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Non-adrenergic, non-cholinergic relaxation of the bovine retractor penis muscle: role of S-nitrosothiols

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1 This study examined the possibility that an S-nitrosothiol, rather than nitric oxide, functions as the non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter in the bovine retractor penis (BRP) muscle.

2 Treatment of BRP muscle with either of two sulphydryl inactivating agents, diamide (1 mM) and N-ethylmaleimide (0.3 mM), inhibited NANC relaxation and this was prevented by pretreating tissues with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM). Inhibition was not specific, however, since the inactivating agents also inhibited the relaxant actions of authentic nitric oxide (0.3 μM), glyceryl trinitrate (0.001–1 μM) and isoprenaline (0.01–1 μM).

3 Reacting nitric oxide with L-cysteine in nominally oxygen-free solution at pH 3, followed by purging to remove free nitric oxide and neutralisation, produced greater and more prolonged relaxant activity when assayed on rabbit aortic rings than could be attributed to nitric oxide alone. H.p.l.c. analysis of the mixture identified a new peak distinct from either L-cysteine or nitric oxide which was responsible for the relaxant activity. The spectral absorption of this new compound had two bands with peaks at 218 and 335 nm.

4 Using a series of structural analogues of L-cysteine (all at 15 mM) it was found that removal of the carboxyl group (L-cysteamine), replacement of the carboxyl with an ester function (L-cysteine methyl ester) or substitution at the amino group (N-acetyl-L-cysteine) had no effect on the ability to generate relaxant activity upon reaction with nitric oxide (0.1 mM). In contrast, substitution at the sulphydryl group (S-methyl-L-cysteine, L-cysteinesulfinic acid and L-cysteic acid), or formation of disulphides (L-cystine and L-cystamine) led to a complete loss of ability to generate relaxant activity. L-Glutathione was also able to react with nitric oxide to produce relaxant activity, and this too was blocked upon substitution of the free sulphydryl group (S-methyl-L-glutathione). A free sulphydryl group was therefore required to generate relaxant activity following reaction with nitric oxide.

5 Reacting L-cysteine (10 mM) with nitric oxide (~3 mM) under more stringent oxygen-free conditions followed by purging to remove free nitric oxide resulted in the generation of low relaxant activity and small absorption peaks at 218 and 335 nm and these were unaffected upon exposure to the air. In contrast, admitting air to the reaction chamber before purging enhanced both relaxant activity and the absorption peaks at 218 and 335 nm by some 40 fold and the solution turned pink due to the appearance of another absorption peak at 543 nm. This enhanced relaxant activity was not due to nitrogen dioxide being the reactive species, since at 0.1 mM this gas failed to react with L-cysteine to generate relaxant activity, and at 1 mM generated less activity than the equivalent concentration of nitric oxide.

6 The relaxant activity generated by reacting nitric oxide with L-cysteine or L-glutathione was abolished following treatment with haemoglobin (3 μM), methylene blue (10 μM) or N-methylhydroxylamine (100 μM), but was unaffected by N^G-nitro-L-arginine (30 μM). Furthermore, two agents that generate superoxide anion, pyrogallol (0.1 mM) and hydroquinone (0.1 mM), also inhibited this relaxant activity as well as that induced by authentic nitric oxide (0.3 μM) but as previously reported, had no effect on relaxation induced by NANC nerve stimulation. Superoxide dismutase (100 u ml^{-1}) reversed the actions of pyrogallol and hydroquinone but had no effect on NANC relaxation.

7 In conclusion, the reaction of nitric oxide with L-cysteine or L-glutathione generates relaxant activity which exceeds that of nitric oxide alone and probably results from formation of S-nitrosocysteine and S-nitrosoglutathione, respectively. The effects of pyrogallol and hydroquinone suggest that the NANC neurotransmitter is a superoxide anion-resistant, nitric oxide-releasing molecule and that neither S-nitrocysteine nor S-nitrosoglutathione is a suitable candidate for this.

Keywords: Nitric oxide; NANC nerves; S-nitrosothiols; S-nitrosocysteine; S-nitrosoglutathione; L-cysteine, bovine retractor penis; pyrogallol; hydroquinone; superoxide anion

Introduction

It is now well established that the L-arginine nitric oxide system (Moncada, 1992) mediates the phenomenon of endothelium-dependent relaxation of blood vessels first reported by Furchgott & Zawadzki (1980). This system is also responsible for the cytostatic and cytotoxic actions of macrophages (Hibbs *et al.*, 1988; Granger *et al.*, 1990), and for the hypotension associated with septic shock (Gray *et al.*, 1991; Kilbourn & Griffith, 1992). Largely on the basis of the actions of analogues of L-arginine that block nitric oxide synthesis (Bowman *et al.*, 1986; Moore *et al.*, 1990; Gibson *et al.*, 1990), we and others have recently proposed that this system can also account for the inhibitory non-adrenergic, non-cholinergic (NANC) transmission process in anococcygeus muscles of the rat and mouse and the retractor penis muscle of the bull (Gillespie *et al.*, 1989; Gibson *et al.*, 1990;

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Hobbs & Gibson, 1990; Martin *et al.*, 1993). Subsequently, this system was shown in a variety of species to mediate NANC relaxant responses in the gastrointestinal tract (Boeckxstaens *et al.*, 1991), the lung (Tucker *et al.*, 1990; Li & Rand, 1991), corpus cavernosus (Ignarro *et al.*, 1990; Pickard *et al.*, 1991), and penile and cerebral arteries (Toda & Okamura, 1990; Liu *et al.*, 1991). Furthermore, the same pathway is known to be present in certain neurones of the central nervous system (Garthwaite *et al.*, 1988; Bredt *et al.*, 1990; 1992), and may play a role in nociception (Moore *et al.*, 1993) as well as in long-term potentiation and inhibition (Schuman & Madison, 1991; Bredt & Snyder, 1992).

Controversy still exists, however, concerning the precise nature of the mediator produced by the L-arginine: nitric oxide system in the vascular endothelium. For example, although some authors have reported that the level of nitric oxide, measured either by chemiluminescence (Palmer *et al.*, 1987) or by oxidation of haemoglobin (Kelm & Schrader, 1988), can account for the activity of endothelium-derived relaxing factor (EDRF), others have challenged this view (Myers *et al.*, 1989; 1990). Indeed, Myers *et al.* (1990) have proposed that the stability and potency of EDRF more closely resembles that of S-nitrosocysteine than of nitric oxide. Doubt has also been cast on the assumption that the NANC transmitter in the anococcygeus and BRP is simply nitric oxide (Gillespie & Sheng, 1990; Gibson *et al.*, 1992). Specifically, generation of the superoxide anion by the xanthine: xanthine oxidase system or by drugs such as pyrogallol or hydroquinone destroys the activity of authentic nitric oxide and of EDRF either in cascade bioassay systems or in arterial rings (Gryglewski *et al.*, 1986; Moncada *et al.*, 1986; Rubanyi & Vanhoutte, 1986), but does not inhibit NANC relaxation (Gillespie & Sheng, 1990; Gibson *et al.*, 1992). It is possible therefore that the NANC neurotransmitter in these tissues may be a superoxide anion-resistant, nitric oxide-releasing molecule rather than free nitric oxide.

The aim of this study was to investigate the possibility that S-nitrosocysteine or a related S-nitrosothiol acts as the NANC neurotransmitter in the BRP muscle. This was attempted first, by examining the actions of two sulphydryl inactivating agents, diamide and N-ethylmaleimide (Minor *et al.*, 1989; Siegle *et al.*, 1993), on NANC relaxation, and secondly, by comparing the properties of the NANC transmitter with those of authentic nitric oxide and those of the relaxants produced following the reaction of nitric oxide with L-cysteine and certain structural analogues.

Methods

Preparation of tissues and tension recording

The bioassay of relaxant activity was assessed either on strips of bovine retractor penis (BRP) muscle or rings of rabbit aorta. BRP muscles were obtained from a local abattoir; some tissues were used that day but others were stored at 4°C in Krebs solution for use the following day. Strips of muscle (2 × 10 mm) were cut and suspended for tension recording either on glass hooks, or within Ag-AgCl ring electrodes if the NANC nerves were to be stimulated. The preparation of rabbit aortic rings was essentially as described previously (Martin *et al.*, 1985). Briefly, male New Zealand rabbits were killed by a lethal injection of pentobarbitone and exsanguination. The aorta was removed, cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings using a razor blade slicing device. Endothelial cells were removed from all rings by gently rubbing the intimal surface with moist filter paper. Rings were suspended for tension recording on stainless steel hooks. Both the aortic rings and BRP muscle strips were suspended under 2 g resting tension in 20 ml organ chambers and bathed at 37°C in Krebs solution comprising (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11 and gassed with 95% O_2 and 5% CO_2. Tension was measured with Grass FTO3C transducers and displayed on a Grass polygraph. Tissues were allowed to equilibrate for 90 min before experiments were begun. Relaxant studies were conducted following submaximal contraction of aortic rings and BRP strips with phenylephrine (0.3 μM). In experiments on the BRP in which NANC nerves were stimulated, adrenergic motor responses were blocked and the tone raised with guanethidine (30 μM). Electrical field stimulation was delivered from a Grass S88 stimulator at 2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage at 3 min intervals.

Nitric oxide solutions and reaction of nitric oxide with L-cysteine and analogues

Solutions of nitric oxide gas were prepared by submerging a brown bottle (volume 18 ml) in a beaker containing 200 ml of distilled, deionised water and gassing with oxygen-free nitrogen (British Oxygen Company) for 1 h. While still submerged, a rubber septum was used to seal the bottle and 4 μl of nitric oxide gas (99.9%, Air Products, U.K.) was injected into the bottle to give a concentration of 20 μM. These solutions of nitric oxide retained their activity for several hours while remaining sealed.

The reaction between nitric oxide and L-cysteine or its analogues (all at 15 mM), was performed under anaerobic conditions but experience showed us how difficult it was to achieve true oxygen-free conditions. Initially, similar precautions to those described in the preceding paragraph for nitric oxide solutions were used. When later experiments showed some oxygen remained and played an important part in the reaction, more elaborate precautions were taken. Specifically, a sealed cuvette containing an aqueous solution of L-cysteine or one of its analogues at pH 3 was submerged in distilled water within a sealed Amicon chamber. Both the cuvette contents and the surrounding distilled water were purged with oxygen-free nitrogen for 1 h. The nitrogen was purified by passing through a series of 300 ml wash bottles containing successively, Fieser's solution to remove any traces of oxygen, saturated lead acetate to remove H_2S from the Fieser's solution and 10% NaOH to remove any nitrogen

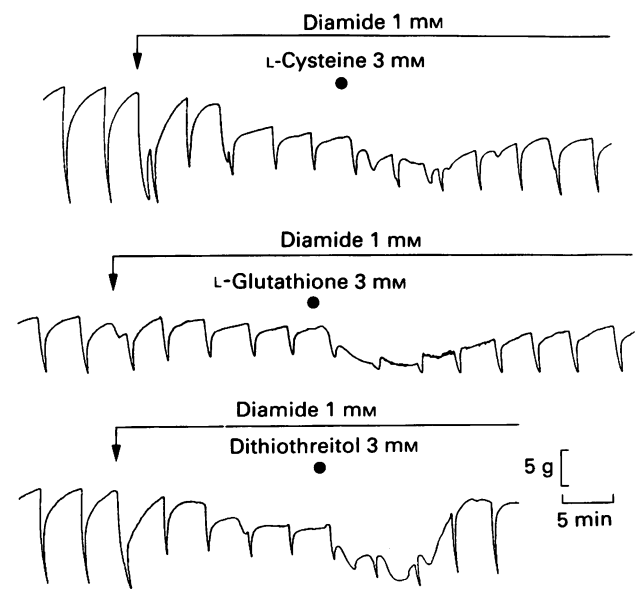

Figure 1 Following induction of tone and blockade of adrenergic motor responses, electrical field stimulation (2 Hz, 20 pulses) of strips of bovine retractor penis muscle elicited NANC relaxant responses. These relaxant responses were inhibited by treatment with diamide (1 mM) and this inhibition was partially reversed upon addition of L-cysteine, L-glutathione or dithiothreitol, all at 3 mM.

dioxide. Nitric oxide gas was passed through the same solutions directly into the cuvette containing the L-cysteine for saturated solutions (~3 mM) or measured volumes of nitric oxide gas were removed with a gas-tight syringe and injected into the L-cysteine solution to give concentrations of 0.1 or 1 mM. The tubing connecting the wash bottles was stainless steel since rubber or plastic tubing absorbed nitrogen dioxide and was permeable to oxygen and the gas-tight syringe was flushed several times with purified oxygen-free nitrogen. Fieser's solution was prepared by adding 60 g of potassium hydroxide, 6 g of sodium anthraquinone-β-sulphonate and 45 g of sodium dithionite to 300 ml of distilled water (Fieser & Fieser, 1967).

In some experiments, nitrogen dioxide (1 mM) was reacted with L-cysteine (10 mM) by the same procedure detailed above for nitric oxide. Nitrogen dioxide was prepared either by mixing purified nitric oxide gas with an equal volume of oxygen (British Oxygen Company) in a gas-tight syringe or liquid nitrogen dioxide was made by heating anhydrous lead nitrate and condensing the evolved nitrogen dioxide gas in a glass coil cooled with dry ice (Moody, 1991).

These solutions were taken for bioassay or spectrophotometric analysis on a Shimadzu UV 240 dual beam spectrophotometer. High performance liquid chromatography (h.p.l.c.) separation was performed using a μBondpac Radial-Pac C18 reverse-phase column (8 × 100 mm) with distilled, deionised water at pH 3 at a flow rate of 1.5 ml min^{-1} and detection was by absorbance at 254 nm.

Drugs

N-acetyl-L-cysteine, L-cystamine, L-cysteamine, L-cysteic acid, L-cysteine, L-cysteine methyl ester, L-cysteinesulphinic acid, L-cystine, L-glutathione, guanethidine sulphate, haemoglobin (bovine), hydroquinone, isoprenaline hydrochloride, L-methionine, S-methyl-L-cysteine, methylene blue, S-methyl-L-glutathione, N-methylhydroxylamine hydrochloride, N^G-nitro-L-arginine, phenylephrine hydrochloride and superoxide dismutase (bovine erythrocyte) were obtained from Sigma (U.K.); diamide, dithiothreitol and N-ethylmaleimide were obtained from Aldrich (U.K.); pyrogallol was obtained from BDH (U.K.) and glyceryl trinitrate (10% w/w in lactose) was obtained from Napp Laboratories (U.K.). All drugs were dissolved and dilutions made in distilled, deionised water.

Solutions of oxyhaemoglobin were prepared as previously described (Martin *et al.*, 1985). Briefly, a 10 fold molar excess of the reducing agent, sodium dithionite, was added to aqueous solutions of commercial haemoglobin (1 mM) and this was followed by dialysis against 100 volumes of distilled water for 2 h at 4°C. The resulting solutions were stored in aliquots at −20°C for up to 14 days.

Results

Effects of diamide and N-ethylmaleimide

Treatment with either of two sulphydryl inactivating agents, diamide (1 mM, Figure 1) and N-ethylmaleimide (0.3 mM), inhibited NANC relaxation of strips of BRP muscle. The blockade induced by diamide was partially reversed by treatment with the sulphydryl agents, L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM), but that induced by N-ethylmaleimide was completely unaffected. Pretreatment with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM) completely prevented inhibition of NANC relaxation by diamide or N-ethylmaleimide. The relaxant actions of authentic nitric oxide (0.3 μM), glyceryl trinitrate (0.001–1 μM), and isoprenaline (0.01–1 μM) were also inhibited by treatment with diamide (1 mM) or N-ethylmaleimide (0.3 mM). In these experiments also, the inhibitory actions of diamide and N-ethylmaleimide were completely prevented by pretreatment with the three sulphydryl compounds. Once established, however, the inhibitory actions of diamide and N-ethylmaleimide were not reversed by the three sulphydryl compounds.

Effects of nitric oxide and L-cysteine

Nitric oxide (1–100 nM) in oxygen-free solution induced concentration-dependent, transient relaxation of phenylephrine-contracted rings of rabbit aorta and strips of BRP muscle (Figures 2 and 8). L-Cysteine (1.5 mM) had no effect by itself, but mixing nitric oxide with L-cysteine in nominally oxygen-free solution at pH 3 followed by purging with oxygen-free nitrogen to remove any free nitric oxide and neutralisation, giving final bath concentrations equivalent to 0.5 μM nitric oxide and 1.5 mM L-cysteine, produced more powerful and sustained relaxation than nitric oxide alone (Figure 2). Clearly, either L-cysteine protected nitric oxide or the two substances combined to form a new compound and these possibilities were tested by h.p.l.c. analysis. L-Cysteine itself produced two peaks in the h.p.l.c., representing its oxidised and reduced forms, respectively (Figure 3). After mixing L-cysteine (10 mM) with nitric oxide (50 μM) a third peak appeared indicating formation of a new compound and bioassay revealed that this peak contained all of the relaxant activity.

Functional groups of L-cysteine reacting with nitric oxide

L-Cysteine has three functional groups which could potentially react with nitric oxide namely, the sulphydryl, carboxyl and amino groups. A series of analogues (all at 15 mM) were used in which each of these groups was modified and the effect on the ability to form the new relaxant activity following reaction with nitric oxide (0.1 mM) examined. The results were clear cut: modification of the sulphydryl always abolished the ability to form the new relaxant activity whereas modification of either of the other two groups had no effect. For example, substitution of the sulphydryl group (S-methyl-L-cysteine, L-cysteinesulphinic acid and L-cysteic acid) or formation of disulphides (L-cystine and L-cystamine)

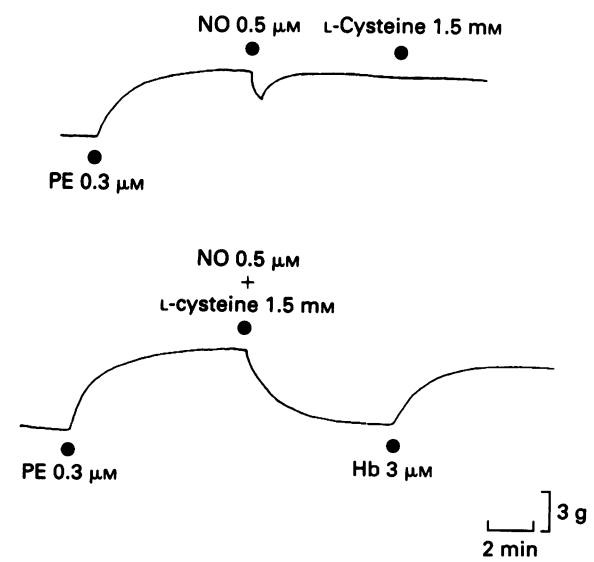

Figure 2 Aqueous solutions of nitric oxide (NO, 0.5 μM) produced transient relaxation of phenylephrine (PE, 0.3 μM)-contracted, endothelium-denuded rings of rabbit aorta, and L-cysteine (1.5 mM) by itself had no effect. Reacting nitric oxide with L-cysteine in nominally oxygen-free solution at pH 3, followed by purging with oxygen-free nitrogen to remove any free nitric oxide and neutralisation, giving final bath concentrations of 0.5 μM nitric oxide and 1.5 mM L-cysteine, produced more powerful and sustained relaxation than could be attributed to nitric oxide alone. This powerful relaxant activity was rapidly abolished upon addition of haemoglobin (Hb 3 μM).

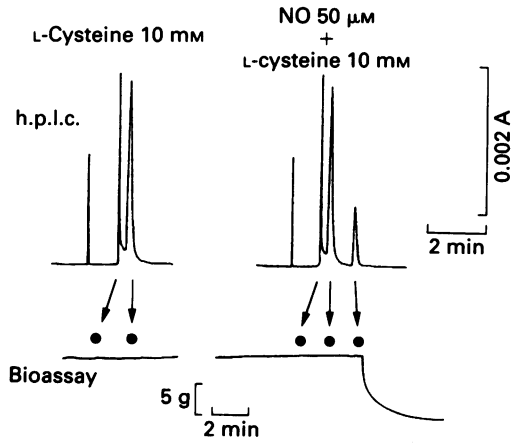

Figure 3 H.p.l.c. analysis split L-cysteine into two peaks representing its oxidised and reduced forms, respectively. Reacting nitric oxide (50 μM) with L-cysteine (10 mM) in nominally oxygen-free solution followed by purging with oxygen-free nitrogen to remove any free nitric oxide and neutralisation resulted in the formation of a new peak which was entirely responsible for relaxant activity as assessed on phenylephrine-contracted endothelium-denuded rings of rabbit aorta.

led to complete loss of the ability to generate relaxant activity, whereas removal of the carboxyl group (L-cysteamine), replacement of the carboxyl with an ester function (L-cysteine methyl ester) or substitution of the amino group (N-acetyl-L-cysteine) had no effect (Figure 4). L-Glutathione was also able to react with nitric oxide to produce relaxant activity, and this too was blocked upon substitution of the free sulphydryl group (S-methyl-glutathione). L-Methionine, which contains a methyl group on the sulphur atom was also unable to generate relaxant activity following reaction with nitric oxide.

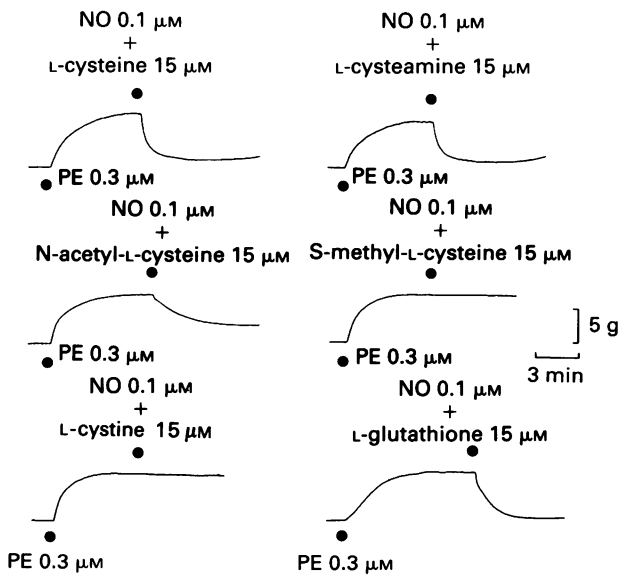

Figure 4 A series of structural analogues (all at 15 mM) was used to determine the functional group of the L-cysteine molecule with which nitric oxide (NO) reacts. Reacting nitric oxide (0.1 mM) with L-cysteine (15 mM) under nominally oxygen-free conditions at pH 3 produced powerful relaxant activity as assessed on phenylephrine (PE, 0.3 mM)-contracted, endothelium-denuded rings of rabbit aorta. L-cysteamine, which lacks the carboxyl group, and N-acetyl-L-cysteine, which has a substituent at the amino group, were able to react with nitric oxide to produce relaxant activity. In contrast, S-methyl-L-cysteine, which has a substituent at the sulphydryl, and L-cystine, the disulphide, failed to react with nitric oxide to produce relaxant activity. L-Glutathione, which also contains a free sulphydryl group reacted with nitric oxide to produce relaxant activity. Final bath equivalents of the reactants are given.

pH dependence

The reaction between L-cysteine and nitric oxide was examined over the range pH 1–8. Generation of powerful relaxant activity was strictly pH-dependent (Figure 5); following purging, neutralisation and assay, little activity had been generated at neutral pH or higher and maximal activity had been obtained at pH 1.

Spectral characteristics and the role of oxygen

As part of the characterization of the new relaxant formed in reacting nitric oxide with L-cysteine, we examined the full spectrum between 190 and 700 nm of each of the reactants and the product formed after mixing. In the course of these experiments it became clear that a low concentration of oxygen was necessary for full activity and that the usual method of producing an oxygen-free environment was inadequate. In our early experiments nitric oxide was reacted with a solution of L-cysteine at pH 3 sealed in a brown bottle immersed in a beaker of distilled water and both the distilled water and the L-cysteine purged with nitrogen before injecting a measured amount of nitric oxide from a gas-tight syringe into the L-cysteine. After allowing 10 min for reaction, free nitric oxide was removed by purging with oxygen-free nitrogen and the mixture was neutralised and assayed. The result was the development of powerful relaxant activity and the appearance of the third peak in the h.p.l.c. shown on Figure 3.

Subsequently, more stringent precautions were taken to exclude oxygen by immersing a sealed cuvette containing

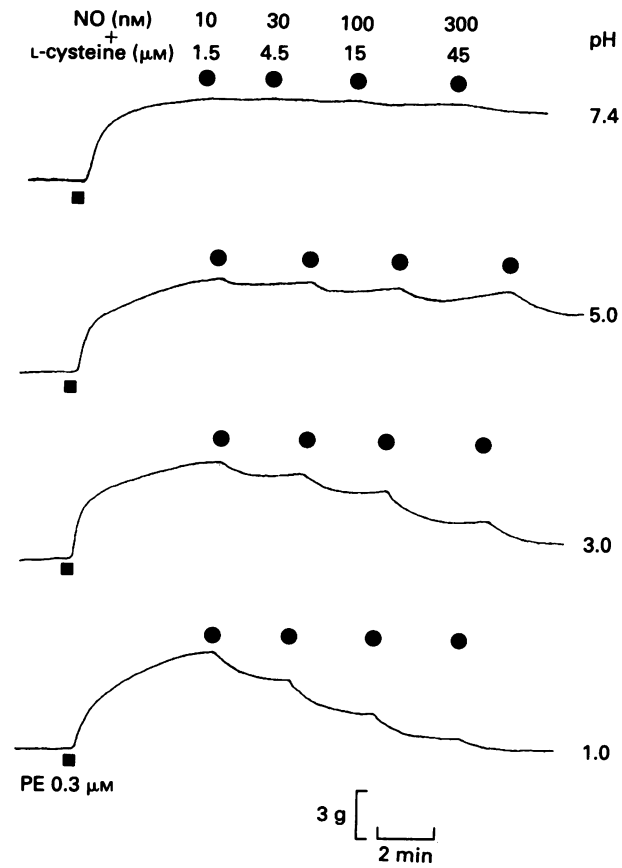

Figure 5 Reacting nitric oxide (NO, 0.1 mM) and L-cysteine (15 mM) in nominally oxygen free solution produced a pH-dependent generation of relaxant activity as assessed on phenylephrine (PE, 0.3 μM)-contracted endothelium-denuded rings of rabbit aorta. Little activity was generated at pH 7.4 and maximal activity was generated at pH 1. Concentrations shown give the final bath equivalent of the two reactants.

L-cysteine (10 mM) at pH 3 in distilled water in an Amicon chamber and purging with nitrogen which had been passed through Fieser's solution followed by bubbling with nitric oxide (~3 mM) for 10 min then purging to remove free nitric oxide. These solutions were clear and their relaxant activity was less (Figure 6) than in the earlier experiments (Figure 2). Exposure of these solutions to air did not affect their relaxant activity. If, however, these solutions were not purged to remove free nitric oxide, opening the cuvette to the air instantly resulted in the development of a pink colour and a ~40 fold increase in relaxant activity (Figure 6).

As would be expected, spectral changes accompanied this change in colour and activity. Where strict precautions were taken to exclude air and free nitric oxide was removed by purging, the clear solution had two new absorption peaks at 218 and 335 nm and these did not change upon exposing the cuvette contents to the air (Figure 6). If, however, the nitric oxide was not removed by purging, opening the cuvette to the air increased these two peaks some 40 fold, the same increase as was seen in relaxant activity. At the same time a third, new absorption peak appeared at 543 nm corresponding to the pink colour of the solution. Other experiments showed that if nitric oxide was not purged but left in contact with L-cysteine in the sealed cuvette than absorbance at 218, 335 and 543 nm increased slowly over a period of hours suggesting a slow entry of oxygen. Furthermore, if the cuvette was opened to the air at any time, the absorbance at these wavelengths increased within seconds to values similar to those shown on Figure 6.

Reactivity of nitric oxide compared with nitrogen dioxide

Since the presence of oxygen greatly increased the generation of relaxant activity, we examined whether or not nitrogen dioxide was the species which reacted with L-cysteine. When either nitric oxide or nitrogen dioxide at 0.1 mM was reacted with L-cysteine (10 mM) at pH 3, relaxant activity and the associated third peak in the h.p.l.c. was only generated with the former (Figure 7). If, however, the concentration of the two gases was increased to 1 mM, relaxant activity and the associated third peak in the h.p.l.c. were also generated with nitrogen dioxide, but the magnitude of these was lower than for nitric oxide. The effectiveness of this higher concentration probably results from the production of some nitrous acid and therefore nitric oxide from the solution of nitrogen dioxide.

Effects of haemoglobin, methylene blue, N-methylhydroxylamine and N^G-nitro-L-arginine

While the relaxant effect of the new compound was presumably due to nitric oxide acting on soluble guanylate cyclase it was possible that this did not need to be released

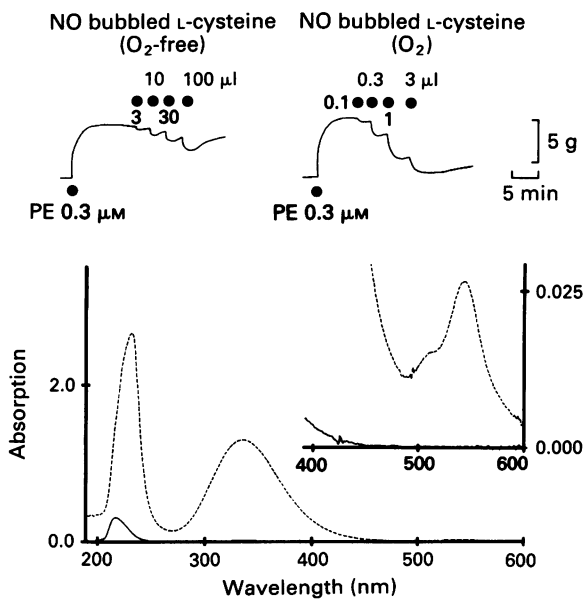

Figure 6 Bubbling nitric oxide gas to saturation (~3 mM) in solutions of L-cysteine (10 mM) in stringent oxygen-free conditions (O_2-free) followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation produced low relaxant activity as assessed on phenylephrine (PE, 0.3 µM)-contracted, endothelium-denuded rings of rabbit aorta. These solutions were colourless but contained two small absorption peaks at 218 and 335 nm (continuous line). If, however, nitric oxide was bubbled to saturation in solutions of L-cysteine (10 mM) and the reaction chamber was opened to the air (O_2) before purging and neutralisation, the relaxant activity generated was greatly (~40 fold) increased. The absorption peaks at 218 and 335 nm (discontinuous line) increased by a proportionate degree and a new peak became visible at 543 nm which was responsible for the development of a pink colour.

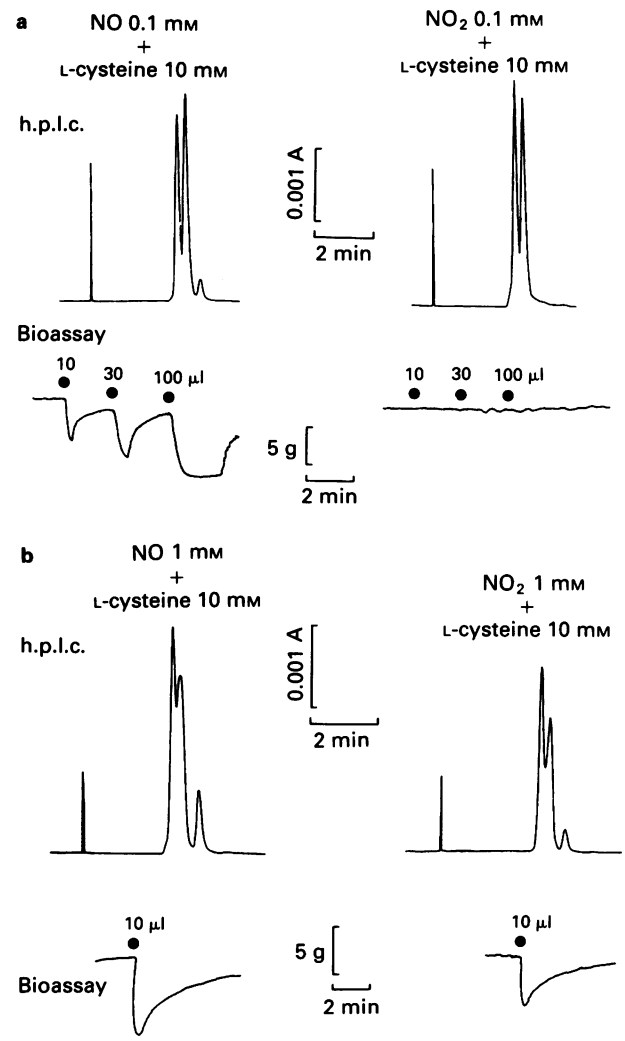

Figure 7 (a) Reacting nitric oxide (NO, 0.1 mM) with L-cysteine (10 mM) under stringent oxygen-free conditions at pH 3 led to the development of relaxant activity as assessed on phenylephrine (0.3 µM)-contracted strips of bovine retractor penis muscle, and h.p.l.c. analysis revealed the presence of three peaks, representing L-cystine, L-cysteine and a new substance that was entirely responsible for relaxant activity. Reacting nitrogen dioxide (NO_2, 0.1 mM) under identical conditions failed to generate relaxant activity or the associated third peak in the h.p.l.c. (b) If the concentration of nitrogen dioxide (NO_2) was increased to 1 mM, relaxant activity and the associated third peak in the h.p.l.c. were generated but the magnitude of these was lower than those generated by nitric oxide (NO) at 1 mM.

from the compound. A more unlikely hypothesis is that the compound could itself stimulate the synthesis of nitric oxide by the NANC nerves. If it releases nitric oxide then haemoglobin should block its action, if it acts via guanylate cyclase then methylene blue and N-methylhydroxylamine should also block, and finally if it activates nitric oxide synthase, N^G-nitro-L-arginine should block. Experimentally the results were clear. Haemoglobin (3 μM, Figure 2), methylene blue (10 μM) and N-methylhydroxylamine (100 μM) abolished the activity of the new relaxant formed from reacting nitric oxide with L-cysteine or L-glutathione but N^G-nitro-L-arginine (30 μM) had not effect.

Effects of pyrogallol and hydroquinone

As previously reported (Gillespie & Sheng, 1990), we found that treatment of strips of BRP with the superoxide anion-generating drugs, pyrogallol (0.1 mM, Figure 8) or hydroquinone (0.1 mM, data not shown), abolished the relaxant actions of authentic nitric oxide (0.3 μM) but had no effect on that to NANC nerve stimulation (2 Hz, 20 pulses). The effect on nitric oxide was blocked by superoxide dismutase (100 u ml^{-1}). Treatment with pyrogallol (0.1 mM) or hydroquinone (0.1 mM, data not shown) also abolished the activity of the new relaxant generated following reaction of nitric oxide (1 mM) with L-cysteine (10 mM) or L-glutathione (10 mM, data not shown), and this inhibition too was reversed by superoxide dismutase (100 u ml^{-1}). Furthermore, superoxide dismutase (100 u ml^{-1}) potentiated the actions of the new relaxant but had no effect on relaxation to NANC nerve stimulation. Other experiments showed that if pyrogallol (0.1 mM) or hydroquinone (0.1 mM) was added to the reaction chamber containing nitric oxide and L-cysteine or L-glutathione rather than to the BRP, then relaxant activity was also destroyed.

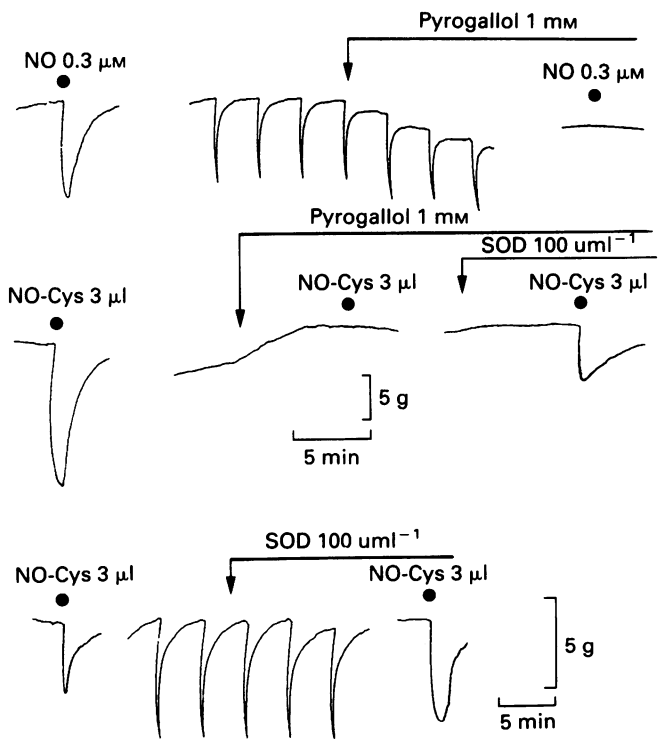

Figure 8 Authentic nitric oxide (NO, 0.3 μM), electrical field stimulation (2 Hz, 20 pulses) of the NANC nerves and the product (NO-Cys) of reacting nitric oxide (1 mM) with L-cysteine (10 mM) all relaxed strips of bovine retractor penis muscle. Treatment with pyrogallol (0.1 mM) abolished the relaxant actions of nitric oxide and of NO-Cys but had no effect on those to NANC nerve stimulation. The inhibitory effects of pyrogallol were reversed following treatment with superoxide dismutase (SOD, 100 u ml^{-1}). Superoxide dismutase also potentiated the relaxant actions of NO-Cys but had no effect on those to NANC nerve stimulation.

Discussion

It is now widely accepted that the L-arginine: nitric oxide system mediates inhibitory NANC transmission in the BRP and the related anococcygeus muscles of the rat and mouse (Gillespie *et al.*, 1989; Gibson *et al.*, 1990; Hobbs & Gibson, 1990; Martin *et al.*, 1993). The finding that superoxide anion-generating drugs such as pyrogallol and hydroquinone inhibit the relaxant actions of authentic nitric oxide but not of NANC nerve stimulation in the BRP and mouse anococcygeus (Gillespie & Sheng, 1990; Gibson *et al.*, 1992), suggests that the neurotransmitter in these tissues may be a superoxide anion-resistant, nitric oxide-releasing molecule rather than free nitric oxide. Indeed, an analogous debate continues concerning the precise nature of EDRF which Myers *et al.* (1990) suggest most closely resembles S-nitrosocysteine than nitric oxide. If a compound does exist which binds nitric oxide and acts as a delivery system between synthesis in the nerve endings or endothelium and action on guanylate cyclase in the effector cells it would ideally meet certain conditions. Specifically, it would bind nitric oxide securely enough to preclude non-specific interactions with proteins and other substances and prevent destruction by superoxide anion. Furthermore, the binding would be reversible so as to deliver nitric oxide to guanylate cyclase.

Sulphydryl inactivating agents have previously been employed to investigate the involvement of S-nitrosothiols in the activity of EDRF, but with equivocal results (Minor *et al.*, 1989; Siegle *et al.*, 1993). We examined the actions of two agents, diamide and N-ethylmaleimide, which respectively oxidise and alkylate sulphydryl groups (Kosower *et al.*, 1969; Siegle *et al.*, 1993). Consistent with involvement of an S-nitrosothiol, we found that both blocked NANC relaxation and this was prevented by pretreating the tissues with excess sulphydryl groups in the form of L-cysteine, L-glutathione or dithiothreitol. These three sulphydryl compounds were also effective in reversing the actions of diamide, presumably through reduction of the -S-S- bonds formed following its action. The actions of N-ethylmaleimide were not reversed, suggesting that blockade had not resulted from covalent inactivation of the sulphydryl groups of endogenous L-cysteine or L-glutathione. The most compelling evidence against the blockade having resulted from loss of the ability to form S-nitrosothiols was obtained when we found that relaxation to nitric oxide, glyceryl trinitrate and isoprenaline was also blocked. It was clear, therefore, that the sulphydryl inactivating agents were blocking the effector pathways for these diverse relaxants and that any additional action in preventing formation of S-nitrosothiols by the NANC nerves would be impossible to discern.

Our next approach was to examine the properties of S-nitrosothiols. A number of methods are available for the synthesis of these compounds, including the reaction of L-cysteine and nitrogen dioxide in cold methanol (Myers *et al.*, 1990), acidification of sodium nitrite in the presence of L-cysteine and other sulphydryl compounds (Kowaluk & Fung, 1990; Gibson *et al.*, 1992; Kerr *et al.*, 1993), and the reaction of nitric oxide with L-cysteine and other sulphydryl compounds under anaerobic conditions (Ignarro & Gruetter, 1980; Ignarro *et al.*, 1981). The S-nitrosothiols produced by each of these routes are powerful smooth muscle relaxants, normally pink in colour, and have a characteristic absorption peak at around 335 nm. There is, however, enormous variability in the reported half-lives of the nitrosothiols produced by these methods ranging from ~30 s (Myers *et al.*, 1990) to 7–10 days (Ignarro & Gruetter, 1980). We initially adopted the method of Ignarro & Gruetter (1980) since we wished to determine if nitric oxide itself rather than higher

oxides of nitrogen could react with the -S-H moiety of L-cysteine. This method has, however, been criticised by Feelisch (1991) who reported that the presence of oxygen was a prerequisite for nitrosation to proceed, and our observations supported this. We found that if nitric oxide was bubbled to saturation in solutions of L-cysteine under rigorous oxygen-free conditions that small absorption peaks were present at 218 and 335 nm, but the solution remained colourless. While still sealed, the absorption peaks at 218 and 335 nm grew slowly with time, but if the solution was opened to the air, they rose roughly 40 fold within a few seconds to stable new values and the solution became pink in colour owing to a new absorbance peak developing at 543 nm. It was likely, therefore, that the presence of oxygen was vital for nitric oxide and L-cysteine to react generating absorption peaks at 218, 335 and 543 nm, and that the small slowly growing peaks at 218 and 335 nm even under our most rigorous oxygen-free conditions were due to the slow entry of trace amounts of oxygen. The development of relaxant activity, as assessed on the BRP and rabbit aorta, was also critically dependent upon the availability of oxygen. Under our rigorous oxygen-free conditions when nitric oxide was allowed to react with L-cysteine, and then unreacted nitric oxide was removed by purging, some relaxant activity was generated, but this increased in direct proportion ($\sim$40 fold) to the increases in absorption at 218 and 335 nm if the solution was exposed to the air before purging.

The requirement for oxygen in the development of relaxant activity and associated absorption peaks showed that nitric oxide itself was unable to react with L-cysteine and that a higher oxide of nitrogen was the reactive species. There is debate over the precise nature of this nitrosating species; some have suggested that it is N_2O_3 or N_2O_4 (Leak *et al.*, 1990), but others dispute this suggesting that it is an as yet uncharacterized oxide of nitrogen (Wink *et al.*, 1993). We also dismissed the possibility that it was nitrogen dioxide, since this gas generated less relaxant activity than nitric oxide following reaction with L-cysteine.

H.p.l.c. analysis confirmed that nitric oxide had indeed reacted with L-cysteine to form a novel compound: the C18 column used fractionated the mixture into three peaks, representing L-cystine (the disulphide), L-cysteine and a third, new peak that was entirely responsible for relaxant activity. Our experiments with a series of structural analogues provided strong evidence that the reaction of nitric oxide or higher oxide was with the sulphydryl group. All derivatives of L-cysteine or L-glutathione in which this group was intact, irrespective of what changes were made in the amino or carboxyl groups, produced powerful relaxants whereas all the compounds in which the sulphydryl was modified were ineffective. It is likely therefore that the relaxants produced were the respective S-nitrosothiols. We confirmed, however, that the smooth muscle relaxant action of the nitrosothiols was derived from the nitric oxide they released (Ignarro *et al.*, 1981; Gibson *et al.*, 1992), by finding that haemoglobin, which binds and inactivates nitric oxide, and methylene blue and N-methylhydroxylamine, which inhibit soluble guanylate cyclase, blocked their activity.

Another striking finding was that the reaction of nitric oxide with L-cysteine was pH-dependent: little relaxant activity was generated at neutral pH, but as the pH was lowered, relaxant activity was increased until an optimum was reached at pH 1. This process is reminiscent of the 'acid-activation' of inhibitory factor extracted from BRP (Gillespie & Martin, 1980). The inactive form of inhibitory factor contains inorganic nitrite and upon acidification generates nitric oxide which binds to a substance which stabilizes its activity (Martin *et al.*, 1988). It has recently been proposed that the acid-activated inhibitory factor is S-nitrosocysteine or S-nitrosoglutathione (Yui *et al.*, 1989; Kerr *et al.*, 1993). Although it is well established that acidification of nitrite leads to liberation of nitric oxide (Furchgott, 1988), it is unclear why acidification should have the additional effect of catalysing the reaction of nitric oxide with L-cysteine. What is clear, however, is that as the chemical reaction between nitric oxide and L-cysteine proceeds so inefficiently at neutral pH, it is unlikely that sufficient S-nitrosocysteine is produced under normal physiological conditions to account for the activity of the NANC neurotransmitter.

Further evidence against S-nitrosocysteine or S-nitrosoglutathione acting as the NANC transmitter in the BRP was obtained using the superoxide anion-generating agents, pyrogallol and hydroquinone. These agents have previously been reported to destroy the activity of authentic nitric oxide but do not affect neurogenic relaxation in the BRP or mouse anococcygeus (Gillespie & Sheng, 1990; Gibson *et al.*, 1992), suggesting that the NANC transmitter involved is a superoxide anion-insensitive, nitric oxide-releasing molecule. We found that pyrogallol and hydroquinone both destroyed the relaxant activity of S-nitrosocysteine, S-nitrosoglutathione and nitric oxide when added to their respective solutions or to the BRP assay tissue, but as before, neither affected relaxation to NANC nerve stimulation. Furthermore, superoxide dismutase potentiated the relaxant actions of nitric oxide and the S-nitrosothiols but had no effect on those to NANC nerve stimulation. Our findings confirm those of Gibson *et al.* (1992) who also found that hydroquinone inhibits the relaxant activity of S-nitrosocysteine, but not of NANC nerve stimulation in the mouse anococcygeus. In contrast to our findings, however, they reported that hydroquinone did not block the actions of S-nitrosoglutathione. Whether this disparity is due to differences in concentrations of drugs used or methods in preparing the nitrosothiol is unclear at present. We are in agreement, however, that the ability of free radical-generating drugs to destroy the activity of S-nitrosocysteine precludes the involvement of this substance in NANC transmission in the BRP and anococcygeus muscles.

In conclusion, although the sulphydryl inactivating agents, diamide and N-ethylmaleimide, inhibit NANC relaxation in the BRP, this occurs through a non-selective action rather than from an inability to form an S-nitrosothiol. Nitric oxide can react in the presence of oxygen and at acid pH with sulphydryl compounds to produce S-nitrosothiols which are potent, long-lasting smooth muscle relaxants. The ability of superoxide anion-generating drugs to inhibit the relaxant actions of these S-nitrosothiols as well as of nitric oxide but not of the NANC nerves, suggests that these substances are not candidates for the inhibitory transmitter. The data suggest that the NANC neurotransmitter is more likely to be a superoxide anion-resistant, nitric oxide-releasing molecule.

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