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Effect of Cu^{2+} on relaxations to the nitrergic neurotransmitter, NO and S-nitrosothiols in the rat gastric fundus

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1 The effects of addition of Cu^{2+} and chelation of Cu^{2+} were studied on relaxations in response to S-nitrosothiols and on relaxations to non-adrenergic non-cholinergic (NANC) nerve stimulation, nitric oxide (NO) and glyceryl trinitrate (GTN) in the rat gastric fundus.

2 The S-nitrosothiols S-nitroso-L-cysteine (NOCys, 1–300 nM), S-nitrosoglutathione (GSNO, 0.01–3 μM) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 0.01–3 μM) induced concentration-dependent relaxations of the rat gastric fundus muscle strip. The relaxant potencies of the S-nitrosothiols were NOCys > SNAP > GSNO. Relaxations to NOCys were transient and comparable to those to NANC nerve stimulation and NO whereas relaxations to GSNO and SNAP were sustained. The relaxations to NOCys, GSNO and SNAP were significantly and concentration-dependently enhanced by $CuSO_4$ (3–30 μM). The order of relaxant potency in the presence of $CuSO_4$ was reversed to GSNO $\approx$ SNAP > NOCys.

3 In the presence but not in the absence of 0.1 μM GSNO, $CuSO_4$ (1 μM) induced a rapid and transient relaxation which was inhibited by the superoxide radical generator, pyrogallol (30 μM). $CuCl_2$ but not $FeSO_4$ mimicked the effect of $CuSO_4$.

4 Electrical stimulation (0.5–8 Hz) of the rat gastric fundus strips induced frequency-dependent relaxations which were previously shown to be nitrergic in nature and which were not affected by $CuSO_4$ (3–30 μM). Relaxations to NO (3–100 nM) and GTN (0.01–1 μM) were not affected by 3 and 10 μM $CuSO_4$ but were inhibited by 30 μM $CuSO_4$.

5 The Cu^{2+} chelator, bathocuproine (3–30 μM) significantly and concentration-dependently inhibited the relaxations to NOCys (0.01–3 μM), GSNO (0.01–10 μM) and SNAP (0.01–3 μM). The inhibitory effect of 10 μM bathocuproine was reversed by 3 μM $CuSO_4$.

6 Bathocuproine (3–30 μM) had no effect on the relaxations to NANC nerve stimulation (0.5–8 Hz) or on the concentration-response curve to NO (0.01–0.3 μM), whereas relaxations to GTN (0.01–1 μM) were significantly inhibited by 30 μM bathocuproine.

7 From these results we conclude that relaxations to S-nitrosothiols and to nitrergic stimulation of the rat gastric fundus are differentially affected by addition and chelation of Cu^{2+}, suggesting that the nitrergic NANC neurotransmitter in the rat gastric fundus is not an S-nitrosothiol but is more likely to be free nitric oxide.

Keywords: Copper; nitrergic neurotransmitter; nitric oxide; non-adrenergic non-cholinergic transmission; rat gastric fundus; S-nitrosothiol

Introduction

We previously proposed nitric oxide (NO) as a non-adrenergic non-cholinergic (NANC) neurotransmitter in the gastrointestinal tract (Bult *et al.*, 1990; Boeckxstaens *et al.*, 1990). Although the involvement of the L-arginine/NO pathway in NANC neurotransmission in the gastrointesintal tract is well established (for reviews see Sanders & Ward, 1992; Stark & Szurszewski, 1992; Rand & Li, 1995b), controversy remains as to the exact nature of the nitrergic NANC neurotransmitter. Gillespie & Sheng (1990) first demonstrated that the superoxide generator, pyrogallol, did not affect NANC nerve-mediated responses of the bovine retractor penis muscle, whereas responses to authentic NO were significantly inhibited. Such a differential effect was also reported in different regions of the gastrointestinal tract, using radical generators (Hobbs *et al*, 1991; Gibson *et al.*, 1992; Barbier & Lefebvre, 1992b; Martin *et al.*, 1994; Lilley & Gibson, 1995) and using NO-trapping compounds (Jenkinson *et al.*, 1995; Rand & Li, 1995a). These results were generally interpreted to suggest that the actual nitrergic NANC neurotransmitter is not free NO but a superoxide-resistant, NO containing molecule, such as an S-nitrosothiol (Thornbury *et al.*, 1991; Gibson *et al.*, 1992; Kerr *et al.*, 1992; Kitamura *et al.*, 1993; Liu *et al.*, 1994). S-nitrosothiols, which are also intermediates in the vasodilator actions of organic nitrates (Ignarro *et al.*, 1981), are shown to be potent relaxants of gastro-intestinal smooth muscle including the rat gastric fundus (Barbier & Lefebvre, 1994). However, in bioassay experiments, we demonstrated that radical generators did not affect the biological activity of S-nitrosothiols whereas the biological activity of the nitrergic NANC neurotransmitter of the canine ileocolonic junction was inhibited to the same extent as the biological activity of authentic NO (Boeckxstaens *et al.*, 1994; De Man *et al.*, 1995c). These results suggest that the nitrergic NANC neurotransmitter in the canine ileocolonic junction behaves pharmacologically like NO and not like an S-nitrosothiol. Similar results were obtained in the guinea-pig colon (Iversen *et al.*, 1994). Recently, it was demonstrated that the biological activity of S-nitrosothiols can be modulated by

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copper (Askew *et al.*, 1995; Gordge *et al.*, 1995). To investigate further whether the nitrergic NANC transmitter is NO or an S-nitrosothiol, we studied the effect of S-nitroso-L-cysteine, S-nitrosoglutathione and S-nitroso-N-acetyl-D,L-penicillamine on isolated muscle strips of the rat gastric fundus and compared the effect of addition and chelation of Cu^{2+} on the relaxations elicited by S-nitrosothiols, authentic NO and the nitrergic NANC neurotransmitter.

Methods

Tissue preparation

Male Wistar rats (250–300 g) were fasted for 48 h with free access to water. The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (60 mg kg^{-1}) and the stomach was removed. Longitudinal muscle strips approximately 1.0 cm long and 0.3 cm wide were prepared and mounted in organ baths (25 ml) filled with Krebs-Ringer solution (in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, Ca ethylenediaminetetracetic acid (CaEDTA) 0.026 and glucose 11.1). In some experiments CaEDTA was omitted from the Krebs solution. The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

Isometric tension recording

One end of the muscle strip was anchored to a glass rod and pulled through two platinum ring electrodes. The other end was connected to a strain gauge transducer (Statham UC2) for continuous recording of isometric tension. The strips were brought at their optimal point of length-tension relationship (Pelckmans *et al.*, 1989) and then allowed to equilibrate for at least 60 min before experimentation.

Experimental protocols

All experiments were performed on muscle strips contracted with 0.1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and in the presence of 1 μM atropine and 30 μM guanethidine. After each $PGF_{2\alpha}$-induced contraction, the muscle strips were washed 4 times at intervals of at least 5 min.

In a first series of experiments, the effect of addition of $CuSO_4$ (3–30 μM, 10 min incubation) was investigated on the frequency-response curve to electrical stimulation (0.5–8 Hz, 1 ms pulse train of 10 s), on the concentration-response curves to NO (3–100 nM), GTN (0.01–1 μM), S-nitroso-L-cysteine (NOCys, 1–300 nM), S-nitrosoglutathione (GSNO, 0.01–3 μM) and S-nitroso-D,L-penicillamine (SNAP, 0.01–3 μM). The effect of $CuSO_4$ was also investigated on relaxations to 10 μM ATP.

In a second series of experiments the effect of the copper chelator, bathocuproine (3–30 μM, 2 h incubation) was investigated on the frequency-response curve to electrical stimulation (0.5–8 Hz) on the concentration-response curves to NO (0.01–0.3 μM), GTN (0.01–1 μM), NOCys (0.01–3 μM), GSNO (0.01–10 μM) and SNAP (0.01–3 μM) and on relaxations induced by 10 μM ATP.

In a separate series of experiments, the muscle strips that were treated with 10 μM bathocuproine were washed three times and then treated for 10 min with 3 μM $CuSO_4$ before the concentration-response curves to S-nitrosothiols were constructed again.

All experiments were performed in parallel with muscle strips that served as time controls and that received saline instead of $CuSO_4$ or bathocuproine.

Drugs used

The following drugs were used: bathocuproine disodium salt (ICN Biomedicals Inc., Aurora, OH, U.S.A.), atropine sulphate, copper sulphate 5-hydrate, copper (II) chloride, iron sulphate 7-hydrate, glyceryl trinitrate (Merck, Darmstadt, Germany), guanethidine monosulphate (Ciba Geigy, Switzerland), adenosine 5′-triphosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Upjohn, Puurs, Belgium). Solutions of NO were prepared freshly for each experiment as described by Kelm *et al.* (1988) and used immediately after preparation. The solutions of S-nitrosothiols

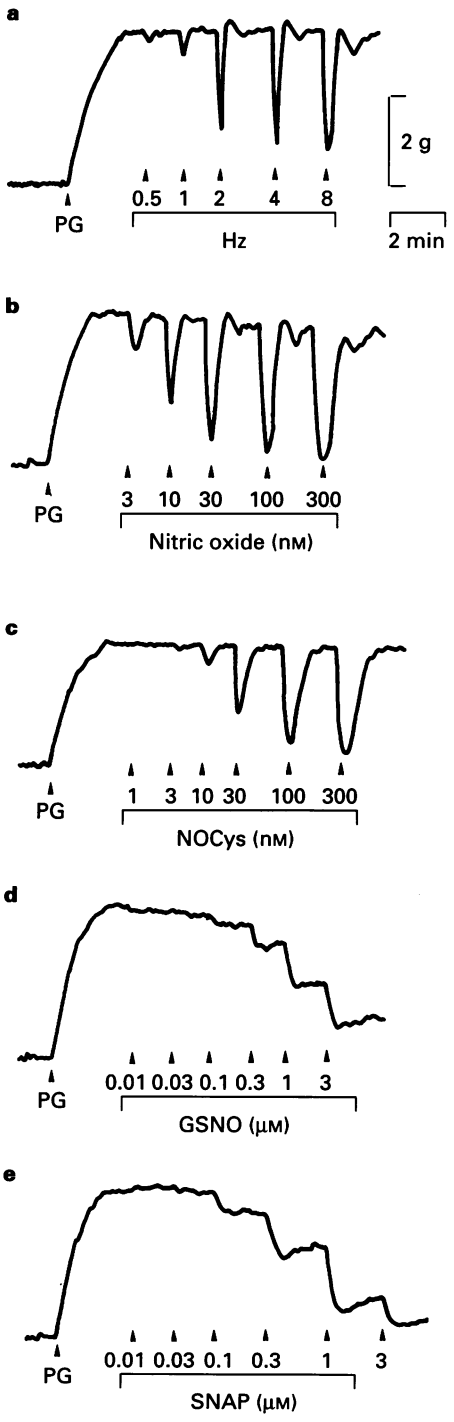

Figure 1 Typical tracings showing (a) the frequency-dependent relaxations to electrical stimulation (1 ms pulses at 0.5–8 Hz for 10 s periods), and the concentration-dependent relaxations to (b) nitric oxide (3–300 nM), to (c) S-nitroso-L-cysteine (NOCys, 1–300 nM), (d) S-nitroso-glutathione (GSNO, 0.01–3 μM) and (e) S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 0.01–3 μM) on prostaglandin $F_{2\alpha}$ (PG)-induced contractions of rat gastric fundus longitudinal muscle strips.

were freshly prepared on the day of experimentation and kept sealed at pH 2 and 0°C as described previously (De Man *et al.*, 1995c). Dilutions of the stock solutions of the S-nitrosothiols were made freshly before each experiment and were used immediately after dilution.

Presentation of results and statistical analysis

Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction of the rat gastric fundus longitudinal muscle strip. Values are shown as mean ± s.e.mean for the number of rats indicated. For statistical analysis, a Student's *t* test for paired and unpaired values was used. *P* values of less than 0.05 were considered to be significant.

Results

Effect of $CuSO_4$ on relaxations to S-nitrosothiols

The S-nitrosothiols, NOCys (1–300 nM), GSNO (0.01–3 μM and SNAP (0.01–3 μM) concentration-dependently relaxed the rat gastric fundus strips. The relaxations to NOCys were rapid in onset and transient and resembled those to authentic NO and NANC nerve stimulation (Figure 1). The relaxations to GSNO and SNAP were less rapid in onset and more sustained than those to NO (Figure 1). The order of relaxant potency was NOCys > SNAP > GSNO. The relaxations to S-nitrosothiols were significantly and concentration-dependently enhanced by $CuSO_4$ (3–30 μM) (Figure 2). The order of relaxant potency in the presence of 30 μM $CuSO_4$ was reversed to GSNO ≈ SNAP > NOCys.

On a $PGF_{2\alpha}$-induced contraction and in the absence of GSNO, $CuSO_4$ (1 μM) did not induce any relaxation (Figure 3a). However, when $CuSO_4$ (1 μM) was added 1 min after GSNO (0.1 μM), which induced a relaxation on its own of 8.0 ± 1.9% ($n=5$), $CuSO_4$ (1 μM) induced a rapid and transient relaxation of 72.2 ± 8.5% ($n=5$) in normal Krebs-Ringer solution (Figure 3a). This relaxation was inhibited to 0.5 ± 0.5% by the superoxide radical generator pyrogallol (30 μM, $n=5$) (Figure 3b). Pyrogallol had no significant effect on the relaxation to 0.1 μM GSNO (from 8.0 ± 1.9% to 5.4 ± 1.8%, $n=5$). Also in the absence of CaEDTA, which is able to bind copper ions, $CuSO_4$ (1 μM) induced a rapid and transient relaxation in the presence of GSNO (0.1 μM) of 82.3 ± 9.9% ($n=3$) which was not different from the relaxation in normal Krebs-Ringer solution (Figure 3c).

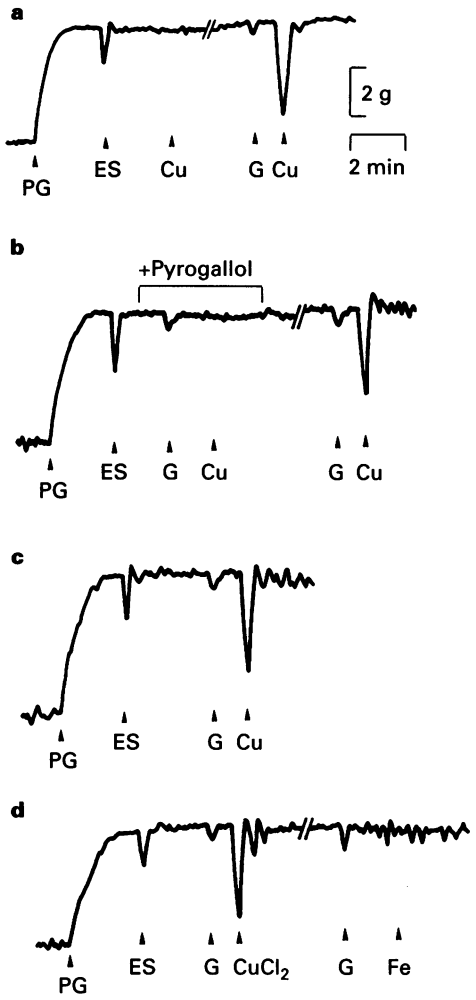

Figure 3 Typical tracings of the rat gastric fundus strip contracted with 0.1 μM prostaglandin $F_{2\alpha}$ (PG) showing (a) the effect of electrical stimulation (ES, 1 Hz for 10 s) and $CuSO_4$ (Cu, 1 μM) in the absence and presence of S-nitrosoglutathione (G, 0.1 μM). (b) Shows the effect of pyrogallol (30 μM) on the relaxation to $CuSO_4$ (Cu, 1 μM) which was added 1 min after S-nitrosoglutathione (G, 0.1 μM). (c) Shows the effect of CaEDTA-free Krebs-Ringer solution on the relaxation to $CuSO_4$ (Cu, 1 μM) in the presence of S-nitrosoglutathione (G, 0.1 μM) and (d) shows the effect of 1 μM $CuCl_2$ and 3 μM $FeSO_4$ (Fe) in the presence of S-nitrosoglutathione (G, 0.1 μM). Tracing breaks represent 3 wash periods of 5 min each with Krebs-Ringer solution that contained 0.1 μM prostaglandin $F_{2\alpha}$.

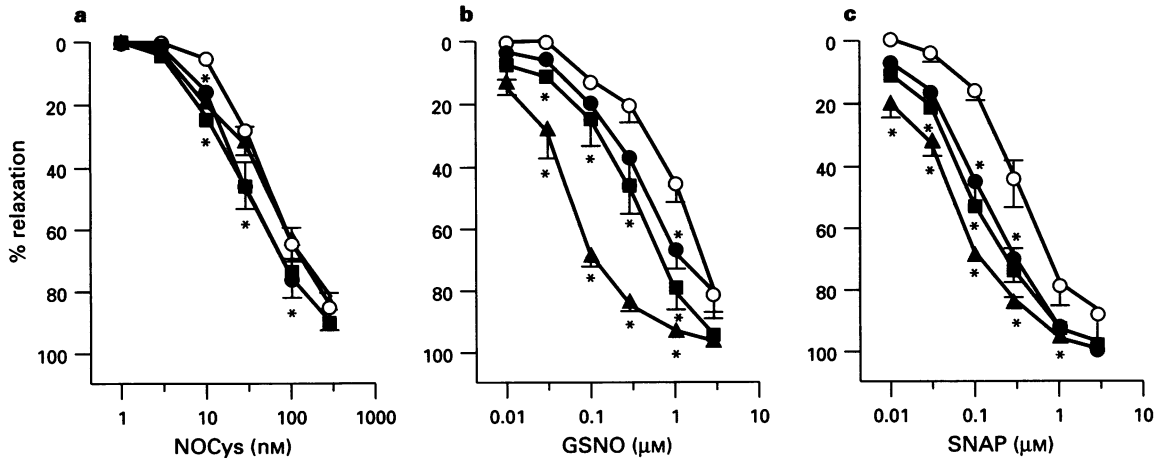

Figure 2 Concentration-response curves to (a) S-nitroso-L-cysteine (NOCys, 1–300 μM), (b) S-nitrosoglutathione (GSNO, 0.01–3 μM) and (c) S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 0.01–3 μM) in control conditions (○) and in the presence of $CuSO_4$ (●, 3 μM; ■, 10 μM and ▲, 30 μM). Results are expressed as percentage decreases of the $PGF_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n=5-8$ experiments. *$P<0.05$ is considered as significantly different from control.

$CuCl_2$ but not $FeSO_4$ mimicked the effect of $CuSO_4$: in the absence of 0.1 μM GSNO, $CuCl_2$ (1 μM) did not induce any relaxation. However, when $CuCl_2$ (1 μM) was added 1 min after GSNO (0.1 μM), which induced a relaxation of 4.9±1.9% ($n=3$), $CuCl_2$ induced a relaxation of 76.8±11.6% ($n=3$) (Figure 3d). In the presence or absence of GSNO (0.1 μM), $FeSO_4$ (1–3 μM) had no effect on the $PGF_{2\alpha}$-induced contraction ($n=3$) (Figure 3d).

Effect of $CuSO_4$ on relaxations to NANC nerve stimulation, NO, GTN and ATP

Electrical stimulation (0.5–8 Hz) of the rat gastric fundus induced frequency-dependent relaxations (Figure 1 and 4), which were previously shown to be mediated by NO as they were abolished by N^G-nitro-L-arginine, a blocker of NO biosynthesis (Boeckxstaens *et al.*, 1991; De Man *et al.*, 1995b). The amplitude or the duration of the relaxations to electrical stimulation were not affected by $CuSO_4$ (3–30 μM) (Figure 4). Also in the absence of CaEDTA, $CuSO_4$ (3–30 μM) had no effect on relaxations to ES (0.5–8 Hz, $n=4$) (results not shown). Authentic NO and GTN both induced concentration-dependent relaxations (Figures 1 and 4). Relaxations to NO (3–100 nM) and GTN (0.01–1 μM) were not affected by 3–10 μM $CuSO_4$ but significantly inhibited by 30 μM $CuSO_4$ (Figure 4). Relaxations to 10 μM ATP were not affected by $CuSO_4$ (3–30 μM ($n=8$). $CuSO_4$ (3–300 μM) had no effect on the basal tension of the muscle strips or on the contraction to $PGF_{2\alpha}$.

Effect of copper chelation on relaxations to S-nitrosothiols

Relaxations to NOCys (0.01–3 μM), GSNO (0.01–10 μM) and SNAP (0.01–3 μM) were significantly and concentration-dependently inhibited by the Cu^{2+} chelator bathocuproine (3–30 μM) (Figure 5). Incubation of the muscle strips for 10 min with 3 μM $CuSO_4$ partly reversed the inhibitory effect of 10 μM bathocuproine on the relaxations to NOCys and GSNO whereas the relaxations to SNAP were enhanced as compared to control values (Figure 6).

Effect of copper chelation on relaxations to NANC nerve stimulation, NO, GTN and ATP

The Cu^{2+} chelator, bathocuproine (3–30 μM), had no effect on the amplitude or the duration of the relaxations to ES (0.5–8 Hz) or on the concentration-response curve to NO (10–

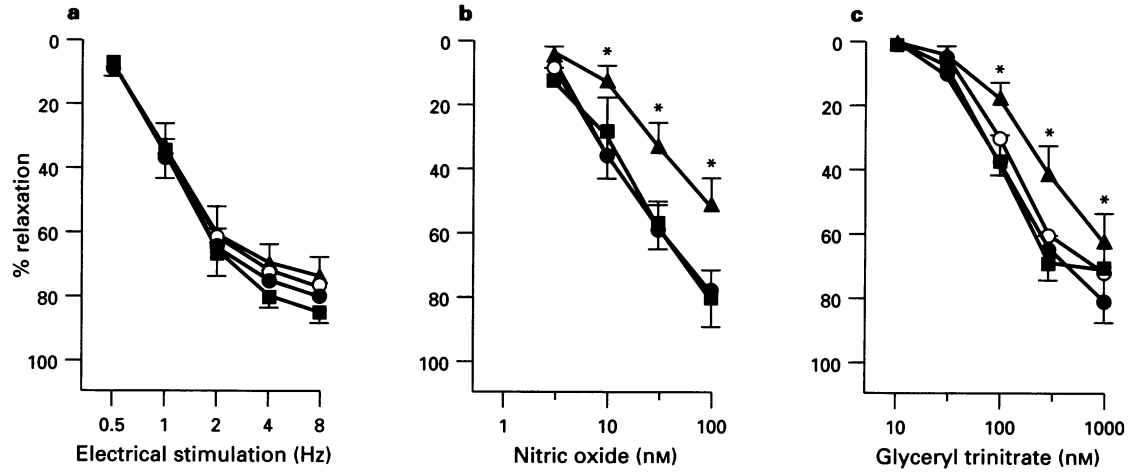

Figure 4 (a) Frequency-response curves to electrical stimulation (1 ms pulses at 0.5–8 Hz for 10 s periods) and concentration-response curves to (b) nitric oxide (3–100 nM) and (c) glyceryl trinitrate (10–1000 nM) in control conditions (○) and in the presence of $CuSO_4$ (●, 3 μM; ■, 10 μM and ▲, 30 μM). Results are expressed as percentage decreases of the $PGF_{2\alpha}$-induced contraction and shown as mean±s.e.mean for $n=6-7$ experiments. $^*P<0.05$ is considered as significantly different from control.

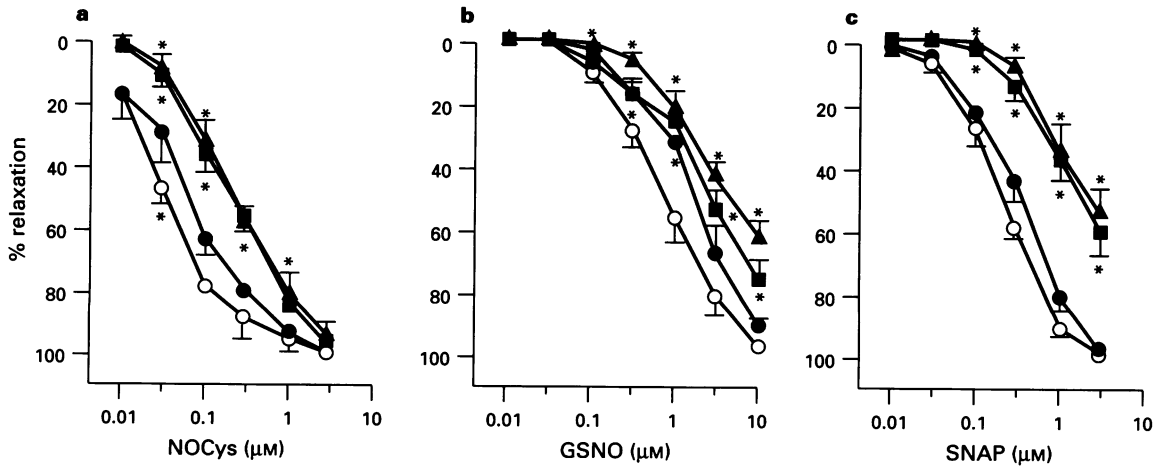

Figure 5 Concentration-response curves to (a) S-nitroso-L-cysteine (0.01–3 μM, NOCys), (b) S-nitrosoglutathione (0.01–10 μM, GSNO) and (c) S-nitroso-N-acetyl-D,L-penicillamine (0.01–3 μM, SNAP) in control conditions (○) and in the presence of bathocuproine (●, 3 μM; ■, 10 μM and ▲, 30 μM). Results are expressed as percentage decrease of the $PGF_{2\alpha}$-induced contraction and shown as mean±s.e.mean for $n=6-9$ experiments. $^*P<0.05$ is considered as significantly different from control.

300 nM) (Figure 7). Relaxations to GTN (0.01–1 μM) were not affected by 3–10 μM bathocuproine but were significantly inhibited by 30 μM bathocuproine (Figure 7). Bathocuproine (3–30 μM) did not affect the basal tension of the muscle strips, the contraction to $PGF_{2\alpha}$ or the relaxations to 10 μM ATP.

Discussion

Although there is compelling evidence that NO is a mediator of NANC neurotransmission in the gastrointestinal tract, there is controversy about the exact identity of the nitrergic NANC neurotransmitter. Superoxide generators and NO-scavengers differentially affect relaxations to NANC nerve stimulation and to authentic NO, suggesting that the actual nitrergic NANC neurotransmitter is not free NO but a superoxide anion-resistant, NO-releasing molecule, such as a S-nitrosothiol. As it was reported that the biological activity of S-nitrosothiols can be modulated by copper ions (Askew *et al.*, 1995; Gordge *et al.*, 1995), we investigated the effect of copper ions on relaxations to S-nitrosothiols and on relaxations to NANC nerve stimulation of the rat gastric fundus. In this study, we have demonstrated that copper ions have a differential effect on relaxations to S-nitrosothiols as compared to relaxations to the nitrergic neurotransmitter and to NO, suggesting that the nitrergic NANC neurotransmitter in the rat gastric fundus is not an S-nitrosothiol.

Indeed, although $CuSO_4$ concentration-dependently enhanced the relaxations to the S-nitrosothiols, NOCys, SNAP and GSNO, it did not affect the relaxations to NANC nerve stimulation and at higher concentrations $CuSO_4$ even inhibited the relaxations to NO and GTN. Conversely, the Cu^{2+} chelator, bathocuproine, concentration-dependently reduced the relaxations to NOCys, SNAP and GSNO whereas relaxations to NANC nerve stimulation and NO were not affected. This inhibitory effect resulted from chelation of Cu^{2+}-ions as addition of $CuSO_4$ restored the relaxant effect of the S-nitrosothiols. These results illustrate that alterations of the Cu^{2+} concentration in the tissue differentially affect relaxations to S-nitrosothiols as compared to those nitrergic stimulation and to NO. These results, which suggest that the nitrergic NANC neurotransmitter in the rat gastric fundus is not an S-nitrosothiol, are in agreement with our previous findings in the canine ileocolonic junction were the nitrergic neurotransmitter behaved pharmacologically like NO and not like an S-nitrosothiol (Boeckxstaens *et al.*, 1994; De Man *et al.*,

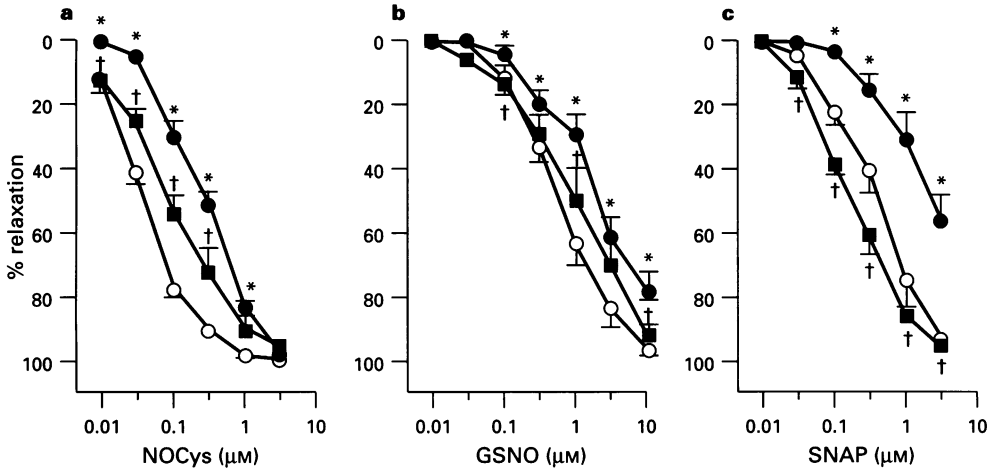

Figure 6 Effect of $CuSO_4$ (■, 3 μM) on the inhibitory effect of bathocuproine (●, 10 μM) on the relaxations to (a) S-nitroso-L-cysteine (○, 0.01–3 μM, NOCys), (b) S-nitrosoglutathione (○, 0.01–10 μM, GSNO) and (c) S-nitroso-N-acetyl-D,L-penicillamine (○, 0.01–3 μM, SNAP). Results are expressed as percentage decrease of the $PGF_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n=5-6$ experiments. $^*P<0.05$ is significantly different from control value, †$P<0.05$ is significantly different from value after bathocuproine.

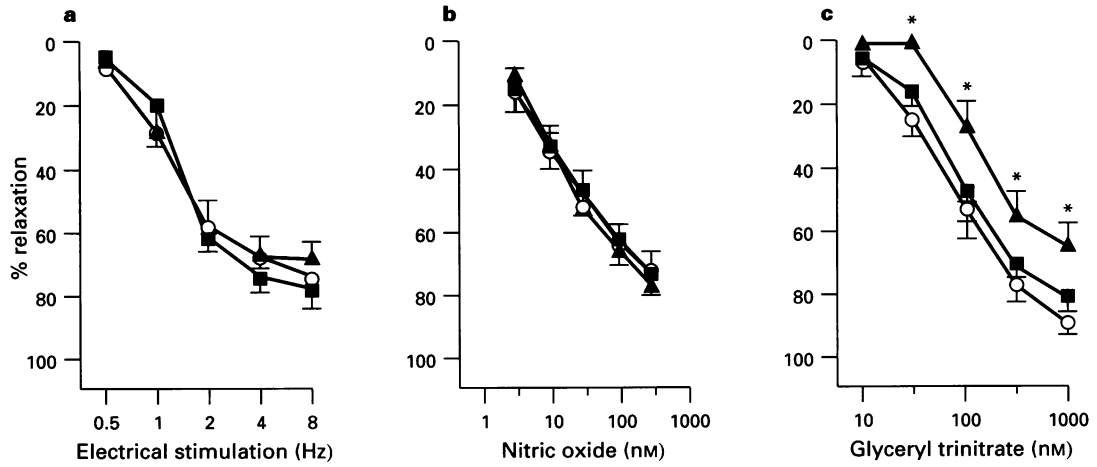

Figure 7 (a) Frequency-response curves to electrical stimulation (1 ms pulses at 0.5–8 Hz for 10 s periods) and concentration-response curves to (b) nitric oxide (1–300 nM) and (c) glyceryl trinitrate (10–1000 nM) in control conditions (○) and in the presence of bathocuproine (●, 3 μM; ■, 10 μM and ▲, 30 μM). Results are expressed as percentage decrease of the $PGF_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n=6-10$ experiments. $^*P<0.05$ is considered as significantly different from control.

1995c). Similar findings were reported for the nitrergic neurotransmitter in the guinea-pig caecum and colon (Wiklund *et al.*, 1993; Iversen *et al.*, 1994) and the bovine retractor penis muscle (Martin *et al.*, 1994) and for the endothelium-derived relaxing factor (Feelisch *et al.*, 1994).

The decomposition of S-nitrosothiols by $CuSO_4$ most likely results from the reaction of Cu^{2+} with the sulphur group of the S-nitrosothiol: it was previously reported that Cu^{2+} decreases the sulphydryl groups of albumin (Di Simplicio *et al.*, 1991) and that Cu^{2+} catalyzes the release of NO from S-nitrosothiols (Askew *et al.*, 1995). Interestingly, we found that $CuSO_4$ itself had a relaxant effect on the rat gastric fundus only in the presence of a low concentration of an S-nitrosothiol. This relaxation was blocked by the radical generator pyrogallol, which potently inhibits relaxations to NO but not those to S-nitrosothiols (De Man *et al.*, 1995a,c) illustrating that $CuSO_4$ induces relaxations by catalysing the release of NO from S-nitrosothiols. As such, Cu^{2+} will have a more pronounced effect on stable S-nitrosothiols. As it was reported that GSNO is more stable than NOCys (Mathews & Kerr, 1993), this might explain our observation that $CuSO_4$ reversed the relaxant potency of the S-nitrosothiols from NOCys > SNAP > GSNO in the absence of $CuSO_4$ to GSNO ≈ SNAP > NOCys in the presence of $CuSO_4$. The effect of $CuSO_4$ was mimicked by $CuCl_2$ but not by $FeSO_4$. These results are in agreement with the observation that copper ions but not iron ions decrease the free thiol groups of albumin (Di Simplicio *et al.*, 1991) and provide evidence that the Cu^{2+} ion but not the SO_4^{2-} ion catalysed the release of NO from S-nitrosothiols.

In contrast to its effect on relaxations to S-nitrosothiols, $CuSO_4$ did not affect the relaxations to nitrergic stimulation. Relaxations to NO and GTN were inhibited, although only at the highest concentration of $CuSO_4$. Similarly, Cu^{2+} inhibited the relaxations to GTN in isolated coronary arteries of the dog (Kamitani, 1984). Relaxations to GTN depend on the availability of intracellular thiols (Needleman *et al.* 1973) and S-nitrosothiols are intermediate compounds in GTN metabolism (Ignarro *et al.*, 1981). Therefore, copper ions most likely inhibit relaxations to GTN by interfering with the binding of NO on the sulphydryl group of the thiol (Askew *et al.*, 1995) thus preventing the formation of intracellular S-nitrosothiols. However, the mechanism by which $CuSO_4$ inhibited the relaxations to free NO is less clear. In bovine lung cells, it was recently demonstrated that $CuSO_4$ inhibited the activity of guanylate cyclase (Schrammel *et al.*, 1995) which is the main target for NO. However, in our study we found that relaxations to NANC nerve stimulation, which are guanylate cyclase-dependent (Barbier & Lefebvre, 1992a), were not affected by $CuSO_4$ suggesting that the muscle strip was still able to relax properly. Alternatively, copper might reduce the biological activity of NO by a direct interaction with the redox state of the NO radical as suggested by Gordge *et al.* (1995). However, addition of $CuSO_4$ in a 1/1 molar ratio to the airtight and oxygen-free stock solution of NO, did not change the relaxant properties of this NO solution (De Man *et al.*, unpublished observations). Finally, copper ions might also react with the oxygen in the Krebs-Ringer solution to form highly reactive hydroxyl or superoxide radicals. Superoxide radicals potently inhibit relaxations to exogenous NO without affecting those to NANC nerve stimulation of the rat gastric fundus (De Man *et al.*, 1995a). This might explain why $CuSO_4$ inhibited the relaxations to free NO but not those to NANC nerve stimulation as superoxide donors inhibit nitrergic NANC relaxations only after inhibition of endogenous superoxide-dismutase (Martin *et al.*, 1994; Lilley & Gibson, 1995; De Man *et al.*, 1995a).

The Cu^{2+} chelator, bathocuproine, inhibited the relaxations to S-nitrosothiols without affecting those to NO or NANC nerve stimulation. These results suggest that removal of Cu^{2+} decreases the biological activity of S-nitrosothiols most likely by inhibiting the copper-catalysed release of NO from S-nitrosothiols. Surprisingly, relaxations to GTN were also inhibited by bathocuproine although only at the highest concentration used. This inhibition was unexpected as we also found that addition of copper inhibited the relaxations to GTN. A possible explanation for this discrepancy is that relaxations to GTN are inhibited by depletion of thiols (De Man *et al.*, 1995a) which is in accordance with the hypothesis that these relaxations depend on the intracellular formation of nitrosothiols (Ignarro *et al.*, 1981). As such, as shown in this study, chelation to Cu^{2+} reduces the biological activity of S-nitrosothiols, resulting in an inhibition of the relaxations to GTN.

In conclusion, we have demonstrated that in the rat gastric fundus, addition or chelation of Cu^{2+} does not affect the nitrergic relaxations to NANC nerve stimulation whereas the relaxations to the S-nitrosothiols NOCys, GSNO and SNAP were enhanced by addition of Cu^{2+} and inhibited by chelation of Cu^{2+}. From these results we conclude that Cu^{2+} has a differential effect on the biological activity of S-nitrosothiols and on that of the nitrergic NANC neurotransmitter in the rat gastric fundus, suggesting that the nitrergic neurotransmitter of the rat gastric fundus is not NOCys, GSNO or SNAP but is more likely to be free NO.

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References

ASKEW, S.C., BARNETT, D.J., MCANINLY, J. & WILLIAMS, D.L.H. (1995). Catalysis by Cu^{++} of nitric oxide release from S-nitrosothiols (RSNO). *J. Chem. Soc. Perkin Trans.*, **2**, 741–745.

BARBIER, A.J. & LEFEBVRE, R.A. (1992a). Effect of 3-isobutyl-1-methylxanthine and zaprinast on non-adrenergic non-cholinergic relaxation in the rat gastric fundus. *Eur. J. Pharmacol.*, **210**, 315–323.

BARBIER, A.J.M. & LEFEBVRE, R.A. (1992b). Effect of LY83583 on relaxation induced by non-adrenergic non-cholinergic nerve stimulation and exogenous nitric oxide in the rat gastric fundus. *Eur. J. Pharmacol.*, **219**, 331–334.

BARBIER, A.J.M. & LEFEBVRE, R.A. (1994). Influence of S-nitrosothiols and nitrate tolerance in the rat gastric fundus. *Br. J. Pharmacol.*, **111**, 1280–1286.

BOECKXSTAENS, G.E., DE MAN, J.G., DE WINTER, B.Y., HERMAN, A.G. & PELCKMANS, P.A. (1994). Pharmacological similarity between nitric oxide and the nitrergic neurotransmitter in the canine ileocolonic junction. *Eur. J. Pharmacol.*, **264**, 85–89.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BOGERS, J.J., BULT, H., DE MAN, J.G., OOSTERBOSCH, L., HERMAN, A.G. & VAN MAERCKE, Y.M. (1991). Release of nitric oxide upon stimulation of nonadrenergic noncholinergic nerves in the rat gastric fundus. *J. Pharmacol. Exp. Ther.*, **256**, 441–447.

BOECKXSTAENS, G.E., PELCKMANS, P.A., BULT, H., DE MAN, J.G., HERMAN, A.G. & VAN MAERCKE, Y.M. (1990). Non-adrenergic non-cholinergic relaxation mediated by nitric oxide in the canine ileocolonic junction. *Eur. J. Pharmacol.*, **190**, 239–246.

BULT, H., BOECKXSTAENS, G.E., PELCKMANS, P.A., JORDAENS, F.H., VAN MAERCKE, Y.M. & HERMAN, A.G. (1990). Nitric-oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. *Nature*, **345**, 346–347.

DE MAN, J.G., BOECKXSTAENS, G.E., DE WINTER, B.Y., HERMAN, A.G. & PELCKMANS, P.A. (1995a). Effect of thiol and superoxide dismutase depletion on non adrenergic non cholinergic responses in the rat gastric fundus. *Neurogastroenterol. Motil.*, **7**, 254.

DE MAN, J.G., BOECKXSTAENS, G.E., DE WINTER, B.Y., MOREELS, T.G., HERMAN, A.G. & PELCKMANS, P.A. (1995b). Inhibition of non-adrenergic non-cholinergic relaxations by nitric oxide donors. *Eur. J. Pharmacol.*, **285,** 269–274.

DE MAN, J.G., BOECKXSTAENS, G.E., DE WINTER, B.Y., MOREELS, T.G., MISSET, M.E., HERMAN, A.G & PELCKMANS, P.A. (1995c). Comparison of the pharmacological profile of S-nitrosothiols, nitric oxide and the nitrergic neurotransmitter in the canine ilecolonic junction. *Br. J. Pharmacol.*, **114,** 1179–1184.

DI SIMPLICIO, P., CHEESEMAN, K.H. & SLATER, T.F. (1991). The reactivity of the SH group of bovine serum albumin with free radicals. *Free Rad. Res. Commun.*, **14,** 253–262.

FEELISCH, M., TE POEL, M., ZAMORA, R., DEUSSEN, A. & MONCADA, S. (1994). Understanding the controversy over the identity of EDRF. *Nature*, **368,** 62–65.

GIBSON, A., BABBEDGE, R.C., BRAVE, S.R., HART, S.L., HOBBS, A.J., TUCKER, J.F., WALLACE, P. & MOORE, P.K. (1992). An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus. *Br. J. Pharmacol.*, **107,** 715–721.

GILLESPIE, J.S. & SHENG, H. (1990). The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and the bovine receptor penis muscles. *Br. J. Pharmacol.*, **99,** 194–196.

GORDGE, M.P., MEYER, D.J., HOTHERSALL, J., NEILD, G.H., PAYNE, N.N. NORONHA-DUTRA, A. (1995). Copper chelation-induced reduction of the biological activity of S-nitrosothiols. *Br. J. Pharmacol.*, **114,** 1083–1089.

HOBBS, A.J., TUCKER, J.F. & GIBSON, A. (1991). Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle: role of superoxide anions. *Br. J. Pharmacol.*, **104,** 645–650.

IGNARRO, L.J., LIPPTON, H., EDWARDS, J.C., BARICOS, W.H., HYMAN, A.L., KADOWITZ, P.J. & GRUETTER, C.A. (1981). Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of S-nitrosothiols as active intermediates. *J. Pharmacol. Exp. Ther.*, **218,** 739–749.

IVERSEN, H.H., GUSTAFSSON, L.E., LEONE, A.M. & WILKLUND, N.P. (1994). Smooth muscle relaxing effects of NO, nitrosothiols and a nerve-induced relaxing factor released in guinea-pig colon. *Br. J. Pharmacol.*, **113,** 1088–1092.

JENKINSON, K.M., REID, J.J. & RAND, M.J. (1995). Hydroxocobalamin and haemoglobin differentiate between exogenous and neuronal nitric oxide in the rat gastric fundus. *Eur. J. Pharmacol.*, **275,** 145–152.

KAMITANI, T. (1984). Inhibitory effect of Cu^{2+} on nitroglycerin-induced vasodilatation. *Eur. J. Pharmacol.*, **100,** 145–154.

KELM, M., FEELISCH, M., SPAHR, R., PIPER, H.M., NOACK, E. & SCHRADER, J. (1988). Quantitative and kinetic characterization of nitric oxide and EDRF released from cultured endothelial cells. *Biochem. Biophys. Res. Commun.*, **154,** 236–244.

KERR, S.W., BUCHANAN, L.V., BUNTING, S. & MATHEWS, W.R. (1992). Evidence that S-nitrosothiols are responsible for the smooth muscle relaxing activity of the bovine retractor penis inhibitory factor. *J. Pharmacol. Exp. Ther.*, **263,** 285–292.

KITAMURA, K., LIAN, Q., CARL, A. & KURIYAMA, H. (1993). S-Nitrosocysteine, but not sodium nitroprusside, produces apamin-sensitive hyperpolarization in rat gastric fundus. *Br. J. Pharmacol.*, **109,** 415–423.

LILLEY, E. & GIBSON, A. (1995). Inhibition of relaxations to nitrergic stimulation of the mouse anococcygeus by duroquinone. *Br. J. Pharmacol.*, **116,** 3231–3236.

LIU, X., GILLESPIE, J.S. & MARTIN, W. (1994). Non-adrenergic non-cholinergic relaxation of the bovine retractor penis muscle: role of S-nitrosothiols. *Br. J. Pharmacol.*, **111,** 1287–1295.

MARTIN, W., MCALLISTER, K.H.M. & PAISLEY, K. (1994). NANC neurotransmission in the bovine retractor penis muscle is blocked by superoxide anion following inhibition by superoxide dismutase with diethyldithiocarbamate. *Neuropharmacology*, **33,** 1293–1301.

MATHEWS, W.R. & KERR, S.W. (1993). Biological activity of S-nitrosothiols: the role of nitric oxide. *J. Pharmacol. Exp. Ther.*, **267,** 1529–1537.

NEEDLEMAN, P., JAKSCHIK, B. & JOHNSON, E.M. JR. (1973). Sulfhydryl requirement for relaxation of vascular smooth muscle. *J. Pharmacol. Exp. Ther.*, **187,** 324–331.

PELCKMANS, P.A., BOECKXSTAENS, G.E., VAN MAERCKE, Y.M., HERMAN, A.G. & VERBEUREN, T.J. (1989). Acetylcholine is an indirect inhibitory transmitter in the canine ileocolonic junction. *Eur. J. Pharmacol.*, **170,** 235–242.

RAND, M.J. & LI, C.G. (1995a). Discrimination by the NO-trapping agent, carboxy-PTIO, between NO and the nitrergic transmitter but not between NO and EDRF. *Br. J. Pharmacol.*, **116,** 1906–1910.

RAND, M.J. & LI, C.G. (1995b). Nitric oxide as a neurotransmitter in peripheral nerves: nature of transmitter and mechanism of transmission. *Annu. Rev. Physiol.*, **57,** 659–682.

SANDERS, K.M. & WARD, S.M. (1992). Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol.*, **262,** G379–G392.

SCHRAMMEL, A., KLATT, P., SCHMIDT, K., KOESLING, D. & MAYER, B. (1995). Inhibition of nitric oxide-stimulated purified soluble guanylyl cyclase by copper ions. *Endothelium*, **3,** S42.

STARK, M.E. & SZURSZEWSKI, J.H. (1992). Role of nitric oxide in gastrointestinal hepatic function and disease. *Gastroenterology*, **103,** 1928–1949.

THORNBURY, K.D., WARD, S.M., DALZIEL, H.H., CARL, A., WESTFALL, D.P. & SANDERS, K.M. (1991). Nitric oxide and nitrosocysteine mimic non adrenergic non cholinergic hyperpolarization in canine proximal colon. *Am. J. Physiol.*, **261,** G553–G557.

WIKLUND, N.P., LEONE, A.M., GUSTAFFSON, L.E. & MONCADA, S.M. (1993). Release of nitric oxide evoked by nerve stimulation in guinea-pig intestine. *Neuroscience*, **53,** 607–611.

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