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Neocuproine potentiates the activity of the nitrergic neurotransmitter but inhibits that of *S*-nitrosothiols

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Abstract

In the present study, we investigated the cellular components that are involved in the release of nitric oxide (NO) from *S*-nitrosothiols and whether these components also modulate the activity of the nitrergic neurotransmitter in the rat gastric fundus. Electrical stimulation of nitrergic nerves induced frequency-dependent transient relaxations which were mimicked by exogenous NO. The *S*-nitrosothiols *S*-nitrosocysteine, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine induced concentration-dependent relaxations which were generally more sustained as compared to those to nitrergic nerve stimulation or NO. The relaxations to nitrergic nerve stimulation and those to NO were significantly enhanced by the copper(I) chelator neocuproine but not affected by the copper(II) chelator cuprizone. The relaxations to the *S*-nitrosothiols were significantly inhibited by neocuproine but not by cuprizone. The antioxidant ascorbate did not affect the tension of the muscle strip. However, in the presence of an *S*-nitrosothiol, ascorbate induced an immediate, sharp and transient relaxation that was significantly inhibited by a low concentration of neocuproine but not by cuprizone. Ascorbate did not induce a relaxation during short-train or prolonged nerve stimulation of the muscle strip. These results suggest that ascorbate interacts with copper to modulate the biological activity of *S*-nitrosothiols but not that of the nitrergic neurotransmitter. The differential effect of neocuproine indicates that *S*-nitrosothiols do not mediate the nitrergic neurotransmission of the rat gastric fundus. As neocuproine is to date the only compound that exerts an opposite effect on the biological activity of the nitrergic neurotransmitter and on that of *S*-nitrosothiols, it may be useful to elucidate the nature of the nitrergic neurotransmitter in the peripheral nervous system.

Keywords: Antioxidant; Ascorbate; Copper; Nitrergic neurotransmission; Nitric oxide (NO); *S*-nitrosothiol

1. Introduction

Nitric oxide (NO) is an important mediator of smooth muscle relaxation in the peripheral nervous system (Boeckxstaens et al., 1990; Bult et al., 1990; for review, see Rand and Li, 1995). However, NO has a short half life which may limit the efficacy and duration of its biological activity (Wood and Garthwaite, 1994). It was therefore suggested that NO may bind intracellularly to a carrier molecule resulting in a more stable NO-containing compound from which NO is released at its site of action. Thiols are likely candidates for such an NO-carrier molecule: they are abundantly present in cells, either as a free thiol or as part of a protein structure. In a cell free system, thiols react with NO or oxides of nitrogen to form *S*-nitrosothiols. Endogenous *S*-nitrosothiols may act as intermediates in the storage and/or transport of NO and consequently enhance the biological potency of NO. Recently, Gordge et al. (1998) suggested that a cell-mediated mechanism for the biotransformation of *S*-nitrosoglutathione is present in a variety of cell types. In addition, *S*-nitrosoglutathione was suggested to be a key molecule in the NO/cGMP signal transduction pathway of bovine lung and rat heart (Mayer et al., 1998) and in the perinatal circulatory transition of stressed newborns (Gaston et al., 1998a,b). Therefore, *S*-nitrosothiols may be involved in the endothelium-dependent, NO-mediated responses in the vasculature. In addition, Goçmen et al. (1998) recently suggested that the relaxant factor released from non-adrenergic non-cholinergic (NANC) nerves in mouse cavernosal tissue is an *S*-nitrosothiol. In the gastrointestinal tract,

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S-nitrosothiols are potent relaxant factors but the role of *S*-nitrosothiols as endogenous mediators of the NO-mediated NANC neurotransmission is controversial (for reviews, see Gibson et al., 1995; Rand and Li, 1995). Possible pathways for the intracellular formation of *S*-nitrosothiols have been proposed (Kharitonov et al., 1995; Gow et al., 1997; Liu et al., 1998) but the cellular components that are involved in the endogenous release of NO from *S*-nitrosothiols remain unclear. As *S*-nitrosothiols may also have therapeutic potential as NO-releasing compounds, it is important to understand the cellular mechanisms involved in NO release from these compounds. In the present study, we investigated which cellular components are involved in the endogenous release of NO from *S*-nitrosothiols in the rat gastric fundus. To elucidate the nature of the nitrergic neurotransmitter in this tissue, we investigated whether these components also modulate the nitrergic neurotransmission in the rat gastric fundus.

2. Materials and methods

2.1. Tissue preparation

Male Wistar rats (250–300 g) were fasted for 24 h with free access to water. The animals were anaesthetised with diethyl ether and exsanguinated from the carotid artery. A laparotomy was performed and the stomach was removed and cut open. After removal of the mucosal layer by sharp dissection, three longitudinal muscle strips of ≈ 10 mm long and 3 mm wide were cut from the gastric fundus. The muscle strips were mounted in organ baths (25 ml) that were filled with Krebs–Ringer solution (1.2 mM KH_2PO_4, 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 11 mM glucose). The solution was maintained at 37°C and aerated with a mixture of 95% O_2 and 5% CO_2.

2.2. 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 (Scaime GM2, France) 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.

2.3. Experimental protocols

All experiments were performed on muscle strips contracted with 0.3 μM prostaglandin $F_{2\alpha}$ and in the presence of 1 μM atropine and 30 μM guanethidine. After finishing

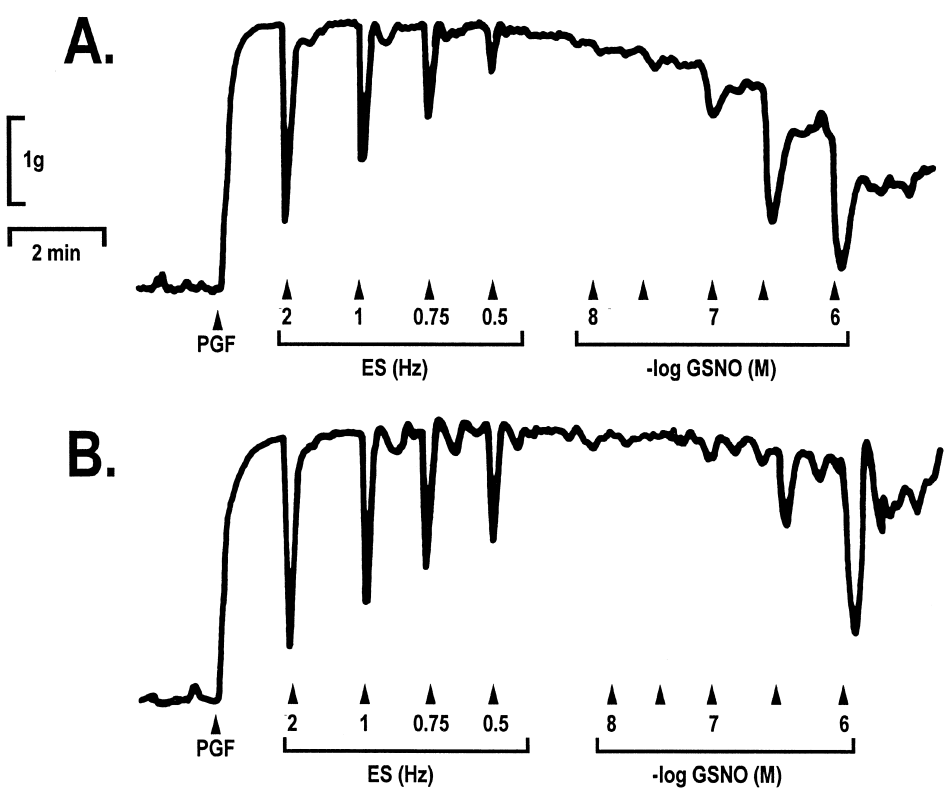

Fig. 1. Typical tracings of the rat gastric fundus contracted with prostaglandin $F_{2\alpha}$ (PGF, 0.3 μM) showing the relaxations induced by electrical stimulation (ES, 0.5–2 Hz, pulses of 10 ms during 10 s) and by *S*-nitrosoglutathione (GSNO, 0.01–1 μM) in (A) control conditions and (B) in the presence of neocuproine (10 μM).

each protocol, the muscle strips were washed at least three times with intervals of 5 min.

We first studied the effect of the copper(I)-specific chelator neocuproine and the copper(II)-specific chelator cuprizone on the frequency–response curve to nitrergic nerve stimulation (0.5–2 Hz, 1 ms, pulse trains of 10 s) and on the concentration–response curve to NO, applied as acidified $NaNO_2$. Then the effect of the copper chelators was studied on the concentration–response curves to the *S*-nitrosothiols *S*-nitrosocysteine, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine. Secondly, the effect of the copper chelators was investigated on the relaxations induced by ascorbate in the presence of an *S*-nitrosothiol. The incubation time of the copper chelators was 10 min except for the experiments where the immediate effect of neocuproine was investigated.

All experiments were performed in parallel with muscle strips that served as time controls receiving saline instead of copper chelators. The relaxations to electrical stimulation, $NaNO_2$, *S*-nitrosocysteine, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetyl penicillamine remained constant over the time course of the experiment.

2.4. Drugs used

The following drugs were used: L-ascorbic acid, atropine sulphate, sodium nitrite (Merck, Darmstadt, Germany); guanethidine monosulphate (Ciba Geigy, Switzerland); *N*-acetyl-D,L-penicillamine, cuprizone, L-cysteine, neocuproine, reduced glutathione, α-tocopherol (Sigma, St. Louis, MO, USA), prostaglandin $F_{2\alpha}$ (Dinolytic® purchased from Upjohn, Puurs, Belgium as a sterile aqueous solution containing 5 mg ml^{11} prostaglandin $F_{2\alpha}$ and 9 mg ml^{11} benzyl alcohol). α-tocopherol was dissolved in dimethylsulfoxide (DMSO). A control solution of DMSO had no effect on its own. Stock solutions of ascorbate, α-tocopherol and *S*-nitrosothiols were prepared freshly on the day of experimentation. *S*-nitrosothiols were synthetised as described previously (De Man et al., 1996a) and kept sealed on ice under argon in the dark. Dilutions of the stock solutions of ascorbate, α-tocopherol and *S*-nitrosothiols were made freshly before each experiment and were used immediately after dilution. A stock solution of 0.1 mM $NaNO_2$ was prepared freshly in distilled water on the day of experimentation. Appropriate dilutions of the stock solution were made and immediately before experimentation the solutions were adjusted to pH 3 with 5 mM HCl. A control solution of acidified distilled water did not affect the tension of the rat gastric muscle strip.

2.5. 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 muscle strip. Values are shown as mean ± S.E.M. for *n* indicating the number of rats used. Statistical significance of differences among values was analysed with one-way analysis of variance followed by Dunnett's test for multiple comparisons with single control or with Student's *t*-test for paired values when appropriate. *P* values of less than 0.05 were considered as significantly different from control.

3. Results

3.1. Effect of neocuproine and cuprizone on relaxations to nitrergic nerve stimulation, $NaNO_2$ and ATP

Short periods of electrical stimulation (0.5–2 Hz, 1 ms duration in trains of 10 s) of the rat gastric fundus, induced frequency-dependent relaxations (Figs. 1 and 2) which are nitrergic in nature as they are abolished by blockers of NO synthase (Boeckxstaens et al., 1991, 1992). These relaxations were mimicked by exogenous NO, applied as acidi-

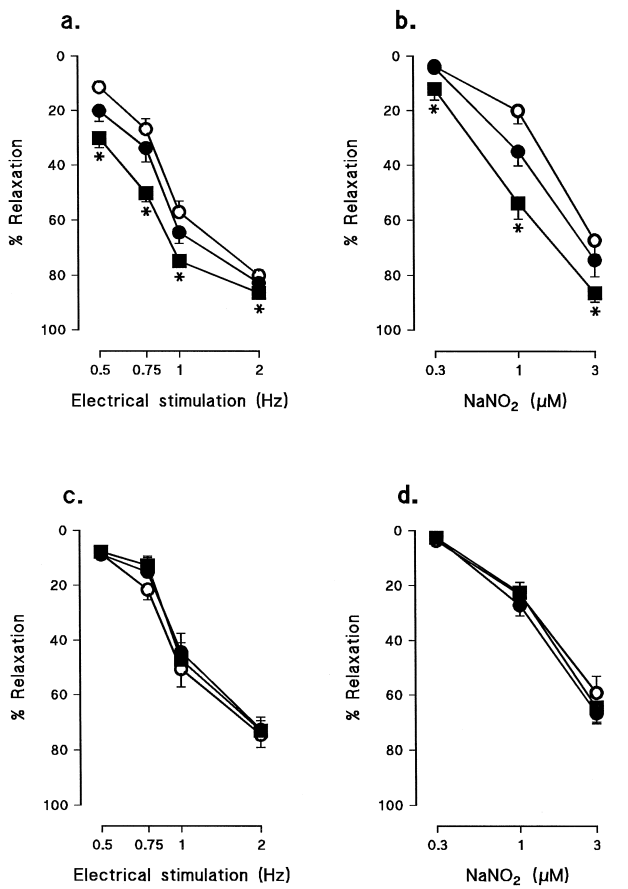

Fig. 2. Frequency–response curves to electrical stimulation (1 ms pulses at 0.5–2 Hz for 10 s periods) and concentration–response curves to NO, applied as acidified sodium nitrite (0.3–3 μM) in control conditions (○) and in the presence of 3–10 μM neocuproine (a and b; ●, 3 μM and ■, 10 μM) and in the presence of cuprizone (c and d; ●, 3 μM and ■, 10 μM). Results are expressed as percentage decreases of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± S.E.M. for $n = 5–7$ experiments. $^*P < 0.05$ is considered as significantly different from control, one-way ANOVA followed by Dunnett's test.

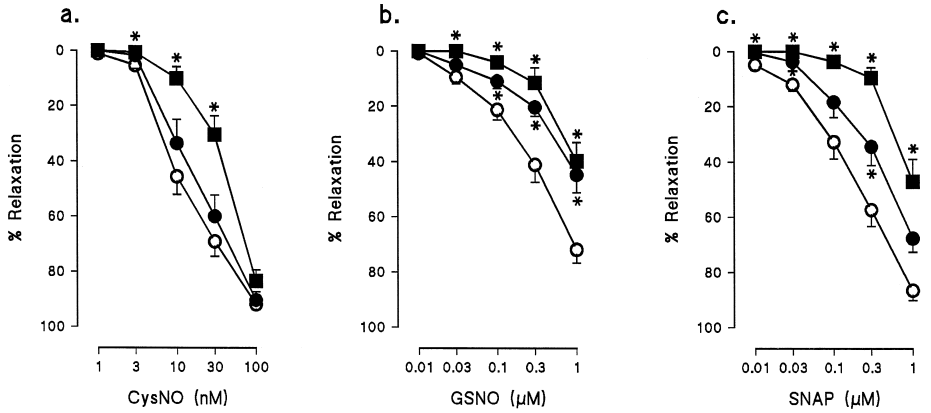

Fig. 3. Concentration–response curves to (a) *S*-nitrosocysteine (1–100 nM), (b) *S*-nitrosoglutathione (0.01–1 μM) and (c) *S*-nitroso-*N*-acetylpenicillamine (0.01–1 μM) in control conditions (○) and in the presence of neocuproine (●, 1 μM and ■, 10 μM). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± S.E.M. for $n = 7$ experiments. $^{*}P < 0.05$ is considered as significantly different from control, one-way ANOVA followed by Dunnett's test.

fied $NaNO_2$ (0.3–3 μM). The copper(I)-specific chelator neocuproine (3–10 μM) significantly enhanced the relaxations to nitrergic nerve stimulation and $NaNO_2$ as compared to control conditions (Figs. 1 and 2) whereas the copper(II)-specific chelator cuprizone (3–10 μM) had no effect (Fig. 2). Neocuproine or cuprizone did not affect the relaxations to ATP, which is a direct smooth muscle relaxant in the rat gastric fundus: relaxations to 10 μM ATP were 43 ± 6% in control conditions and 44 ± 6% in the presence of 10 μM neocuproine ($n = 5$) and 39 ± 6% in control conditions and 37 ± 2% in the presence of 10 μM cuprizone ($n = 5$). Neocuproine or cuprizone did not affect the contraction to prostaglandin $F_{2\alpha}$ or the basal tension of the rat gastric fundus strip.

3.2. Effect of neocuproine and cuprizone on relaxations to S-nitrosothiols

The *S*-nitrosothiols *S*-nitrosocysteine, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylpenicillamine induced concentration-dependent relaxations of the rat gastric fundus (Figs. 1 and 3). These relaxations were significantly and concentration-dependently inhibited by the copper(I)-specific chelator neocuproine (1–10 μM) (Figs. 1 and 3) but not by the copper(II)-specific chelator cuprizone (1–10 μM) (Fig. 4). The effect of neocuproine was immediate: when neocuproine (10 μM) was injected in the organ bath while the strip was regaining tone from a *S*-nitrosoglutathione-induced relaxation, the tension of the muscle strip immediately returned to the contraction level that was reached before addition of *S*-nitrosoglutathione (Fig. 5A). The same was observed for *S*-nitroso-*N*-acetylpenicillamine but not for *S*-nitrosocysteine as relaxations to *S*-nitrosocysteine were generally not sustained.

To mimic the relaxations to *S*-nitrosothiols, the muscle strip was stimulated continuously at 2 Hz, which induced a sustained relaxation. Ninety seconds after starting the stimulation, neocuproine (10 μM) was injected in the organ bath. In contrast to the relaxations to *S*-nitrosothiols,

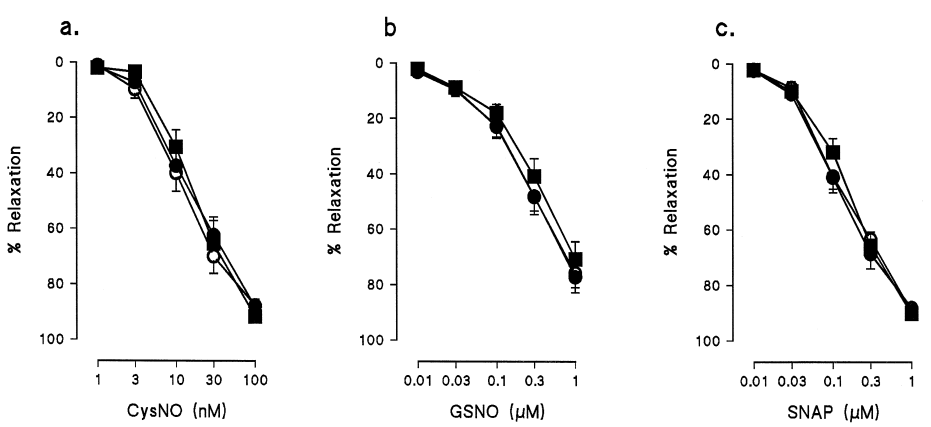

Fig. 4. Concentration–response curves to (a) *S*-nitrosocysteine (1–100 nM), (b) *S*-nitrosoglutathione (0.01–1 μM) and (c) *S*-nitroso-*N*-acetylpenicillamine (0.01–1 μM) in control conditions (○) and in the presence of cuprizone (●, 1 μM and ■, 10 μM). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± S.E.M. for $n = 7$ experiments. $^{*}P < 0.05$ is considered as significantly different from control, one-way ANOVA followed by Dunnett's test.

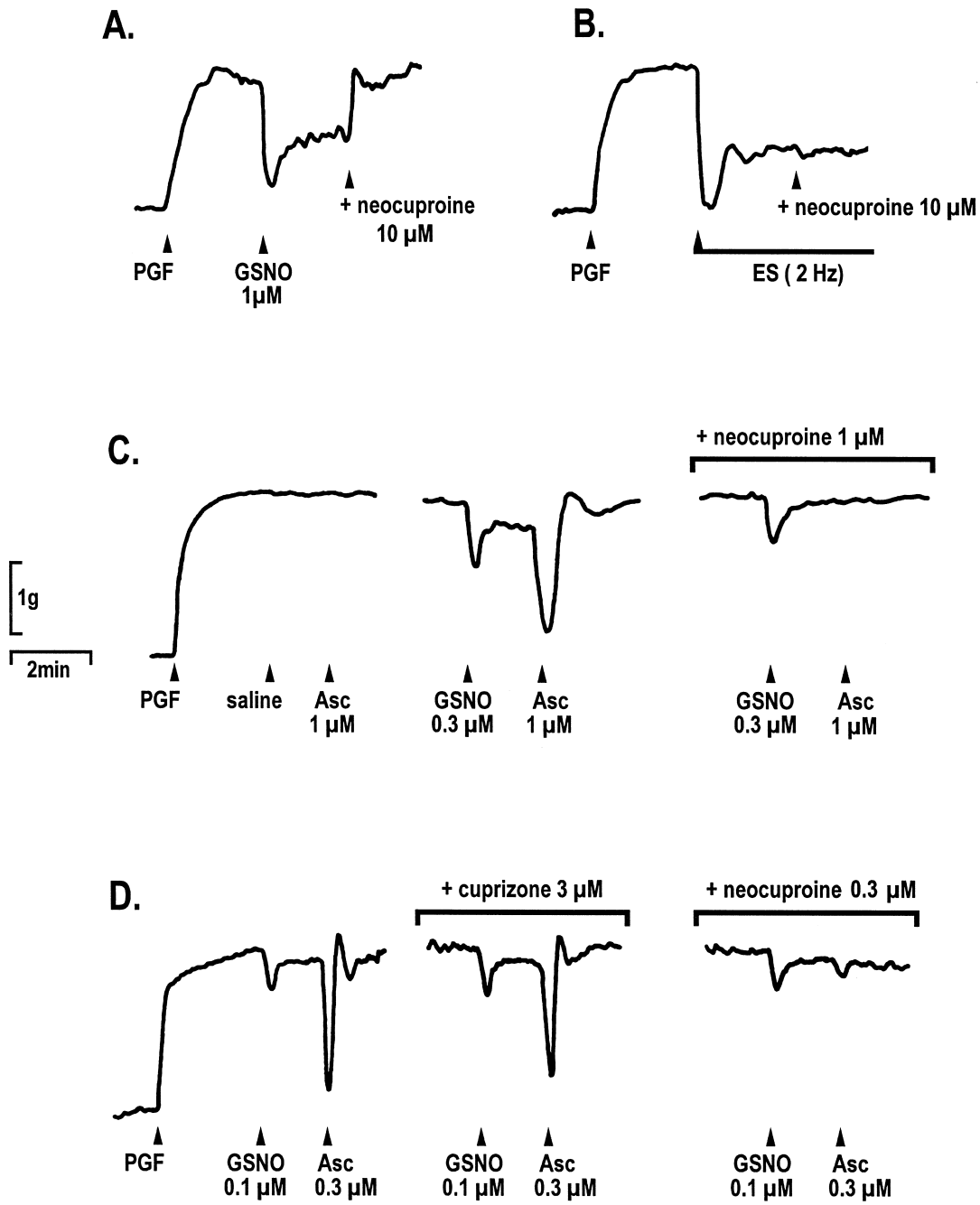

Fig. 5. Typical tracings of the rat gastric fundus strip contracted with prostaglandin $F_{2\alpha}$ (PGF, 0.3 µM) showing the effect of 10 µM neocuproine on the sustained relaxation to (A) *S*-nitrosoglutathione (GSNO, 1 µM) and (B) continuous electrical stimulation at 2 Hz. (C) Shows the effect of ascorbate (Asc) in the presence and absence of *S*-nitrosoglutathione and the effect of 1 µM neocuproine on the relaxation to ascorbate and on that to *S*-nitrosoglutathione. (D) Shows the effect of 3 µM cuprizone and 0.3 µM neocuproine on the relaxation to ascorbate in the presence of *S*-nitrosoglutathione.

neocuproine did not abolish this sustained relaxation to prolonged nerve stimulation (Fig. 5B, $n = 4$).

3.3. Effect of neocuproine and cuprizone on relaxations to ascorbate

In control conditions, the antioxidant ascorbate (0.1–100 µM) did not relax the rat gastric fundus muscle strip. However, in the presence of an *S*-nitrosothiol, ascorbate induced an immediate, sharp and transient relaxation (Fig. 5C): in the presence of 0.3 µM *S*-nitrosoglutathione, ascorbate (1 µM) induced a relaxation of $88 \pm 5\%$ ($n = 6$) that was abolished by the radical generators pyrogallol (0.3 µM) and duroquinone (0.3 µM) ($n = 4$ each, results not shown). The relaxation to ascorbate was significantly inhibited from $88 \pm 5\%$ to $4 \pm 2\%$ ($n = 6$) by the copper(I) chelator neocuproine (1 µM) (Fig. 5C) indicating that this relaxation is modulated by copper ions. However,

Table 1
Effect of neocuproine (0.3 μM) and cuprizone (3 μM) on the relaxation to 0.3 μM ascorbate that was observed in the presence of 0.1 μM *S*-nitrosoglutathione (GSNO) or 0.1 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP)
Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± S.E.M. for $n = 6$ experiments.

	GSNO	+ Ascorbate	SNAP	+ Ascorbate
Control	18 ± 2%	51 ± 8%	33 ± 5%	63 ± 5%
+ Neocuproine	16 ± 3%	5 ± 2%[a]	29 ± 4%	20 ± 2%[a]
Control	17 ± 2%	56 ± 7%	34 ± 5%	57 ± 4%
+ Cuprizone	21 ± 2%	52 ± 5%	35 ± 3%	52 ± 6%

[a] $P < 0.05$ is considered as significantly different from control, Student's *t*-test for paired values.

neocuproine also inhibited the initial relaxation to *S*-nitrosoglutathione from 52 ± 8% to 33 ± 6% ($n = 6$) (Fig. 5C). To exclude the possibility that the inhibition of the ascorbate-induced relaxation by neocuproine resulted from the inhibition of the initial relaxation to *S*-nitrosoglutathione, additional experiments were performed with a lower concentration of neocuproine. In these experiments, 0.3 μM ascorbate induced a sharp and transient relaxation in the presence of 0.1 μM *S*-nitrosoglutathione (Fig. 5D). This relaxation to ascorbate was significantly inhibited by 0.3 μM neocuproine whereas the initial relaxation to 0.1 μM *S*-nitrosoglutathione was not affected (Fig. 5D and Table 1). Similarly, 0.3 μM ascorbate induced a sharp relaxation in the presence of 0.1 μM *S*-nitroso-*N*-acetylpenicillamine. This relaxation to ascorbate was significantly inhibited by 0.3 μM neocuproine but the initial relaxation to *S*-nitroso-*N*-acetylpenicillamine was not affected (Table 1). Ascorbate (1 μM) did not induce a relaxation in the presence of SIN-1 (1 μM, $n = 4$), which is a non-thiol NO donor. Also on the sustained response to prolonged nerve stimulation at 2 Hz, ascorbate (1 μM) did not produce a relaxation ($n = 4$).

The copper(II) chelator cuprizone had no effect: cuprizone (3 μM) did not affect the relaxation to ascorbate (0.3 μM) or the initial relaxation to *S*-nitrosoglutathione or *S*-nitroso-*N*-acetyl penicillamine (Fig. 5D and Table 1).

The relaxation to ascorbate in the presence of an *S*-nitrosothiol was mimicked by the antioxidant α-tocopherol: in the presence of 0.1 μM *S*-nitroso-*N*-acetylpenicillamine, α-tocopherol (0.3 μM) induced a sharp relaxation of 40 ± 7% ($n = 4$) which was significantly inhibited to 14 ± 2% ($n = 4$) by neocuproine (0.3 μM) but not affected by cuprizone (3 μM, $n = 4$).

4. Discussion

In the present study on the rat gastric fundus, the copper(I) chelator neocuproine enhanced the relaxations to nitrergic nerve stimulation and to NO whereas the relaxations to *S*-nitrosothiols were inhibited. The copper(II) chelator cuprizone had no effect. To our knowledge, neocuproine is the only compound to date that exerts an opposite effect on relaxations to nitrergic nerve stimulation and on those to *S*-nitrosothiols which makes it a useful tool to differentiate between endogenous and exogenous NO. In many tissues, a number of compounds inhibited the activity of exogenous NO but not that of the nitrergic neurotransmitter suggesting that the nitrergic neurotransmitter might not be free NO (for reviews, see Gibson et al., 1995; Rand and Li, 1995). Alternatively, this discrepancy may result from the inability of exogenously added compounds to react with the endogenously released nitrergic transmitter. Martin et al. (1994) showed for instance that the nitrergic neurotransmitter is protected against breakdown by endogenously present antioxidants. La and Rand (1999), however, recently suggested that this protective mechanism may not fully explain the resistance of the nitrergic neurotransmitter to oxidative breakdown. This demonstrates the need for compounds that affect both the responses to endogenously released and exogenously added NO. In our study, the copper(I) chelator neocuproine was active towards both the endogenously released nitrergic neurotransmitter and the exogenously applied *S*-nitrosothiols: it had an opposite effect on these responses suggesting that *S*-nitrosothiols do not act as intermediates in the nitrergic neurotransmission in the rat gastric fundus. Neocuproine enhanced the relaxations to exogenous NO in a similar manner as those to nitrergic nerve stimulation indicating that the activity of the nitrergic neurotransmitter in this tissue resembles that of free NO. The underlying mechanism in which neocuproine enhances these relaxations is not clear. The auto-oxidation of copper(I) to copper(II) is associated with the formation of radicals which may persistently inhibit the biological activity of the nitrergic neurotransmitter and this may be prevented by removing copper(I) with neocuproine. However, it is expected that tissue antioxidants protect the nitrergic neurotransmitter against breakdown by superoxide radicals (Martin et al., 1994; De Man et al., 1996b; Lilley and Gibson, 1996). Alternatively, Schrammel et al. (1996) demonstrated that endogenous copper may inhibit the activity of guanylyl cyclase, which is the target enzyme of NO, and therefore, chelation of copper may result in an enhanced response to endogenously released and exogenously applied NO.

There is recent evidence that *S*-nitrosothiols are endogenously present and that they play a role as stable NO donors in the vasculature (Kharitonov et al., 1995; Gow et al., 1997; Gaston et al., 1998a,b; Gordge et al., 1998; Liu et al., 1998; Mayer et al., 1998). In the gastrointestinal tract, *S*-nitrosothiols are potent smooth muscle relaxants with therapeutic potential but the cellular mechanisms by which NO is released from *S*-nitrosothiols are largely unknown. We therefore investigated which cellular components are involved in the metabolism of *S*-nitrosothiols in the rat gastric fundus and whether these components also

affect the biological activity of the nitrergic neurotransmitter in this tissue.

The inhibitory effect of neocuproine on the relaxations to *S*-nitrosothiols was immediate: the sustained part of an *S*-nitrosothiol-induced relaxation was quickly abolished by neocuproine but not by cuprizone. This indicates that copper(I) and not copper(II) modulates the biological activity of *S*-nitrosothiols in the rat gastric fundus. In a cell free system, Dicks et al. (1996) previously also observed that neocuproine inhibits the copper-induced liberation of NO from *S*-nitrosothiols. Similarly, copper(I) is involved in the anti-platelet aggregation action of *S*-nitrosothiols (Gordge et al., 1996) and in the vasodilator responses to *S*-nitrosothiols in rat tail artery (Al-Sa'doni et al., 1997). In an aqueous solution, copper(I) is unstable and it is spontaneously oxidised to copper(II). In tissue however, endogenous antioxidants may prevent this oxidation suggesting that antioxidants, in association with copper, may play a role as endogenous modulators of *S*-nitrosothiol degradation. We found that the antioxidant ascorbate by itself did not affect the tension of the rat gastric fundus muscle. Also, in the presence of the non-thiol NO donor SIN-1, ascorbate did not induce a relaxation. However, in the presence of an *S*-nitrosothiol, ascorbate induced an immediate and transient relaxation. The relaxation to ascorbate in the presence of an *S*-nitrosothiol was inhibited by radical generators pyrogallol and duroquinone, which destroy NO, and also by the copper(I) chelator neocuproine: in higher concentrations (≥ 1 μM), neocuproine almost abolished the relaxation to ascorbate but it also inhibited the initial response to the *S*-nitrosothiol. However, a lower concentration of neocuproine (0.3 μM) significantly inhibited the relaxation to ascorbate without affecting the initial relaxation to the *S*-nitrosothiol. These results suggest that ascorbate induced the breakdown of *S*-nitrosothiols through a copper(I) specific mechanism. In normal conditions, cellular copper is complexed with proteins but even protein-bound copper(II) can be reduced to copper(I) with thiols acting as antioxidants (Dicks and Williams, 1996). In the present study, the antioxidant ascorbate affected the relaxations to *S*-nitrosothiols, most likely through a copper(I)-dependent mechanism. The antioxidant α-tocopherol mimicked the relaxations to ascorbate indicating that this effect resulted from the antioxidant capacities of these compounds. These results suggest that the interaction between antioxidants and copper may represent a biological pathway for the metabolism of *S*-nitrosothiols in cells.

Lilley and Gibson (1997) recently demonstrated a tetrodotoxin-sensitive release of ascorbate from isolated muscle strips of the rat anococcygeus. As this tissue contains a dense network of nitrergic nerves, ascorbate may be released simultaneously with the nitrergic transmitter to protect it from oxidative breakdown. We previously demonstrated that exogenously added ascorbate does not affect the relaxations to nitrergic nerve stimulation in the rat gastric fundus (De Man et al., 1998). However, our present observation that ascorbate and tissue-copper interact to induce the release of NO from *S*-nitrosothiols, raises the possibility that the nitrergic transmitter is stored and released as an *S*-nitrosothiol from which NO is liberated by the simultaneously released ascorbate. However, the differential effect of neocuproine on relaxations to *S*-nitrosothiols and on those to short-trains of nitrergic nerve stimulation argues against this hypothesis as discussed above. In addition, neocuproine immediately abolished the sustained part of the relaxations to *S*-nitrosothiols but it did not affect the sustained part of the relaxation to prolonged electrical stimulation. Surprisingly, neocuproine did not enhance the relaxations to prolonged nitrergic nerve stimulation. Possibly, the incubation time of neocuproine was too short to exert an immediate effect on the responses to endogenously released NO whereas it may have reacted directly with the exogenously added *S*-nitrosothiols. Alternatively, the sustained relaxations to prolonged electrical stimulation of the rat gastric fundus are not purely nitrergic in nature and involve, in addition to NO, also vasoactive intestinal polypeptide (De Beurme and Lefebvre, 1987; Li and Rand, 1990; Boeckxstaens et al., 1992). This may explain why neocuproine did not affect these sustained relaxations to prolonged nerve stimulation.

The concentration of ascorbate in gut tissue is not known but in human gastric juice it is reported to be in the mM range (Waring et al., 1996). In such an environment, *S*-nitrosothiols will readily decompose. As recently demonstrated, *S*-nitrosothiols also decompose in the presence of superoxide anions (Aleryani et al., 1998; Jourdheuil et al., 1998; Trujillo et al., 1998), which are generated continuously during the aerobic cell cycle. Overall, these results suggest that in tissue with a strong oxidant/antioxidant activity, such as nitrergically innervated tissue (Martin et al., 1994; Lilley and Gibson, 1996, 1997), *S*-nitrosothiols can not act as stable intermediates for storage and transport of NO. Possibly, as suggested by Hogg et al. (1996), the anti-oxidative effect of thiols may enable free NO to diffuse through cells in close association with thiols. This may also explain why nitrergic relaxations in the rat gastric fundus are relatively resistant to superoxide stress even after inhibition of endogenous Cu/Zn superoxide dismutase (De Man et al., 1996a, 1996b; Lefebvre, 1996).

In summary, in the rat gastric fundus neocuproine enhanced the relaxations to nitrergic nerve stimulation and free NO and inhibited those to *S*-nitrosothiols. In the presence of an *S*-nitrosothiol, ascorbate induced an NO-mediated relaxation that was inhibited by neocuproine but not by cuprizone. During prolonged nerve stimulation, ascorbate did not induce a relaxation. These results suggest that copper and ascorbate interact to modulate the biological activity of *S*-nitrosothiols but not that of the nitrergic neurotransmitter. The opposite effect of neocuproine on relaxations to *S*-nitrosothiols and on those to nitrergic nerve stimulation suggests that *S*-nitrosothiols do not mediate the nitrergic neurotransmission in the rat gastric

fundus and that neocuproine is a useful tool to elucidate the nature of the nitrergic neurotransmitter in the peripheral nervous system.

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