British Journal of Pharmacology (1998) 123, 1039–1046

S-nitrosothiols and the nitrergic neurotransmitter in the rat gastric fundus: effect of antioxidants and metal chelation

Joris G. De Man, Benedicte Y. De Winter, Tom G. Moreels, Arnold G. Herman & [1]Paul A. Pelckmans

Divisions of Gastroenterology and Pharmacology, Faculty of Medicine, University of Antwerp (UIA), B-2610 Antwerpen-Wilrijk, Belgium

1 The effects of the antioxidants ascorbic acid and α-tocopherol and of the metal chelator ethylenediaminetetraacetic acid (EDTA) were studied on relaxations in response to S-nitrosothiols, authentic nitric oxide (NO) and nitrergic non-adrenergic non-cholinergic stimulation of the rat gastric fundus.

2 The S-nitrosothiols S-nitrosocysteine (1–100 nM), S-nitrosoglutathione (0.01–3 μM) and S-nitroso-N-acetylpenicillamine (0.01–3 μM) induced concentration-dependent relaxations of the rat gastric fundus muscle strips, which were precontracted with prostaglandin $F_{2\alpha}$. The relaxations to all S-nitrosothiols were concentration-dependently enhanced by the antioxidants ascorbic acid (0.1–3 μM) and α-tocopherol (3–30 μM) and inhibited by the metal chelator EDTA (26 μM).

3 Ascorbic acid and α-tocopherol alone did not induce a relaxation of the precontracted rat gastric fundus muscle strip. However, when ascorbic acid (1 μM) or α-tocopherol (1 μM) were injected in the organ bath 1 minute after S-nitrosoglutathione (0.1 μM) or after S-nitroso-N-acetylpenicillamine (0.1 μM), they induced an immediate, sharp and transient relaxation. This relaxation was inhibited by the superoxide generator pyrogallol (2 μM). Such a relaxation to ascorbic acid or α-tocopherol was not observed in the presence of S-nitrosocysteine (10 nM).

4 Electrical field stimulation (0.5–4 Hz) of the precontracted rat gastric fundus strips induced frequency-dependent nitrergic relaxations which were mimicked by authentic NO (3–300 nM) and by acidified sodium nitrite $NaNO_2$ (0.3–10 μM). Ascorbic acid (0.3–3 μM), α-tocopherol (3–30 μM) or EDTA (26 μM) did not affect the relaxations to nitrergic stimulation, NO or $NaNO_2$.

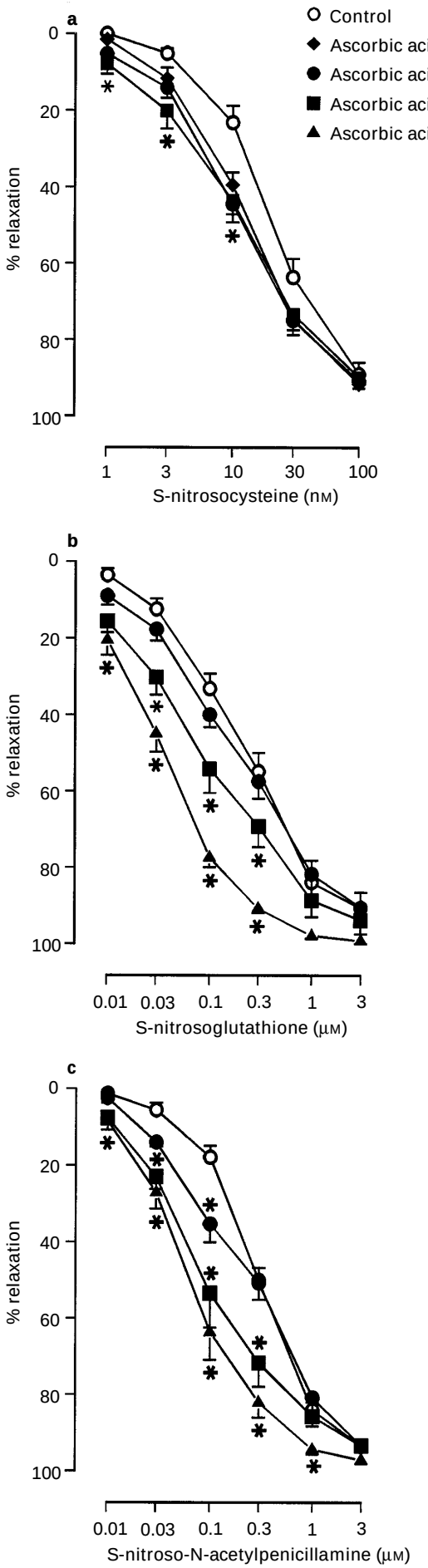
5 In summary, relaxations to S-nitrosothiols in the rat gastric fundus are enhanced by the antioxidants ascorbic acid and α-tocopherol and inhibited by the metal chelator EDTA. However, relaxations to nitrergic stimulation of the rat gastric fundus or those to authentic NO were not affected by the antioxidants or by the metal chelator. These results indicate that antioxidants and metal chelators have a different effect on the biological activity of S-nitrosothiols and on that of the nitrergic neurotransmitter. Therefore, our results suggest that S-nitrosothiols do not act as intermediate compounds in nitrergic neurotransmission in the rat gastric fundus.

Keywords: Antioxidant; ascorbic acid; EDTA; nitrergic neurotransmission; nitric oxide; S-nitrosothiols; non-adrenergic non-cholinergic; gastric fundus; α-tocopherol; superoxide

Introduction

Nitric oxide (NO) is an important mediator of the inhibitory non-adrenergic non-cholinergic (NANC) neurotransmission in the gastrointestinal tract (for reviews see: Sanders & Ward, 1992; Lefebvre, 1995; Rand & Li, 1995b). However, the exact nature of the nitrergic neurotransmitter has remained a matter of debate. Early studies on the nature of the nitrergic NANC neurotransmitter showed that relaxations to nitrergic stimulation of the mouse anococcygeus and bovine retractor penis muscle were resistant to superoxide anions, whereas relaxations to authentic NO were fully blocked (Gibson & Mirzazadeh, 1989; Gillespie & Sheng, 1990). Generally the same results were later found in almost all nitrergically innervated tissues, indicating that the actual nitrergic neurotransmitter might not be free NO but a superoxide resistant, NO-carrying molecule. Low molecular weight thiols such as cysteine and glutathione were proposed to act as an NO-carrier. These thiols are abundantly present in cells and they may react with NO or adducts of NO to form S-nitrosothiols (Ignarro *et al*., 1981; Gow *et al*., 1997), which are regarded as more stable compounds as compared to free radical NO (Matthews & Kerr, 1993). Therefore, S-nitrosothiols were proposed to act as intermediates that stabilize and transport NO, thereby enhancing its biological efficacy. In the rat gastric fundus, a number of authors have rejected free radical NO as the nitrergic neurotransmitter or suggested S-nitrosothiols as likely mediators of nitrergic neurotransmission (Hobbs *et al*., 1991; Kitamura *et al*., 1993; Barbier & Lefebvre, 1994; Rand & Li, 1995a). However, in our own studies, we found evidence to suggest free radical NO and not an S-nitrosothiol as the nitrergic neurotransmitter in the rat gastric fundus and the canine ileocolonic junction (Boeckxstaens *et al*., 1991; 1994; De Man *et al*., 1995; 1996a) and the same results were also found for the guinea-pig colon (Iversen *et al*., 1994). In spite of this, the failure of superoxide generators to inhibit nitrergic responses of the rat gastric fundus remained unexplained. However, recently Martin *et al*. (1994) discovered that the nitrergic neurotransmitter is protected from oxidative attack by the enzyme antioxidant CuZn superoxide dismutase. In addition, non-enzymatic antioxidants such as ascorbic acid and α-tocopherol also protect NO-mediated responses against oxidative breakdown (Lilley & Gibson, 1996), suggesting that tissue antioxidants may play an important role in protecting the biological activity of the nitrergic neurotransmitter. However, the antioxidant ascorbic acid was also shown to catalyze the decomposition of S-nitrosothiols (Kashiba-

[1] Author for correspondence.



Iwatsuki *et al*., 1996; Singh *et al*., 1996; Scorza *et al*., 1997). If antioxidants protect the biological activity of the nitrergic neurotransmitter on the one hand but catalyze the decomposition of S-nitrosothiols on the other hand, it seems unlikely that the nitrergic neurotransmitter is an S-nitrosothiol. The aim of the present study was to investigate the effect of the antioxidants ascorbic acid and α-tocopherol on relaxations induced by nitrergic stimulation of the rat gastric fundus and on relaxations induced by authentic NO and S-nitrosothiols. As metal ions such as copper may modulate the biological activity of S-nitrosothiols (Askew *et al*., 1995; Gordge *et al*., 1995), we also investigated the effect of the metal chelator ethylenediaminetetraacetic acid (EDTA) on relaxations to nitrergic stimulation, NO and S-nitrosothiols.

Methods

Tissue preparation

Male Wistar rats (250–300 g) were fasted for 48 h with free access to water. The animals were anaesthetized with pentobarbitone (60 mg kg^{-1}). A laparotomy was performed and the stomach was removed and cut open. After removal of the mucosa 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 (composition in mM: NaCl 118.3, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11). 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 to 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 0.3 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and in the presence of 1 μM atropine and 30 μM guanethidine. After each protocol had finished, the muscle strips were washed at least three times with intervals of 5 min.

In the first series of experiments, the effect of the antioxidants ascorbic acid (0.3–3 μM) and α-tocopherol (3–30 μM) was investigated on the frequency-response curve to electrical stimulation (0.5–4 Hz, 1 ms, pulse trains of 10 s), on the concentration-response curves to NO (3–300 nM), $NaNO_2$ (0.3–10 μM, pH 2), S-nitrosocysteine (1–100 nM), S-nitroso-

Figure 1 Concentration-response curves to (a) S-nitrosocysteine (1–100 nM), (b) S-nitrosoglutathione (0.01–3 μM) and (c) S-nitroso-N-acetylpenicillamine (0.01–3 μM) in control conditions and in the presence of ascorbic acid (0.1–3 μM). Results are expressed as percentage decrease of the $PGF_{2\alpha}$-induced contraction and shown as mean for $n=7-9$ experiments; vertical lines indicate s.e.mean. *$P<0.05$, significantly different from control, one-way ANOVA followed by Dunett's test; the asterisks in (a) refer to the curve after 1 μM ascorbic acid.

glutathione (0.01 – 3 μM) and S-nitroso-N-acetylpenicillamine (0.01 – 3 μM).

In the second series of experiments the effect of ethylenediaminetetraacetic acid (EDTA, 26 μM) was investigated on the frequency-response curve to electrical stimulation (0.5 – 4 Hz), on the concentration-response curves to NO (3 – 100 nM), S-nitrosocysteine (1 – 30 nM), S-nitrosoglutathione (0.01 – 0.3 μM) and S-nitroso-N-acetylpenicillamine (0.01 – 0.3 μM).

All experiments were performed in parallel with muscle strips that served as time controls receiving saline or dimethyl sulphoxide (DMSO, the solvent of α-tocopherol) instead of antioxidants or EDTA. The relaxations to electrical stimulation, NO, $NaNO_2$, S-nitrosocysteine, S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine remained constant over the time course of the experiment and they were not affected by DMSO.

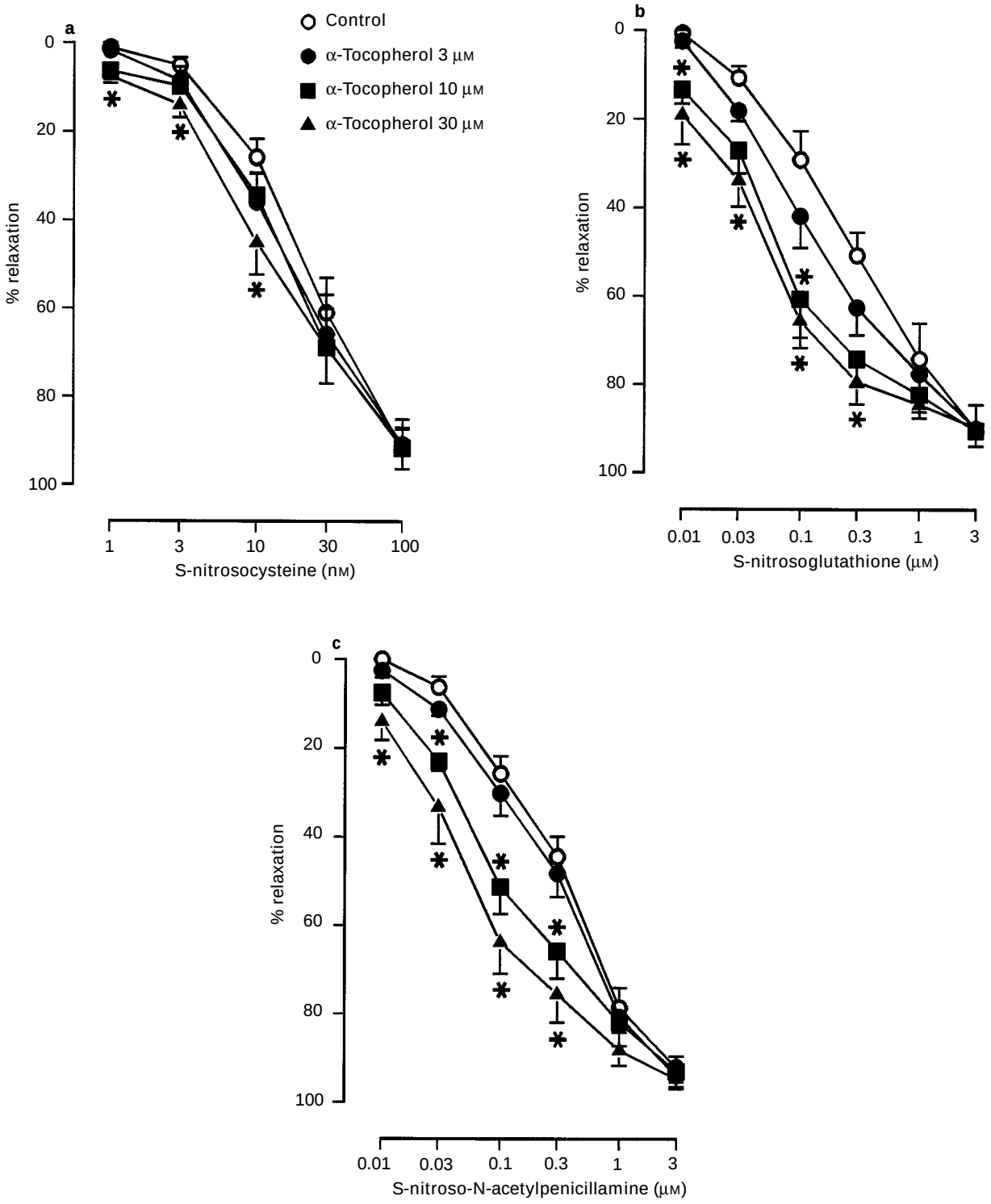


Figure 2 Concentration-response curves to (a) S-nitrosocysteine (1 – 100 nM), (b) S-nitrosoglutathione (0.01 – 3 μM) and (c) S-nitroso-N-acetylpenicillamine (0.01 – 3 μM) in control conditions and in the presence of α-tocopherol (3 – 30 μM). Results are expressed as percentage decrease of the $PGF_{2\alpha}$-induced contraction and shown as mean for $n=7$ experiments; vertical lines show s.e.mean. $^*P<0.05$, significantly different from control, one-way ANOVA followed by Dunett's test.

Drugs used

The following drugs were used: atropine sulphate, ethylenediaminetetraacetic acid (EDTA), citric acid, sodium nitrite (Merck, Darmstadt, Germany); guanethidine monosulphate (Ciba Geigy, Switzerland); N-acetyl-D,L-penicillamine, L-cysteine, diethyldithiocarbamate, reduced glutathione, pyrogallol, sodium dithionite, α-tocopherol (Sigma Chemical Co., St. Louis, MO, U.S.A.), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Dinolytic purchased from Upjohn, Puurs, Belgium as a sterile aqueous solution containing 5 mg ml^{-1} $PGF_{2\alpha}$ and 9 mg ml^{-1} benzyl alcohol); nitric oxide gas (L'Air Liquide, Belgium). α-Tocopherol was dissolved in dimethylsulphoxide (DMSO). Solutions of NO were prepared freshly before each experiment as described by Kelm *et al.* (1988) and used immediately after preparation. Stock solutions of S-nitrosothiols were prepared freshly on the day of experimentation as described previously (De Man *et al.*, 1995) and kept sealed on ice under argon in the dark. Dilutions of the stock solutions of the S-nitrosothiols were made up fresh 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 muscle strip. Values are shown as mean ± s.e.mean for the number of rats indicated. Statistical significance of differences between values was analysed with one-way analysis of variance followed by Dunett'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 to be significantly different from control.

Results

Effect of ascorbic acid and α-tocopherol 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 as previously found (Barbier & Lefebvre, 1994; De Man *et al.*, 1996a). The relaxations to S-nitrosocysteine, S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine were concentration-dependently enhanced by ascorbic acid (0.1–3 μM) (Figure 1) and by α-tocopherol (3–30 μM) (Figure 2). Ascorbic acid or α-tocopherol alone did not affect the $PGF_{2\alpha}$-induced contraction of the rat gastric fundus muscle strip (Figure 3). However, when ascorbic acid (1 μM) was injected 1 min after S-nitrosoglutathione (0.1 μM) or after S-nitroso-N-acetylpenicillamine (0.1 μM), it induced an immediate transient relaxation of 90 ± 3% and 80 ± 7%, respectively ($n = 5–8$) (Figure 3). Such a relaxation was not seen in the presence of S-nitrosocysteine (10 nM) or in the presence of the native thiols glutathione or N-acetylpenicillamine ($n = 4$ each, results not shown). The relaxation to ascorbic acid in the presence of S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine was inhibited to 19 ± 5% and 6 ± 2%, respectively, by the superoxide generator pyrogallol (2 μM) (Figure 3). Also α-tocopherol (30 μM) induced a rapid and transient relaxation in the presence of S-nitrosoglutathione (0.1 μM) and S-nitroso-N-acetylpenicillamine (0.1 μM) but not in the presence of S-nitrosocysteine (10 nM) (results not shown).

Ascorbic acid up to 100 μM had no effect on the pH of the Krebs-Ringer solution. In addition, citric acid (1 μM) did not affect the relaxations to S-nitrosothiols and it did not induce relaxation in the presence of the S-nitrosothiols ($n = 4$, results not shown). As ascorbic acid or α-tocopherol may exert their effect on S-nitrosothiols via their reducing capacities, we also investigated the effect of the reducing agent sodium dithionite. However, sodium dithionite (30 μM) did not affect the relaxations to the S-nitrosothiols: relaxation to 10 nM S-nitrosocysteine: from 35 ± 8% to 31 ± 8%; relaxation to 0.1 μM S-nitrosoglutathione: from 20 ± 4% to 22 ± 4% and relaxation to 0.1 μM S-nitroso-N-acetylpenicillamine: from 28 ± 5% to 31 ± 5%; saline vs 30 μM sodium dithionite (all $n = 6$).

Effect of ascorbic acid and α-tocopherol on relaxations to nitrergic stimulation, NO and $NaNO_2$

Electrical stimulation (0.5–4 Hz, 1 ms duration in trains of 10 s) of the rat gastric fundus induced frequency-dependent relaxations which were nitrergic in nature as they were abolished by blockers of NO synthase (Boeckxstaens *et al.*, 1991; De Man *et al.*, 1995). These relaxations were mimicked by authentic NO (3–100 nM) and by acidified $NaNO_2$ (0.3–10 μM) which keeps NO in solution. The relaxations to nitrergic stimulation, NO and $NaNO_2$ were not affected by ascorbic acid (0.3–3 μM) (Figure 4) or α-tocopherol (10–30 μM) ($n = 7–8$, results not shown). As it was previously demonstrated that the nitrergic neurotransmitter is protected from breakdown by endogenous CuZn superoxide dismutase (Martin *et al.*, 1994), we re-examined the effect of ascorbic acid after inactivation of CuZn superoxide dismutase with diethyldithiocarbamate (DETC). As described previously (De Man *et al.*, 1996b), treatment of the strips for 45 min with 1 mM DETC did not affect the relaxations to nitrergic stimulation. Also after DETC treatment, ascorbic acid (1–3 μM) still did not affect the nitrergic relaxations to 0.5–4 Hz electrical stimulation ($n = 6$) (for 0.5 Hz: from 10 ± 2% to 12 ± 5%; for 1 Hz: from 43 ± 6% to 46 ± 9%; saline vs 1 mM DETC plus 3 μM ascorbic acid). Also the reducing agent sodium dithionite (30–100 μM) did not affect the frequency-

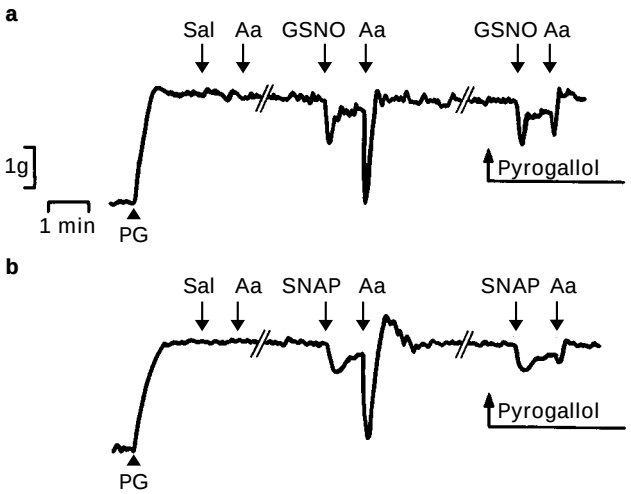


Figure 3 Typical tracings of the rat gastric fundus strip contracted with 0.1 μM prostaglandin $F_{2\alpha}$ (PG). (a) The effect of ascorbic acid (Aa, 1 μM) injected into the organ bath 1 min after saline (Sal), 1 min after S-nitrosoglutathione (GSNO, 0.1 μM) and 1 min after GSNO (0.1 μM) in the presence of 2 μM pyrogallol. (b) The effect of ascorbic acid (Aa, 1 μM) injected in the organ bath 1 min after saline (Sal), 1 min after S-nitroso-N-acetylpenicillamine (SNAP, 0.1 μM) and 1 min after SNAP (0.1 μM) in the presence of 2 μM pyrogallol. Tracing breaks represent 3 wash periods of 5 min each with Krebs-Ringer solution that contained 0.1 μM prostaglandin $F_{2\alpha}$.

response curve to 0.5–4 Hz electrical stimulation ($n=4$) (for 0.5 Hz: from $21\pm6\%$ to $14\pm3\%$; for 1 Hz: from $46\pm12\%$ to $41\pm12\%$; saline vs 100 μM sodium dithionite).

Effect of EDTA on relaxations to S-nitrosothiols, nitrergic stimulation and NO

Relaxations to S-nitrosocysteine (1–30 nM), S-nitrosoglutathione (0.01–0.3 μM) and S-nitroso-N-acetylpenicillamine (0.01–0.3 μM) were significantly inhibited by the metal chelator EDTA (26 μM) (Figure 5). However, EDTA (26 μM) did not affect the frequency-response curve to 0.5–4 Hz electrical stimulation ($n=6$, results not shown) or the concentration-response curve to NO ($n=8$, results not shown).

Discussion

There is compelling evidence that the L-arginine/NO pathway plays a major role in the inhibitory autonomic neurotransmis-

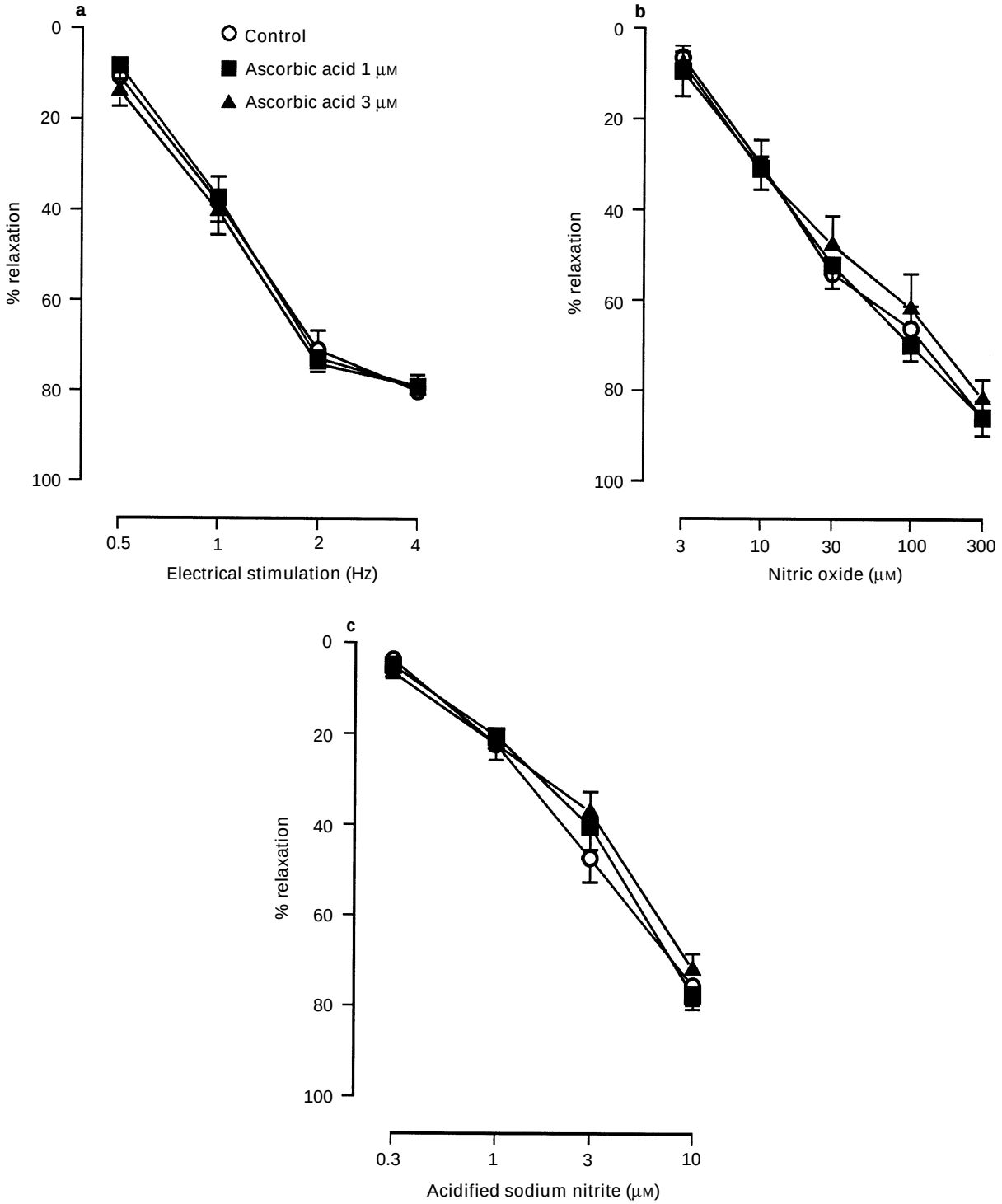


Figure 4 (a) Frequency-response curves to electrical stimulation (1 ms pulses at 0.5–4 Hz for 10 s periods) and concentration-response curves to (b) nitric oxide (3–300 nM) and (c) acidified sodium nitrite (0.3–10 μM) in control conditions and in the presence of ascorbic acid (1–3 μM). Results are expressed as percentage decreases of the $PGF_{2\alpha}$-induced contraction and shown as mean for $n=5-7$ experiments; vertical lines indicate s.e.mean. One-way ANOVA followed by Dunett's test showed no significant differences.

sion of the gastrointestinal tract, but the exact nature of the nitrergic neurotransmitter remains unclear. Several studies have proposed S-nitrosothiols rather than free NO as the actual relaxing factor mediating the nitrergic neurotransmission of the enteric nervous system. In the present study on isolated muscle strips of the rat gastric fundus, we demonstrated that relaxations to S-nitrosothiols were enhanced by antioxidants and inhibited by chelation of metals. However, relaxations to nitrergic stimulation or to authentic NO were not affected. These results indicate that S-nitrosothiols do not act as intermediates in nitrergic neurotransmission in the rat gastric fundus.

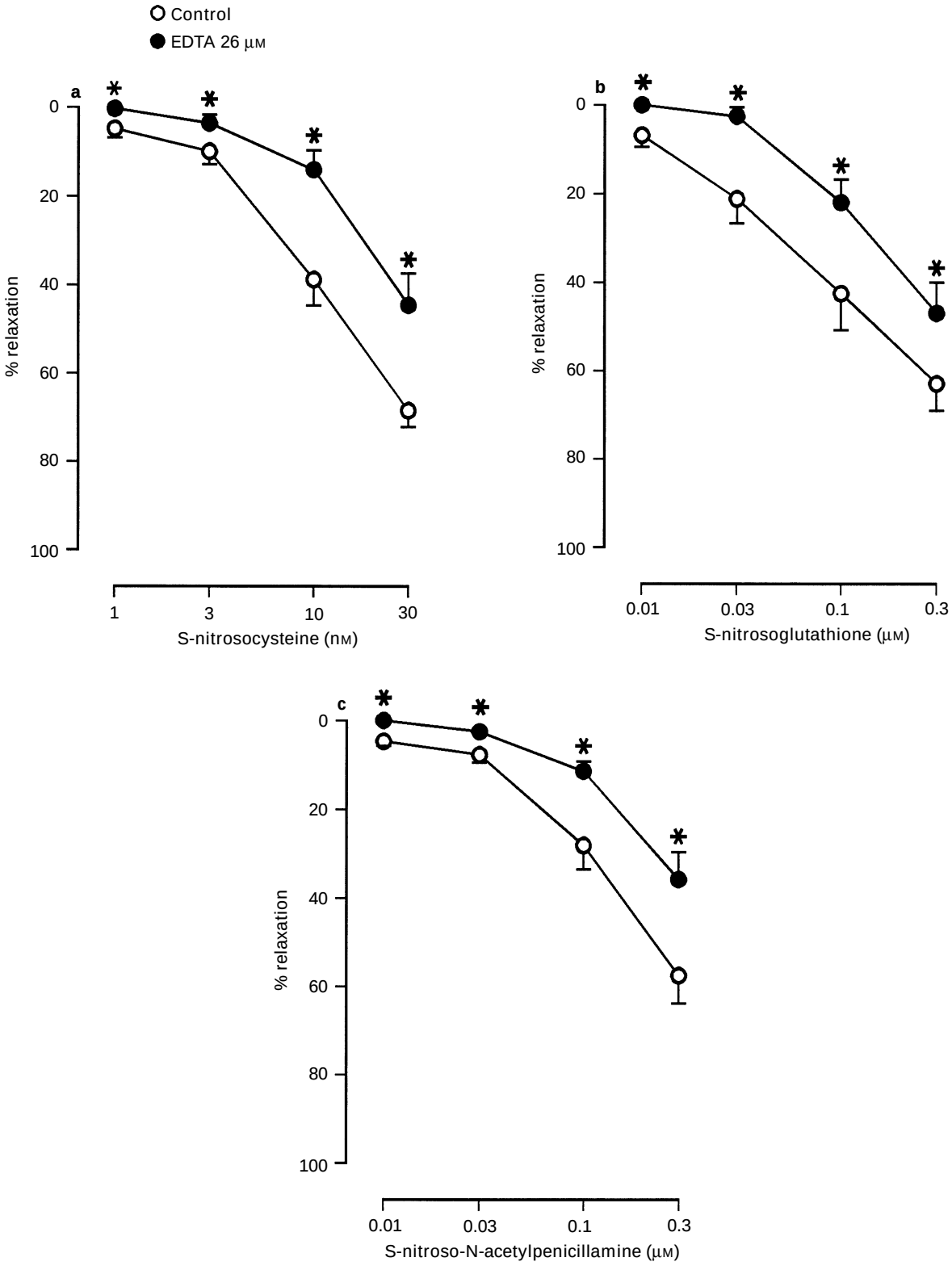


Figure 5 Concentration-response curves to (a) S-nitrosocysteine (1–30 nM), (b) S-nitrosoglutathione (0.01–0.3 μM) and (c) S-nitroso-N-acetylpenicillamine (0.01–0.3 μM) in control conditions and in the presence of 26 μM EDTA. Results are expressed as percentage decreases of the $PGF_{2\alpha}$-induced contraction and shown as mean for $n=6-8$ experiments; vertical lines indicate s.e.mean. $^*P<0.05$, significantly different from control, Student's t test for paired values.

The nitrosothiols S-nitrosocysteine, S-nitrosoglutathione and S-nitroso-N-acetylpenicillamine concentration-dependently relaxed the rat gastric fundus. These relaxations were enhanced by the antioxidants ascorbic acid and α-tocopherol. In addition, we observed that in the absence of S-nitrosothiols, ascorbic acid and α-tocopherol did not induce a relaxation. However, in the presence of a low concentration of S-nitrosoglutathione or S-nitroso-N-acetylpenicillamine, ascorbic acid and α-tocopherol fully relaxed the rat gastric fundus muscle strip. This relaxation, which was rapid in onset and transient, is most likely mediated by NO as it was sensitive to the superoxide generator pyrogallol, which potently inhibits relaxations to NO but not those to stable S-nitrosothiols (De Man *et al*., 1995). Interestingly, a relaxation to ascorbic acid or α-tocopherol was not observed in the presence of S-nitrosocysteine. However, S-nitrosocysteine readily releases NO in solution and it is the least stable of the three S-nitrosothiols used in this study (Matthews & Kerr, 1993). Most likely, the unstable S-nitrosocysteine was already largely decomposed at the moment that ascorbic acid was injected, whereas more stable S-nitrosothiols decompose slowly and are therefore more susceptible to further decomposition by ascorbic acid.

The exact mechanism by which ascorbic acid and α-tocopherol break down S-nitrosothiols is not clear. The acidic nature of the ascorbic acid solution cannot explain this effect, since α-tocopherol mimicked the effect of ascorbic acid whereas citric acid did not. There is evidence that low levels of ascorbic acid break down S-nitrosoglutathione in physiological solution (Kashiba-Iwatsuki *et al*., 1996) and in human plasma (Scorza *et al*., 1997). Scorza *et al*. (1997) suggested that the antioxidant-induced decomposition of S-nitrosothiols is regulated by a reductive activation of NO^+ to free radical NO. In our experiments, we noticed that the relaxation induced by ascorbic acid in the presence of an S-nitrosothiol was very rapid in onset, transient and pyrogallol-sensitive, suggesting that antioxidants rather induce a rapid release of NO from S-nitrosothiols. We previously observed the same effect with copper: in the rat gastric fundus, copper induces a transient relaxation in the presence but not in the absence of S-nitrosothiols (De Man *et al*., 1996a), most likely resulting from the copper-induced release of NO from S-nitrosothiols (Askew *et al*., 1995; Gordge *et al*., 1995). This release is mediated by copper(I) rather than copper(II) ions (Dicks *et al*., 1996; Gordge *et al*., 1996) but copper(I) is unstable in solution. Possibly, reducing agents such as ascorbate may reduce copper(II) to copper(I) thereby accelerating the copper(I)-induced release of NO from the S-nitrosothiol. However, in our study, the well known reducing agent sodium dithionite had no effect on relaxations to S-nitrosothiols and it did not induce a relaxation in the presence of an S-nitrosothiol. In addition, in physiological pH conditions, ascorbic acid is not able to reduce the transition metal iron(III) to iron(II) (Hsieh & Hsieh, 1997). These results suggest that other mechanisms than oxido-reduction reactions may be involved in the antioxidant-induced decomposition of S-nitrosothiols. Several decades ago, it was shown that ascorbic acid and α-tocopherol were able to prevent the formation of N-nitrosocompounds (Mirvish *et al*., 1972), a mechanism by which antioxidants may prevent cancer (Mirvish, 1986). However, via this mechanism antioxidants prevent the formation of N-nitrosocompounds but they do not induce the decomposition of N-nitrosocompounds.

Although the antioxidants used in this study clearly enhanced the relaxations to S-nitrosothiols, they did not affect the relaxations to NO or to the NO donor $NaNO_2$. Moreover, relaxations to nitrergic stimulation of the rat gastric fundus were also not affected by the antioxidants, even after inhibition of CuZn superoxide dismutase, an enzyme which is colocalized with neuronal NO synthase (Liu *et al*., 1997) and which may protect the nitrergic neurotransmitter from breakdown (Martin *et al*., 1994; Paisley & Martin, 1996). Lilley & Gibson (1996) recently demonstrated that ascorbic acid and α-tocopherol also protect NO-induced relaxations of the mouse anococcygeus against attack by superoxide ions. High concentrations of ascorbic acid are normally present in the cytosol and in plasma, but also in human upper gastrointestinal biopsies (Waring *et al*., 1996), and in gastric juices of man (Sobola *et al*., 1989) and rats (Muto *et al*., 1997) high concentrations of ascorbic acid have been found. All together, these results suggest that antioxidants are important protective mediators with various physiological functions, including protection of the nitrergic neurotransmitter. However, as antioxidants protect the nitrergic neurotransmitter on the one hand but catalyze the decomposition of S-nitrosothiols on the other, it is unlikely that an S-nitrosothiol is the actual nitrergic neurotransmitter.

This was further illustrated by the effect of the metal chelator EDTA: EDTA did not affect the relaxations to nitrergic stimulation, NO or $NaNO_2$, but inhibited the relaxations to S-nitrosothiols. We previously showed that relaxations of the rat gastric fundus to S-nitrosothiols are enhanced by copper (De Man *et al*., 1996a), which is in agreement with the hypothesis that transition metals decompose S-nitrosothiols (Askew *et al*., 1995; Gordge *et al*., 1995). Therefore, the inhibitory effect of the metal chelator EDTA on responses to S-nitrosothiols most likely results from chelation of copper ions, thereby inhibiting the copper-induced release of NO from S-nitrosothiols. As also observed with the antioxidants, EDTA was less effective in inhibiting relaxations to S-nitrosocysteine as compared to relaxations to S-nitrosoglutathione or S-nitroso-N-acetylpenicillamine. As discussed above, compounds that modulate the release of NO from S-nitrosothiols will be less effective towards unstable S-nitrosothiols, such as S-nitrosocysteine, as these rapidly release NO in solution (Matthews & Kerr, 1993). In support of previous findings, our results also demonstrate that trace amounts of metals, either present as free ions in the buffer solution or enzyme bound in the tissue (Gordge *et al*., 1996), may affect the biological activity of S-nitrosothiols, an observation which should be taken in account when the effects of S-nitrosothiols are studied.

In summary, we demonstrated that antioxidants enhance relaxations to S-nitrosothiols in the rat gastric fundus whereas chelation of metals inhibits these relaxations. However, relaxations to nitrergic stimulation of the rat gastric fundus or those to authentic NO were not affected. From these differential effects we conclude that S-nitrosothiols do not act as stabilizing mediators of nitrergic neurotransmission and that the nitrergic neurotransmitter in the rat gastric fundus is not an S-nitrosothiol but more likely free NO.

This work was supported by the Belgian Fund for Medical Scientific Research (Grant nr G.0220.96).

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(Received November 7, 1997
Revised November 24, 1997
Accepted November 26, 1997)