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Biochemical Pharmacology 66 (2003) 2365–2374
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Inhibition of arterial contraction by dinitrosyl–iron complexes: critical role of the thiol ligand in determining rate of nitric oxide (NO) release and formation of releasable NO stores by *S*-nitrosation

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Received 15 May 2003; accepted 28 July 2003

Abstract

The inhibition of arterial tone produced by two nitric oxide (NO) derivatives of biological relevance, dinitrosyl–iron complexes with cysteine (DNIC-CYS) or with glutathione (DNIC-GSH), was compared. Both compounds induced vasorelaxation within the same concentration range (3–300 nM) in endothelium-denuded rat aortic rings. Consistent with a faster rate of NO release from DNIC-CYS than from DNIC-GSH, the relaxant effect of DNIC-CYS was rapid in onset and tended to recover with time, whereas the one of DNIC-GSH developed slowly and was sustained. In addition, DNIC-GSH (0.3 and 1 μM) but not DNIC-CYS (1 μM) induced, even after washout of the drug, a persistent hyporesponsiveness to vasoconstrictors and a relaxant effect of low molecular weight thiols like *N*-acetylcysteine (NAC, which can displace NO from preformed NO stores). Both effects of DNIC-GSH were associated with elevation of cyclic GMP content and were attenuated by NO scavengers or a cyclic GMP-dependent protein kinases inhibitor. In rings previously exposed to DNIC-GSH, addition of mercuric chloride (which can cleave the cysteine–NO bond of *S*-nitrosothiols) elicited relaxation, completely blunted the one of NAC and also abolished the persistent elevation of NO content. In conclusion, this study shows that whereas both DNIC-CYS and DNIC-GSH elicited a NO release-associated relaxant effect in isolated arteries, only DNIC-GSH induced an inhibition of contraction which persisted after drug removal. The persistent effect of DNIC-GSH was attributed to the formation of releasable NO stores in arterial tissue, most probably as *S*-nitrosothiols. Thus, the nature of the thiol ligand plays a critical role in determining the mechanisms and duration of the effect of LMW-DNIC in arteries.

Keywords: Nitric oxide; Dinitrosyl–iron complexes; *S*-Nitrosation; *N*-Acetylcysteine; Vascular contraction; NO stores

1. Introduction

DNIC are NO derivatives of biological relevance. They are formed in various cells and tissues, especially during NO overproduction by the inducible NOS (NOS-2) (for reviews, see [1,2]). High molecular weight and LMW-DNIC exist, with cysteine residues of proteins or with LMW thiols like cysteine or glutathione as ligand, respectively. LMW-DNIC are much more instable than the protein-bound species [2,3] and, as demonstrated in acellular systems, easily release NO or transfer it, alone or together with iron, to various targets [2,4–9]. In cells or

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Abbreviations: NO, nitric oxide; DNIC, dinitrosyl–iron complexes; DNIC-CYS, dinitrosyl–iron complexes with cysteine; DNIC-GSH, dinitrosyl–iron complexes with glutathione; LMW, low molecular weight; NAC, *N*-acetylcysteine; NOS, NO synthase; GSNO, *S*-nitrosoglutathione; oxyHb, oxyhemoglobin; metHb, methemoglobin; NE, norepinephrine; *p*-HMBA, *para*-hydroxymercuribenzoic acid; Rp-8Br-cGMPS, Rp-8-bromo-guanosine-3′5′-cyclic monophosphorothioate; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; EPR, electron paramagnetic resonance; DETC, diethyldithiocarbamate.

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doi:10.1016/j.bcp.2003.07.017

tissues, LMW-DNIC exert cyclic GMP-dependent [4,10] and -independent effects [11–13]. The latter have been attributed to nitrosative modification of proteins via the transfer of the NO or $Fe(NO)_2$ group, forming protein *S*-nitrosothiols or protein-bound DNIC, respectively [2,12,13]. Besides being responsible for post-translational modification of various proteins, formation of *S*-nitrosothiols or DNIC on proteins are also involved in NO transport and storage in blood and tissues [14–17].

In arteries expressing NOS-2, protein-bound DNIC are formed and may contribute to vascular hyporesponsiveness to vasoconstrictors [18,19]. LMW-DNIC exert vasorelaxant effects which are likely due to the activation of the cyclic GMP pathway [10,20] and have been postulated as the 'endothelium-derived relaxing factor' released by endothelium-dependent agonists [21]. LMW-DNIC can also enhance the release of NE from perivascular nerves by cyclic GMP-independent mechanisms [13]. The transfer of the $Fe(NO)_2$ group of LMW-DNIC, resulting in the formation of NO stores as protein-bound DNIC, has been also demonstrated in isolated arteries [4]. However, the potential role of *S*-nitrosation of cysteine residues in the effects of LMW-DNIC on vascular tone is not documented. As recently demonstrated [22], persistent *S*-nitrosation of protein is another mechanism of formation of releasable NO stores in arteries. It accounts for the long-lasting inhibition of arterial tone induced by GSNO, another LMW NO derivative of biological significance [23].

The aim of the present study was to compare the effect of two LMW-DNIC of biological relevance, DNIC-CYS and DNIC-GSH, on vascular tone. Their ability to induce or not persistent inhibition of contraction by *S*-nitrosation reactions was especially investigated. For this purpose, responses to contractile agonists were assessed in rat aortic rings previously exposed to the LMW-DNIC, and then carefully washed out. The potential role of *S*-nitrosation of tissue thiol was investigated using thiol-modifying reagents, LMW thiols and mercuric chloride. LMW thiols can displace NO bioactivity from protein *S*-nitrosothiols [15,22,24] and/or from protein-bound DNIC [4,18,25], whereas mercuric chloride is known to cleave the cysteine-NO bond of *S*-nitrosothiols [26].

2. Materials and methods

2.1. Rate of NO release from DNIC

Conversion of oxyHb (10 μM) to methemoglobin (metHb) in 100 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4 100 mM, pH 7.4, 37°) was applied to study the release of NO from LMW-DNIC. MetHb formation was continuously monitored in a diode array spectrophotometer (Hewlett-Packard 8453) by recording the absorbance changes at 401 nm (isosbestic point at 411 nm) and calculated from the absorbance changes ($\varepsilon_{401-411} = 38\ mM^{-1}\ cm^{-1}$) [27]. The initial rate of metHb formation was determined between 0.5 and 5 min after the start of the reaction and was corrected for the blank (metHb formation in the absence of LMW-DNIC).

2.2. Preparation of arteries

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health (agreement number B 67900, given by French authorities). Thoracic aorta was removed from male Wistar rats (12–14 weeks old, 300–380 g, bred from genitors provided by Iffa Credo) after anesthesia with pentobarbital (60 mg/kg, i.p.). It was cleaned of connective and fat tissues in 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) and cut into rings. The endothelium was removed by gently rubbing the intimal surface of the rings with forceps.

2.3. Contraction/relaxation experiments

Rings (2–3 mm length) were mounted under a basal tension of 2 g in organ chambers filled with Krebs solution (at 37°, bubbled with 95% O_2/5% CO_2) for isometric tension recordings. After equilibration, rings were precontracted with NE (1 μM) and the absence of functional endothelium was verified by the lack of relaxant effect of subsequent addition of acetylcholine (1 μM). After washout, the relaxant effect of DNIC was determined in NE (0.1 μM)-precontracted aortic rings, by addition of the compounds either at a single concentration, or in a cumulative manner. In other experiments (performed in the dark), rings were exposed or not to DNIC for 30 min. In some cases, aortic rings were exposed to DNIC-GSH together with the thiol-modifying agent *p*-HMBA (10 μM). Rings were then extensively washed out (over a 60-min period, during which time the Krebs solution was changed every 20 min). They were then contracted by cumulative addition of NE and when a steady-state level of contraction was reached, LMW thiols or mercuric chloride was added in a cumulative manner. In some aortic rings, the effects of NE and NAC were studied in the presence of oxyHb (10 μM) or Rp-8Br-cGMPS (100 μM). When the influence of carboxy-PTIO (100 μM) was studied, phenylephrine (PHE) was used as contractile agonist since a stable level of contraction could not be obtained using NE. The contractile effect of NE (or PHE) was expressed in g of developed tension. The EC_{50} values of LMW-DNIC (concentration that produced 50% relaxation of precontracted rings) were determined by log-logit regression. The relaxant effect of LMW thiols or mercuric ions was expressed in percentage of contraction, 100% being the tone induced by the contractile agonist.

2.4. Cyclic GMP determination

Rat aortic rings were exposed or not to DNIC-GSH for 30 min (at 37° in aerated Krebs solution) and then extensively washed out (during 1 hr). They were then incubated for 30 min at 37° in aerated Krebs solution supplemented with isobutylmethylxanthine (100 μM). In some cases, NAC (10 mM) was added during the last 10 min of incubation. After that, rings were transferred into 1 mL of ice-cold $HClO_4$ (1.07 M). Samples for cyclic GMP and DNA determination were prepared as previously described [10]. The cyclic GMP content was assessed by radioimmunoassay and was expressed as fmol/μg DNA. DNA was determined as described by Brunk *et al.* [28].

2.5. NO spin-trapping and EPR spectroscopy

Aortic rings (6–8 mm length) were exposed or not to DNIC-GSH (in the dark, for 30 min at 37° in aerated Krebs solution) and then extensively washed out (during 60 min). The NO content was assayed after formation of the electron paramagnetic resonance (EPR)-detectable paramagnetic spin adduct Fe(II)NO(diethyldithiocarbamate)$_2$ [Fe(II)NO(DETC)$_2$] in rings treated (for 30 min at 37°) with 0.5 mM [Fe(II)(DETC)$_2$] complex as colloid [29]. Some arteries rings were treated with mercuric chloride (1 mM for 5 min) before being exposed to [Fe(II)(DETC)$_2$]. All samples were carefully washed out after each treatment and before NO trapping. Tissues were then rapidly frozen in calibrated tubes (0.3 mL) and kept in liquid nitrogen until EPR measurements. EPR spectra were recorded on a MS100 spectrometer (Magnettech) under the following conditions: temperature 77 K, microwave frequency 9.34 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 0.5 mT, time constant 100 ms. After EPR measurements, the tissue samples were dried and weighted. The relative [Fe(II)NO(DETC)$_2$] concentrations (A/W_{ds}) were determined dividing the third component amplitude (A) of the three-lines EPR signal by the weight of the dried sample (W_{ds}).

2.6. Mercuric chloride-induced formation of nitrite in solutions of DNIC-GSH or GSNO

The Saville–Griess assay was used to assess mercuric chloride-induced cleavage of S–NO bonds, with formation of nitrites as end-product (resulting from oxidation of released NO). In brief, 100 μL sample (DNIC-GSH or GSNO) diluted in Griess buffer (50 mM Tris–HCl, pH 8.8, 150 mM NaCl, 5 mM KCl, 1% Nonidet P40, 1 mM phenylmethylsulfonyl fluoride, 1 mM diethylene triamine pentaacetic acid) were incubated for 20 min at room temperature with 1% sulfanilamide and 0.1% *N*-(1-naphthyl-ethylene diamine), in the presence or absence of 500 μM mercuric chloride. The absorbance was read at 540 nm and the amount of nitrites was calculated from standard curve.

2.7. Drugs and reagents

Unless otherwise indicated, drugs were purchased from Sigma Chemical Co. Sodium pentobarbital was purchased from Sanofi Santé Animale. NAC (5 g/25 mL solution of EDTA/NaOH, pH 6.5, Fluimucil®) was a generous gift from Zambon laboratory. Rp-8Br-cGMPS was purchased from Biolog Life Science Institute. OxyHb was prepared as described by Murphy and Noack [27]. Briefly, Hb from bovine blood was dissolved in phosphate buffer in the presence of sodium hydrosulfite and exposed to a stream of O_2 for 10 min. The oxyHb solution was then desalted and purified through a Sephadex G-25 column and its concentration was calculated from the optical absorbance at 415 nm (molar extinction coefficient: 131 $mM^{-1}\,cm^{-1}$). Kit for cyclic GMP determination was obtained from Immunotech. DNIC-CYS and DNIC-GSH (1:20 molar ratio) were synthesized in a Thunberg tube apparatus by treatment of $FeSO_4{\cdot}7H_2O$ (2 mM) and L-cysteine or glutathione (40 mM) solutions for 5 min with gaseous NO (obtained by the reaction of $FeSO_4$ with $NaNO_2$ in 0.1 M HCl and then purified by the method of low-temperature sublimation in an evacuated system) in 10 mM Hepes buffer (pH 7.4) in oxygen free conditions. This was followed by 1-min evaporation to remove unbound NO [20]. Stock solutions of DNIC were stored in liquid nitrogen. DNIC was thawed and diluted in deoxygenated Krebs solution just before use. GSNO was prepared as initially described [30] and its effective concentration was calculated by optical absorbance [30].

2.8. Statistical analysis

Results are expressed as mean ± SEM of n experiments. Concentration–response curves were compared by the multi-analysis of variance (MANOVA). Other statistical comparisons were performed with one-way ANOVA. P values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Rate of NO release from LMW-DNIC

The rate of release of NO from DNIC-CYS and DNIC-GSH was compared using the oxyHb assay in buffer solution. As illustrated in Fig. 1, DNIC-CYS released NO at significant higher rates (about 3.5 times) than DNIC-GSH.

3.2. Vasorelaxant effect of LMW-DNIC

In rat aortic rings precontracted with NE, DNIC-CYS and DNIC-GSH produced concentration-dependent relaxant effect, with similar potencies (Fig. 2A). The EC_{50} values

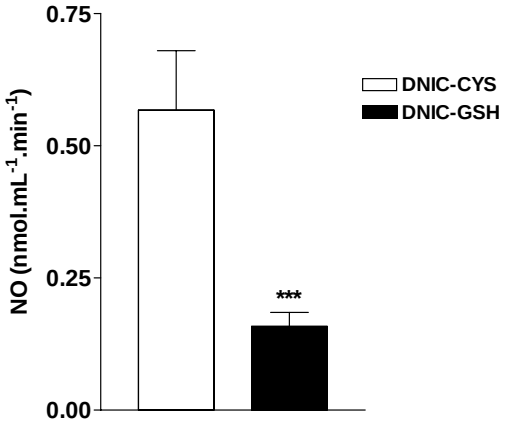

Fig. 1. Histograms showing the rate of release of NO from DNIC-CYS and DNIC-GSH (1 μM) in phosphate buffer. NO release was determined using the oxyHb assay. Results are expressed as mean ± SEM of at least three experiments. $^{***}P < 0.001$ vs. DNIC-CYS.

of DNIC-CYS and DNIC-GSH were 31 ± 4 nM (N = 8) and 32 ± 8 nM (N = 8), respectively. The relaxant effect of DNIC-CYS was rapid in onset and the tension tended to recover within few minutes (Fig. 2B). By contrast, the decrease in contraction evoked by DNIC-GSH developed more slowly and was more sustained (Fig. 2C).

3.3. Persistent effect of DNIC-GSH on arterial tone

In order to demonstrate persistent effect, the response to NE and LMW thiols were determined in aortic rings which were pre-exposed for 30 min to DNIC and then extensively washed out to remove the drug. Rings pre-exposed to DNIC-CYS (1 μM) exhibited neither modification of NE-induced contraction (Fig. 3A) nor relaxant response upon addition of NAC (Fig. 3B). By contrast, rings previously exposed to 0.3 or 1 μM DNIC-GSH (but not those exposed to 0.01 μM) displayed, even after washout, a persistent reduction of the contractile effect of NE (Fig. 3C) and a relaxant response to NAC (Fig. 3D). In rings pre-exposed to DNIC-GSH (0.3 μM), but not in controls, not only NAC but also L-cysteine, D-cysteine, and glutathione elicited relaxation (Fig. 3E).

3.4. Role of the NO-cyclic GMP pathway in the persistent effect of DNIC-GSH on arterial tone

The role of the NO-cyclic GMP pathway in the persistent changes of aortic reactivity induced by DNIC-GSH was analyzed using oxyHb and carboxy-PTIO (NO scavengers), and Rp-8Br-cGMPS (an inhibitor of cyclic GMP-dependent protein kinases). OxyHb (Fig. 4A and B), carboxy-PTIO (Fig. 4C and D), and Rp-8Br-cGMPS (Fig. 4E and F), all restored the effect of contractile agonists (Fig. 4A, C, and E) and inhibited the relaxant effect of NAC (Fig. 4B, D, and F). None of these inhibitors affected agonist-induced contraction in rings not previously exposed to DNIC-GSH (not shown). The cyclic GMP content of aortic rings was also determined. As illustrated in Fig. 5, aortic rings pre-exposed to DNIC-GSH (0.3 μM) displayed, even after washout, a significant (about 3.2-fold) increase in cyclic GMP content. Addition of NAC (10 mM) to these rings induced a further increase in cyclic GMP content (Fig. 5).

3.5. Role of thiols and S-nitrosothiols in the persistent effect of DNIC-GSH on arterial tone

The role of tissue thiols in DNIC-GSH-induced changes in contractility was analyzed by exposing aortic tissue to the thiol-modifying agent *p*-HMBA. Inclusion of *p*-HMBA (10 μM) together with DNIC-GSH (0.3 μM) resulted in a decrease of the relaxant effect of subsequent

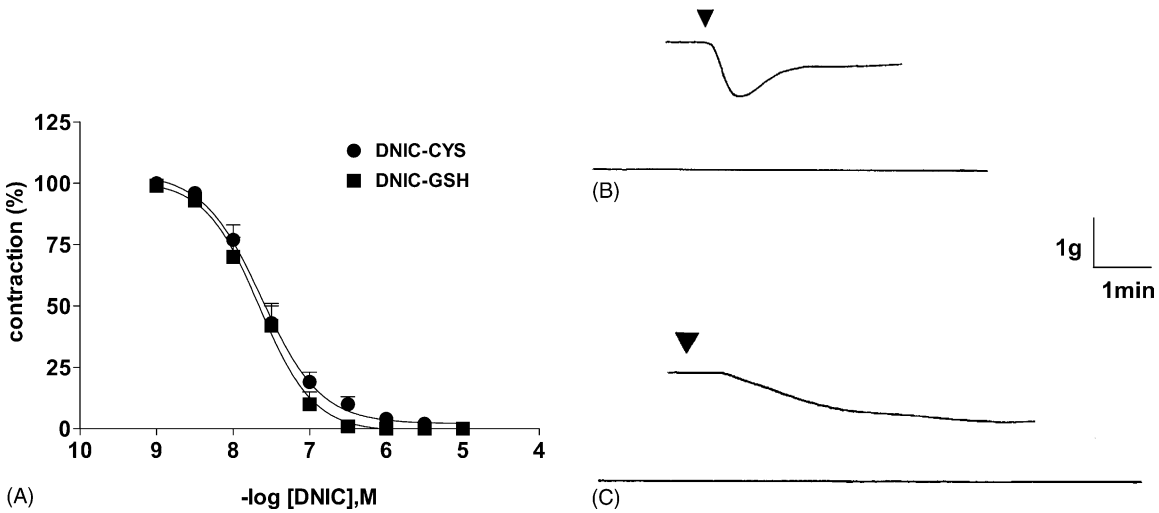

Fig. 2. (A) Concentration-dependent relaxation elicited by DNIC-CYS and DNIC-GSH in norepinephrine-precontracted rat aortic rings. Results are expressed as mean ± SEM of eight experiments. Representative traces of the relaxant effect of (B) DNIC-CYS (0.03 μM) and (C) DNIC-GSH (0.03 μM) in norepinephrine-precontracted rat aortic rings.

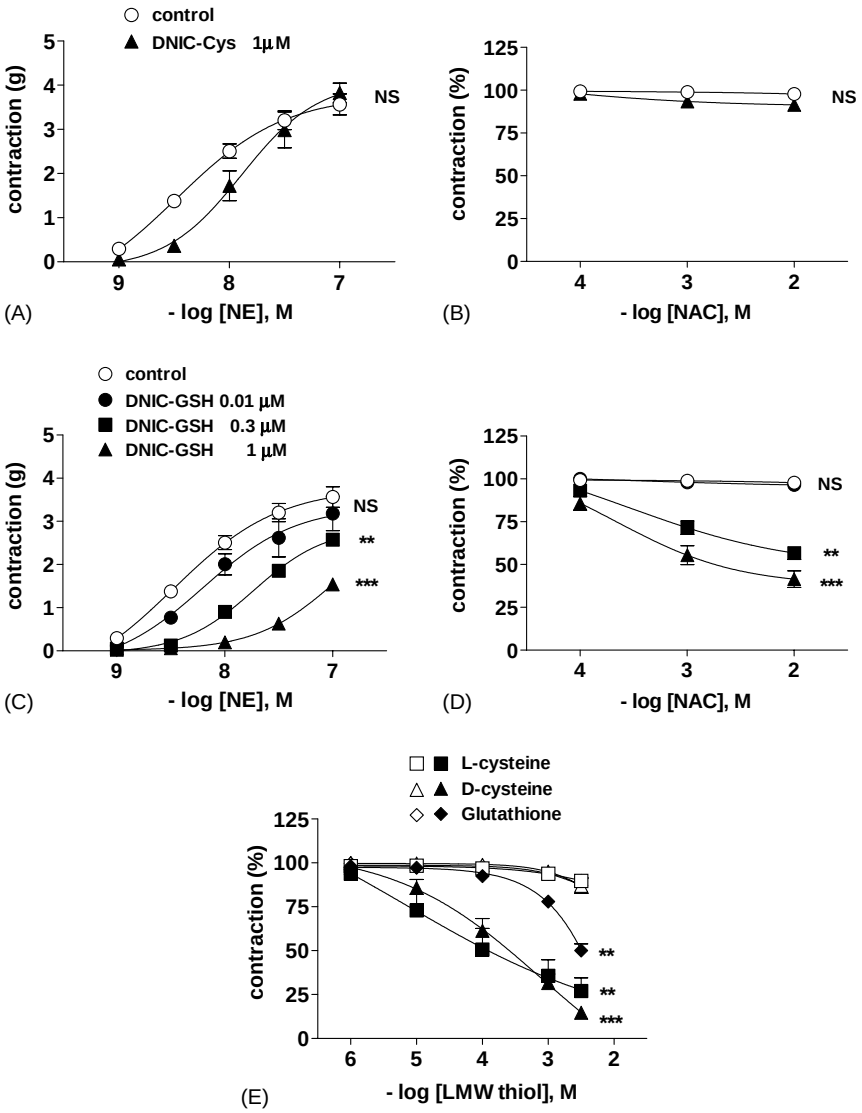

Fig. 3. (A and C) Contractile effect of norepinephrine and (B and D) effect of subsequent addition of *N*-acetylcysteine in rat aortic rings pre-exposed to DNIC-CYS (1 μM; A and B) or DNIC-GSH (0.01, 0.3, or 1 μM; C and D) followed by several washout. (E) Effect of various LMW thiols in rat aortic rings pre-exposed (closed symbols) or not (open symbols) to DNIC-GSH (0.3 μM followed by several washout) and precontracted with norepinephrine. Results are expressed as mean ± SEM of 4–10 experiments. Error bars are not shown when the size of the symbol exceeds the value of the SEM. NS: not significant; $^{**}P < 0.01$; $^{***}P < 0.001$

addition of NAC (Fig. 6A). In some experiments, the effect of mercuric chloride (which is known to cleave the cysteine-NO bound of *S*-nitrosothiols) was also investigated. Addition of mercuric chloride induced marked relaxation in rings pre-exposed to 0.3 μM DNIC-GSH, but not in controls (Fig. 6B). The relaxant effect of mercuric chloride was rapid in onset (within 2 min) and transient, with tension returning close to initial precontraction values within 5–10 min. Once tension partially recovered after addition of mercuric chloride, NAC failed to elicit a relaxant response (Fig. 6C). However, subsequent addition of the NO-donor 3-morpholinosydnonimine (10 μM) induced full relaxation, indicating that mercuric chloride-treated arteries were still responsive to NO (not shown).

3.6. Role of S-nitrosothiols in the persistent increase of NO content induced by DNIC-GSH in arteries

EPR spectroscopy using [Fe(II)$(DETC)_2$] as spin trap was applied for the detection of NO in rat aortic rings. A large increase of NO content was detected in rings pre-exposed to DNIC-GSH (100 μM), even after washout (Fig. 7). DNIC-GSH-induced increase in NO content was abolished when mercuric chloride (1 mM) was applied before addition of the spin trap (Fig. 7).

3.7. Selectivity of mercuric chloride for S-nitrosothiols

The selectivity of mercuric chloride for the decomposition of RSNO vs. DNIC was further investigated. In one

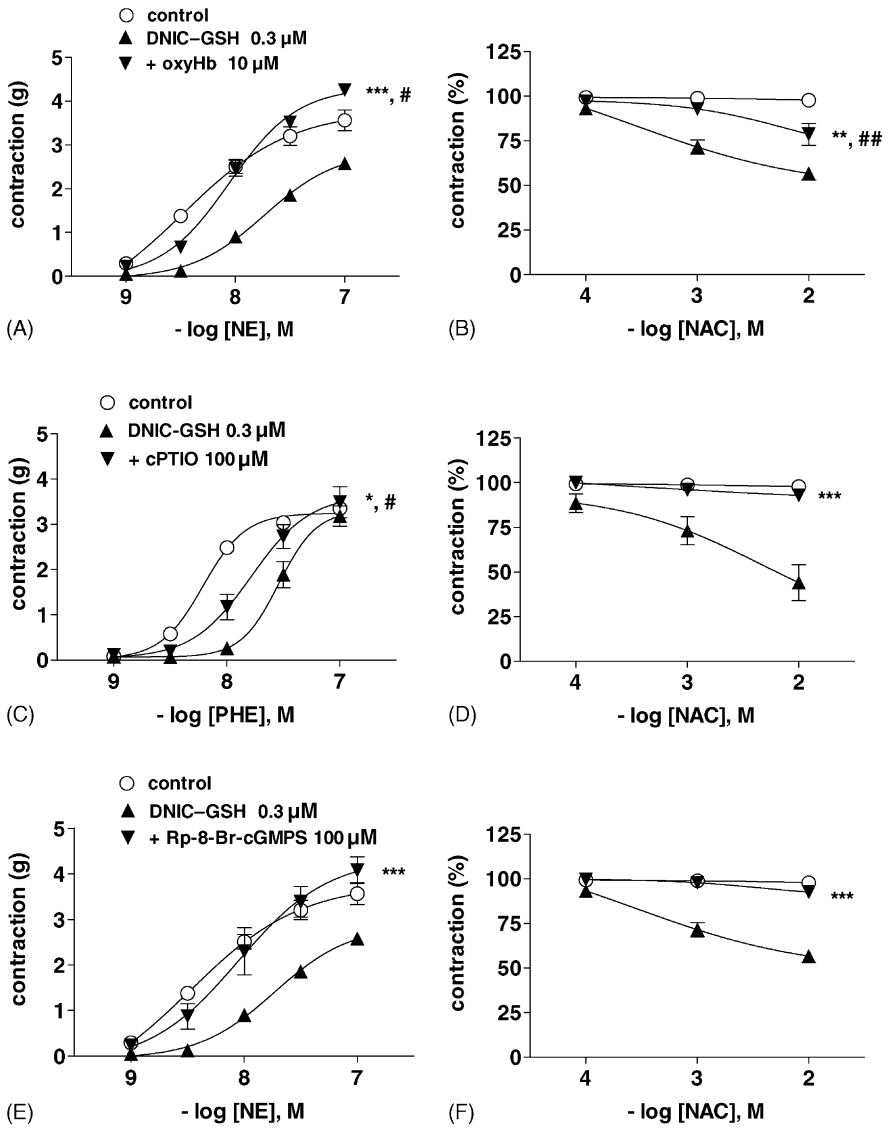

Fig. 4. (A and B) Influence of oxyHb (10 μM), (C and D) carboxy-PTIO (100 μM), and (E and F) Rp-8Br-cGMPS (100 μM) on the contractile effect of norepinephrine (A and E) or phenylephrine (C) and on the relaxant effect of *N*-acetylcysteine (B, D, and F) in rat aortic rings pre-exposed or not to DNIC-GSH (0.3 μM followed by several washout). Results are expressed as mean ± SEM of at least three experiments. Error bars are not shown when the size of the symbol exceeds the value of the SEM. NS: not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. DNIC-GSH; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. control.

series of experiments, either GSNO (as a model *S*-nitrosothiol) or DNIC-GSH were first mixed or not with mercuric chloride. Aliquots were then applied to NE

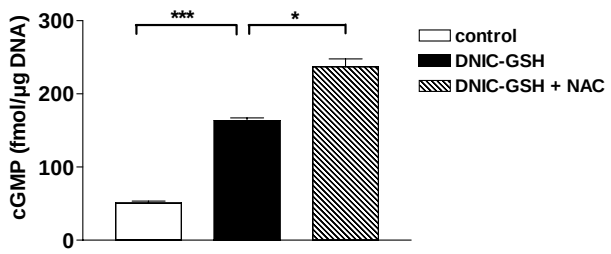

Fig. 5. Histograms showing the cyclic GMP content of rat aortic rings pre-exposed or not to DNIC-GSH (0.3 μM followed by several washout) and subsequently treated or not with *N*-acetylcysteine (10 mM). Results are expressed as mean ± SEM of at least 6–11 experiments. $^{*}P < 0.05$; $^{***}P < 0.001$.

(0.1 μM)-precontracted rat aortic rings. As expected, addition of GSNO (final concentration 1 μM, Fig. 8A) and DNIC-GSH (final concentration 0.1 μM, Fig. 8C) produced almost complete relaxation. Addition of GSNO + mercuric chloride (final concentration 1 and 10 μM, respectively) did not induce a vasorelaxant effect (Fig. 8B). However, addition of DNIC-GSH + mercuric chloride (final concentration 0.1 and 10 μM, respectively) evoked a relaxant effect which was comparable to the one obtained by addition of DNIC-GSH alone (Fig. 8D). In another series of experiments, increasing concentrations of GSNO and DNIC-GSH were exposed or not to mercuric chloride (500 μM) and nitrite concentration was determined using the Griess assay. GSNO gave rise to nitrites only to a minimal extent. Consistent with a cleavage of the cysteine-NO bond of GSNO, mercuric chloride produced complete conversion of GSNO into nitrites (Fig. 8E).

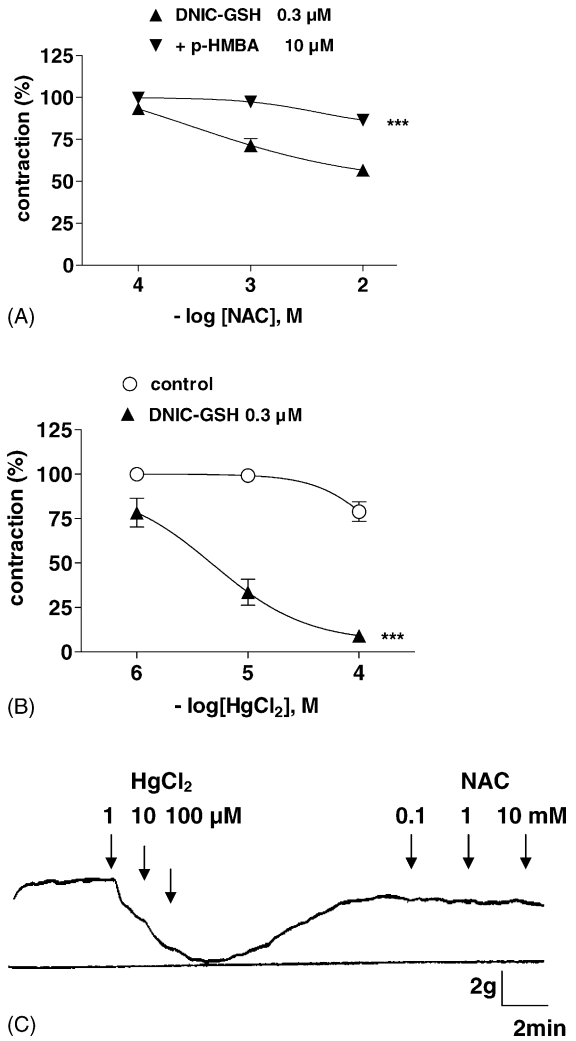

Fig. 6. (A) Effect of *N*-acetylcysteine in rat aortic rings pre-exposed to DNIC-GSH (0.3 μM) in the absence or presence of *p*-HMBA (10 μM) followed by several washout. Results are expressed as mean ± SEM of five experiments. $^{***}P < 0.001$ vs. DNIC-GSH alone. (B) Effect of mercuric chloride in rat aortic rings pre-exposed or not to DNIC-GSH (0.3 μM followed by several washout). Results are expressed as mean ± SEM of five experiments. $^{***}P < 0.001$ vs. control. (C) Representative traces of the effect of mercuric chloride and subsequent addition of *N*-acetylcysteine in rings pre-exposed or not to DNIC-GSH (0.3 μM followed by several washout). Traces are representative of three experiments. (A and B) Error bars are not shown when the size of the symbol exceeds the value of the SEM.

By contrast, mercuric chloride did not influence the production of nitrites from DNIC-GSH (Fig. 8E). Inclusion of mercuric chloride did not influence by itself nitrite determination (not shown).

4. Discussion

The major finding of the present study is that the LMW-DNIC of biological relevance, DNIC-CYS and DNIC-GSH, differ in their ability to induce or not an inhibition of arterial tone which persists after washout of the drug.

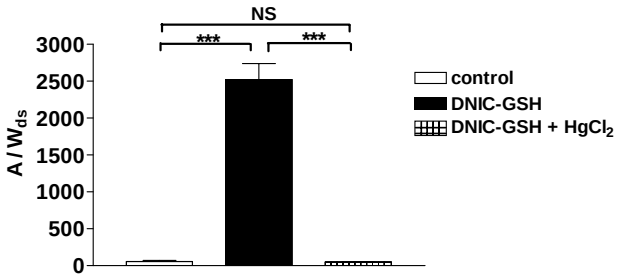

Fig. 7. NO content of rat aortic rings pre-exposed or not to DNIC-GSH (100 μM, followed by several washout) and subsequently treated or not with mercuric chloride (1 mM). NO content is expressed as amplitude of the [Fe(II)NO(DETC)$_2$] signal (*A* in relative units) per milligram of the dried sample (W_{ds}). Results are expressed as mean ± SEM of six experiments. NS: not significant; $^{***}P < 0.001$.

The persistent inhibition of contraction obtained with DNIC-GSH is attributed to the formation of releasable NO stores on tissue thiols, most probably as *S*-nitrosothiols.

The vasorelaxant properties of LMW-DNIC have been previously reported [4,10,13,20] and attributed to the activation of the cyclic GMP pathway [10,13]. In accordance with a previous report [31], the present study shows that the nature of the thiols ligand influences both the stability of LMW-DNIC and the time-course of their vasorelaxant effect. Indeed, DNIC-CYS which gave rise very rapidly to NO in buffer solution, induced a rapid and relatively transient relaxant effect. By contrast, DNIC-GSH which released NO at slower rate than DNIC-CYS, elicited a slowly developing and sustained relaxation. The present study demonstrates that LMW-DNIC differ not only in their time-course of NO release and associated vasorelaxation, but also in their ability to induce or not an inhibition of arterial tone which persists after washout of the drug. When rat aortic rings were exposed to DNIC-CYS (at a concentration producing full relaxation) and then washed out for drug removal, the contractile effect of subsequent addition of NE was not modified. Such a short-lasting and easily reversible effect is expected from the reversible binding of NO to the heme group of guanylyl-cyclase and from the short biological half-life of NO. Contrary to DNIC-CYS, pre-exposure to DNIC-GSH resulted, even after washout of the drug, in an inhibition of the response to NE and in a relaxant effect of various LMW thiols, including NAC. The decrease of the response to α-adrenergic agonists and the relaxant effect of NAC induced by DNIC-GSH were both associated with elevation of cyclic GMP level in aortic tissue and were both attenuated in the presence of NO scavengers (oxyHb or carboxy-PTIO) or a cyclic GMP-dependent protein kinases inhibitor (Rp-8-Br-cGMPS). This demonstrates the role of the activation of the NO-cyclic GMP pathway in the persistent alterations of arterial tone induced by DNIC-GSH. Direct evidence of persistent NO elevation in arteries pre-exposed to DNIC-GSH was obtained by EPR spectroscopy, using Fe(II)NO(DETC)$_2$ as spin trap.

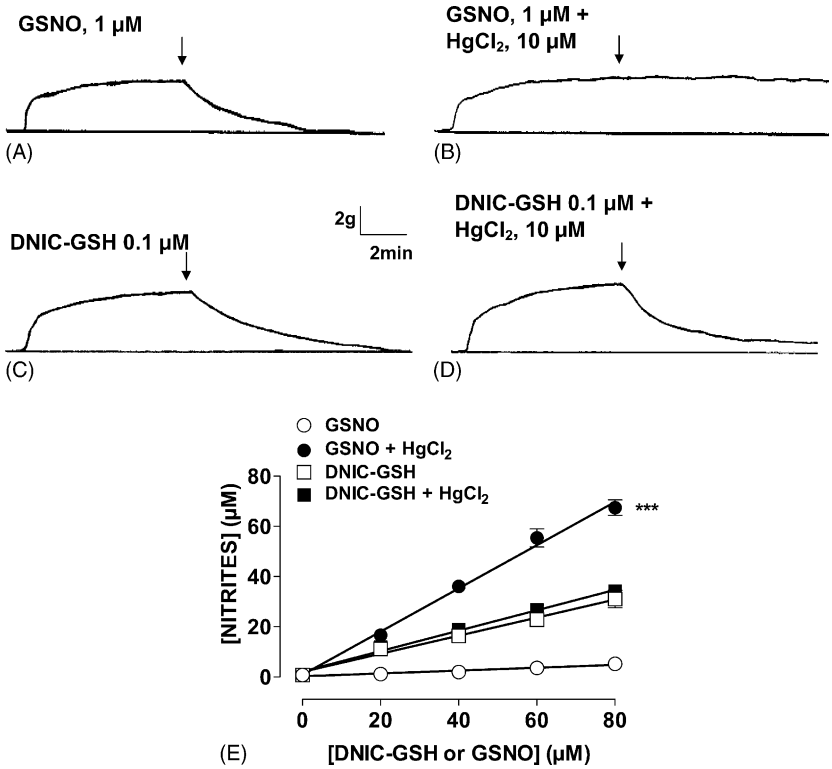

Fig. 8. Representative traces of the effect of addition of (A) GSNO (final concentration 1 μM), (B) premixed solution of GSNO + mercuric chloride (added at the final concentration of 1 and 100 μM, respectively), (C) DNIC-GSH (final concentration 0.1 μM), and (D) premixed solution of DNIC-GSH + mercuric chloride (added at the final concentration of 0.1 and 100 μM, respectively) in norepinephrine-precontracted rat aortic rings. Traces are representative of at least three experiments. (E) Effect of addition of mercuric chloride (500 μM) on nitrite formation in solutions of GSNO or DNIC-GSH. Results are expressed as mean ± SEM of three experiments. NS: not significant; $^{***}P < 0.001$.

The difference between two DNIC in relaxant activity is determined by different stability of the complexes. As previously proposed [31], the higher stability of DNIC-GSH is likely due to high stability of GSNO, which is a component of the chemical equilibrium between DNIC-GSH and its constituents. Conversely, lower stability of DNIC-CYS is determined by the low stability of CYS-NO. Steric hindrance due to molecular bulk of glutathione seems to determine slow process of the decomposition of GSNO, catalyzed by transition metal ions. The effects of DNIC-GSH on arterial tone reported in the present study were very similar to those produced by GSNO [22]. The persistent inhibition of contraction and relaxant effect of LMW thiols induced by pre-exposure to GSNO have been attributed to formation of NO stores by *S*-nitrosation of tissue thiols, and subsequent release of NO (or NO-related vasorelaxant compounds) from *S*-nitrosated thiols, respectively [22]. The involvement of these mechanisms in the persistent effects of DNIC-GSH on arterial tone was investigated in the present study using *p*-HMBA (an agent that modifies thiols groups) and mercuric chloride (an agent that cleaves the S-NO bond of *S*-nitrosothiols). Addition of *p*-HMBA (together with DNIC-GSH) almost completely prevented the effect of subsequent addition of NAC. Addition of mercuric chloride in arteries pre-exposed to DNIC-GSH produced a marked relaxant effect, abrogated the effect of subsequent addition of NAC, and also completely blunted the EPR-detectable increase in tissue NO content. These data are consistent with formation of releasable NO stores by *S*-nitrosation of tissue thiols in arteries pre-exposed to DNIC-GSH. LMW-DNIC are carriers of NO in its oxidized state, NO^+ [2,9], a well-known *S*-nitrosating agent. The ability of LMW-DNIC to induce *S*-nitrosation of proteins in acellular models has been previously reported [2,5]. From the present study, it appears that the nature of the thiol ligand of LMW-DNIC determined not only the time-course of NO release in solution and of immediate vasorelaxation, but also their ability to induce or not persistent inhibition of contraction. DNIC-CYS, which rapidly decomposed into NO, elicited only short-lasting and easily reversible effects. The relative stability of DNIC-GSH can allow sufficient amount of NO^+ to be transferred to tissue thiols, thereby inducing persistent inhibition of contraction. The localization and nature of the *S*-nitrosated thiols which serves as releasable NO stores remain to be elucidated in arteries. Indirect evidence argues for localization in the extracellular space, or at the external side of cell membranes. Indeed in aorta pre-exposed to DNIC-GSH, the effect of NAC was attenuated by pretreatment with a thiol reagent which does not cross cell membranes [30]. In addition in these arteries, not only the membrane-permeant NAC, but also glutathione

which has a very limited cellular uptake [32,33] elicited vasorelaxation.

The transfer of the $Fe(NO)_2$ group from LMW-DNIC to protein thiols results in the formation of protein-bound DNIC [2,4]. Evidence has been reported that the latter can behave as NAC-releasable NO stores in arteries after exposure to relatively high concentrations (>30 μM) of DNIC-CYS [4] or during endotoxin-induced NO overproduction by the NOS-2 [18]. In the present study, it is shown that NO release from preformed stores in arteries pre-exposed to DNIC-GSH can be triggered not only by NAC and other LMW thiols, but also by mercuric chloride. Furthermore, because mercuric chloride abrogated the effect of subsequent addition of NAC in these arteries, these agents probably interact with the same stores. It is well known that mercuric chloride efficiently cleaves the S-NO bond of *S*-nitrosothiols [26]. Using two different experimental approaches (vascular relaxation as bioassay for NO-mediated effect in isolated arteries and nitrites determination in physiological solution), the present study demonstrates that mercuric chloride selectively decomposed *S*-nitrosothiols, without affecting DNIC. Thus, although the formation of protein-bound DNIC cannot be excluded, experimental data do not support their contribution as NAC-releasable NO stores in arteries pre-exposed to DNIC-GSH.

Together, these data demonstrate that the LMW-DNIC of biological relevance, DNIC-CYS and DNIC-GSH, both elicited a relaxant effect in isolated arteries in the same concentration range and with a time-course consistent with the rate of NO release in solution. However, an inhibition of contraction which persisted after drug removal was obtained with DNIC-GSH but not with DNIC-CYS. The persistent inhibition of contraction obtained with DNIC-GSH was attributed to the formation of releasable NO stores in arterial tissue, most probably as *S*-nitrosothiols. Thus, the mechanisms and duration of the effect of LMW-DNIC in arteries are critically dependent on the nature of their thiol ligand. The formation of NO stores in arteries by LMW-DNIC is probably not restricted to *in vitro* models. Indeed after infusion of LMW-DNIC, the sustained phase of hypotension which follows the immediate drop in blood pressure has been attributed to the formation of releasable NO stores [34]. Moreover, arteries removed from these animals exhibited a relaxant response to DETC [35] which as NAC, can displace NO or NO related relaxant species from both protein-bound DNIC and *S*-nitrosothiols [22,36,37]. The formation of NO stores from endogenous NO in arteries has been proposed as an adaptative mechanism towards various stress conditions [35,37]. Interestingly, it has been recently reported that endothelium-derived NO accumulates in rat aorta as a LMW *S*-nitroso species (most probably GSNO) and nitrites [38]. These NO stores which are photosensitive [38] are probably different from the LMW thiol-releasable stores characterized in the present study and in previous ones [18,22], as NAC failed to induce relaxation in control vessels. Besides some physiological or patho-physiological roles, formation of releasable NO stores might be also of pharmacological interest to compensate the impaired production of endogenous NO that occurs during various vascular diseases.

Acknowledgments

The authors thank Véronique Freund for helpful contribution in contractile experiments. This work was partially supported by a grant from Fondation de France. J.L.A. is recipient of a fellowship from CAPES-Brazil.

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