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Role of *S*-Nitrosation of Cysteine Residues in Long-Lasting Inhibitory Effect of Nitric Oxide on Arterial Tone

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ABSTRACT

S-Nitrosation of cysteine residues plays an important role in nitric oxide (NO) signaling and transport. The aim of the present study was to investigate the role of *S*-nitrosothiols as a storage form of NO, which may account for the long-lasting effects in the vasculature. Rat aorta exposed to *S*-nitrosoglutathione (GSNO) displayed, even after washout of the drug, a persistent increase in cysteine-NO residues (detected by immunostaining using an antiserum that selectively recognized *S*-nitrosoproteins) and in NO content (detected by NO spin-trapping), a persistent attenuation of the effect of vasoconstrictors, and a relaxant response upon addition of low molecular weight (LMW) thiols. Rat mesenteric and porcine coronary artery exposed in vitro to GSNO, as well as aorta and mesenteric arteries removed from rats treated in vivo with GSNO, displayed similar modifications of contraction. In isolated aorta exposed to GSNO, the decrease of the contractile response and the relaxant effect of LMW thiols were both blunted by NO scavengers (oxyhemoglobin or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) or by a cyclic GMP-dependent protein kinase inhibitor (Rp-8-bromoguanosine-3′,5′-cyclic monophosphorothioate). In these arteries, mercuric chloride (which cleaves the cysteine-NO bond) exerted a transient relaxation, completely abolished the one of LMW thiols, and blunted the increase in cysteine-NO residues and NO content. Together, these data support the idea that *S*-nitrosation of cysteine residues is involved in long-lasting effects of NO on arterial tone. They suggest that *S*-nitrosation of tissue thiols is a mechanism of formation of local NO stores from which biologically active NO can subsequently be released.

S-Nitrosation of cysteine residues plays an important role in nitric oxide (NO) signaling (for review, see Broillet, 1999; Eu et al., 1999; Lane et al., 2001; Stamler et al., 2001). In various cells or tissues, it is associated with stimulation of expression or activity of NO synthases (NOS) (Gow et al., 2002). Besides being an important mechanism for post-translational modification of proteins, *S*-nitrosation reactions may also play a role in blood transport and remote effects of NO (Stamler et al., 1992; 1997; Jia et al., 1996; Gow and Stamler, 1998; Stoclet et al., 1998; Gross and Lane, 1999; Rassaf et al., 2002). By analogy, *S*-nitrosation has been also postulated as a mechanism for local storage of NO in tissues (Lovren and Triggle, 1998; Megson et al., 2000; Muller et al., 2002). *trans*-Nitrosation reactions, consisting of transfer of NO from one cysteine residue to another, are involved in both generation and fate of *S*-nitrosothiols. Indeed, low molecular weight (LMW) *S*-nitrosothiols can transfer NO to cysteine residues (Arnelle and Stamler, 1995; Butler et al., 1995; Hughes, 1999; Hogg, 2000; Tsikas et al., 2001) and conversely, LMW thiols can displace NO from stable *S*-nitrosoproteins (with formation of LMW *S*-nitrosothiols as intermediates) and transfer it to different targets (Scharfstein et al., 1994; Jia et

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; LMW, low molecular weight; GSNO, *S*-nitrosoglutathione; NAC, *N*-acetylcysteine; DETC, diethyldithiocarbamate; PBS, phosphate-buffered saline; EPR, electron paramagnetic resonance; NE, norepinephrine; PHE, phenylephrine; *p*-HMBA, *para*-hydroxymercuribenzoic acid; *p*-CMPS, *para*-chloromercuriphenylsulfonic acid; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; g-BSA, glutaraldehyde-conjugated bovine serum albumin; U46619, U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin; oxyHb, oxyhemoglobin; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; MANOVA, multivariate analysis of variance; ANOVA, analysis of variance; Rp-8-Br-cGMPS, Rp-8-bromoguanosine 3′,5′-cyclic monophosphorothioate; SOD, superoxide dismutase.

al., 1996; Stoclet et al., 1998; Vanin, 1998; Muller et al., 2002).

In the vasculature, long-lasting hyporeactivity to vasoconstrictors can be induced by in vitro (Megson et al., 1997; 1999; Sogo et al., 2000; Terluk et al., 2000) or in vivo (da Silva-Santos et al., 1999) treatment with LMW *S*-nitrosothiols. It is unlikely that this effect is primarily a result of the release from *S*-nitrosothiols of the free radical NO activating soluble guanylyl cyclase. Indeed, NO as free radical possesses a relatively short biological half-life and the binding of the NO radical to the heme group of guanylyl cyclase is a reversible process (Butler et al., 1995). The present study was designed to investigate the possibility that *S*-nitrosation of cysteine residues is involved in long-lasting effects of NO in the vasculature. For this purpose, arteries were exposed to *S*-nitrosoglutathione (GSNO), a LMW *S*-nitrosothiol of physiological relevance as possible intermediate in NO/cyclic GMP signaling (Mayer et al., 1998). After careful washout, arteries were processed for immunostaining and electron paramagnetic resonance (EPR) spectroscopy (for detecting cysteine-NO residues and NO, respectively) and for assessment of contractility. Various reagents were used to displace NO from cysteine-NO bonds: *N*-acetylcysteine (NAC) and other LMW thiols (Scharfstein et al., 1994; Jia et al., 1996; Stoclet et al., 1998; Vanin, 1998; Muller et al., 2002), mercuric chloride (which is known to cleave the cysteine-NO bond; Saville, 1958) and diethyldithiocarbamate (DETC, which also decomposes *S*-nitrosothiols; Arnelle et al., 1997). Preliminary reports of this study have been presented as abstracts (Alencar et al., 2002a,b).

Materials and Methods

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 and mesenteric bed were removed from male Wistar rats (12–14 weeks old; 300–380 g) after anesthesia with pentobarbital (60 mg/kg, i.p.). Some rats were previously infused with saline or GSNO. In this case, after pentobarbital-induced anesthesia and tracheotomy, rats were intubated and the left jugular vein was catheterized for drug administration (infusion rate of 17 μl/kg/min). Rectal temperature was monitored and maintained constant with a heating lamp. After drug infusion, rats were killed with an overdose of pentobarbital. Porcine hearts were obtained from local slaughterhouse, placed immediately into an ice-cold Krebs' solution (119 mM NaCl, 4.7 mM KCl, 1.17 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.18 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose) and transported to the laboratory. Rat aorta and mesenteric arteries and porcine left coronary artery were cleaned of connective and fat tissues in Krebs' solution. The endothelium was removed by rubbing the intimal surface of the rings with forceps.

Immunostaining and Confocal Microscopy. Rings (2–3 mm length) of aorta and superior mesenteric artery (from rats bred from genitors provided by Iffa-Credo, Abresles, France) were exposed or not to GSNO for 30 min (at 37°C in 95% O_2/5% CO_2-aerated Krebs' solution, in the dark) and then extensively washed out (over a 60-min period, during which time the Krebs' solution was changed every 20 min). Some preparations were subsequently treated with mercuric chloride (1 mM for 5 min, followed by washout). Preparations were then fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.4), treated for at least 3 h with saccharose (20% in PBS), embedded in Tissue Tek (Miles Laboratories, Elkhart, IN) and frozen in isopentane (−50°C). Cross sections (about 16 μm thick) were prepared on a cryostat microtome (Frigocut 2800; Reichert, Vienna, Austria) and thaw-mounted on gelatin-coated slides. Slices were incubated overnight at room temperature with rabbit polyclonal antibodies directed against conjugated NO-cysteine (Mnaimneh et al., 1997; Lorch et al., 2000) [1:100 dilution in PBS-Triton 0.5% (v/w)]. The secondary antibody was goat anti-rabbit IgG (Alexa Fluor 488, 1/200 dilution in PBS-Triton). The sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined under a confocal microscope (1024 MRC; Bio-Rad, Hercules, CA) with a 40× epifluorescence objective (Nikon, Tokyo, Japan). Z-series were collected in 1-μm steps, and final images were obtained after stacking. After excitation at 488 nm with an Argon laser, emission signal was recorded with a Zeiss 515–565 nm filter. Nonspecific fluorescence was assessed after incubating slices with the secondary antibody and measuring the average intensity value of fluorescence. All images were corrected for nonspecific fluorescence.

ELISA. Glutaraldehyde-conjugated bovine serum albumin and *S*-nitrosated derivatives were synthesized as described previously (Boullerne et al., 1995). Briefly, cysteine was linked to BSA via glutaraldehyde and, after a reduction step, cys-g-BSA was nitrosated using $NaNO_2$ in acid medium. Nonglutaraldehyde-conjugated BSA and glutathione were nitrosated in the same manner. ELISA experiments were conducted according Boullerne et al. (1995). Briefly, plates were coated with *S*-NO-cys-g-BSA or cys-g-BSA (10 μg/ml). Saturation of free binding sites was performed with PBS containing Tween 20 (0.05%), glycerol (10%), and BSA (0.1%). Mixture of competing antigens (*S*-NO-cys-g-BSA, *S*-NO-BSA, cys-g-BSA, or GSNO) and rabbit polyclonal antibodies directed against conjugated NO-cysteine (final dilution, 1:10,000) were incubated overnight at 4°C. Mixture (200 μl) was added to coated plates, which were then incubated for 2 h at 37°C. After rinses with PBS-Tween, well plates were exposed for 1 h at 37°C to horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG, 1:10,000 dilution). After rinses, immunobinding was revealed by peroxidase assay using *ortho*-phenylenediamine as substrate (10-min exposure in the dark). After stopping the reaction with H_2SO_4 (4 N), optical density at 492 nm was read. Values were corrected for the blank (well plates coated with cys-g-BSA). Ratio between absorbances with (B) and without (Bo) competitors is expressed versus the concentration of competitor.

NO Spin-Trapping and EPR Spectroscopy. Rings (6 to 8 mm length) of aorta (from rats obtained from Harlan, Gannat, France) and porcine coronary artery were exposed or not to GSNO (in the dark, for 30 min at 37°C in aerated Krebs' solution) and then extensively washed out (during 1 h). The NO content was assayed after formation of the paramagnetic spin adduct $[Fe(II)NO(DETC)_2]$ detectable by EPR in rings treated (for 30 min at 37°C) with the $[Fe(II)(DETC)_2]$ complex (0.5 mM). The $[Fe(II)(DETC)_2]$ complex was prepared as a colloid solution (Kleschyov et al., 2000a) after mixing of 2 mM NaDETC and 1 mM $FeSO_4$ dissolved in Krebs' solution containing 5 mM HEPES, pH 7.6. Some arteries were treated with $HgCl_2$ (1 mM for 5 min) or with NAC (10 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, Berlin, Germany) 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 the 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-line EPR signal by the weight of the dried sample (W_{ds}).

Cyclic GMP Determination. Rat aortic rings were exposed or not to 1 μM GSNO for 30 min (at 37°C in aerated Krebs' solution) and then extensively washed out (during 1 h). They were then incubated for 30 min at 37°C in aerated Krebs' solution supplemented with isobutylmethylxanthine (100 μM). After that, rings

were transferred into 1 ml of ice-cold $HClO_4$ (1.07 M). Samples for cGMP and DNA determination were prepared as described previously (Muller et al., 1998). The cyclic GMP content was assessed by radioimmunoassay and was expressed as femtomoles per microgram of DNA. DNA was determined as described by Brunk et al. (1979).

Contraction/Relaxation Experiments. Rings (2 to 3 mm length) of aorta, superior mesenteric arteries (from rats bred from genitors provided by Iffa-Credo), and porcine coronary arteries were mounted in organ chambers filled with Krebs' solution (at 37°C; bubbled with 95% O_2/5% CO_2) for isometric tension recordings. The passive tension was 1, 2, and 5 g for mesenteric artery, aorta, and coronary artery, respectively. After equilibration, rings were precontracted with norepinephrine (NE; 1 μM, for aortic rings), phenylephrine (PHE; 1 μM, for mesenteric artery rings), or the thromboxane mimetic U46619 (10 nM, for coronary artery rings). The absence of functional endothelium was verified by the lack of relaxant effect of acetylcholine (1 μM) in the aorta and mesenteric artery or of bradykinin (1 μM) in the coronary artery. After washout, rings were exposed or not to GSNO for 30 min in the dark. All subsequent experimental procedures were also performed in the dark. In some experiments, aortic rings were exposed to GSNO together with the thiol-modifying agent, *para*-hydroxymercuribenzoic acid (*p*-HMBA, 10 μM) or *para*-chloromercuriphenylsulfonic acid (*p*-CMPS, 10 μM). Rings were then extensively washed out (for 1 h, during which time the Krebs' solution was changed every 20 min) and then submaximally contracted (with increasing concentrations of NE, PHE, or U46619). When a stable level of contraction was obtained, low molecular weight thiols, DETC, 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 oxyhemoglobin (oxyHb, 10 μM) or Rp-8-Br-cGMPS (100 μM). When the influence of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO, 100 μM) was studied, PHE was used as contractile agonist because a stable level of contraction could not be obtained using NE. Experiments in rat arteries were completed within 3.5 to 4 h after removal from the animal. In preliminary experiments, it has been checked that the NO synthase inhibitor N^{ω}-nitro-L-arginine methylester (300 μM) did not significantly enhance the contractile response to NE in endothelium-denuded aortic rings mounted for 4 h in organ bath. Thus, the inducible NOS activity, if any, was not significantly involved in the regulation of contractile response to the agonist, in the experimental conditions. In some experiments, aorta was also removed from rats infused with NaCl (0.9%) or GSNO (0.6 μmol/kg/min). The thoracic aorta was removed, and rings were prepared and suspended in organ chambers as described above. After equilibration (30 min), rings were precontracted with PHE; once the contraction was stable, NAC was added. Contractile effects are expressed in grams of developed tension and relaxant ones in percentage of contraction (100% being the tone induced by the contractile agonist).

Drugs and Reagents. Unless otherwise indicated, drugs were purchased from Sigma Chemical Co or Aldrich (Saint Quentin-Fallavier, France). Rabbit polyclonal antibodies directed against conju-

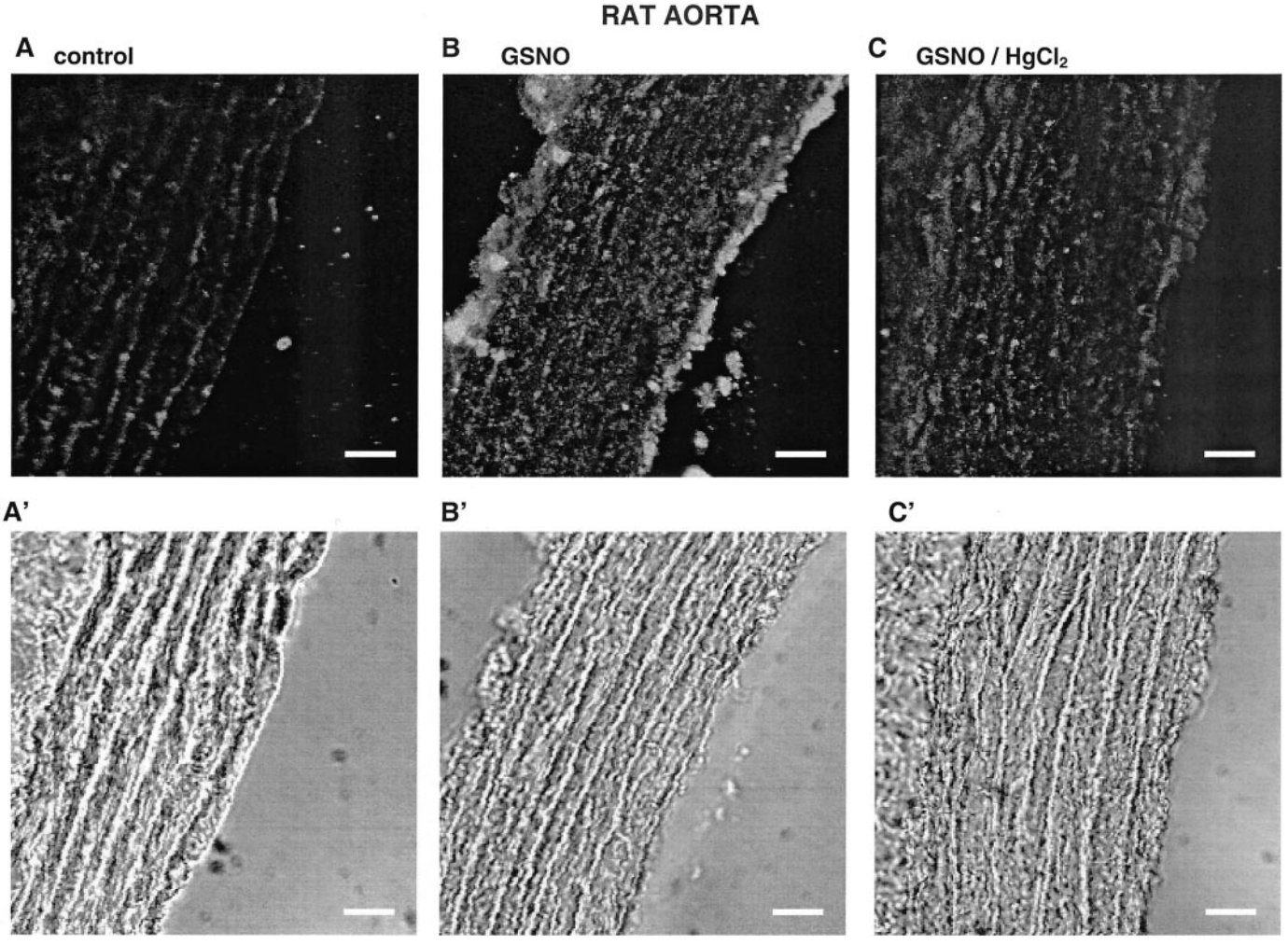

Fig. 1. Immunolabeling of *S*-nitrosated cysteine residues in rat aortic rings. A, control rings (not pre-exposed to GSNO). B, rings exposed to GSNO (100 μM followed by several washouts). C, rings treated with mercuric chloride (1 mM) after exposure to GSNO (100 μM). The respective light transmission figures are shown in A′, B′, and C′. Calibration bars, 25 μm. Representative pictures from three independent experiments.

gated NO-cysteine were obtained as described previously (Mnaimneh et al., 1997; Lorch et al., 2000). Alexa Fluor 488 and U46619 were purchased from Molecular Probes (Leiden, The Netherlands) and Cayman Chemical Company (Ann Arbor, MI), respectively. Kit for cyclic GMP determination was obtained from Immunotech (Marseille, France). Horseradish peroxidase-labeled antibody (goat anti-rabbit IgG) was purchased from Diagnostic Pasteur (Paris, France). NAC (Fluimucil) was a generous gift from Zambon laboratory (Antibes, France). GSNO was prepared as initially described (Gordge et al., 1998), and its effective concentration was calculated by optical absorbance (Gordge et al., 1998). Sodium pentobarbital was purchased from Sanofi Santé Animale (Libourne, France) and sodium heparin from Laboratoires Léo S.A. (St-Quentin-Yvelines, France).

Statistical Analysis. Results are expressed as mean ± S.E.M. of *n* experiments. Concentration-response curves were compared by the multivariate 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.

Results

Persistent Increase in Cysteine-NO Residues in Arteries Pre-exposed to GSNO. *S*-nitrosated cysteine residues were detected using polyclonal antibodies directed against the *S*-NO moiety. A weak staining was observed in some aortic rings after exposure to 1 μM GSNO and washout (not shown). However, compared with controls (Fig. 1A), a marked staining was detected after exposure of aortic rings to 100 μM GSNO, even after extensive washout of the tissues (Fig. 1B). Staining was not observed in rings treated with mercuric chloride after exposure to GSNO (Fig. 1C). In the rat mesenteric artery, staining was detected even after pre-exposure to 1 μM GSNO and washout (not shown). It was more intense after exposure to 100 μM GSNO (Fig. 2B) and, as in the aorta, no staining could be demonstrated in mesenteric arteries exposed to mercuric chloride after GSNO (Fig. 2C).

Selectivity of the Antibodies for *S*-Nitrosated Proteins. Antibodies selectivity for *S*-nitrosoproteins versus non-nitrosated or LMW species was evaluated by ELISA. As depicted in Fig. 3, *S*-nitrosated proteins (*S*-NO-BSA and *S*-NO-cys-g-BSA) displayed high affinity for the antibodies used. By contrast GSNO, a LMW *S*-nitrosothiol, had much lower affinity (concentration required for 50% inhibition of binding greater than 1 mM). In accordance with previous findings (Boullerne et al., 1995), no displacement was obtained using non-nitrosated cys-g-BSA.

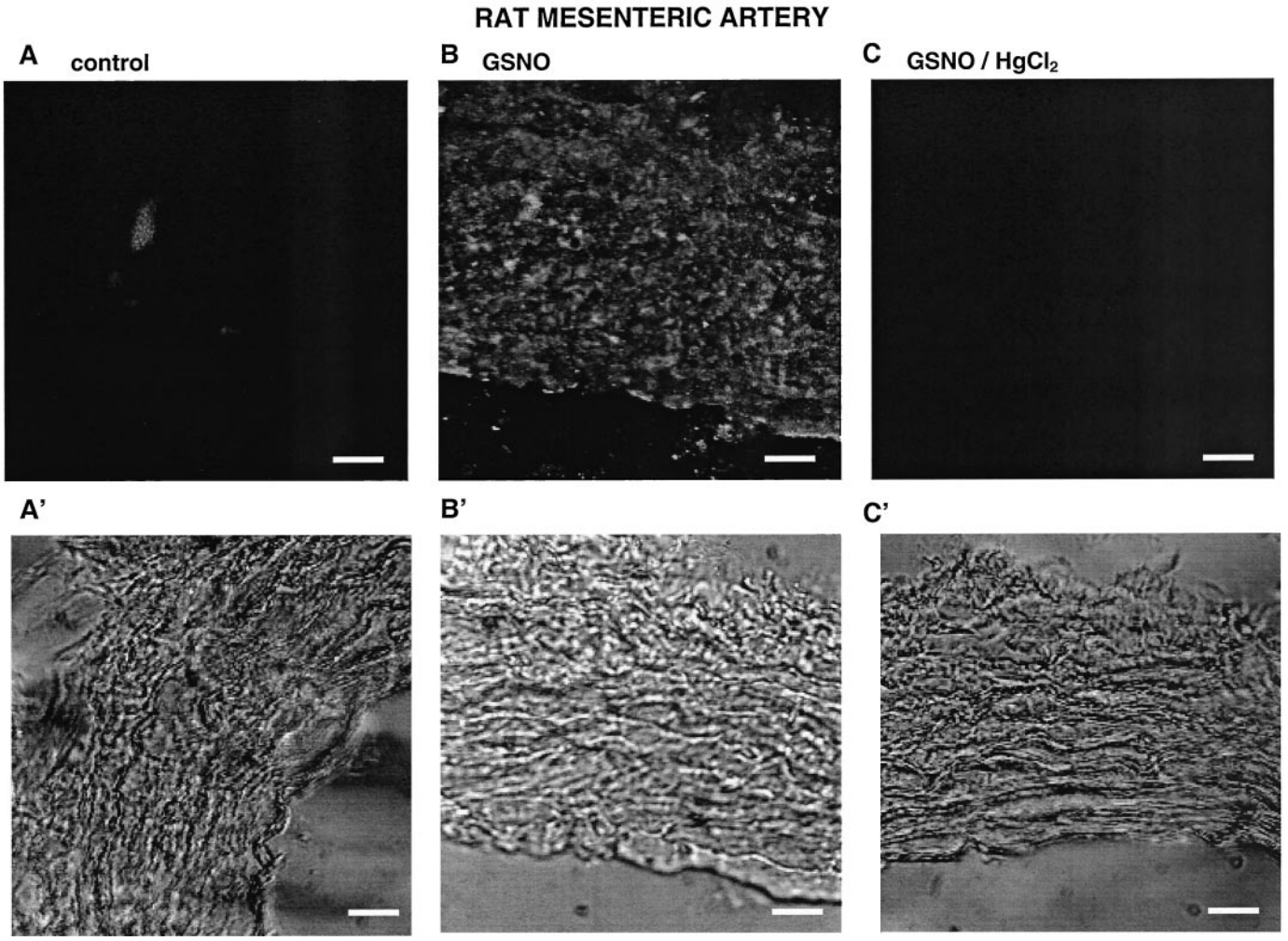

Fig. 2. Immunolabeling of *S*-nitrosated cysteine residues in rat mesenteric artery. A, control rings (not pre-exposed to GSNO); B, rings exposed to GSNO (100 μM followed by several washouts). C, rings treated with mercuric chloride (1 mM) after exposure to GSNO (100 μM). The respective light transmission figures are shown in A′, B′, and C′. Calibration bars, 25 μm. Representative pictures from two independent experiments.

Persistent Increase in NO Content in Arteries Pre-Exposed to GSNO. The NO content of arterial rings was evaluated using EPR spectroscopy and [Fe(II)$(DETC)_2$] as spin trap (Fig. 4). Exposure of rat aortic rings to 100 μM GSNO resulted, even after washout, in the appearance of the typical three-lines signal of the [Fe(II)NO$(DETC)_2$] complex at g = 2.04 after incubation with the NO trapping agent (see Fig. 4A, b, for representative spectra and Fig. 4B for quantification of the signals). Aortic rings pre-exposed to 1 μM GSNO displayed a very weak signal for [Fe(II)NO$(DETC)_2$]. However, consistent with an increase of NO level in these arteries, their cyclic GMP content was increased about 1.6-fold (from 36.56 ± 7.26 in controls to 57.95 ± 5.69 fmol/μg of DNA in rings exposed to 1 μM GSNO and washed out; n = 5–6, $p < 0.05$). In aortic rings pre-exposed to GSNO (100 μM), the signal of the [Fe(II)NO$(DETC)_2$] complex was markedly decreased when mercuric chloride was applied before addition of the spin trap (Fig. 4, A, c, and B). The EPR signal intensity was also decreased in GSNO-pretreated aortic rings that were exposed to the LMW thiol NAC before addition of the spin trap (Fig. 4, A, d, and B). In porcine coronary arteries pre-exposed to GSNO (100 μM, followed by washout), the typical signal for [Fe(II)NO$(DETC)_2$] was also observed (Fig. 4, C, b, and D). As in the aorta, a significant decrease of the signal was observed in GSNO-treated coronary arteries, which were exposed to mercuric chloride before the spin trap (Fig. 4, C, c, and D).

Persistent Changes in Contractility in Arteries Pre-Exposed to GSNO. In rat aortic rings pre-exposed to 1 or 100 μM GSNO and then extensively washed-out (but not in those pre-exposed to 0.1 μM GSNO), the contractile response to NE was diminished (Fig. 5A) and addition of NAC further depressed contraction (Fig. 5, B for a representative trace and C for concentration-response curves). The effect of NAC was rapid in onset (a peak was reached within 1 min) and was transient (the tension returned back to initial precontraction values within 10 min). An attenuation of the contractile response to NE and a relaxant effect of NAC were still observed when the washout period after GSNO exposure was expanded to 3 h (not shown). In aortic rings pre-exposed to GSNO (but not in controls), not only NAC but also D-cysteine, L-cysteine, and glutathione caused relaxation (Fig. 5D).

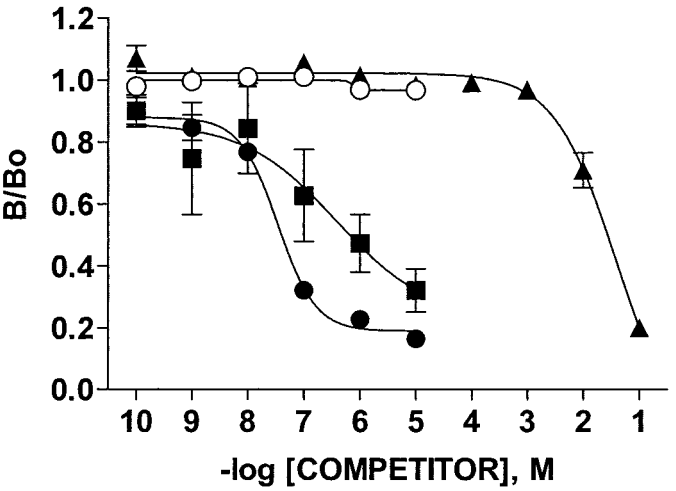

Fig. 3. Displacement curves obtained with rabbit antiserum directed against *S*-NO moiety from competition experiments between *S*-NO-cys-g-BSA and *S*-NO-cys-g-BSA (●), *S*-NO-BSA (■), cys-g-BSA (○), and GSNO (▲). B/Bo [ratio between absorbances with (B) and without (Bo) competition] is plotted versus the concentration of competitor. Results are expressed as mean ± S.E.M. of three to nine experiments. Error bars are not shown when the size of the symbol exceeds the value of the S.E.M.

Similar experiments were conducted in rat mesenteric and porcine coronary arteries pre-exposed in vitro to 1 μM GSNO. These arteries (but not control ones) exhibited an attenuation of the effect of vasoconstrictors (PHE in rat mesenteric artery, Fig. 6A; U46619 in porcine coronary artery, Fig. 6C) and a relaxant effect of NAC (Figs. 6, B and D). The effects of PHE and NAC were also studied in aortic rings removed from rats treated in vivo with GSNO (0.6 μmol/kg/min for 45 min). In these rings, the contractile response to PHE was diminished compared with controls (Fig. 6E) and NAC exerted a significant relaxant effect (Fig. 6F).

Role of NO and *S*-Nitrosation of Tissue Thiols in GSNO-Induced Changes in Contractility. The role of the NO-cGMP pathway in GSNO-induced persistent changes in aortic reactivity was analyzed using the NO scavengers oxyHb and carboxy-PTIO and the inhibitor of cGMP-dependent protein kinases Rp-8-Br-cGMPS (Fig. 7). oxyHb (Fig. 7, A and B), carboxy-PTIO (Fig. 7, C and D), and Rp-8-Br-cGMPS (Fig. 7, E and F) all fully restored the responses to contractile agonists (Fig. 7, A, C, and E) and abrogated the relaxant effect of NAC (Fig. 7, B, D, and F). None of these inhibitors affected agonist-induced contraction in rings not previously exposed to GSNO (not shown). Variations in the amplitude of the relaxant effect of NAC in GSNO-exposed aortic rings might be related to the use of either NA (Fig. 7, B and F) or PHE (Fig. 7D) as contractile agonist.

The role of *S*-nitrosation of tissue thiols in GSNO-induced changes in contractility was analyzed by exposing aortic tissue to thiol-modifying agents. Inclusion of either *p*-HMBA or *p*-CMPS together with GSNO attenuated the relaxant effect of subsequent addition of 10 mM NAC (39 and 65% inhibition by 10 μM of *p*-HMBA and *p*-CMPS, respectively). Experiments were also performed using DETC and mercuric chloride, both of which were able to displace NO (or NO-related compounds) from *S*-nitrosothiols. In control aortic rings pre-contracted with NE, neither DETC (Fig. 8A) nor mercuric chloride (up to 10 μM, Fig. 8B) affected tone. By contrast, DETC and mercuric chloride caused marked relaxation in rings pre-exposed to 1 μM GSNO (Figs. 8, A and B). The relaxant effect of DETC or mercuric chloride was rapid in onset (within 2 min) and transient, with tension returning near initial precontraction values within 10 min. Once tension recovered after addition of DETC or mercuric chloride, subsequent addition of NAC failed to elicit a relaxant response (Fig. 8C). Conversely, previous addition of NAC abolished the relaxant effect of subsequent addition of mercuric chloride or DETC (data not shown).

Discussion

The present study demonstrates that exposure of various arteries to GSNO, in vitro or in vivo, induced a long-lasting attenuation of contractile responses and a relaxant effect of LMW thiols. GSNO produced also a persistent increase in NO and cyclic GMP content, and in *S*-nitrosated cysteine residues in arteries. Furthermore, evidences are provided for the implication of *S*-nitrosation of cysteine residues and sub-

sequent release of NO from *S*-nitrosated thiols in the persistent inhibition of arterial tone.

In arteries, *S*-nitrosation of cysteine residues has been recently demonstrated during activation of endothelial NOS, using an antiserum that recognized the *S*-NO moiety (Gow et al., 2002). Using the same antiserum, the present study shows a marked immunostaining for *S*-nitrosated cysteine residues in rat aorta and mesenteric artery pre-exposed to GSNO. It should be emphasized that staining was observed even after careful washout of the tissue. Because staining was not observed in control arteries or in those exposed to mercuric chloride [which cleaves the *S*-NO bond (Saville, 1958)] after GSNO, it can be attributed to GSNO-induced increase in tissue *S*-NO content. It has been previously suggested that LMW *S*-nitrosothiols could be retained within vascular tissue and be responsible for long-lasting hyporeactivity to vasoconstrictors (Megson et al., 1997; 1999). Although the present study cannot totally rule out the possibility that immunostaining was caused by GSNO accumulated in tissue, the antibodies used exhibited very low affinity for GSNO. Indeed, concentration of GSNO of 1 mM (Gow et al., 2002) or higher (ELISA experiments of the present study) competed effectively with *S*-NO-cys-g-BSA for antibody binding, whereas submicromolar concentrations of *S*-nitrosoproteins did so. Thus, the used antibodies displayed a large selectivity for *S*-nitrosoproteins. This favors the hypothesis of a transnitrosation mechanism (i.e., transfer of NO from GSNO to cysteine residues of tissue protein) in GSNO-induced increase in arterial *S*-NO content. The ability of LMW *S*-nitrosothiols such as GSNO to directly transfer NO to cysteine residues of proteins has been previously demonstrated in nonvascular models (Arnelle and Stamler, 1995; Butler et al., 1995; Hogg, 2000; Tsikas et al., 2001).

NO content in arteries was determined by EPR spectroscopy using the [Fe(II)$(DETC)_2$] complex as NO trapping agent. Control arteries (endothelium-denuded) exhibited EPR spectra indicating small formation of the [Fe(II)NO-$(DETC)_2$] complex. This EPR signal was not caused by an

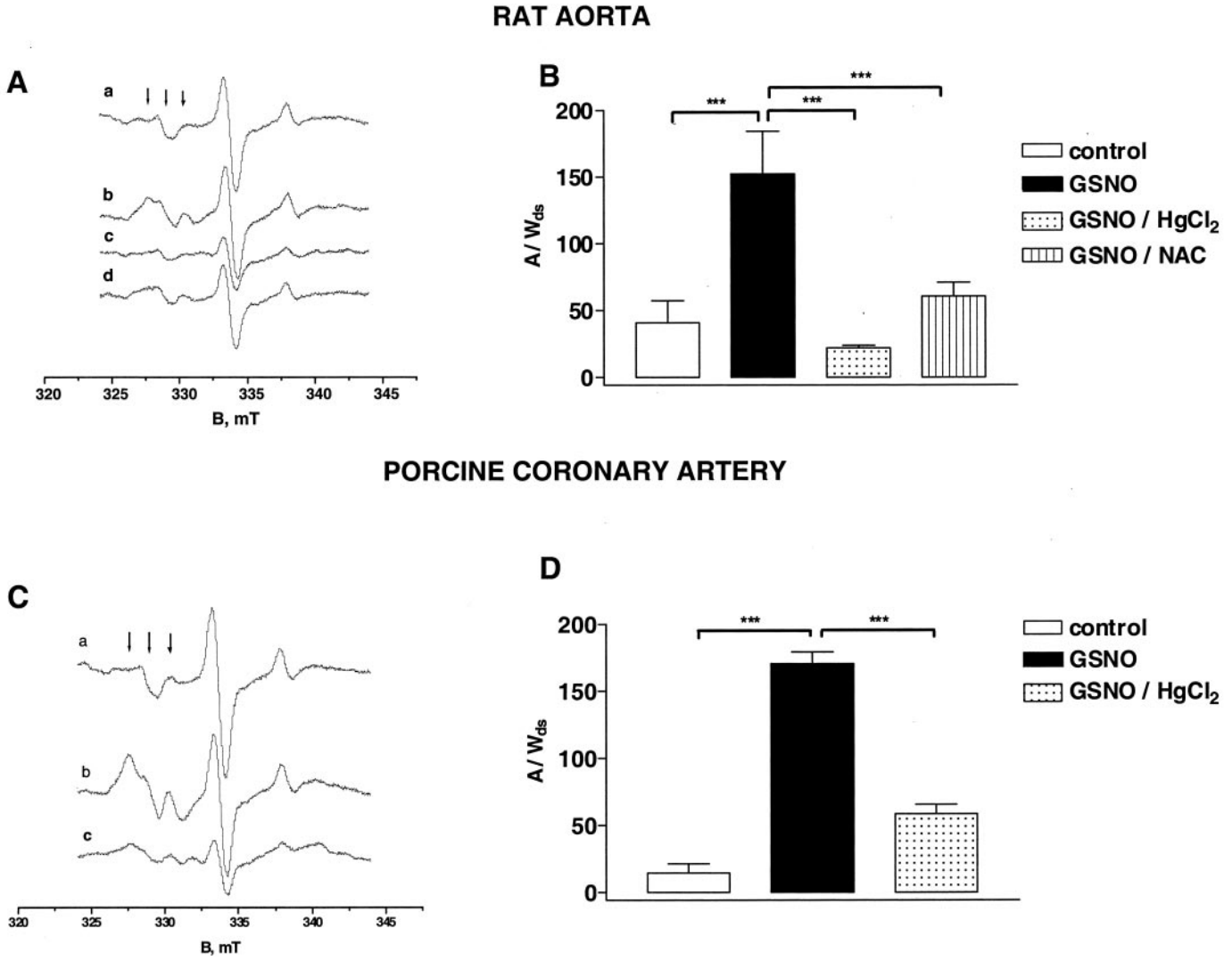

Fig. 4. Typical EPR spectra of rings from rat aorta (A) and porcine coronary artery (C) after incubation with [Fe(II)$(DETC)_2$]. a, control rings; b, rings pre-exposed to GSNO (100 μM followed by several washouts; c, rings treated with mercuric chloride (1 mM) after exposure to GSNO; and d, rings treated with NAC (10 mM) after exposure to GSNO. Arrows indicate the three hyperfine components of the [Fe(II)NO$(DETC)_2$] EPR signal. Quantification of the amplitude of the [Fe(II)NO$(DETC)_2$] signal (A/W_{ds} in relative units per mg, where A is amplitude and W_{ds} is weight of the dried sample) in rings from rat aorta (B) and porcine coronary artery (D). Results are expressed as mean ± S.E.M. of four (aortic rings) or three (coronary artery rings) experiments. ***, $P < 0.001$ (ANOVA).

interaction of the spin trap with contaminating nitrite. Actually, nitrite anions (up to 300 μM) in presence of the lipophilic [Fe(II)(DETC)$_2$] complex do not give an EPR signal in physiological conditions (I. Lobysheva and F. Nepveu, personal communication). The EPR signal in controls may originate from interaction of the [Fe(II)(DETC)$_2$] trap with a small amount of endogenous nitrosated thiols that could exist in tissue. Nevertheless, GSNO, even after careful washout (i.e., in the same conditions in which a persistent increase in *S*-nitrosated cysteine residues was detected in arteries), induced a marked increase in NO content over control values in rat aorta and porcine coronary arteries. In both arteries, the GSNO-induced increase in NO content was blunted by mercuric chloride, indicating that it was probably caused by *S*-nitrosated cysteine residues. However, the precise source of NO, which is trapped by the Fe(II)(DETC)$_2$ complex, is not determined. Indeed, the Fe(II)(DETC)$_2$ complex can trap free NO, which could be progressively released from *S*-nitrosated proteins, and NO from *S*-nitrosated thiols as well (Arnelle et al., 1997).

Exposure of various arteries to GSNO in vitro but also in vivo induced a persistent attenuation of the response to vasoconstrictors that was clearly caused by the activation of the NO-cGMP pathway. Indeed, not only were cyclic GMP levels elevated in GSNO-exposed rat aorta but also scavengers of NO (oxyHb and carboxy-PTIO) and an inhibitor of cGMP-dependent protein kinases (Rp-8-Br-cGMPS) restored contractile responses. The attenuation of the effect of vasoconstrictors induced by GSNO was invariably associated with a relaxant response upon addition of LMW thiols. The latter can be explained by the ability of LMW thiols to displace NO from stable *S*-nitrosated proteins and thus to facilitate the transfer of NO to soluble guanylyl cyclase. Accordingly, the effect of NAC was blunted by oxyHb, carboxy-PTIO, and Rp-8-Br-cGMPS in GSNO-exposed rat aorta. Furthermore, in these arteries, no elevation of NO content could be detected after pre-exposure to NAC. The ability of DETC to decompose *S*-nitrosothiols (Arnelle et al., 1997) precluded its use as spin trap for EPR demonstration of an increase in tissue NO content immediately after addition of NAC.

In the present study, several experimental data support the proposal that GSNO-induced changes in vascular contractility were caused by persistent *S*-nitrosation of cysteine residues. The role of thiol groups was demonstrated by the ability of agents that modify thiol groups (i.e., *p*-HMBA and *p*-CMPS) to prevent NAC-induced relaxation. Moreover, re-

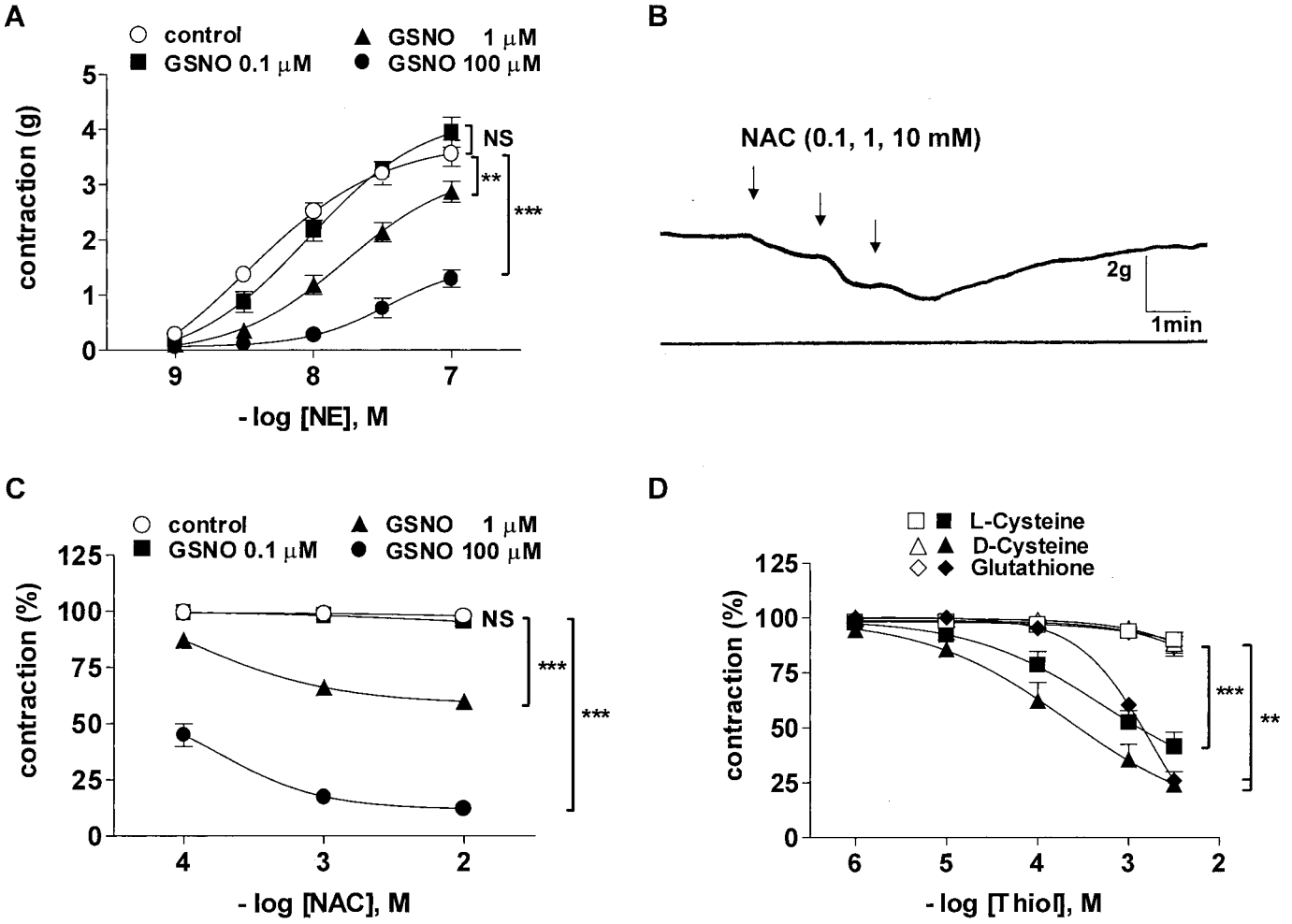

Fig. 5. Contractile effect of NE (A) and effect of subsequent addition of NAC (B and C) in rat aortic rings pre-exposed or not to GSNO (0.1, 1, or 100 μM, followed by several washouts). D, effect of various LMW thiols in rat aortic rings pre-exposed (closed symbols) or not (open symbols) to GSNO (1 μM followed by several washouts) and precontracted with NE. Results are expressed as mean ± S.E.M. of 4 to 17 experiments. Error bars are not shown when the size of the symbol exceeds the value of the S.E.M. NS, not significant; **, $P < 0.01$; ***, $P < 0.001$ (MANOVA).

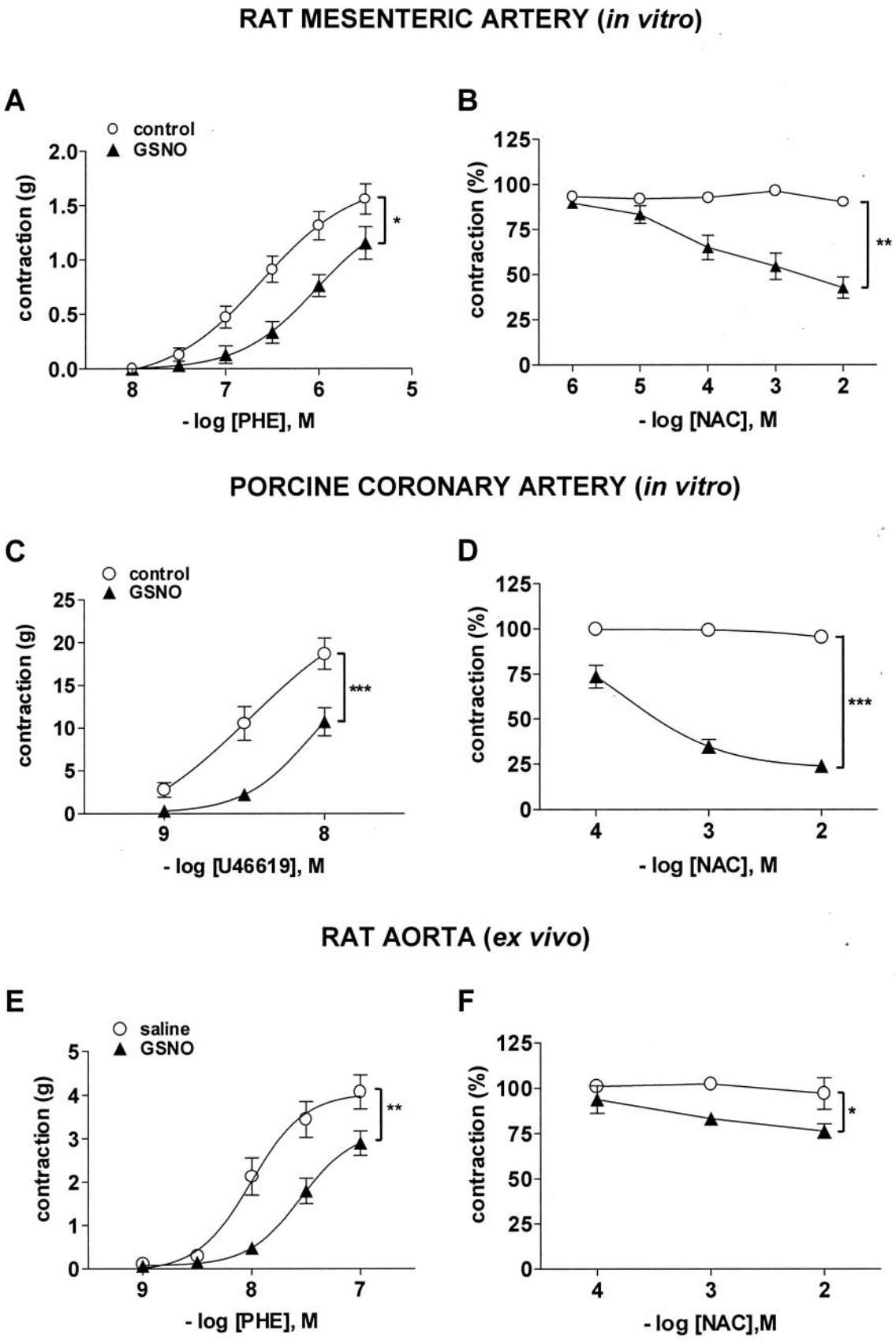

Fig. 6. Contractile effect of PHE or U46619 (A, C, E) and effect of subsequent addition of NAC (B, D, F) in rings from rat mesenteric artery (A, B) or porcine coronary artery (C, D) pre-exposed or not to GSNO (1 μM followed by several washouts) or in aortic rings removed from rats previously infused with saline or GSNO (0.6 μmol/kg/min). Results are expressed as mean ± S.E.M. of five to eight experiments. Error bars are not shown when the size of the symbol exceeds the value of the S.E.M. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (MANOVA).

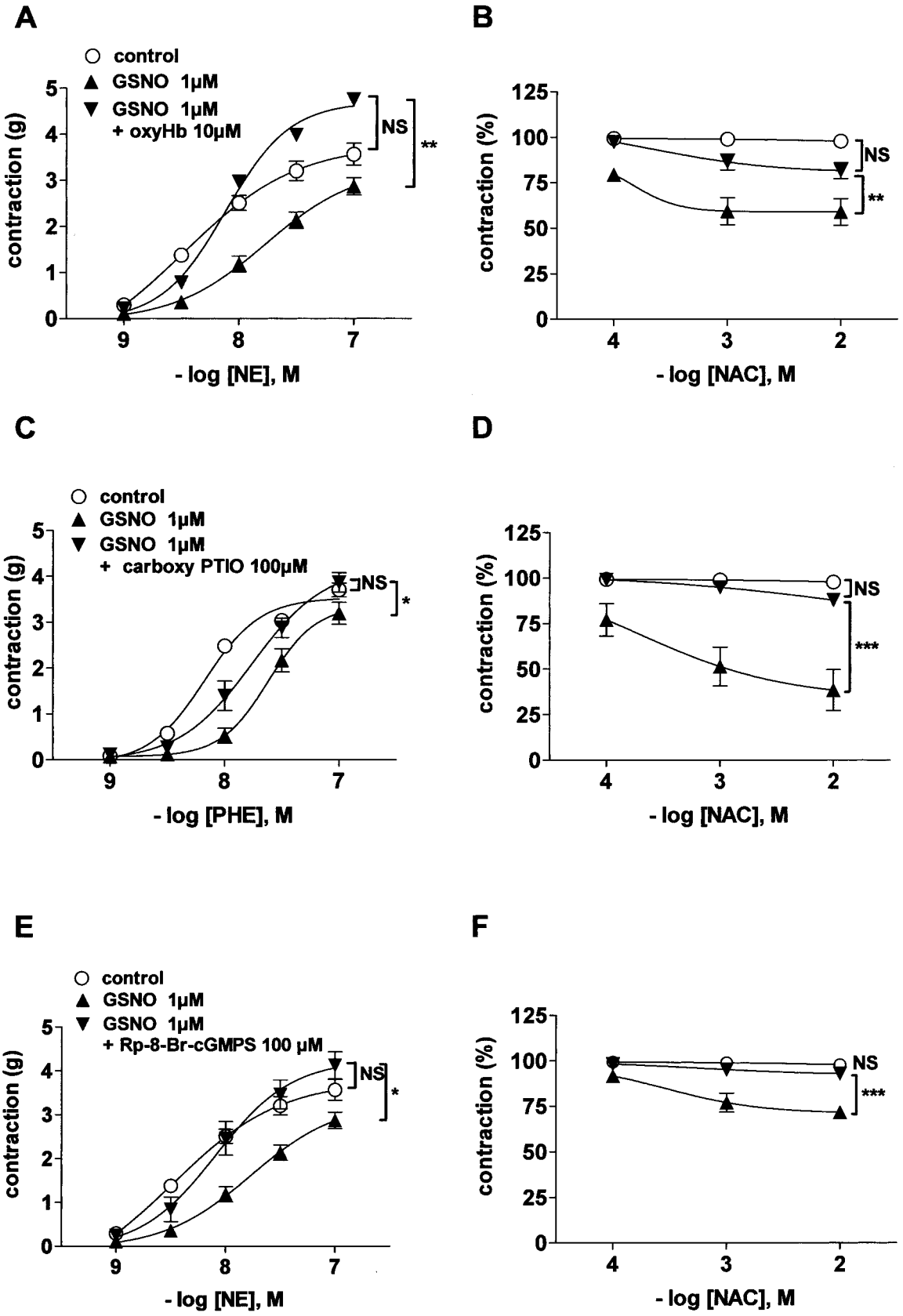

Fig. 7. Influence of oxyHb (10 μM) (A, B), carboxy- PTIO (100 μM) (C, D) and Rp-8-Br-cGMPS (100 μM) (E, F) on the contractile effect of NE (A, E) or PHE (C) and on the relaxant effect of NAC (B, D, F) in rat aortic rings pre-exposed or not to GSNO (1 μM followed by several washouts). Results are expressed as mean ± S.E.M. of at least three experiments. Error bars are not shown when the size of the symbol exceeds the value of the S.E.M. NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (MANOVA).

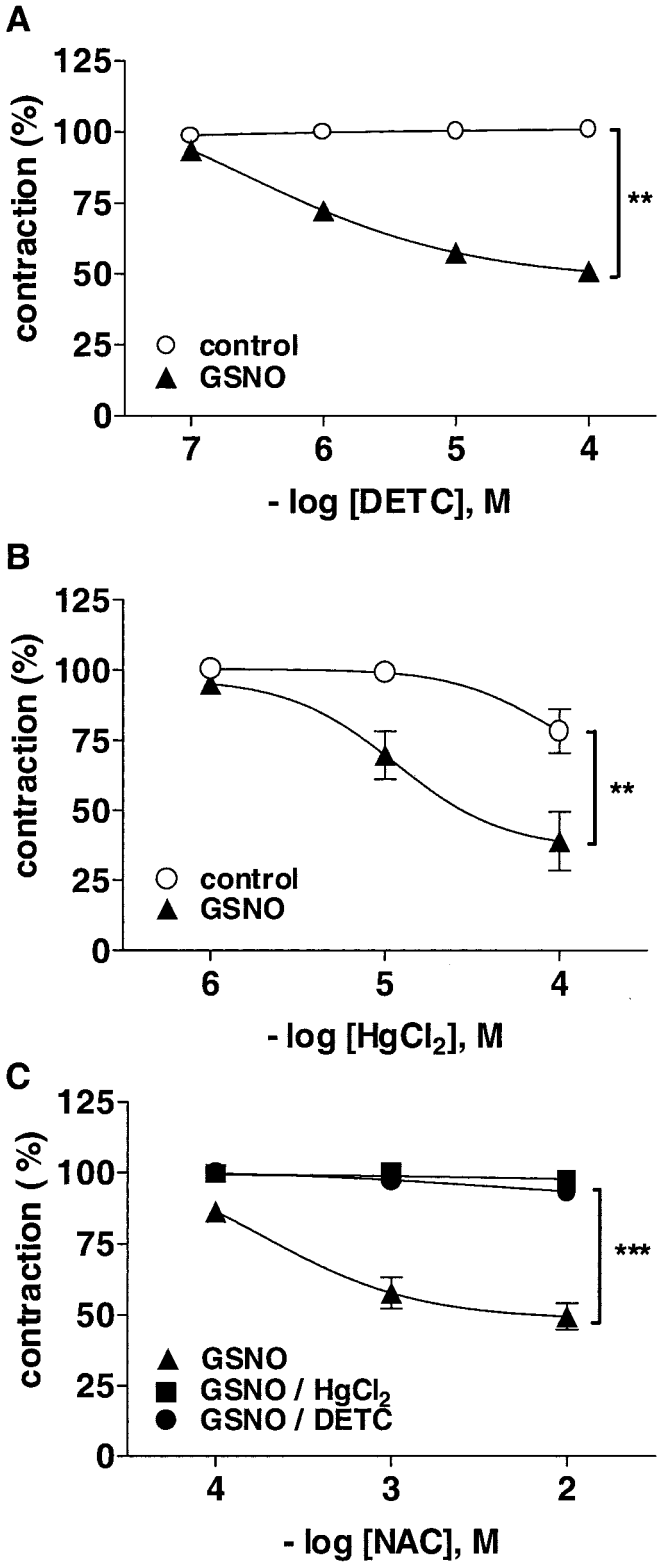

Fig. 8. Effect of DETC (A) and mercuric chloride (B) in rat aortic rings pre-exposed or not to GSNO (1 μM followed by several washouts) and precontracted with NE. C, effect of NAC before (▲) and after addition of DETC (100 μM, ●) or mercuric chloride (100 μM, ■) in rings pre-exposed or not to GSNO (1 μM followed by several washouts) and precontracted with NE. Results are expressed as mean ± S.E.M. of five experiments. Error bars are not shown when the size of the symbol exceeds the value of the S.E.M. **, $P < 0.01$; ***, $P < 0.001$ (MANOVA).

laxation was elicited in GSNO-exposed aortic rings, not only by NAC but also by DETC, which can decompose *S*-nitrosated cysteine residues and release NO or NO-related compounds (Arnelle et al., 1997). DETC is also an inhibitor of superoxide dismutase (SOD) (Heikkila et al., 1976). Relatively large concentrations (0.1–10 mM) of DETC and/or prolonged incubation time (0.5–2 h) have been used by different authors to irreversibly inhibit SOD and subsequently influence vascular tone (see, for instance, MacKenzie and Martin, 1998; Gupte et al., 1999). In the present study, DETC affected contraction in a concentration range of 1 to 100 μM, with a rapid onset and in a transient manner. Furthermore, SOD inhibition and subsequent increase in superoxide levels would increase rather than decrease contraction in NO-exposed vessels. Therefore, in the present study, a role of SOD inhibition in the effect of DETC on contraction seems unlikely. It should be noted also that vascular SOD activity is not affected by the colloid [Fe(II)$(DETC)_2$] complex used as spin trap in EPR experiments (Kleschyov et al., 2000a). In addition to DETC and NAC, mercuric chloride [which is known to cleave the *S*-NO bond (Saville, 1958)] also produced relaxation in GSNO-exposed arteries. Furthermore, mercuric chloride (as well as DETC) completely abrogated the effect of subsequent addition of NAC in GSNO-exposed aortic rings, and it also blunted the increase in NO content and in *S*-nitrosated cysteine residues. Altogether, these data are consistent with the existence of a common pool of *S*-nitrosothiols in tissues with which NAC, DETC, and mercuric chloride can interact and thus promote the release of vasoactive NO. The persistent attenuation of the response to contractile agonist (in the absence of exogenously added LMW thiols) after in vitro or in vivo exposure of arteries to GSNO (this study) or other LMW *S*-nitrosothiols (Megson et al., 1997; 1999; da Silva-Santos et al., 1999; Sogo et al., 2000; Terluk et al., 2000) might be attributed to a progressive release of NO from *S*-nitrosated proteins, triggered by endogenous LMW thiols. This warrants further investigation. The localization and nature of the *S*-nitrosated proteins remain to be elucidated as well. Indirect evidence argues for localization in the extracellular space or at the external side of cell membranes. Indeed, in aorta pre-exposed to GSNO, not only the membrane-permeant NAC but also glutathione, which has a very limited cellular uptake (Hiraishi et al., 1994; Deneke et al., 1995), elicited vasorelaxation. Furthermore, in these arteries, the effect of NAC was attenuated by thiol reagents (*p*-HMBA and *p*-CMPS) that did not cross cell membranes (Gordge et al., 1998).

In conclusion, the present data provide evidence that persistent *S*-nitrosation of cysteine residues is involved in long-lasting inhibitory effect of NO on arterial tone. They suggest that *S*-nitrosated tissue thiols may serve as local NO stores from which bioactive NO can subsequently be released. The role of these NO stores deserves future investigations. In arteries, endothelial NO production is associated with *S*-nitrosation of tissue thiols (Gow et al., 2002), and evidence was obtained for the formation of LMW thiol-releasable NO stores during endotoxin-induced NO overproduction by the inducible NOS (Muller et al., 1996, 1998; Kleschyov et al., 2000b). The formation of vascular NO stores has also been proposed as a protective mechanism toward various stress conditions (Manukhina et al., 2000). Besides some physiological or pathophysiological roles, formation of releasable NO

stores might be also of pharmacological interest to balance the impaired production of endogenous NO that occurs during various vascular diseases.

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