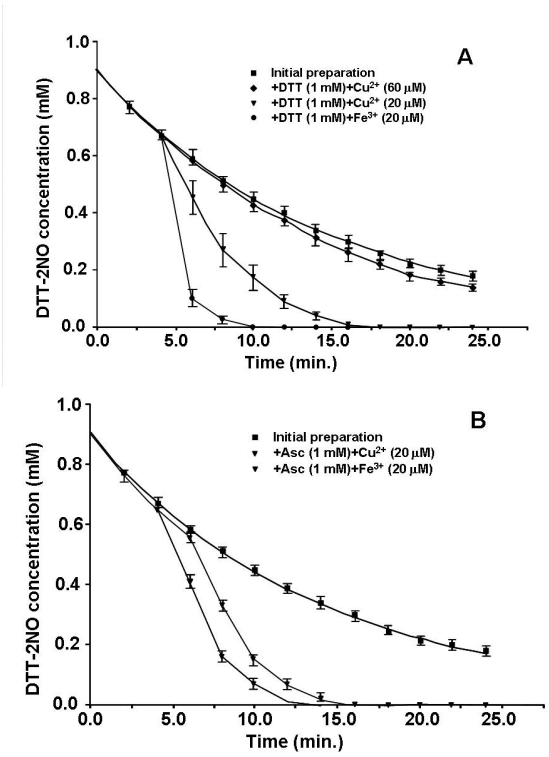


Fig.6. Influence of transition metal additions (shown by arrows) on stability of DTT-2NO in the presence of DTT (top panel) or ascorbate (bottom panel). Results are expressed as mean ± SEM of three to five experiments.

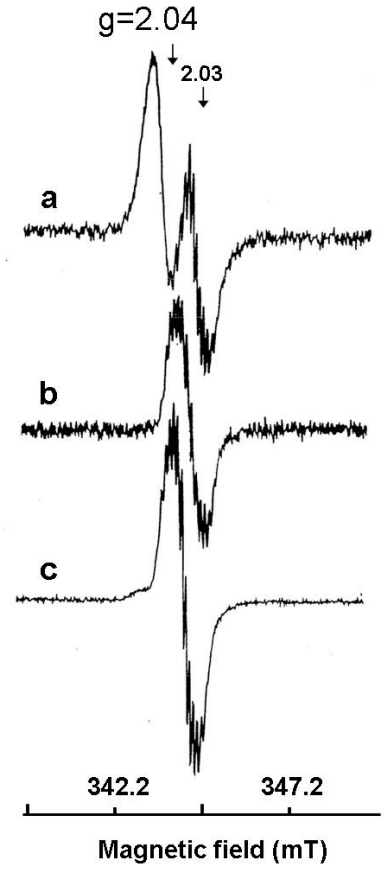


Fig.7. EPR spectra from the solution of DTT-2NO (2mM) + DTT (5mM) + $FeSO_4$ (0.1 mM) (**a**), from the solution of DNIC with DTT (**b**) or from DNIC with cysteine (**c**).Recordings were made at ambient temperature.

iron ions, experiments demonstrating a slight increase of the destroying action of 50 μM ascorbate by the addition of 2μM iron (data not shown) allow to suggest that the concentration of intrinsic iron was nearly 1-2 μM.

Effect of L-cysteine or GSH on the stability of cys-NO or GS-NO, respectively

Addition of L-cysteine induced either acceleration of cys-NO decomposition (at concentration of 50 μM) or stabilization of the compound (at concentration of 1 mM) (Fig. 3, curves 1 and 2, top and bottom panels). At low concentrations, L-cysteine induced the degradation of cys-NO probably by acting as a reducing agent similarly to ascorbate. Copper and iron attenuated the stabilizing effect of 1 mM L-cysteine on cys-NO (Fig. 4,curves 3, top and bottom panels). Distinctly from the process catalyzed with copper, the achievement of a plateau in the amount of cys-NO (not complete decomposition of the latter) was characteristic of iron-induced process in the presence of 1 mM cysteine (Fig. 3, curve 3, bottom panel).

EPR assays of cys-NO solutions treated by L-cysteine and iron reveal the appearance of paramagnetic (monomeric) DNIC with cysteine. The complex was detected by its characteristic EPR signal which had a shape of isotropic singlet at g = 2.03 with 13-component hyperfine structure (HFS) at ambient temperature (Fig. 4e). These parameters were identical with those for the EPR signal from DNIC with cysteine obtained by treating the $FeSO_4$ and L-cysteine solutions with gaseous NO (Fig. 4d) (Vanin *et al.*, 1996). When recorded at 77 K, the EPR signal from DNIC with cysteine formed in the cys-NO solution treated by L-cysteine and iron was characterized with anisotropic shape with $g_{\perp} = 2.04$ and $g_{||} = 2.01$ (Fig. 4a,c). The weak signal which was recorded at 77 K immediately after addition of iron to the solution of cys-NO + L-cysteine was followed by an increase in its intensity and achievement of a plateau (Fig. 4b,c). The concentration of DNIC increased with elevation of the amount of added iron.

The appearance of DNIC with cysteine was in accordance with the above-mentioned mechanism of RS-NO decomposition catalyzed by iron (Scheme 2). Another fact supporting the mechanism was the detection of less stable species of DNIC, i.e. DNIC with phosphate, in 150 mM phosphate buffer containing cys-NO (0.45 mM) after addition of 100 μM Fe^{2+} (Fig. 4g,h). The formation of this DNIC was observed in the solution only under anaerobic conditions. Kinetic assay demonstrated low stability of the complex. In contrast to DNIC with cysteine, DNIC with phosphate appeared in high amount immediately after process initiation and then rapidly decomposed in parallel with cys-NO decomposition. The parameters of the EPR signal of the DNIC formed in phosphate buffer were identical with those of DNIC with phosphate which was synthesized by treating the $FeSO_4$ with gaseous NO in phosphate buffer solutions (Fig. 4f).

Upon addition of GSH (10 mM) to GS-NO solution (2-3 mM), GS-NO began to decay slowly (Fig. 5B) and addition of 100 μM Fe^{2+} accelerated the decomposition of GS-NO in the presence of GSH (Fig. 5A). Iron-induced degradation of GS-NO in this case was also associated with the formation of DNIC with GSH (Fig. 5C). The EPR signal coincided by its parameters with the EPR signal of DNIC with GSH which was obtained by treatment of the $FeSO_4$ and GSH solutions with gaseous NO (not shown). The parameters of the signal were identical with those for DNIC with cysteine (Fig. 4d). At the end of GS-NO decomposition, when the two weak absorbance bands typical for the diamagnetic (dimeric) form of DNIC with GSH (Vanin *et al.*, 1996) appeared at 310 and 360 nm (Fig. 5A), the EPR signal from the solution decreased to a level 5-6 times below the initial. The intensity of the EPR signal was increased 5-6 times when the pH of the solution was increased from 7.4 to 11-12. This was due to transformation of the dimeric (diamagnetic) form of DNIC to the paramagnetic (monomeric) form (Vanin *et al.*, 1996).

S-Dinitrosodithiotreitol (DTT-2NO) decomposition in aqueous solutions at neutral pH

According to spectrophotometrical measurements, both SH-groups in DTT molecules were S-nitrosated even at the ratio of nitrite to DTT equal 2.2: 1. This makes reasonable to consider the preparation as S-dinitrosodithiotreitol (DTT-2NO). In contrast to GS-NO, DTT-2NO synthesized even at a high ratio of nitrite to SH-group (1.5:1) in the solution did not display high stability in the solution at neutral pH. The decrease of DTT-2NO amount followed the first-order rate low quite well (Fig. 6). BCS or BPDS had no influence on the stability of DTT-2NO (data not shown). This allows to suggest that the process of DTT-2NO decomposition was due to intrinsic mechanism characteristic of this compound. As previously proposed, the decomposition of S-mononitroso-DTT as well as DTT-2NO is determined by intramolecular processes of mutual reduction-oxidation of S-nitroso-groups, resulting in the

release of NO, nitrous oxide and nitrite, and oxidation of DTT to a disulfide derivative (Arnelle & Stamler, 1995). Similar mechanism was also proposed for S-nitroso-derivative of thioredoxin which contains, as with DTT, two nearby located SH-groups in the molecule (Nikitovich & Holmgren, 1996).

Ascorbate or DTT (1 mM), copper or iron ions (20 μM) when added alone practically did not affect the stability of DTT-2NO (data not shown). However, as with GS-NO, addition of copper (20-60 μM) simultaneously with reducing agents (1 mM of ascorbate or DTT) sharply accelerated DTT-2NO decomposition (Fig. 6). Half-life time dropped from 10 min to 1 min. BCS (1 mM) but not BPDS (1 mM) abolished the effect of copper ions (40 μM) (data not shown). Addition of 20 μM iron to a solution of DTT-2NO notably induced DTT-2NO decomposition in the presence of ascorbate (1 mM) similarly to the action of copper ions (Fig. 6, bottom panel). Both BPDS and BCS (1 mM) completely eliminated the effect (data not shown). However a destructive effect of the same concentration of iron was negligible when DTT (1 mM) was used as reducing agent (Fig. 6, top panel). In the presence of DTT, the destroying action of became apparent at 100 μM iron concentration (data not shown).

Similarly to the experiments on other RS-NO, the formation of DNIC with DTT should be expected. This was the case when the solution of DTT-2NO (2 mM) + DTT (5 mM) + Fe^{2+} (100 μM) was assayed by the EPR method at ambient temperature. Two EPR signals were recorded (Fig. 7a). First at g-factor of 2.04 was structureless, while second at g-factor 2.03 was characterized with 13-component HFS. The first signal was observed also from the solution of DTT-2NO + DTT without added iron. The identification of the center responsible for the signal remains obscure. Concerning with second signal, its parameters were identical to the signal characteristic of DNIC with DTT, synthesized with above-described method (Fig. 7b). Interestingly, both signals were identical to that from DNIC with cysteine recorded at ambient temperature (Fig. 7c). As previously considered (Vanin *et al.*, 1996), the 13-component HFS characteristic of the EPR signal from DNIC with cysteine is due to hyperfine interaction of unpaired electron with nuclear spin of nitrogen atom ($I = 1$) from two NO^+ ligands and with four protons ($I = 1/2$) from methylene groups located nearby sulfur atoms in two cysteine ligands. The identity of HFS in EPR signal from DNIC with DTT with that of DNIC with cysteine indicated the similarity of electron and space structures of both complexes. It means that iron atom in DNIC with DTT is coordinated with two NO^+ ligands and two sulfur atoms which are in vicinity with methylene groups. The conclusion supports the idea that contact of DTT-2NO with iron in the presence of DTT results in NO liberation from DTT-2NO in accordance with afore-mentioned Scheme 3.

Studies of vasorelaxing activity of RS-NOs and DNICs

Cys-NO (30 nM) produced relaxant effect in aortic rings precontracted with NA. The duration of the effect was correlated with the stability of cys-NO preparations (Fig. 8a,b). Indeed, the relaxation evoked by the more stable cys-NO prepa-

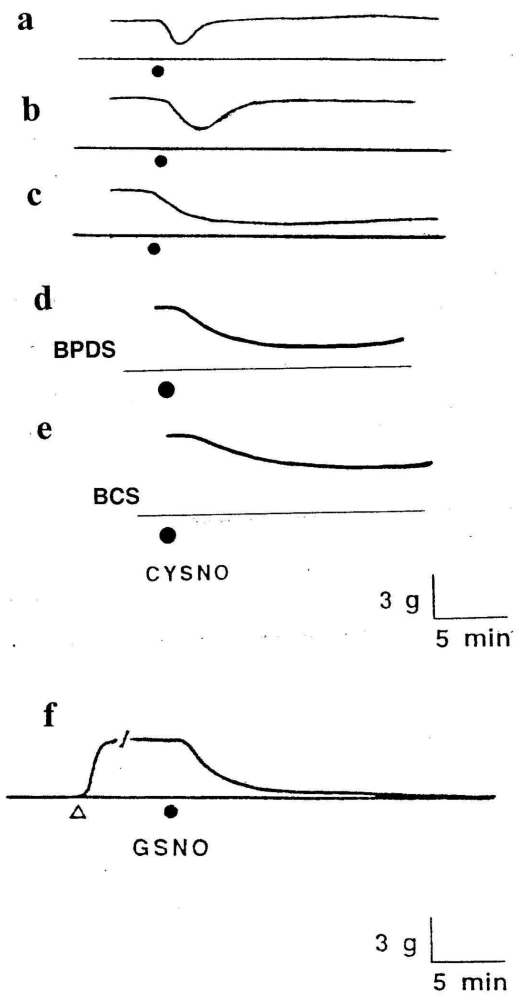


Fig.8. Relaxant effect of cys-NO or GS-NO in endothelium-denuded rat aortic rings precontracted with noradrenaline (0.1μM). **a** or **b** - Representative traces of the effect of the preparations **B** and **A** of cys-NO (30 nM) alone, respectively. **c-e** -Representative traces of the effect of preparation **A** in the presence of L-cysteine (10μM), BPDS or BCS (100μM), respectively. **f** - Representative traces of the effect of GS-NO (100nM) alone.

ration (synthesized at the ratio $[HNO_2]$:[RSH] = 1.5:1 followed by dilution with 150 mM Hepes buffer, final pH 7) was sustained about 5 min period (see Fig. 8b for representative traces). The less stable preparation (synthesized at the ratio $[HNO_2]$:[RSH] = 1.5: 1 followed by treatment with strong basic solution to increase pH from acid to neutral values) produced short lasting relaxant effect (not more than 2 min, Fig. 8a). In the presence of L-cysteine (10 μM), the duration of relaxing effect of most unstable cys-NO preparation was markedly prolonged: it remained sustained at least over 15 min period (Fig. 8c for representative traces). By contrast, ascorbate (500 μM) significantly reduced the amplitude and duration of relaxing effect of cys-NO (Fig. 9a,b for representative traces).

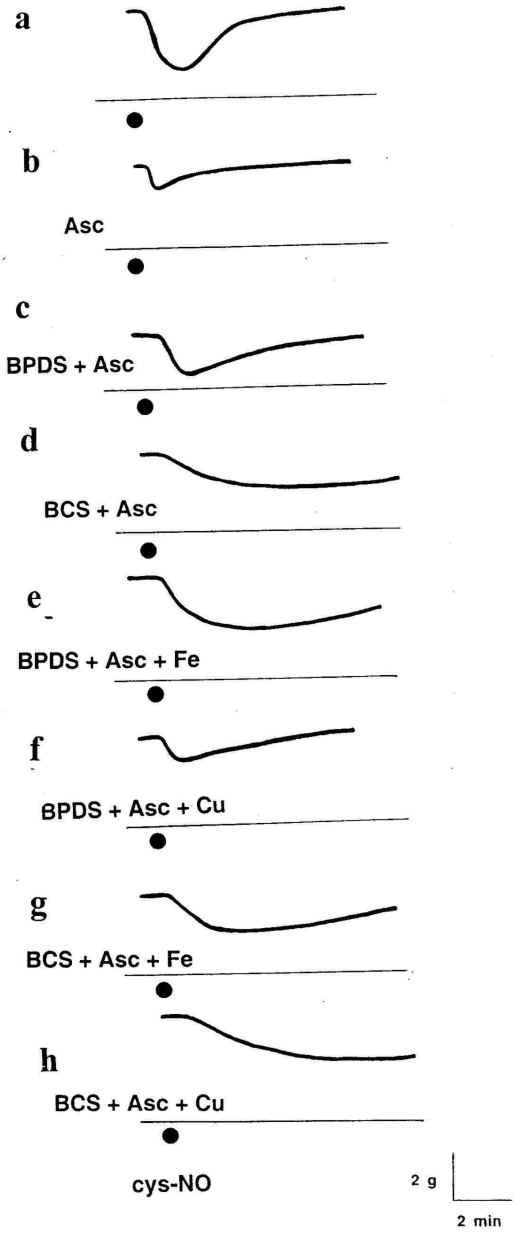


Fig.9. Representative traces of the relaxant effect of cys-NO (preparations **A**, 30 nM) in endothelium-denuded rat aortic rings precontracted with noradrenaline (0.1μM). Effect of cys-NO alone (**a**) or in the presence of ascorbate (0.5 mM,), BPDS (0.1 mM), BCS (0.1 mM), iron (250 nM) and copper (250 nM) (**b-h**).

In contrast to the transient aspect of the relaxations evoked by cys-NO preparations synthesized at the ratio $[HNO_2]$:[RSH] = 1.5:1, those elicited by GS-NO preparation synthesized at the ratio $[HNO_2]$:[RSH] = 1.35: 1 were sustained over a 15 min period (Fig. 8f for representative traces).

The addition of BPDS or BCS (100 μM) prolonged the duration of the relaxing effect of cys-NO (Fig. 8d,e) even in the presence of 500 μM ascorbate (Fig.9,c,d for representative traces). When Cu^{2+} or Fe^{2+} was added to the bathing medium containing BPDS or BCS (100 μM) + ascorbate (500 μM), their influence on cys-NO-induced vasorelaxation was similar to their action on cys-NO stability in solutions containing metal chelators + ascorbate. Indeed, BCS protected against the inhibitory effect of both copper and iron on cys-NO relaxant effect (Fig. 9g,h), whereas BPDS revealed a marked protection only towards iron ions (Fig.9e,f)

In accordance with previous studies (Vedernikov, Mordvintcev, Malenkova & Vanin, 1992; Muller, Kleschyov, Malblanc & Stoclet, 1998), monomeric paramagnetic DNICs also produced a concentration-dependent relaxing effect in aortic rings precontracted with NA (Fig. 10A). Although the potency of DNIC with cysteine and DNIC with GSH was similar (the EC_{50} values were 31 ± 4 and 32 ± 8 nM, respectively, n = 8, Fig. 10C), the time-course of their relaxant response was different. Whereas addition of DNIC with cysteine resulted in a quasi-immediate relaxant response which tended to partially recover within few minutes (Fig. 11A), the relaxant response of DNIC with GSH developed more slowly and was more sustained (Fig. 11B). Interestingly, the potency of DNIC with cysteine was not significantly different from the potency of cys-NO in the presence of L-cysteine (EC_{50} =25 ± 6 nM) (Fig. 10A,B).

DNIC with phosphate and DNIC with BCS also produced relaxant effect in aortic rings (Fig. 10D). DNIC with phosphate produced more short lasting relaxant response than DNIC with BCS (Fig. 11, C,D).

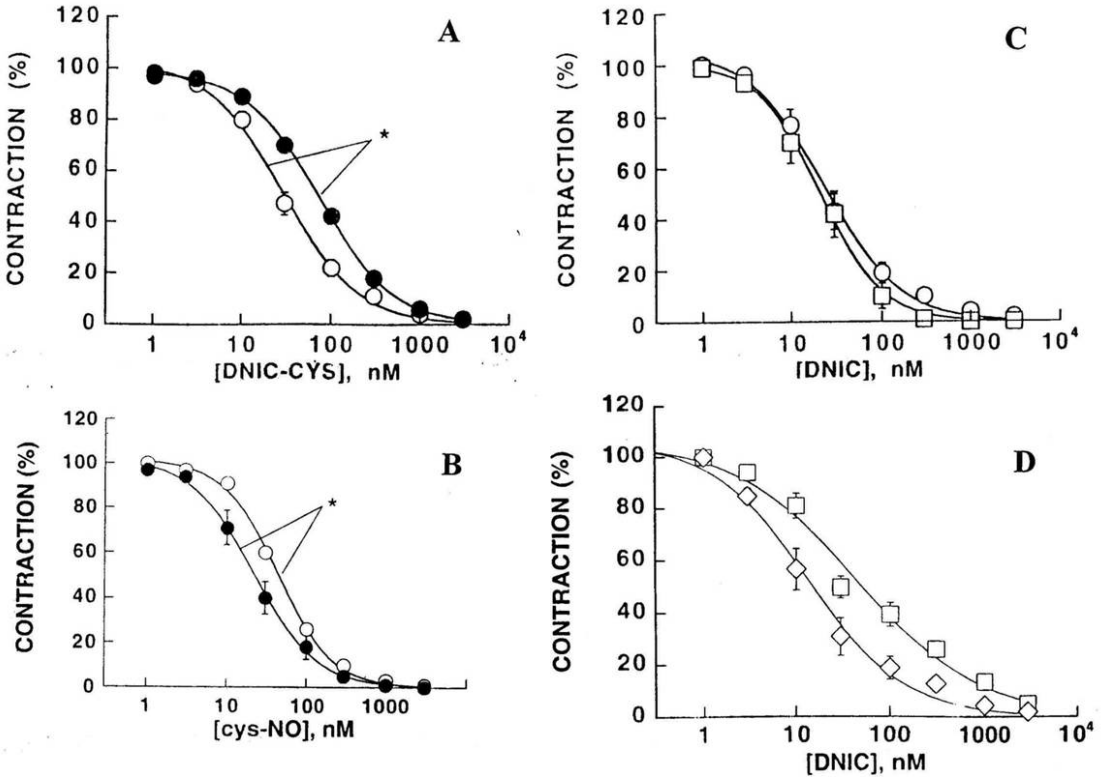


Fig.10.Concentration-response curves of the relaxant effect of DNIC with cysteine in the absence (open circles) and in the presence of L-cysteine (10 μM, closed circles) (panel **A**); cys-NO (preparation **B**) in the absence (open circles) and in the presence of L-cysteine (10 μM, closed circles) (panel **B**); DNIC with GSH (open squares) or with cysteine (open circles) (panel **C**) or DNIC with phosphate (open diamonds) or BCS (open squares) (panel **D**). Endothelium-denuded rat aortic rings were precontracted with noradrenaline (0.1 μM). *P < 0.05, in comparison to controls.

DISCUSSION

The main conclusions from the present study can be summarized as follows:

(i) the process of decomposition of the most unstable RS-NO cys-NO, was determined by interaction with intrinsic iron; the amount of intrinsic copper in cys-NO solution was rather too small to produce any effect on cys-NO stability;

(ii) the concentrations of intrinsic copper/iron in GS-NO solution was not large enough to influence GS-NO stability;

(iii) DTT-2NO was decomposed spontaneously in the solution without any effect of intrinsic copper/iron ions; when added at the concentration of 20 μM, the ions became to sharply accelerate the process in the presence of ascorbate;

(iv) the process of iron-induced decomposition of all RS-NO proceeded through the formation of DNIC with non-thiol (less stable) or thiol-containing (more stable) ligands, both of which released free NO molecules;

(v) there existed a direct correlation between the stability of RS-NOs in the solutions and the duration of their relaxant effect on isolated blood vessels.

The finding that BPDS was capable to completely block the destroying action of Fe^{2+} ions but not of Cu^{+} on cys-NO was decisive for above enumerated conclusions. BPDS as well as 1,10-phenantroline are well-known chelators not only for Fe^{2+} but also for Cu^{2+} ions (Sammes & Yahioglu, 1994; Bush, Whitehead, Pink, Gramm, Eglin, Watton & Pence, 2001). It is likely that Cu^{2+}/Cu^{+}-BPDS complexes similarly to copper ions alone are able to ensure catalytical decomposition of cys-NO as redox agent in accordance with Scheme 1. As previously shown (Bush *et al.*, 2001), copper ions can form Cu(phenantroline)$_2$ complexes which are able to include a third ligand. Therefore, it is reasonable to suggest that cys-NO could penetrate into Cu(BPDS)$_2$ complexes to be reduced and thereby decomposed. This seemed not to be case for interaction of cys-NO with Cu-BCS complexes which provided cys-NO stability. As to Fe^{2+}-BPDS complexes, their lack of effect on cys-NO stability could be explained by full saturation of coordination sphere (binding three chelator molecules to each iron ion). This made impossible

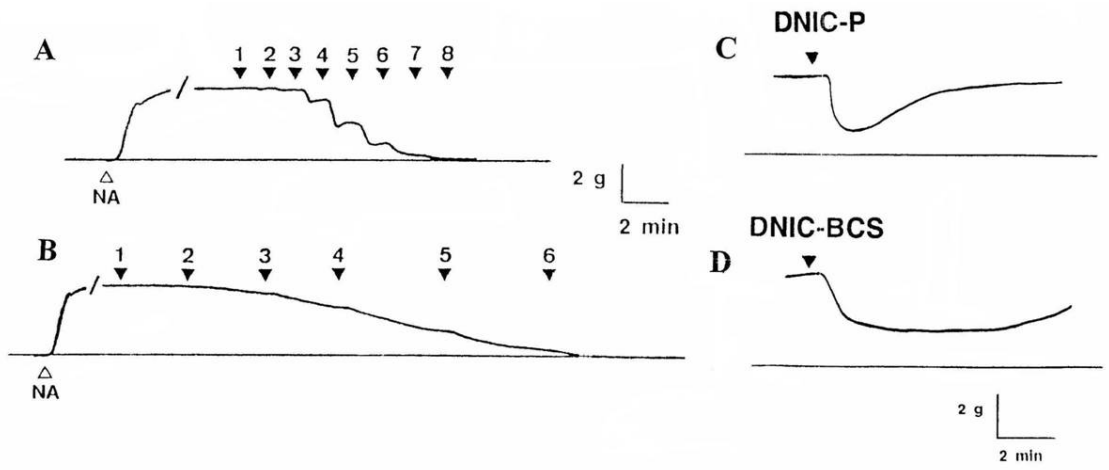


Fig.11.Representative traces of the relaxant effect of DNIC with cysteine (**A**), GSH (**B**), phosphate (**C,** 100 nM) or BCS (**D**, 100 nM) in endothelium-denuded rat aortic rings precontracted with noradrenaline (0.1 μM).. In panels **A** and **B,** numbers indicate the addition of increasing concentrations of DNICs (1 : 1nM; 2 ; 3nM; 3 : 10nM; 4 : 30 nM; 5 : 0.1μM; 6 : 0.3μM; 7 : 1μM; 8 : 3μM).

the interaction between cys-NO and iron in the complexes. Altogether, the present data make questionable the idea that intrinsic copper can be responsible for the decomposition of cys-NO and probably of other RS-NOs. The admixture of this transition metal in sample solutions is rather too small to affect the process. Our data are rather consistent with the view that intrinsic iron influences the stability of RS-NOs. The catalytical activity of Fe^{2+} ions in the process of cys-NO degradation did not significantly differ from that for Cu^{+}. So, only higher amount of intrinsic iron in the sample solutions ensures the predominant role of iron in the process of cys-NO decomposition.

The demonstration of the formation of unstable DNIC with phosphate in cys-NO solutions after Fe^{2+} ion addition is in favour of the mechanism of RS-NO degradation initiated by Fe^{2+} ions in accordance with Scheme 2. It is noteworthy that the decay of the complexes closely paralleled the decomposition of cys-NO. This evidences that the formation of DNIC with non-thiol ligand, (with phosphate) occured directly in the course of cys-NO decomposition on its binding to Fe^{2+} (Scheme 2), but not as a result of the binding of neutral NO molecules released from cys-NO with Fe^{2+} and phosphate ligands, i.e. during the secondary reaction following the cys-NO decomposition. It is obvious that the rate of RS-NO decomposition via this pathway is determined by DNIC stability: the process would be attenuated with increasing complex stability. This was the case when the more stable DNIC with cysteine were formed in the solution of cys-NO in the presence of cysteine. Stabilization of cys-NO was observed in this case. Obviously, the inclusion of intrinsic iron into stable DNIC with cysteine deprived it the opportunity to destroy cys-NO. Thus altogether, the present data allow to suggest that the process of cys-NO decomposition really proceeds through the formation of DNIC with non-thiol or thiol-containing ligands. The subsequent decomposition of DNIC ensures appearance of free NO molecules in the solutions.

From this point, the high stability of GS-NO in the solution can be explained by low rate of reaction with Fe^{2+} ions, probably due to steric hindrance. The content of intrinsic iron was not large enough to initiate the process. Notable decomposition of GS-NO was observed when Fe^{2+} ions were added in a higher amount (100 μM) in the presence of reducing agents (GSH, ascorbate). Second possible reason is a high stability of DNIC with GSH comparatively with that of DNIC with cysteine (Vanin *et al.*, 1996). This means that the great bulk of intrinsic iron could be bound to the complexes, which attenuated its destructive influence on RS-NO to a considerable extent.

The conclusion about predominant role of intrinsic iron in cys-NO decomposition is fully consistent with the data obtained for cys-NO vasorelaxant activity. In accordance with proposed mechanism of the destroying effect of iron ions on the RS-NO, vasorelaxation was induced mainly by NO liberated from formed DNIC. It is likely that NO released from unstable DNIC (for example, DNIC with phosphate or other non-thiol ligands) was dispersed over the volume of incubation medium far from the vessel and, due to oxidation and escaping to head space, could not reach efficiently vascular tissue. This resulted in a weak and transient relaxation. By contrast, long living DNIC

with cysteine or BCS formed in the medium following addition of cysteine or BCS respectively, ensured sustained relaxant response due to protection of NO from oxidation and escaping from the medium (Fig. 8c or Fig. 11D). In this case, the formation of protein bound DNIC as well as S-nitrosylated protein during the interaction of low molecular weight DNIC with vascular tissue can not be excluded.

One feature of the bioassay should be taken into consideration. The studies of cys-NO stability in the solution were performed with a concentration of cys-NO which was much more than that of intrinsic iron, whereas the opposite ratio was characteristics of the evaluation of cys-NO vasorelaxant activity. Under this condition, all cys-NO could be rapidly transformed into DNIC which then serve as the main NO donor. Only addition of iron chelators, BPDS or BCS, could ensure the manifestation of vasorelaxant activity of cys-NO itself. Similar situation could also take place when the most stable cys-NO preparation or GS-NO was added to the medium. In both cases, cys-NO was efficiently protected against destroying action of intrinsic iron. In both cases, the vasorelaxant effect evoked by cys-NO (or GS-NO) was sustained, over a period of at least 15 min. In the latter cases, the effect was probably initiated by interaction of RS-NOs with vascular tissues, that could provide enzymatic decomposition of the molecules. As afore mentioned, the role of such type of enzymatic systems in cells are documented now (Kowaluk & Fung, 1990; Gorge *et al.*, 1996; Gaston, 1999; Zai *et al.*, 1999; Sorenson *et al.*, 2000; Ramachandran *et al.*, 2001).

The enzymatic mechanism of RS-NO decomposition proposed for one type of enzyme, protein disulfide isomerase (PDI) (Ramachandran *et al.*, 2001), is shown in Scheme 4. According to the Scheme, the way of NO liberation from the S-nitrosylated PDI formed in the process is similar to that for S-nitrosylated DTT proposed elsewhere (Arnelle & Stamler, 1995). It is ensured by intramolecular mutual reduction-oxidation of S-nitroso groups in the compound. Similar mechanism of NO release was proposed also for S-nitrosothioredoxin (Nikitovich & Holmgren, 1996). According to our data, the rate of the process for DTT-2NO is not too large: half-life time of DTT-2NO was equal to 10 min. However the rate was sharply increased (nearby 10 times) by copper or iron ions in the presence of ascorbate, or by copper in the presence of DTT. The effect of iron was associated with the formation of DNIC with DTT when DTT was added to the solution. So it is reasonable to propose similar effect of copper or iron ions on the decomposition of RS-NO catalyzed by PDI or other enzymatic systems. Interestingly, the participation of iron ions was recently demonstrated for some enzymes ensuring RS-NO decomposition (Sorenson *et al.*, 2000).

Acknowledgement

This work was partially supported by INTAS (Grant INTAS/OPEN 97/524) and Russian Foundation of Basic Researches (Grant 02-04-48456). J.L. Alencar was supported by Capes (Brazil).

REFERENCES

Al-Sa'doni H. H., Megson I. L., Bisland S., Butler A. R. & Flitney F. W. (1997). Neocuproine, a selective Cu(I). chelator and the relaxation of rat vascular smooth muscle by S-nitrosothiols. *Br. J. Pharmacol*., **121**, 1047-1050.

Arnelle D. R. & Stamler J. S. (1995). No+, NO and NO- donation by S- nitrosothiols: Imlications for regulation of physiological functions by S- nitrosylation and acceleration of disulfide formation. *Arch. Biochem. Biophys.*, **318**, 279-285.

Beloso P. H. & Williams D. L. H. (1997). Reversibility of S-nitrosothiol formation. *Chem. Comm.*, **1**, 89–91.

Bush P. M., Whitehead J. P., Pink C. C., Gramm E. C., Eglin J. L., Watton S. P. & Pence L. E. (2001). Electronic and structural variation among copper(II). complexes with substituted phenatrolines. *Inorg. Chem*., **40**, 1871-1877.

Butler A. R. & Rhodes P. (1997). Chemistry, anazlysis and biological roles of S-nitrosothiols. *Anal. Biochem*., **249**, 1-9.

Dicks A. P., Swift H. R., Williams D. L. H., Butler A. R., Al-Sa'doni H. H. & Cox B. G. (1996). Identification of Cu^{+} as the effective reagent in nitric oxide formation from S-nitrosothiols (RSNO*). J. Chem. Soc. Perkin Trans*., **2,** 481-487.

Dicks A. P., Li E., Munro A. P., Swift H. R. & Williams D. L. H. (1997). The reaction of S-nitrosothiols with thiols at high thiol concentration. *Can. J. Chem*., **76,** 789-794.

Dicks A. P., Beloso P. H. & Williams D. L. H. (1997). Decomposition of S- nitrosothiols: the effect of added thiols*. J. Chem. Soc. Perkin Trans*., **2**, 1429-1434.

Feelish M. & Stamler J. S. (1996). Donors of nitrogen oxides. [In:] Feelisch, M., Stamler, J. S. (Eds.), *Methods in Nitric Oxide Re*s (p. 84-85). New York: J. Willey and Sons Ltd.

Gaston B. (1999). Nitric oxide and thiol groups. *Biochim. Biophys. Acta*, **1411,** 323-333.

Gorge M. P., Meyer D. J., Hothersall J., Neild G. H., Payne N. N. & Noronha-Dutra A. (1995). Copper chelator-induced reduction of the biological activity of S-nitrosothiols. *Br. J. Pharmacol*., **114**, 1038-1049.

Gorge M. P., Hothersall J. S., Neild G. H. & Noronha-Dutra A. A. (1996). Role of copper(I). -dependent enzyme in anti-platelet action of S- nitrosoglutathione. *Br. J. Pharmacol*., **119**, 536-538.

Gorren A. F., Schrammel A., Schmidt K. & Mayer B. (1996). Decomposition of S- nitrosoglutathione in the presence of copper and glutathione. *Arch. Biochem. Biophys*., **330**, 219-228.

Kashibo-Iwatsuki M., Yamaguchi M. & Inoue M. (1996). Role of ascorbic acid in the metabolism of S-nitrosoglutathione. *FEBS Lett*., **389**, 149-152.

Kowaluk E. A. & Fung H.-L. (1990). Spontaneous liberation of nitric oxide cannot account for in vitro vascular relaxation by S-nitrosothiols. *J. Pharmacol. Exp. Ther*., **255**, 1256-1264.

McAninly J., Williams D. L. H., Askew S. C., Butler A. R. & Russel C. (1993). Metal ions catalysis in nitrosothiol (RSNO) decomposition. *J. Chem. Soc. Chem. Comm*., 1758-1759.

Muller B., Kleschyov A., Malblanc S. & Stoclet J.-C. (1998). Nitric oxide- related cyclic GMP-independent relaxing effect on N-acetylcysteine in LPS-treated rat aorta. *Br. J. Pharmacol*., **123**, 1221-1229.

Nikitovich D. & Holmgren A. (1996). S-nitrosoglutathione is cleaved by the thioredoxin system with liberation of glutathione and redox regulating nitric oxide. *J. Biol. Chem*., **271**, 19180-19185.

Ramachandran N., Root P., Jiang X.-M., Hogg P. J. & Mutus B. (2001). Mechanism of transfer of NO from extracellular S-nitrosothiols into the cytosol by cell-surface protein disulfide isomerase. *Proc. Natl. Acad. Sci. USA*, **98**, 9539-9544.

Rubanyi G. M., Johns A., Wilcox D., Bates F. V. & Harrison D. (1991). Evidence that a S- nitrosothiol, but not nitric oxide, may be identical with endothelium-derived relaxing factor. *J. Cardiovasc. Pharmacol*., **17 (Suppl. 3),** S41-S45.

Sammes P. G. & Yahioglu G. (1994). 1. 10-phenantroline: a versatile ligand. *Chem. Soc. Rev*., **23**, 327-334.

Scorza G., Pietraforte D. & Minetti M. (1985). Role of ascorbate and proteine thiols in the release of nitric oxide from S-nitroso- albumin and S-nitroso-glutathione in human plasma. *Free Rad. Biol. &Med*., **22,** 633-642 1997.

Sheu F.-W., Zhu W. & Fung P. C. W. (2000). Direct observation of trapping and release of nitric oxide by glutathione and cysteine with electron paramagnetic resonance spectroscopy. *Biophys. J*., **78,** 1216-1226.

Singh R. J., Hogg N., Joseph J. & Kalyanaraman B. (1996). Mechanism of nitric oxide release from S-nitrosothiols. *J. Biol. Chem*., **271**, 18596-18603.

Sorenson E., Skiles E. H., Xu B., Aleryani S. & Kostka P. (2000). Role of redox-active iron ions in the decomposition of S-nitrosocysteine in subcellular fractions of porcine aorta. *Eur. J. Biochem*., **267**, 4593-4599

Vanin A. F., Stukan R. A. & Manukhina E. B. (1996). Physical properties of dinitrosyl-iron complexes with thiol-containing ligands in relation to their vasodilartor activity. *Biochim. Biophys. Acta*, **1259**, 5-12.

Vanin A. F., Malenkova I. V. & Serezhenkov V. A. (1997). Iron catalyzes both decomposition and synthesis of S-nitrosothiols: optical and EPR studies. *Nitric Oxide:Biol. &Chem*., **1**, 191-203.

Vanin A. F. (1998). Dinitrosyl iron complexes and S-nitrosothiols are two possible forms for stabilization and transport of nitric oxide in biological systems. *Biochemistry (Moscow)*, **67**, 782-793.

Vanin A. F., Serezhenkov V. A. & Malenkova I. V. (2001). Nitric oxide initiates iron binding to neocuproine. *Nitric Oxide: Biol. &Chem*, **5**, 166-175.

Vedernikov Y. P., Mordvintcev P. I., Malenkova I. V. & Vanin A. F. (1992). Similarity between the vasorelaxing activity of dinitrosyl iron cysteine complexes and endothelium-derived relaxing factor. *Eur. J. Pharmacol*., **211**, 313-317.

Williams D. L. H. (1985). S-nitrosation and the reactions of S-nitroso compounds. *Chem. Soc. Rev*., **14**, 171-196.

Williams D. L. H. (1996). The mechanism of nitric oxide formation from S- nitrosothiols (thionitrites). *Chem. Comm*., 1085-1091.

Xu A., Vita J. A. & Keaney J. F. (2000). Ascorbic acid and glutathione modulate the biological activity of S-nitrosoglutathione. *Hypertension*, **36**, 149-152.

Zai A., Rudd M. A., Scriber A. W. & Loscalzo J. (1999). Cell-surface protein disulfide isomerase catalyzes transnitrosation and regulates intracellular transfer of nitric oxide. *J. Clin. Invest*., **103**, 393-399.