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Short communication

Formation of releasable NO stores by *S*-nitrosoglutathione in arteries exhibiting tolerance to glyceryl-trinitrate

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Abstract

S-Nitrosating nitric oxide (NO) donors like *S*-nitrosoglutathione (GSNO) induce a persistent inhibition of vascular tone, through the formation of releasable NO stores. In this study, we investigate whether GSNO also induces NO stores-related effects in vessels exhibiting tolerance to glyceryl-trinitrate. Rat aortic rings treated with glyceryl-trinitrate (100 μM for 1 h) exhibited increased level of superoxide and a decrease in NO elevation and relaxation induced by subsequent addition of glyceryl-trinitrate. In glyceryl-trinitrate-treated rings as in controls, pre-exposure to GSNO (1 μM for 30 min) induced a persistent hyporesponsiveness to noradrenaline and a relaxant response to *N*-acetylcysteine (a low molecular weight thiol which can displace NO from NO stores), both of which being inhibited by guanylyl-cyclase or cyclic GMP-dependent protein kinase inhibitors. These data indicate that GSNO can promote the formation of releasable NO stores in arteries exhibiting increased superoxide level and tolerance to glyceryl-trinitrate. Formation of releasable NO stores is of potential interest to restore the protective effect of NO in organic nitrate-tolerant blood vessels.

Keywords: *N*-Acetylcysteine; Nitric oxide; Nitric oxide store; Organic nitrate; Superoxide anion; Tolerance

1. Introduction

Nitric oxide (NO) delivery to blood vessels is of particular interest in pathologies associated with impaired production of endogenous NO. As NO donors, organic nitrates require enzymatic biotransformation, and despite acute vasodilating properties, their long-term use is limited by development of tolerance. Reduced biotransformation of glyceryl-trinitrate by mitochondrial aldehyde dehydrogenase plays a role in tolerance, and increased mitochondrial production of reactive oxygen species likely contributes to this phenomenon (Chen et al., 2002; Sydow et al., 2004).

Low molecular weight *S*-nitrosothiols do not induce tolerance and their relaxant effect is retained in glyceryl-trinitrate-tolerant arteries (Miller et al., 2000). It is commonly accepted that reversible activation of soluble guanylyl cyclase is involved in their relaxant effect (Brunner et al., 1996; Tseng et al., 2000). Moreover, *S*-nitrosothiols also induce a persistent hyporesponsiveness to vasoconstrictors (Megson et al., 1997; Terluk et al., 2000) which is associated with a relaxant effect of low molecular weight thiols like *N*-acetylcysteine (Alencar et al., 2003a,b). We provide evidence that formation of releasable NO stores accounts for the persistent effect of *S*-nitrosating agents on arterial tone (Alencar et al., 2003a,b). Since formation of releasable NO stores is of potential interest in organic nitrate-tolerant vessels to restore the protective effect of NO, we investigated here whether GSNO induces NO stores-related effects in arteries exhibiting tolerance to glyceryl-trinitrate.

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2. Materials and methods

2.1. Preparation of arteries

Thoracic aorta was removed from male Wistar rats (11–14 weeks old) after anesthesia with pentobarbital (60 mg/kg, i.p.). Endothelium-denuded rings were mounted in organ chambers as previously described (Alencar et al., 2003a,b). For tolerance induction, rings were exposed to 100 μM glyceryl-trinitrate (or solvent) for 1 h, in the absence or presence of superoxide dismutase (SOD 50 U/ml, added 30 min before glyceryl-trinitrate). After washout (during at least 1 h), rings were processed as described below.

2.2. Dihydroethidium staining

Serial sections of aortic rings (30 μm thick) were prepared as previously described (Alencar et al., 2003b). Slides were then incubated with dihydroethidium (2.5 μM for 30 min) in phosphate-buffered saline. Dihydroethidium produces red fluorescence when oxidized to ethidium bromide by superoxide (Mulsch et al., 2001). Sections were then rinsed, air dried, mounted in Vectashield®, cover-slipped and examined under a laser scanning confocal microscope (Bio-Rad MRC-1024) with a 60× epifluorescence objective. Images were obtained as previously described (Alencar et al., 2003b). Emission signal was recorded with a Zeiss 573–637 nm filter. Fluorescence was quantified using Confocal Assistant Application™, CAS40 4.02 software.

2.3. NO spin-trapping and electron paramagnetic resonance spectroscopy

The NO content was assayed after formation of electron paramagnetic resonance-detectable [Fe(II)NO(diethyldithiocarbamate)$_2$] ([Fe(II)NO(DETC)$_2$]) (Kleschyov et al., 2000). Rings (6–8 mm length) were exposed for 30 min at 37 °C to colloid [Fe(II)(DETC)$_2$] (0.5 mM) and glyceryl-trinitrate (0.5 mM) or solvent, and then rapidly frozen in liquid nitrogen. Spectra were recorded as previously described (Alencar et al., 2003b).

2.4. Isometric tension recordings

Glyceryl-trinitrate, GSNO or 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEA-NO) was added cumulatively to rings precontracted with noradrenaline (0.1 μM), in the absence or presence of SOD (50 U/ml, added 30 min before noradrenaline). In some experiments, rings were exposed or not to GSNO (1 μM for 30 min). After washout (during 1 h), concentration–response curves to noradrenaline and *N*-acetylcysteine were constructed, in the absence or presence of the inhibitor of guanylyl cyclase 1*H*[1,2,4,]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ, 1 μM, added 15 min before noradrenaline) or cyclic GMP-dependent protein kinase Rp-8-bromoguanosine 3′5′-cyclic monophosphorothioate (Rp-8Br-cGMPS, 100 μM, added 45 min before noradrenaline).

2.5. Drugs and reagents

Glyceryl-trinitrate was obtained from Besins International, *N*-acetylcysteine from Zambon laboratory, DEA-NO from Alexis Corporation, ODQ from Tocris-Cookson and Rp-8Br-cGMPS from Biolog. GSNO was prepared as

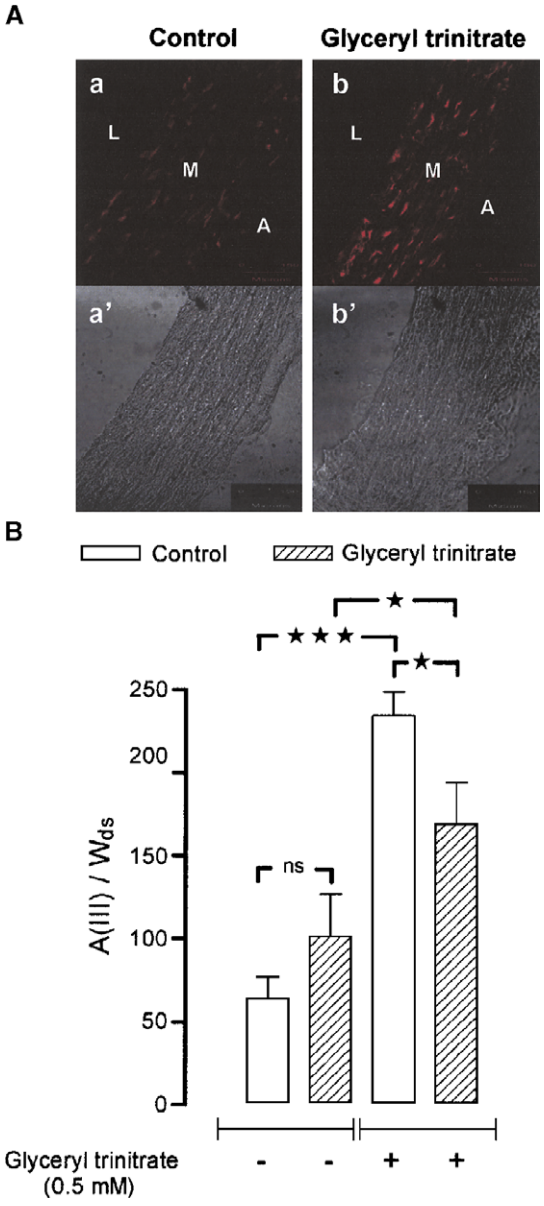

Fig. 1. (A) Dihydroethidium staining in (a) control and (b) glyceryl-trinitrate-treated rat aortic segments. Respective light transmission pictures are shown in a′ and b′. Representative pictures from 3 experiments. L: lumen ; M: media; A: adventitia. (B) Effect of glyceryl-trinitrate on NO content in rings previously exposed or not to glyceryl-trinitrate. A(III)/W_{ds}: amplitude of the third component of the three-lines EPR signal divided by the weight of the dried sample. ns: not significant , $^*P<0.05$, $^{***}P<0.001$ (one-way ANOVA; $n=3$–4).

previously described (Alencar et al., 2003a,b). Other drugs were purchased from Sigma Chemical or Aldrich.

2.6. Statistical analysis

Results are expressed as mean ± S.E.M. of *n* experiments (*n*: number of rats). Concentration–response curves were compared by two-way analysis of variance (ANOVA). Other statistical comparisons were performed with one-way ANOVA. *P* values less than 0.05 were considered statistically significant.

3. Results

Rat aortic rings pretreated with glyceryl-trinitrate (100 μM for 1 h) exhibited an increase in dihydroethidium staining (in arbitrary units, from 7.6 ± 2.3 to 28.2 ± 7.2,

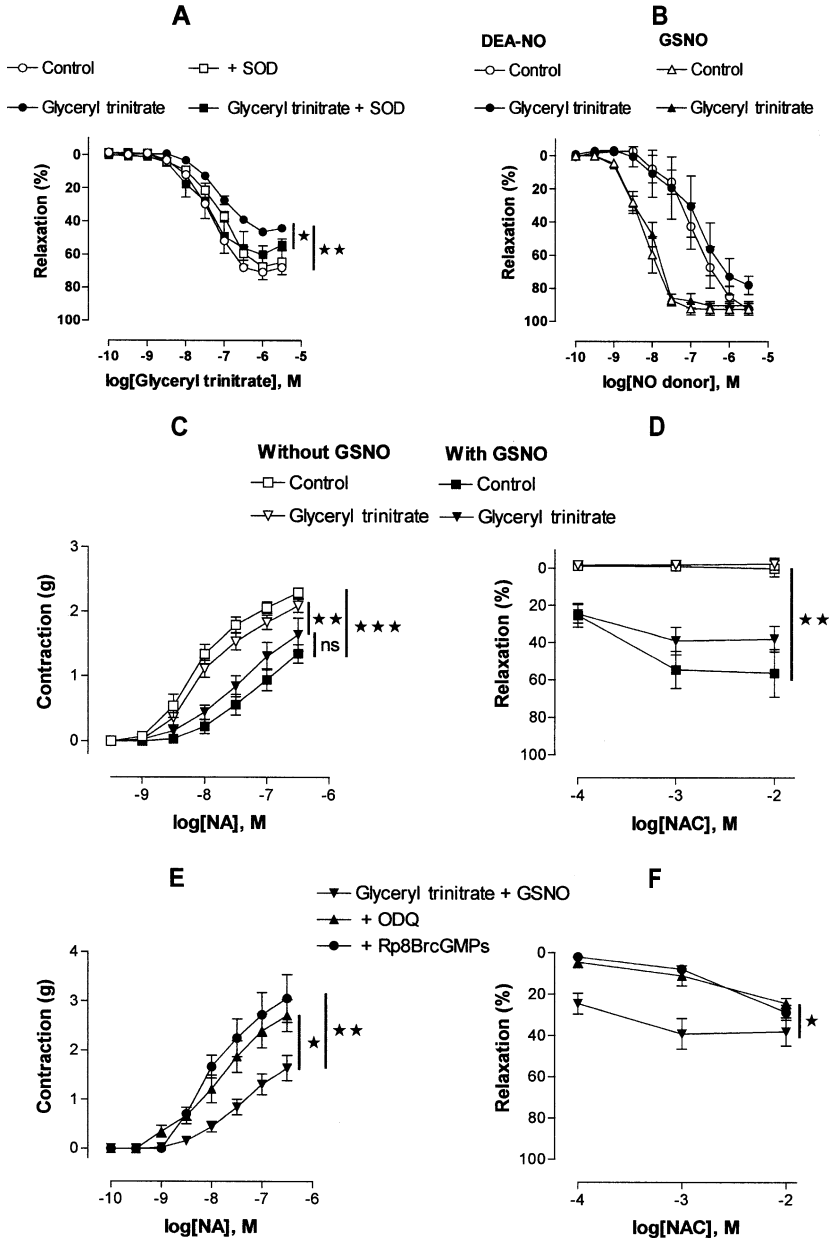

Fig. 2. (A) Relaxant effect of glyceryl-trinitrate in rat aortic rings previously exposed or not to glyceryl-trinitrate, in the absence or presence of SOD (present throughout pre-incubation period and relaxant experiments with glyceryl-trinitrate). *$P<0.05$; **$P<0.01$ compared with respective control (two-way ANOVA; $n=4$–9). (B) Relaxant effect of GSNO and DEA-NO in rings previously exposed or not to glyceryl-trinitrate ($n=4$). (C) Contractile effect of noradrenaline and (D) relaxant effect of *N*-acetylcysteine in rings previously exposed or not to glyceryl-trinitrate and/or GSNO (in panel D, rings were all precontracted with 0.3 μM noradrenaline; the corresponding precontraction value can be drawn from panel C). ns: not significant; **$P<0.01$ compared with respective control (two-way ANOVA; $n=6$–9). (E) Influence of ODQ (1 μM) and Rp-8Br-cGMPS (100 μM) on the contractile effect of noradrenaline and (F) on the relaxant effect of *N*-acetylcysteine in rings previously exposed to glyceryl-trinitrate and GSNO. *$P<0.05$; **$P<0.01$ compared with respective control (two-way ANOVA; $n=4$–9).

$n=3$; $P<0.05$), which was predominantly localized in the media (Fig. 1A). These rings also displayed a decrease of NO elevation (Fig. 1B) and relaxation (Fig. 2A) induced by subsequent addition glyceryl-trinitrate. SOD did not modify the relaxant effect of glyceryl-trinitrate in control rings, but restored it to control values in glyceryl-trinitrate pretreated ones (Fig. 2A). Relaxation to GSNO or DEA-NO was not impaired in glyceryl-trinitrate pretreated rings (Fig. 2B).

Pre-exposure to GSNO (1 μM for 30 min) resulted in a comparable decrease of noradrenaline-induced contraction (Fig. 2C) and in a relaxant effect of *N*-acetylcysteine (Fig. 2D) in rings pretreated or not with glyceryl-trinitrate. In rings pretreated with glyceryl-trinitrate and GSNO, ODQ and Rp-8Br-cGMPS inhibited the persistent hyporesponsiveness to noradrenaline (Fig. 2E) and the relaxant effect of *N*-acetylcysteine (Fig. 2F). In GSNO-untreated rings, contraction was not affected by these inhibitors (not shown). In GSNO-untreated rings precontracted with low concentration of noradrenaline (0.03 μM), *N*-acetylcysteine (10 mM) decreased tone by only $6 \pm 2\%$ ($n=3$).

4. Discussion

The present study shows that GSNO retains its ability to induce long lasting hyporesponsiveness to noradrenaline and relaxant effect of *N*-acetylcysteine in arteries exhibiting elevation of superoxide level and tolerance to glyceryl-trinitrate.

Dihydroethidium staining in glyceryl-trinitrate-treated rings was attributed to superoxide. Indeed, dihydroethidium is relatively specific for superoxide (Munzel et al., 2002) and staining was decreased by cell permeant superoxide dismutase mimetics (not shown). Increase of superoxide was also demonstrated using the same probe in rat aorta after in vivo treatment with glyceryl-trinitrate (Mulsch et al., 2001). In accordance with some previous studies (Unger et al., 1993; Hasegawa et al., 1999; Miller et al., 2000; Chen et al., 2002), no cross-tolerance was found here between glyceryl-trinitrate and other NO donating agents (GSNO and DEA-NO). In glyceryl-trinitrate-treated rings, the lack of alteration of DEA-NO-induced relaxation despite elevated superoxide level suggests that NO fluxes coming from spontaneous decomposition of DEA-NO in the bathing solution largely exceeded those of superoxide, and thus masked the influence of NO degradation by superoxide. It also indicates that, contrary to what was observed in in vivo model of tolerance (Mulsch et al., 2001), the cyclic GMP pathway remained unaffected in glyceryl-trinitrate-treated rings. Moreover, these data also suggest that biotransformation of glyceryl-trinitrate was altered in tolerant rings, as previously proposed (Unger et al., 1993; Hasegawa et al., 1999). The lower elevation of NO induced by glyceryl-trinitrate, together with the unchanged effect of DEA-NO in tolerant rings compared to controls, support this possibility. According to recent reports, reduced mitochondrial aldehyde dehydrogenase activity accounts for nitrate tolerance (Chen et al., 2002) and increased production of reactive oxygen species by mitochondria contributes to inhibition of aldehyde dehydrogenase activity (Sydow et al., 2004). The present study shows that SOD restored relaxation to glyceryl-trinitrate in tolerant arteries. Even though SOD is not expected to directly interact with intracellular superoxide, this supports a role of superoxide in tolerance to glyceryl-trinitrate in the present model.

In glyceryl-trinitrate- treated rings as in controls, GSNO induced a persistent hyporesponsiveness to noradrenaline and a relaxant effect of *N*-acetylcysteine. In control arteries, several lines of evidence indicate that formation of NO stores by *S*-nitrosation of cysteine residues and subsequent release of vasoactive NO account for these effects (Alencar et al., 2003a,b). Accordingly, it is shown here in tolerant arteries that the persistent effects of GSNO are blunted by inhibitors of soluble guanylyl cyclase (ODQ) or cyclic GMP-dependent protein kinases (Rp-8Br-cGMPS). Thus, GSNO fully retained its ability to induce NO stores-related effects in glyceryl-trinitrate-tolerant arteries exhibiting increased superoxide levels. This implies that in this model, elevated superoxide impaired neither the formation of NO stores, nor the effect of NO released from stores. This might be explained by differential localisation of NO stores and superoxide production. *S*-Nitrosated proteins are likely located at the external side of cell membranes or in the extracellular space, because formation could be prevented by thiols reagents that do not cross cell membranes (Alencar et al., 2003a,b). Thus, as for DEA-NO, the relaxant effect of extracellularly generated NO was not affected despite increased intracellular level of superoxide. It has been shown that tolerance to glyceryl-trinitrate can be reversed by *N*-acetylcysteine (Torresi et al., 1985). This property is unlikely to account for the effect of *N*-acetylcysteine observed here, since *N*-acetylcysteine did not affect tone in tolerant arteries which were not exposed to GSNO.

In conclusion, this study shows that GSNO can promote the formation of releasable NO stores in arteries exhibiting increased level of superoxide and tolerance to glyceryl-trinitrate. Formation of releasable NO stores is an alternative way of NO delivery to blood vessels exhibiting tolerance to organic nitrates.

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