S-Nitrosating Nitric Oxide Donors Induce Long-Lasting Inhibition of Contraction in Isolated Arteries

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ABSTRACT

The ability of various nitric oxide (NO) donors to induce long-lasting inhibition of contraction in isolated arteries was compared. All the studied compounds elicited a relaxant effect in rat aortic rings precontracted with norepinephrine (NE). Almost maximal relaxation was obtained within 1 μM of each compound. The S-nitrosating agents S-nitrosoglutathione (GSNO), S-nitroso-N-acetylpenicillamine, S-nitroso-N-acetylcysteine, and sodium nitroprusside (1 μM) produced a decrease of the maximal effect of NE that persisted after removal of the drug. This hyporesponsiveness to NE was associated with a relaxant effect that persisted after removal of the drug. The same differential effects of GSNO and DEA-NO on contraction were also observed in porcine coronary arteries. Rat aortic rings previously exposed to 100 μM GSNO, but not to 100 μM DEA-NO, displayed a persistent increase in NO content (determined by NO spin trapping) and cysteine-NO residues (determined by immunostaining with an anti-cysteine-NO antiserum). The GSNO-induced increase in cysteine-NO residues in aortic tissue was prevented by the thiol-modifying agent p-hydroxymercuribenzoic acid. This study shows that in isolated arteries, the effects of S-nitrosating agents differed from those of other NO-donating agents. S-Nitrosating agents induced a persistent inhibition of contraction, which was attributed to the formation of releasable NO stores by S-nitrosation of tissue thiols. These differential effects of NO donors may be important for orientating their therapeutic indications.

Some nitric oxide (NO) donors such as sodium nitroprusside (SNP) or low-molecular weight (LMW) S-nitrosothiols (RSNO) cause long-lasting vasodilatation in vitro (after washing out the drug) or in vivo (after drug elimination).

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ABBREVIATIONS: NO, nitric oxide; SNP, sodium nitroprusside; LMW, low-molecular weight; RSNO, S-nitrosothiols; GSNO, S-nitrosothiolate; NONOates, diazeniumdiolates; SNP, sodium nitroprusside; NAC, N-acetylcysteine; EPR, electron paramagnetic resonance; NE, norepinephrine; U46619, 9,11-dideoxy-11α, 9α-epoxymethanoprostaglandin F(2α); SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; p-HMBA, para-hydroxymercuribenzoic acid; S-NO-cys-g-BSA, S-nitroso-glutaraldehyde-conjugated bovine serum albumin; DETC, diethylthiocarbamate; oxyHb, oxymyoglobin; methHb, methemoglobin; DEA-NO, 2-(N,N-diethy lamino)-diazenolate-2-oxide; SNAP, S-nitroso-N-acetylpenicillamine; CysNO, S-nitrosocysteine; SNAC, S-nitroso-N-acetylcysteine; MANOVA, multianalysis of variance.
al., 2001). This mechanism may result in long-lasting effects due to post-translational protein modification (Stamler et al., 2001). In arteries, protein S-nitrosation is increased by exposure to exogenous NO or stimulation of endothelial NO synthase activity (Gow et al., 2002). Recently, we have demonstrated the role of S-nitrosation of cysteine residues in the inhibitory effect of S-nitrosoglutathione (GSNO) on arterial tone, which persisted after removal of the drug (Alencar et al., 2003). This led us to propose that S-nitrosation of tissue thiols is a mechanism of formation of local NO stores in arteries from which biologically active NO can be released subsequently (Alencar et al., 2003).

From one compound to the other, NO donors greatly differ in the pathways and/or redox forms by which NO or NO\textsuperscript+ is produced (Butler et al., 1995; Feelisch, 1998). These characteristics may result in substantial differences in the reactivity of NO toward targets and may also explain some differences among NO donors for their biological properties (Feelisch, 1998). The objective of the present study was to compare the ability of NO donors to induce persistent inhibition of contraction in isolated arteries. For this purpose, arterial preparations were first exposed to various NO donors. The used compounds release the free radical NO spontaneously (NONOates or 3-morpholinosydnonimine) or after enzymatic metabolism (glyceryl trinitrate), or release preferentially NO\textsuperscript+ (SNP) or are able to transfer NO\textsuperscript+ to cysteine residues by redox mechanisms (LMW RSNO). After washout of the NO-donating agent, the response of the arteries to an appropriate vasoconstrictor and to N-acetylcysteine (NAC) were determined. NAC is a LMW thiol that can displace NO from cysteine-N0 bonds (Scharfstein et al., 1994; Alencar et al., 2003). Most experiments were conducted in rat aortic rings, which have been used in a previous study to demonstrate the role of S-nitrosation in the long-lasting effects of GSNO (Alencar et al., 2003). Some experiments were also performed in porcine coronary arteries, to determine whether the effects of NO donors obtained in rat aorta can be extended to other vascular beds and animal species. Electron paramagnetic resonance (EPR) spectroscopy and immunostaining were applied to evaluate elevation in NO content and formation of cysteine-NO residues in rat aorta, respectively. A preliminary report of this study has been presented in abstract form (Alencar et al., 2001).

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 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 Ifla Crede, Aburessles, France) after anesthesia with pentobarbital (60 mg/kg i.p.). Porcine hearts were obtained from local slaughterhouse, placed immediately into an ice-cold Krebs' solution (composition 119 mM NaCl, 4.7 mM KCl, 1.17 mM MgSO\textsubscript{4}, 1.25 mM CaCl\textsubscript{2}, 1.18 mM KH\textsubscript{2}PO\textsubscript{4}, 25 mM NaHCO\textsubscript{3}, and 11 mM glucose) and transported to the laboratory. Rat aorta and left coronary artery from porcine heart were cleaned of connective and fat tissues in Krebs' solution, and cut into rings. The endothelium was removed by rubbing the intimal surface of the rings with forceps.

Contraction/Relaxation Experiments. Rings (2- to 3-mm length) of aorta and porcine coronary arteries were mounted in organ chambers for isometric tension recording as described previously (Alencar et al., 2003). After equilibration, rat aortic rings were precontracted with norepinephrine (NE, 1 µM), and the absence of dilution of endothelium was verified by the lack of relaxant effect of acetylcholine (1 µM). In rings of porcine coronary arteries, NE and acetylcholine elicited only weak effects. Thus, the thromboxane mimetic U46619 (10 nM) and bradykinin (1 µM) were used in these preparations for contraction and endothelium-dependent relaxation, respectively. After washout, the relaxant effect of the NO donors was determined in NE (0.1 µM)-precontracted aortic rings, by addition of the compounds in a cumulative manner. In other experiments (performed in the dark), rings were exposed or not to various NO donors for 30 min. When SIN-1 was used, superoxide dismutase (SOD, 100 U/ml) was added for removal of superoxide anions that are generated during SIN-1 decomposition (Feelisch et al., 1989). Rings were then extensively washed out (over a 60-min period, during which time the Krebs' solution was changed every 20 min) and contracted by cumulative addition of NE (in rat aortic rings) or U46619 (in rings of porcine coronary artery). NAC was added in a cumulative manner when a steady-state level of contraction was reached. The EC\textsubscript{50} values of the NO donors (concentration that produced 50% relaxation of precontracted rings) were determined by log-logit regression. The contractile effects of NE or U46619 were expressed in g of developed tension. The relaxant effect of NAC was expressed in percentage of contraction. The relaxant effect being the tone induced by the contractile agonist.

Immunostaining and Confocal Microscopy. Aortic rings (2- to 3-mm length) were exposed or not to NO donors for 30 min (in the dark, at 37°C in Krebs' solution). In some experiments, rings were exposed to GSNO in the presence of the thiol-modifying agent para-hydroxymercuribenzoic acid (p-HMBA, 1 mM). Rings were then extensively washed out (over a 60-min period, during which time the Krebs' solution was changed every 20 min) and handled for immunostaining and confocal microscopy as described previously (Alencar et al., 2003). Slices were incubated overnight at room temperature with rabbit polyclonal antibodies directed against conjugated NO-cysteine (Mnaimeh et al., 1997; Lorch et al., 2000) (1:100 dilution). The selectivity of this antiserum for S-nitrosoproteins has been previously documented. Submicromolar concentrations of S-nitrosoproteins competed effectively with S-nitroso-glutaraldehyde-conjugated bovine serum albumin (S-NO-cys-g-BSA) for antibody binding, whereas concentrations of GSNO of 1 mM (Gow et al., 2002) or higher (Alencar et al., 2003) were necessary for competition. The secondary antibody was goat anti-rabbit IgG (Alexa Fluor 488, 1:200 dilution). Nonspecific fluorescence was suppressed by preincubating slices with the secondary antibody and measuring the average intensity value of fluorescence. All images were corrected for nonspecific fluorescence.

NO Spin-Trapping and EPR Spectroscopy. Aortic rings (6- to 8-mm length) were exposed or not to NO donors (in the dark, for 30 min at 37°C in aerated Krebs' solution) and then extensively washed out (during 60 min). The NO content was assayed as described previously (Alencar et al., 2003) after formation of the paramagnetic adduct Fe(II)NO(diethyldithiocarbamate)\textsubscript{2} ([Fe(II)NO(DETC)\textsubscript{2}]) detectable by EPR in rings treated (for 30 min at 37°C) with 0.5 mM [Fe(II)NO(DETC)\textsubscript{2}] complex as colloids (Kleschyov et al., 2000). 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; and time constant, 100 ms. After EPR measurements, the tissue samples were dried and weighted. The relative [Fe(II)NO(DETC)\textsubscript{2}] concentrations (A/W\textsubscript{m}) were determined dividing the third component amplitude (A) of the three-lines EPR signal by the weight of the dried sample (W\textsubscript{m}).

Rate of NO Release from RSNO. Conversion of oxyhemoglobin (oxyHb, 10 µM) to methemoglobin (metHb) in 100 mM phosphate buffer (NaH\textsubscript{2}PO\textsubscript{4}/Na\textsubscript{2}HPO\textsubscript{4}, 100 mM, pH 7.4, 37°C) was used to study
the release of the free radical NO from RSNO. OxyHb was prepared as described by Murphy and Noack (1994). Briefly, hemoglobin from bovine blood was dissolved in phosphate buffer in the presence of sodium hydrosulphite and exposed to a stream of O₂ for 10 min. The oxyHb solution was then deaerated 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). 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 (ε₄₀₁-₄₁₁ = 38 mM⁻¹ cm⁻¹) (Murphy and Noack, 1994). 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 NO-releasing compounds).

**Drugs and Reagents.** Unless otherwise indicated, drugs were purchased from Sigma Chemical (Saint Quentin-Fallavier, France). Rabbit polyclonal antibodies directed against conjugated 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). Sodium pentobarbital was purchased from Sanofi Santé Animal (Libourne, France). NAC (5 g/25 ml solution of EDTA/NaOH, pH 6.5, Fluimucil) was a generous gift from Zambon Laboratory (Antibes, France). SIN-1 (a generous gift of Aventis, Paris, France) was stored at −20°C as a 10 mM stock solution in 5% glucose. GTN (1.5 mg/ml in propylene glycol, a generous gift from Zambon Laboratory (Antibes, France). SIN-1 (154Alencar et al., 2003) was synthesized by mixing equimolar amounts of cysteine and sodium nitrite in 0.5 N HCl for 10 min at room temperature. CysNO was freshly prepared in Krebs’ before each experiment, whereas GSNO and SNAC were stored at 20°C as a 10 mM stock solution in NaOH (10 mM). S-nitroso-N-acetylcysteine (SNAP) was stored at −20°C as a 10 mM stock solution in heparin (10 mM). S-Nitrosocysteine (CysNO), GSNO, and S-nitroso-N-acetylcysteine (SNAC) were synthesized from equimolar concentrations (100 mM) of L-cysteine, glutathione, or NAC with NaN₃ in 0.5 N HCl for 10 min at room temperature. CysNO was freshly prepared before each experiment, whereas GSNO and SNAC were stored at −20°C for 1 week. The effective concentration of the CysNO, GSNO, and SNAC was calculated from their optical absorbance at 338 nm and the reported molar extinction coefficients (Gordge et al., 1998). Subsequent dilutions of all drugs were freshly prepared in Krebs’ solution and kept protected from light on ice.

**Statistical Analysis.** Results are expressed as mean ± S.E.M. of n experiments. Concentration-response curves were compared by the multianalysis of variance (MANOVA). Other statistical comparisons were performed with one-way ANOVA. P values less than 0.05 were considered statistically significant.

**Results**

**Effects of NO Donors on Arterial Contraction.** In rat aortic rings precontracted with NE, all the studied NO donors produced concentration-dependent relaxation. Their EC₅₀ values are listed in Table 1; they varied between 11 ± 4 (for SNP) and 245 ± 38 nM (for GSNO). For each compound, full (100%) relaxation was obtained in a concentration range varying from 0.3 to 10 μM. In another set of experiments, arterial rings were exposed or not to NO donors for 30 min. After several washouts for removal of the drug, concentration-effect curves of NE (in rat aortic rings) or U46619 (in rings of porcine coronary artery) were constructed and NAC was added when steady-state level of contraction was reached. In these conditions, NAC did not induce a relaxant effect in control rings (Figs. 1, 2, and 3). In rat aortic rings pre-exposed for 30 min to GTN (1 μM) or SIN-1 (1 μM, in the presence of 100 U/ml SOD) and then extensively washed out, the contractile effect of NE was not affected (Fig. 1, A and C), and NAC did not exert a relaxant effect (Fig. 1, B and D). After exposure to DEA-NO (1 μM), although the contractile responses to low concentrations of NE (3 and 10 nM) were diminished, those obtained at higher concentrations of NE (30 and 100 nM) were not significantly affected (Fig. 1E). Addition of NAC in rings pre-exposed to DEA-NO failed to induce a relaxant response (Fig. 1F). In contrast, rat aortic rings previously exposed to 0.1 or 1 μM SNP (but not those exposed to 0.01 μM SNP) displayed a significant reduction of the effect of NE (Fig. 1G) and a relaxant effect upon NAC addition (Fig. 1H). The relaxant 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).

Similar experiments were performed with various RSNO (Fig. 2). In rat aortic rings previously exposed to CysNO (1 μM) and then extensively washed out, the contractile effect of NE was not affected in comparison with controls (Fig. 2A), and NAC did not produce vasorelaxation (Fig. 2B). However, as reported previously (Alencar et al., 2003), preexposure to 1 μM GSNO induced a persistent attenuation of the response to NE (Fig. 2C) and a relaxant effect of NAC (Fig. 2D). In aortic rings pre-exposed to low concentration of SNAP or SNAC (0.01 or 0.1 μM), there was no significant alteration of NE-induced contraction (Fig. 2, E and G), and NAC did not affect contraction (Fig. 2, F and H). In contrast, rings exposed to 1 μM SNAP or SNAC exhibited a significant decrease of the contractile effect of NE (Fig. 2, E and G), and NAC elicited a marked relaxant effect (Fig. 2, F and H).

Some experiments were also conducted in porcine coronary artery (Fig. 3). Similar modifications of contraction were observed, compared with the rat aorta. In contrast to GSNO, DEA-NO (1 μM) failed to induce a persistent hyporesponsiveness to U46619 (Fig. 3A) or a relaxant response upon addition of NAC (Fig. 3B).

**Rate of NO Release from RSNO.** In an attempt to explain differences among RSNO on arterial contraction, depending on the nature of the ligand, the various RSNO were compared for their rate of release of NO as free radical in solution, using the oxyHb assay. As summarized in Table 2, GSNO, SNAP, and SNAC released NO at significant slower rates (about 2 to 3 times) than CysNO.

**Effects of GSNO and DEA-NO on NO Content and Cysteine-NO Residues in Rat Aorta.** NO content and S-nitrosated cysteine residues were determined in rat aortic rings by EPR spectroscopy (using [Fe(II)(DETC)]₃ as spin
trap) and immunostaining (using polyclonal antibodies directed against the S-NO moiety), respectively. In these experiments, GSNO and DEA-NO were used as prototypes of compounds producing persistent depression of contractile response to vasoconstrictor agonists or not, respectively. As reported previously (Alencar et al., 2003), exposure of rings to 100 μM GSNO resulted, even after extensive washout of the tissue, in persistent elevation of NO content (Fig. 4) and S-nitrosated residues (Fig. 5B). The addition of p-HMBA (1 mM) inhibited GSNO-induced increase in cysteine-NO residues (Fig. 5C). In contrast, rings pre-exposed to 100 μM DEA-NO and washed out displayed neither elevation of NO content (Fig. 4) nor increase in the staining of cysteine-NO residues (Fig. 5E).

Discussion

In this study, the ability of various NO donors to induce long-lasting inhibition of contraction in isolated arteries was compared. Despite the fact that they all elicited rapid vasorelaxation, the used NO donors can be classified in two categories, on the basis of the present data. The first category comprises GSNO, SNAP, SNAC, and SNP. Preexposure of rat aortic rings to these drugs, followed by washout, resulted in persistent decrease in the maximal response of NE. This effect was associated with a relaxant response to NAC, and as demonstrated in the case of GSNO, with a persistent increase in contents of NO and S-nitrosated cysteine residues. The second category of drugs, comprising DEA-NO,
SIN-1, GTN, and CysNO, produced none of these effects (except a moderate decrease in potency of NE without alteration of its maximal response in aortic rings pretreated with DEA-NO). Preexposure to GSNO and DEA-NO produced similar effects on contraction in rat aorta and porcine coronary artery, indicating that their differential effects were not restricted to one vascular bed and species.

The mechanisms of NO-induced rapid vasorelaxation are well documented. Although cGMP-independent mechanism(s) are implicated in some vessels (Boilotina et al., 1994), it is commonly accepted that reversible activation of soluble guanylyl cyclase mainly accounts for the relaxant effect of the NO donors used here (Morley and Keefer, 1993; Brunner et al., 1996; Homer et al., 1999; Tseng et al., 2000). This implies the production of NO as free radical. The first category compounds (GSNO, SNAP, SNAC, and SNP) have in common to be also able to induce S-nitrosation of thiols. CysNO is an exception among RSNO. Although it should be able to transfer NO \(^{\text{ radical}}\) to other thiols, CysNO is much less stable than other LMW RSNO (Mathews and Kerr, 1993; Vanin et al., 2002a,b), and, as shown here using the oxyHb assay, it released NO radical more rapidly than other LMW RSNO. These data strongly suggest that, unlike other LMW RSNO, CysNO was not stable enough to transfer sufficient amount of NO \(^{\text{ radical}}\) to tissue thiols.

EPR spectroscopy was applied here and previously (Alencar et al., 2003) to demonstrate NO elevation in arteries. Probably because of limited sensitivity of the EPR method of...
Nitrosated proteins in arteries were directly de-

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vasoconstrictors induced by S-nitrosating agents. Thus, the

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persistent effect of S-nitrosating agents on contraction was likely mediated by NO release from S-nitrosoproteins and subsequent activation of the cGMP pathway rather than by persistent modification of target(s) via S-nitrosation. Involvement of a cGMP-independent mechanism has been suggested to explain the persistent decrease in sensitivity to vasoconstrictors in rat aortic rings after 2- to 4-h exposure to SNAP, GSNO, or DEA-NO (Kanagy et al., 1996). Such mechanism might be implicated in the rightward shift in NE concentration-effect curve seen here in rat aorta after DEA-NO, but elucidating the mechanism of this effect requires further investigations.

Addition of the LMW thiol NAC in the organ bath was used in the present study to trigger the release of NO from S-nitrosoproteins, after exposure of tissue to NO donors. NAC can enter into cells where it produces glutathione, thereby exerting antioxidant effects. Synthesis of glutathione from NAC is a relatively slow metabolic process. In contrast, the relaxing effect of NAC shown here was rapid (within 1 min), and similar relaxant effect could also be produced in GSNO pretreated arteries by glutathione, which has a very limited cellular uptake (Alencar et al., 2003). Furthermore, the relaxant effect of NAC was abolished when SNO bonds were disrupted by mercuric chloride, and it was markedly attenuated in tissues pretreated with nonpermeant thiol reagents (Alencar et al., 2003). In addition, both NAC and mercuric chloride abolished the persistent elevation in tissue NO content in GSNO-pretreated arteries (Alencar et al., 2003). Together, these data indicate that the effect of NAC reported here did not result from antioxidant properties, but rather from displacement of NO from cysteine-NO residues. The mechanism(s) by which NAC releases NO from S-nitrosoproteins may be transnitrosation, leading to formation of an unstable RSNO and to the release of NO by homolytic cleavage of the latter. Recently, another efficient mechanism has been suggested to explain catalysis of LMW RSNO formation by serum albumin exposed to NO radical (Rafikova et al., 2002). It seems that the hydrophobic compartment of albu-

min can serve as a sink for the NO radical and O₂, where NO is oxidized to NO⁺; subsequently, NO⁺ can be transferred directly to LMW thiols, resulting in the formation of vasodilatating RSNO. Similar mechanisms could perhaps participate in the relaxing effect of LMW thiols seen here and previously (Alencar et al., 2003). However, abolition of the effect of NAC after disruption of S-NO bonds or inhibition of their formation rather supports the view that NAC elicited vasorelaxation by NO release from S-nitrosated proteins. It has been reported that LMW RSNO, probably GSNO, and nitrite represent storage forms of endogenous endothelium-derived NO in rat aorta (Rodriguez et al., 2003). Release NO from these NO storage forms was triggered by light in rings with endothelium. These stores were likely not identical to those released by NAC, because NAC did not produce relaxation in aortic rings with endothelium, not pre-exposed to NO donors (data not shown).

In conclusion, this study shows that in isolated arteries, short-lasting and reversible relaxant effects were obtained with drugs producing the free radical NO, without increasing in a persistent manner NO content and cysteine-NO residues in arterial tissue. In contrast, S-nitrosating agents induced a persistent inhibition of contraction that was attributed to the formation of releasable NO stores by S-nitrosation of tissue thiols. The ability of various NO donors to induce either short- or long-lasting effects may be important for orientating their therapeutic use.

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