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Smooth muscle relaxing effects of NO, nitrosothiols and a nerve-induced relaxing factor released in guinea-pig colon

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1 The aim of the present study was to compare the biological activity of S-nitroso-L-cysteine (CYSNO), S-nitrosoglutathione (GSNO), S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and hydroxylamine to that of nitric oxide (NO) and a vascular relaxing factor released by nerve stimulation in the guinea-pig intestine. The biological activity was examined in a bioassay system with guinea-pig colon as donor tissue and a series of spiral strips of rabbit aorta without endothelium as detector tissues.

2 Electrical stimulation of the guinea-pig colon released a vascular relaxing factor. The half-life of the relaxing factor down the bioassay cascade was the same as exogenously applied NO. N^{ω}-nitro-L-arginine (L-NOARG) inhibited the release of bioactivity.

3 The relaxations of the assay tissues caused by exogenous CYSNO also declined during the passage down the cascade. However, in the presence of L-cysteine (10^{-5} M) the half-life of CYSNO increased and there was no significant breakdown through the cascade. In contrast, the half-life of applied NO and the vascular relaxing factor released by nerve stimulation was unaffected by the presence of L-cysteine.

4 Exogenously applied GSNO (20–50 nM), SNAP (2–4 nM) and hydroxylamine (300–600 nM) caused relaxations that did not decline during the passage down the cascade.

5 In summary, the relaxation of the bioassay tissues during nerve stimulation was indistinguishable from the relaxation induced by NO, whereas relaxations induced by CYSNO, GSNO, SNAP and hydroxylamine showed different pharmacological profiles. The released bioactivity is thus likely to be NO itself.

Keywords: Non-adrenergic non-cholinergic; nitric oxide; S-nitroso-L-cysteine; bioassay; smooth muscle

Introduction

Nitric oxide (NO) is formed enzymatically from L-arginine in mammalian tissue (Palmer *et al.*, 1988), where it has roles in cell-to-cell signalling and in host-defence reactions (Moncada *et al.*, 1991). NO released from neurones has been suggested as a non-adrenergic non-cholinergic (NANC) mediator of relaxation in intestinal smooth muscle (Bult *et al.*, 1990; Desai *et al.*, 1991). In support, NO synthase is present within enteric neurones (Bredt *et al.*, 1991; Belai *et al.*, 1992; Ward *et al.*, 1992) and nerve-stimulation elicits the release of a factor with similar biological activity to NO (Bult *et al.*, 1990; Wiklund *et al.*, 1993). Furthermore, nerve-stimulation induces a frequency-dependent release of the NO-metabolites, nitrite and nitrate in guinea-pig intestine (Wiklund *et al.*, 1993). Inhibitors of NO synthase reduce NANC relaxations of the anococcygeus muscle (Gillespie *et al.*, 1989; Li & Rand, 1989), canine duodenum (Toda *et al.*, 1990) and guinea-pig caecum (Shuttleworth *et al.*, 1991), but evidence on the effect of the inhibitors on bioactivity- or metabolite-overflow is often lacking.

It has been suggested that NO, a highly reactive and unstable molecule is stabilized in tissues by a reaction with a carrier molecule R-SH, thus prolonging its half-life *in vivo* and preserving its biological activity (Myers *et al.*, 1990; Stamler *et al.*, 1992a). Accordingly, the NO-like factor released by NANC-nerves, as well as the endothelium-derived relaxing factor (EDRF), has been propsed to be a NO-containing molecule from which NO is subsequently released to the target cells (Myers *et al.*, 1990). Different S-nitrosothiols have been suggested as possible endogenous NO-donors, such as S-nitroso-L-cysteine (CYSNO; Myers *et al.*, 1990), dinitrosyl-iron-cysteine complex (DNIC; Vanin, 1991), and S-nitrosoglutathione (GSNO). Another suggested endogenous compound is hydroxylamine or an analogue thereof (Thomas & Ramwell, 1989). If NO can be stored as a nitrosothiol compound in nerve-terminal vesicles it may be able to function as a classical neurotransmitter, stored and released from vesicles (Thornbury *et al.*, 1991; Sanders & Ward, 1992). Cysteine is the most abundant source of free sulphydryl groups in mammalian tissue (Jocelyn, 1972), and at normal extracellular pH, CYSNO has a biological half-life similar to that of NO. Thus, CYSNO has been a favoured candidate for an alternative, endogenously released NO-like compound (Myers *et al.*, 1990). L-Cysteine recently has been shown to prolong the half-life of CYSNO, but not of NO or EDRF, released from vascular tissue (Feelisch *et al.*, 1994).

The aim of the present study was to compare the biological activity of NO, CYSNO, GSNO, S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and hydroxylamine to that of a vascular relaxing factor released by stimulation of nerves in the guinea-pig intestine, in order to elucidate whether NO is released as a free radical or in a more stabilized form.

Methods

Bioassay

Guinea-pigs (250–400 g) of either sex were stunned and bled. The mesenteric artery was cannulated and the large intestine perfused with saline. The distal part of the colon was removed and a 25 cm long strip of longitudinal muscle, together with the underlying myenteric plexus was isolated, folded five times and used as donor tissue. The preparation was mounted in a heated (37°C) glass chamber and superfused (5 ml min^{-1}) with Tyrode solution (concentration in mM: Na^+ 161, K^+ 2.8, Ca^{2+} 1.8, Mg^{2+} 0.5, Cl^- 144, HCO_3^- 24, $H_2PO_4^-$ 0.4 and glucose 5.6) heated to 37°C and continuously gassed with 5% CO_2 in O_2. Transmural nerve

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stimulation (20 Hz, 1.0 ms, 1200 pulses) was applied by needle-shaped silver electrodes. New Zealand White rabbits (1.5–2.5 kg) of either sex were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.v.) and the aorta was removed and cut into 3–4 cm long spiral strips. The endothelium was removed by gentle rubbing and the detector arteries (RbAs) were mounted in glass chambers arranged in a cascade as previously described (Gryglewski *et al.*, 1986a). The effluent from the donor tissue was used to superfuse the detector arteries and reached detector artery 1 at 2 s, artery 2 at 5 s and artery 3 at 8 s delay. The superfusate also contained guanethidine 3×10^{-6} M and L-arginine 3×10^{-5} M. The detector arteries were in addition superfused with atropine (10^{-6} M), N^{ω}-nitro-L-arginine methyl ester (L-NAME) (5×10^{-5} M) and phenylephrine (5×10^{-7} M); phenylephrine was used to increase smooth muscle tone.

Glyceryl trinitrate (GTN), applied by 20 s injection of 5–50 pmol, caused submaximal relaxations of the detector arteries. The relaxations by GTN did not decline down the cascade, and were used to calibrate the relative sensitivity of the detector arteries to guanylyl cyclase-mediated relaxation. Only detector arteries with less than 10% variability in the relaxation to GTN were used. All experiments were made in the presence of 10 units ml^{-1} superoxide dismutase (SOD; Gryglewski *et al.*, 1986b).

Mechanical muscular activity of the donor tissue was recorded isometrically by a Grass FT03 transducer (Grass Instruments, Quincy, MA, U.S.A.) at a load of 10 mN. Responses of the detector arteries were monitored isotonically from a load of 30 mN by Harvard Bioscience transducers type 52-9511 (Harvard Apparatus Company, Inc., Millis, MA, U.S.A.). Recordings were displayed by a Grass Polygraph (mod. 7P1).

Drugs

Nitric oxide solutions were prepared as previously described (Palmer *et al.*, 1987). Solutions of S-nitroso-L-cysteine (CYSNO) were synthesized according to Gibson *et al.* (1992) and were prepared immediately before application. L-Arginine, atropine chloride, N^{ω}-nitro-L-arginine (NOARG), N^{ω}-nitro-L-arginine methyl ester (L-NAME), L-cysteine, guanethidine, phenylephrine, tetrodotoxin (TTX), hydroxylamine and superoxide dismutase (SOD) (3900 units mg^{-1} solid) were purchased from Sigma Co (St Louis, MO, U.S.A.). S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and S-nitrosoglutathione (GSNO) were purchased from Alexis Co (Läufelfingen, Switzerland) and were prepared on ice and diluted with deoxygenated H_2O immediately before use.

Statistics

Experimental data were expressed as mean values ± s.e.mean. Statistical significance was tested according to Student's *t* test for paired observations.

Results

Electrical stimulation (20 Hz, 1.0 ms, 1200 pulses) of the guinea-pig colon elicited a contraction (35 ± 3 mN, $n = 4$) that was blocked by tetrodotoxin (10^{-6} M; $n = 3$) (not shown). Furthermore, there was a concomitant release of a vascular relaxing factor. The bioactivity of this factor rapidly declined during the passage down the cascade. When the relaxation in detector artery 1 was defined as 100% there was 13 ± 7% of the relaxing bioactivity left in the superfusate at detector artery 2 and 8 ± 7% left at detector artery 3 ($n = 6$–7, $P < 0.001$; Figures 1a and 2a). L-NOARG (5×10^{-5} M) almost completely inhibited the nerve-induced vascular relaxing activity (16 ± 7% left at detector artery 1 and no activity left at detector artery 2 or 3, $n = 3$, $P < 0.005$). Concomitantly, L-NOARG (5×10^{-5} M) increased the nerve-induced contractile responses in the donor tissue by 39 ± 13% ($P < 0.05$, $n = 4$). TTX (10^{-6} M) totally abolished the stimulation-induced vascular relaxing activity ($n = 3$).

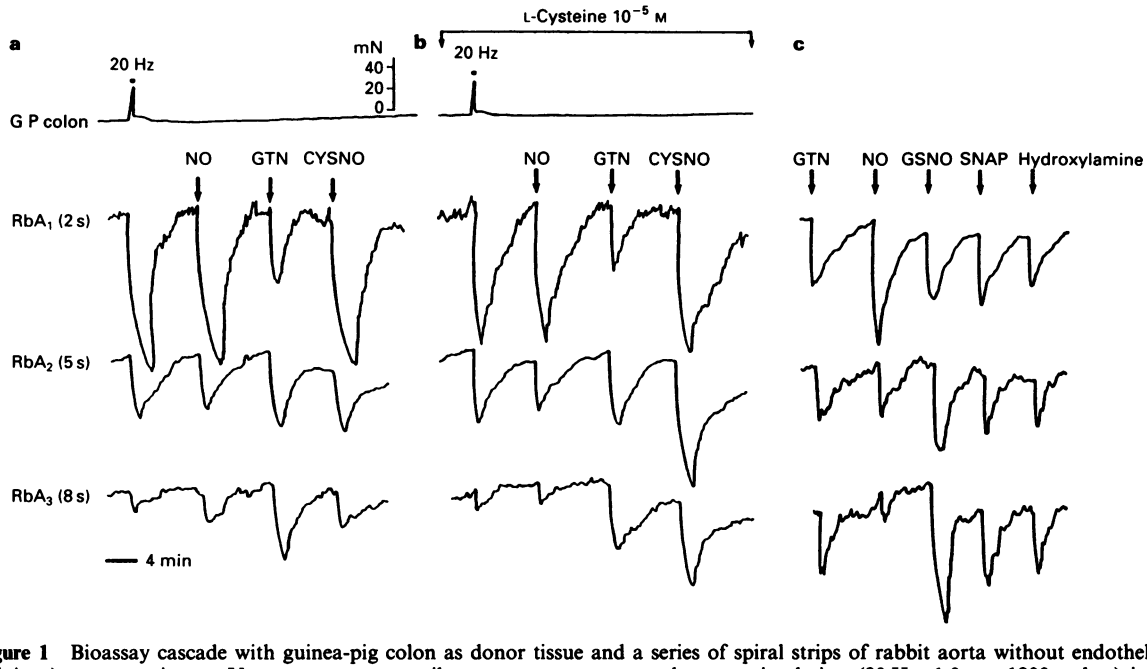

Figure 1 Bioassay cascade with guinea-pig colon as donor tissue and a series of spiral strips of rabbit aorta without endothelium (RbA_{1-3}) as assay tissues. Upper trace: contractile response to transmural nerve stimulation (20 Hz, 1.0 ms, 1200 pulses) in the donor tissue. Lower traces: inhibitory responses in the assay tissues, induced by a factor released during nerve stimulation of the donor tissue, or by application of exogenous compounds. (a) The inhibitory effect of glyceryl trinirate (GTN) was unaffected down the cascade while the inhibitory effects of the nerve-induced factor, nitric oxide (NO) and S-nitroso-L-cysteine (CYSNO) were degraded down the cascade at approximately the same rate. (b) In the presence of 10^{-5} M L-cysteine there was a significant prolongation of the relaxing bioactivity by CYSNO down the cascade, while the vascular relaxing bioactivity released by nerve stimulation, or NO was still rapidly degraded down the cascade. (c) Application of GTN, nitrosoglutathione (GSNO), S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and hydroxylamine by injection over the assay tissues caused relaxations in the rabbit aortic strips that did not decline down the cascade while the inhibitory effect of exogenous NO was degraded down the cascade.

When NO (10–50 pmol) was applied by 20 s injection over the assay tissues, the detector arteries relaxed with the same time course as previously observed for the vascular relaxing factor released by nerve stimulation (Figure 1a and Table 1). NO was applied at a dose that caused a relaxation in detector artery 1 of the same size as that elicited by nerve stimulation. When the relaxation in detector artery 1 was defined as 100% there was 17 ± 13% left at detector artery 2 and 2 ± 2% left at detector artery 3 (Figures 1a and 2c).

Exogenous application of CYSNO (25–75 pmol), by 20 s injection, resulted in relaxations in the detector arteries, that declined down the cascade ($n = 4$, $P < 0.005$). The amount of CYSNO applied was chosen to cause a relaxation in detector artery 1 of the same magnitude as those elicited by nerve stimulation and NO. When the relaxation in detector artery 1 was defined as 100% there was 63 ± 19% left at detector artery 2 and 35 ± 11% left at detector artery 3 (Figures 1a and 2e, and Table 1).

In the presence of L-cysteine (10^{-5} M) there was no change in biological half-life of the vascular relaxing bioactivity induced by nerve stimulation or exogenous NO (Table 1). When the relaxation to nerve stimulation in detector artery 1 was defined as 100% there was 19 ± 9% remaining in superfusate at detector artery 2 and 5 ± 4% left at detector artery 3 (Figures 1b and 2b). When the relaxation to NO in detector artery 1 was defined as 100% there was 20 ± 9% of bioactivity left at detector artery 2 and 8 ± 6% at detector artery 3 (Figures 1b and 2d). In contrast, there was in the presence of 10^{-5} M L-cysteine, a significant increase in half-life of the relaxing effect caused by CYSNO ($P < 0.01$, $n = 4$) and there was no longer a breakdown during its passage down the cascade (Table 1). When the relaxation in detector artery 1 was defined as 100% there was 104 ± 18% of bioactivity remaining at detector artery 2 and 110 ± 18% remaining at detector artery 3 (Figures 1b and 2f).

Application of GSNO (40–80 pmol), SNAP (3–7 pmol) or

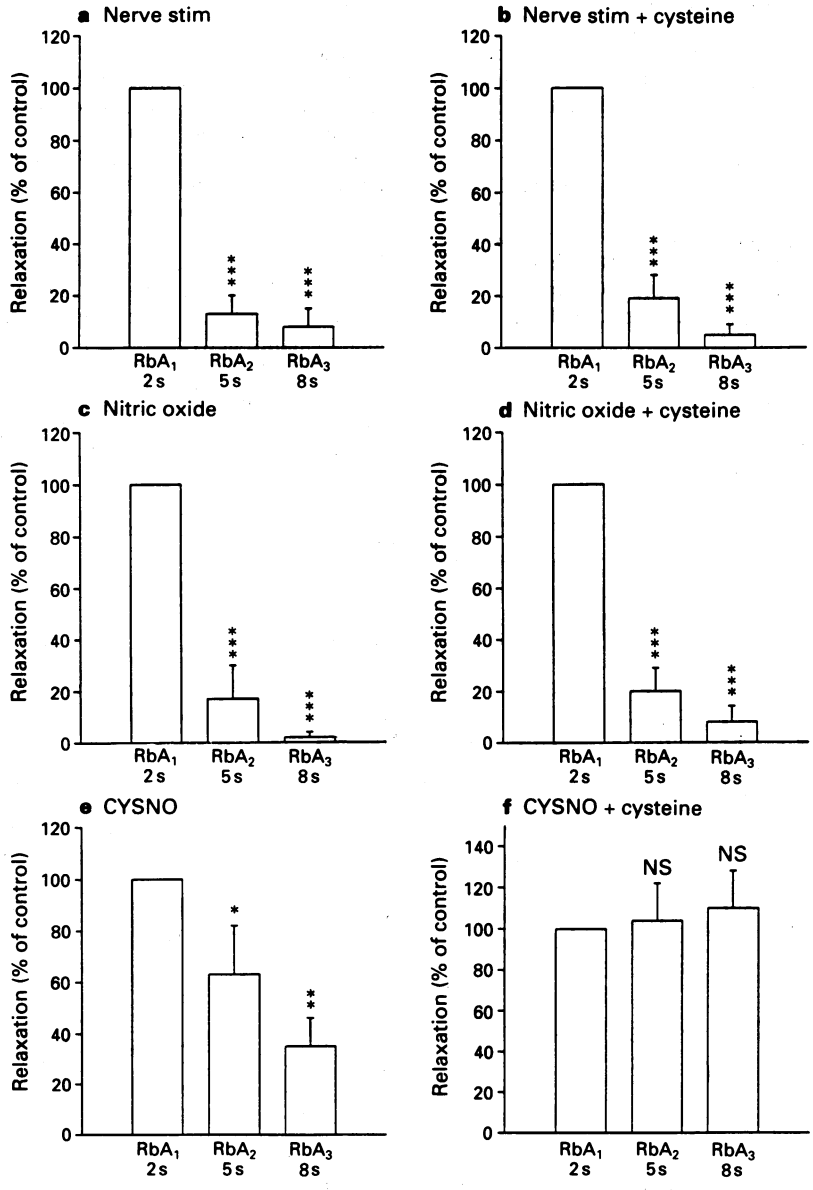

Figure 2 Diagrams illustrating the vascular relaxing activities of a nitric oxide (NO)-like factor released by nerve stimulation, and by exogenous NO and by S-nitroso-L-cysteine (CYSNO), in a bioassay cascade where the donor tissue was guinea-pig colon and the assay tissues were rabbit aortic strips. The superfusate reached detector artery 1 (RbA1) at 2 s, RbA2 at 5 s and RbA3 at 8 s delay. The relaxations in RbA1 were defined as 100%. (a) Relaxing activity released by nerve stimulation of the donor tissue. (b) Relaxing activity released by nerve stimulation of the donor tissue, in the presence of 10^{-5} M L-cysteine. (c) Relaxing effect of exogenous NO applied over the detector arteries. (d) Relaxing effect of exogenous NO applied over the detector arteries, in the presence of 10^{-5} M L-cysteine. (e) Relaxing effect of exogenous CYSNO applied over the detector arteries. (f) Relaxing effect of exogenous CYSNO, in the presence of 10^{-5} M L-cysteine. The half-life of the relaxing effect by CYSNO increased significantly in the presence of L-cysteine. (*$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$).

Table 1 Characteristics of a NANC-factor, released from guinea-pig colon and of some candidate molecules for this entity

Compound	*Half-life in bioassay cascade* (s)	*Half-life in bioassay cascade in the presence of* L-*cysteine (s)*
NANC factor	~2	~2
NO	~2	~2
CYSNO	5–6	>>NANC factor*
GSNO	>>NANC factor*	ND
SNAP	>>NANC factor*	ND
Hydroxylamine	>>NANC factor*	ND

*There was no detectable loss of vascular relaxing activity during passage down the cascade. ND = not determined. CYSNO: S-nitroso-L-cysteine; GSNO: S-nitrosoglutathione; SNAP: S-nitroso-N-acetyl-D,L-penicillamine.

hydroxylamine (0.5–1.0 nmol) by 20 s injection caused relaxations of the rabbit aortic strips. However, there were no measurable losses of vascular relaxing activity down the cascade when they were administered at concentrations causing relaxations, in detector artery 1, of the same magnitude as those elicited by nerve stimulation ($n = 4$) (Figure 1c and Table 1).

Discussion

NANC inhibitory nerves in the colon are likely to mediate the inhibitory component of the peristaltic reflex (Sanders & Ward, 1992). In the present study, contractile responses to nerve stimulation in the guinea-pig colon were studied. However, when the muscle tone is increased transmural stimulation elicits NANC relaxations which are inhibited by L-NOARG (Iversen *et al.*, 1994).

This study has shown that S-nitroso-L-cysteine (CYSNO) presents certain pharmacological properties in a bioassay-system that distinguish it from the arginine-derived NANC inhibitory factor in the guinea-pig colon. Thus, the half-life of the vascular relaxing activity of CYSNO in our bioassay system was significantly prolonged in the presence of L-cysteine, which was not the case for exogenous NO or the arginine-derived inhibitory factor released from the guinea-pig colon. The prolongation of CYSNO-mediated relaxation by cysteine is in agreement with previous observations on EDRF/NO released from rabbit aorta endothelium cells (Feelisch *et al.*, 1994), in which the half-life of EDRF/NO bioactivity from these cells was in fact found to be shortened by L-cysteine. This has also been observed in intact aortic strips (Jia & Furchgott, 1993), where SOD treatment largely attenuated the shortening effect of L-cysteine on EDRF and NO. The shortening effect is thought to be due to auto-oxidation of L-cysteine, with generation of O_2^-, and this might well explain why in our experiments, where SOD was present throughout, L-cysteine did not shorten the half-life of NO or the released bioactivity. The present data thus indicate a resemblance in bioactivity between authentic nitric oxide (NO) and the arginine-derived NANC inhibitory factor, providing further evidence that, during activation of NANC-nerves, NO is released as a free molecule, rather than as a S-nitrosothiol.

The biological activities of the NO-generating compounds GSNO, SNAP and hydroxylamine were examined in the bioassay cascade. The half-lives of these compounds were found to be much longer than those of NO and the nerve-induced vascular relaxing factor, with no loss of activity down the cascade. This is consistent with previous studies on hydroxylamine and DNIC (Feelisch *et al.*, 1994), and on GSNO and S-nitrosomercaptoethanol (Myers *et al.*, 1990), thereby making them unlikely candidates for the inhibitory principle of intestinal NANC-neuroeffector transmission.

The relatively short-lasting biological activity of exogenous CYSNO in the bioassay cascade (in the absence of L-cysteine) is consistent with the previous observation that CYSNO spontaneously liberates substantially more NO than other S-nitrosothiols, when measured by headspace during 10 min in oxygenated Krebs buffer (Kowaluk & Fung, 1990). This spontaneous liberation of NO does not seem to account for the major part of *in vitro* vascular relaxation by S-nitrosothiols (Kowaluk & Fung, 1990). It has been suggested that metal ion catalysis is the predominant pathway for the release of NO from S-nitrosothiols (McAninly *et al.*, 1993). The stabilization by L-cysteine of CYSNO is proabably due to the ability of thiols to complex metal ions present in the buffer solution (Feelisch *et al.*, 1994).

Conversion of NO to other oxidation states (NO^+ or NO^-) has recently been suggested (Stamler *et al.*, 1992b). However NO^+ as such is not active as a dilator, and NO^- is only relaxant at very high concentrations and is considerably more stable than NO in a bioassay cascade (Feelisch *et al.*, 1994).

In conclusion, the relaxation of bioassay tissues to nerve stimulation of a donor tissue from guinea-pig colon was indistinguishable from the relaxation induced by NO, whereas relaxations by CYSNO, GSNO, SNAP and hydroxylamine showed different pharmacological profiles. The data suggest that intact NO is a major nerve-induced relaxing principle in the guinea-pig colon.

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