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Effect of thiol modulators and Cu/Zn superoxide dismutase inhibition on nitrergic relaxations in the rat gastric fundus

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1 The effects of superoxide anion generators before and after treatment with inhibitors of Cu/Zn superoxide dismutase (Cu/Zn SOD) and the effects of thiol-modulating agents were investigated on nitrergic relaxations to electrical stimulation of non-adrenergic non-cholinergic (NANC) nerves of the rat gastric fundus and on relaxations to authentic nitric oxide (NO) and nitroglycerin.

2 The superoxide anion generators, pyrogallol (30 μM) and duroquinone (30–60 μM), significantly inhibited the relaxations to NO (0.03–3 μM) but not nitrergic relaxations to NANC nerve stimulation (0.5–8 Hz) or those to ATP (10 μM). Treatment of the rat gastric fundus with the inhibitors of Cu/Zn SOD, diethyldithiocarbamate (DETC, 1 mM for 2 h) or triethylenetetramine (TETA, 100 μM for 2 h) had no effect on the relaxations to NANC nerve stimulation (1–8 Hz), NO (0.03–3 μM) or on those to ATP (10 μM).

3 After treatment of the rat gastric fundus with DETC (1 mM) but not after treatment with TETA (100 μM), pyrogallol (30 μM) and duroquinone (30–60 μM) significantly inhibited the nitrergic relaxations to electrical stimulation (0.5–8 Hz) and those to NO (0.03–3 μM). This inhibitory effect of pyrogallol and duroquinone was prevented by addition of exogenous SOD (250 units ml^{-1}). Pyrogallol but not duroquinone also inhibited the NO-independent relaxations to ATP (10 μM).

4 The thiol modulators, buthionine sulphoximine (1 mM for 2 h) and ethacrynic acid (30 μM for 2 h), significantly inhibited the relaxations to nitroglycerin (0.03–3 μM) but had no effect on the nitrergic relaxations to electrical stimulation (0.5–8 Hz) or on those to NO (0.03–10 μM) and ATP (10 μM). The thiol modulators, sulphobromophthalein (100 μM for 2 h) and diamide (30–100 μM for 2 h) did not affect the relaxations to nitroglycerin, or those to NANC nerve stimulation and NO.

5 In summary, thiol modulators significantly inhibited the thiol-dependent relaxations to nitroglycerin but not those to NANC nerve stimulation or NO. Relaxations to nitrergic stimulation were decreased by superoxide anion generators only after inhibition of Cu/Zn SOD. These results suggest that the nitrergic NANC neurotransmitter in the rat gastric fundus is not a nitrosothiol but more likely free NO,which is protected from breakdown by tissue SOD.

Keywords: Nitrergic neurotransmitter; nitric oxide; non-adrenergic non-cholinergic transmission; rat gastric fundus; superoxide anion; thiol modulator; S-nitrosothiol

Introduction

Nitric oxide (NO) is a mediator with an important physiological role in the gastrointestinal tract. We previously provided evidence that NO is a non-adrenergic non-cholinergic (NANC) neurotransmitter in different regions of the gastrointestinal tract (Boeckxstaens *et al.*, 1990; Bult *et al.*, 1990; De Man *et al.*, 1991) including the rat gastric fundus (Boeckxstaens *et al.*, 1991). However, in different studies that were carried out in a variety of tissues, the exact identity of the nitrergic NANC neurotransmitter was questioned. A number of pharmacological compounds had a different effect on responses to authentic NO and on responses to NANC nerve stimulation (Gillespie & Sheng, 1990; Hobbs *et al.*, 1991; Barbier & Lefebvre, 1992; Jenkinson *et al.*, 1995; Lilley & Gibson, 1995; Rand & Li, 1995), suggesting that the nitrergic NANC neurotransmitter is not free NO but an NO-releasing compound such as a nitrosothiol (Gibson *et al.*, 1992; Thornbury *et al.*, 1991; Kerr *et al.*, 1992; Kitamura *et al.*, 1993; Barbier & Lefebvre, 1994; Liu *et al.*, 1994). An alternative explanation for this differential effect is that the nitrergic neurotransmitter is free NO which is protected from breakdown by tissue antioxidants such as superoxide dismutase (SOD), which scavenges superoxide radicals. Recently, Thomas *et al.* (1996) demonstrated that the intramural neurones in the opossum oesophagus contain a variety of antioxidant enzymes. In addition, in the rat stomach manganese SOD is located in nerve cells and smooth muscle cells (Fang & Christensen, 1995) and it is partially colocalized with NADPH diaphorase, a marker for nitric oxide synthase, in the rat colon (Ceilley *et al.*, 1995). These results might suggest that antioxidant enzymes may have an important protective role on the NO-mediated neurotransmission. This was recently illustrated by the finding that an inhibitor of Cu/Zn SOD made the nitrergic neurotransmitter sensitive to superoxides in the bovine retractor penis muscle and in the mouse anococcygeus muscle (Martin *et al.*, 1994; Lilley & Gibson, 1995; Paisley *et al.*, 1996). In addition, in bioassay experiments, we previously demonstrated that responses to the nitrergic neurotransmitter of the canine ileocolonic junction were inhibited by superoxide anion generators to an comparable extent as the responses to authentic NO, whereas those to nitrosothiols were not affected (Boeckxstaens *et al.*, 1994; De Man *et al.*, 1995b). These findings provide evidence in favour of free NO as the nitrergic NANC neurotransmitter in these tissues. To investigate whether the nitrergic NANC neurotransmitter in the rat gastric fundus is free NO or

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an nitrosothiol, we studied the effect of the superoxide anion generators, pyrogallol and duroquinone, before and after treatment of the rat gastric fundus with the Cu/Zn SOD inhibitors, diethyldithiocarbamate (DETC) and triethylenetetramine (TETA) on the relaxations to nitrergic stimulation, authentic NO and nitroglycerin. To investigate whether thiols are involved in the nitrergic neurotransmission in the rat gastric fundus, we also studied the effect of the thiol modulators, ethacrynic acid, buthionine sulphoximine, sulphobromophthalein and diamide on the relaxations to nitrergic stimulation, authentic NO and nitroglycerin.

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 via a midline incision. Longitudinal muscle strips of approximately 1.0 cm in length 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, CaEDTA 0.026 and glucose 11.1). 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 attached 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 with an interval of at least 5 min.

In a first series of experiments the effects of the superoxide anion generators, pyrogallol (30 μM) and duroquinone (30–60 μM), were investigated on the frequency-response curve to electrical stimulation (0.5–8 Hz, 1 ms, 9 V, pulse trains lasting 10 s), on the concentration-response curve to NO (0.03–3 μM) and on the relaxation to ATP (10 μM).

In a second series of experiments, the rat gastric fundus was treated for 2 h with the inhibitors of Cu/Zn superoxide dismutase (Cu/Zn SOD), diethyldithiocarbamate (1 mM, Martin *et al.*, 1994; Lilley & Gibson, 1995) and triethylenetetramine (0.1 mM, Abdalla & Will, 1995). After wash-out, the effect of this treatment in the presence and absence of pyrogallol or duroquinone was investigated on the frequency-response curve to electrical stimulation (1–8 Hz, 1 ms), on the concentration-response curve to NO (0.03–3 μM) and on the relaxation to ATP (10 μM).

In a third series of experiments, the effect of a 2 h incubation of the rat gastric fundus with buthionine sulphoximine, an inhibitor of glutathione production (1 mM, Murphy *et al.*, 1991), ethacrynic acid, a sulphydryl alkylator (30 μM, Li *et al.*, 1994), sulphobromophthalein, an inhibitor of glutathione S-transferase (100 μM, Yeates *et al.*, 1989) or diamide, a glutathione oxidator (30–100 μM, Murphy *et al.*, 1991) was investigated on the frequency-response curve to electrical stimulation (1–8 Hz, 1 ms), on the concentration-response curve to NO (0.03–30 μM) and nitroglycerin (0.3–3 μM) and on relaxations to ATP (10 μM) of the rat gastric fundus.

Drugs used

The following drugs were used: atropine sulphate, nitroglycerin (Merck, Darmstadt, Germany); guanethidine monosulphate (Ciba Geigy, Switzerland); adenosine 5'-triphosphate, L-buthionine-[S,R]-sulphoximine, diamide, diethyldithiocarbamic acid sodium salt, duroquinone, ethacrynic acid, pyrogallol, sulphobromophthalein, triethylenetetramine dihydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, Upjohn, Puurs, Belgium). Superoxide dismutase was a gift from Grunenthal GmbH (Aachen, Germany). All drugs were dissolved in distilled water except duroquinone and ethacrynic acid which were dissolved in 10% dimethylsulphoxide. Solutions of NO were prepared freshly for each experiment as described by Kelm *et al.* (1988) and used immediately after preparation.

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, 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 superoxide anion generators and Cu/Zn SOD inhibition on relaxations to nitrergic stimulation and NO

Electrical stimulation of the rat gastric fundus induced frequency-dependent relaxations, which were previously shown to be mediated by NO (Boeckxstaens *et al.*, 1991; De Man *et al.*, 1995a) and which were mimicked by authentic NO. The superoxide anion generators, pyrogallol (30 μM) and duroquinone (30–60 μM), did not affect the relaxations to nitrergic stimulation (0.5–8 Hz, 1 ms) whereas those to authentic NO (0.3–30 μM) were significantly inhibited (Figure 1). In time control experiments, the solvent of duroquinone, dimethylsulphoxide, had no effect on the relaxations to nitrergic stimulation or NO. Treatment of the rat gastric fundus with the Cu/Zn SOD inhibitors, DETC (1 mM) or TETA (100 μM), did not affect the relaxations to electrical stimulation (1–8 Hz, 1 ms), NO (0.03–3 μM) (Figure 2) or ATP (10 μM, data not shown). The superoxide anion generators and Cu/Zn SOD inhibitors did not affect the basal tension, the $PGF_{2\alpha}$-induced contraction or the relaxation to 10 μM ATP (data not shown).

After treatment of the rat gastric fundus with DETC (1 mM) but not after treatment with TETA (100 μM), duroquinone (30–60 μM) concentration-dependently inhibited the relaxations to nitrergic stimulation (0.5–8 Hz, 1 ms) (Figure 3). In addition, after DETC treatment, duroquinone (60 μM) further inhibited the relaxations to 0.3 and 1 μM NO as compared to the effect of duroquinone alone (Student's *t* test for unpaired values) (Figures 1 and 3) whereas the NO-independent relaxations to ATP (10 μM) were not affected (data not shown). After DETC (1 mM) but not after TETA (100 μM) treatment, pyrogallol (30 μM) inhibited the relaxations to nitrergic stimulation (1–8 Hz, 1 ms) (Figure 3) but treatment with DETC plus pyrogallol had no additional effect on the relaxations to NO (0.03–3 μM) as compared to the effect of pyrogallol alone (Student's *t* test for unpaired values) (Figures 1 and 3). In addition, DETC plus pyrogallol also significantly inhibited the relaxations to 10 μM ATP (ATP; from 52 ± 4% to 35 ± 4%, $n=6$). Washing the strips with pyrogallol-free or duroquinone-free Krebs-Ringer solution reversed the inhibitory effect of the superoxide anion generators on the nitrergic relaxations, whereas addition of exogenous SOD (250–1000 units ml^{-1}) did not reverse this effect

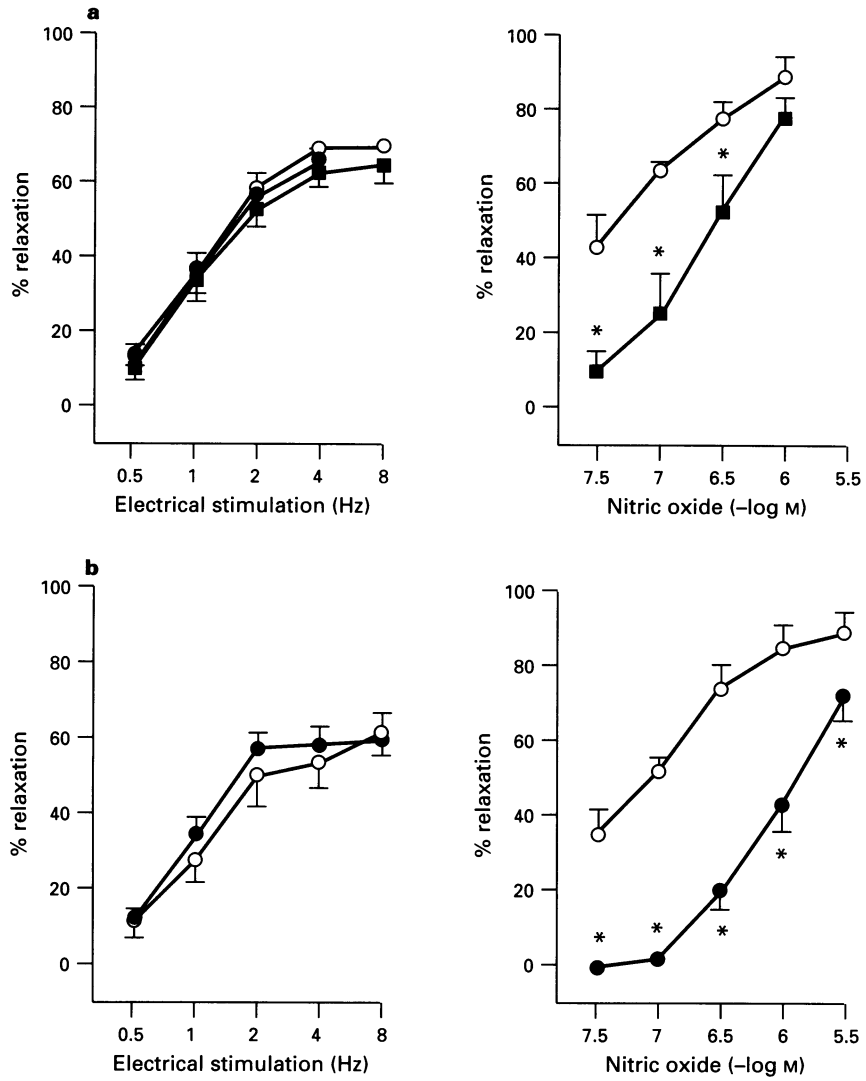

Figure 1 Frequency-response curves to electrical stimulation (0.5–8 Hz, 1 ms) and concentration-response curves to nitric oxide (0.03–3 μM) in control conditions (○) and in the presence of (a) duroquinone (●, 30 μM and ■, 60 μM) and (b) pyrogallol (●, 30 μM). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n = 4-6$ experiments. $^*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

($n = 6$, data not shown). However, when SOD (250 units ml^{-1}) was added after DETC treatment but before the superoxide generators, it completely prevented the inhibitory effect of duroquinone and pyrogallol on the relaxations to nitrergic stimulation (0.5–8 Hz, 1 ms) and on the relaxations to NO (0.03–1 μM): relaxations to 1 Hz were 31 ± 5% in control conditions and 28 ± 5% in the presence of SOD plus 60 μM duroquinone after DETC treatment ($n = 5$); relaxations to 0.1 μM NO were 64 ± 4% in control conditions and 62 ± 7% in the presence of SOD plus 60 μM duroquinone after DETC treatment ($n = 4$). Similarly, relaxations to 1 Hz were 32 ± 9% in control conditions and 28 ± 8% in the presence of SOD plus 30 μM pyrogallol after DETC treatment ($n = 4$); relaxations to 0.1 μM NO were 49 ± 4% in control conditions and 52 ± 4% in the presence of SOD plus 30 μM pyrogallol after DETC treatment ($n = 4$). After DETC treatment, pyrogallol but not duroquinone significantly reduced the $PGF_{2\alpha}$-induced contraction from 6.0 ± 1.7 g to 3.5 ± 1.2 g ($n = 9$).

Effect of thiol modulators on relaxations to nitroglycerin, nitrergic stimulation and NO

Nitroglycerin induced concentration-dependent relaxations of the rat gastric fundus strips. The relaxations to nitroglycerin (0.03–3 μM) were significantly inhibited by the thiol modulators, buthionine sulphoximine (1 mM) (Figure 4) and ethacrynic acid (30 μM) (Figure 5) but not by sulphobromophtaleine (100 μM) or diamide (30–100 μM) (data not shown). In contrast, the relaxations to electrical stimulation (0.5–8 Hz, 1 ms) or to authentic NO (0.03–10 μM) were not affected by buthionine sulphoximine (1 mM) (Figure 4), ethacrynic acid (30 μM) (Figure 5), sulphobromophthalein (100 μM) or diamide (30–100 μM) (data not shown). None of the thiol modulators had an effect on the basal tension, the $PGF_{2\alpha}$-induced contraction or the relaxation to ATP (10 μM). Dimethylsulphoxide, the solvent of ethacrynic acid, had no effect on the relaxations to nitroglycerin, nitrergic stimulation or NO.

Discussion

The importance of NO as a mediator of NANC neurotransmission is now widely accepted, but there is controversy about the exact chemical identity of the nitrergic neurotransmitter as it was reported that relaxations to authentic NO but not those to nitrergic NANC stimulation are sensitive to superoxide anions. This led to the suggestion that the actual

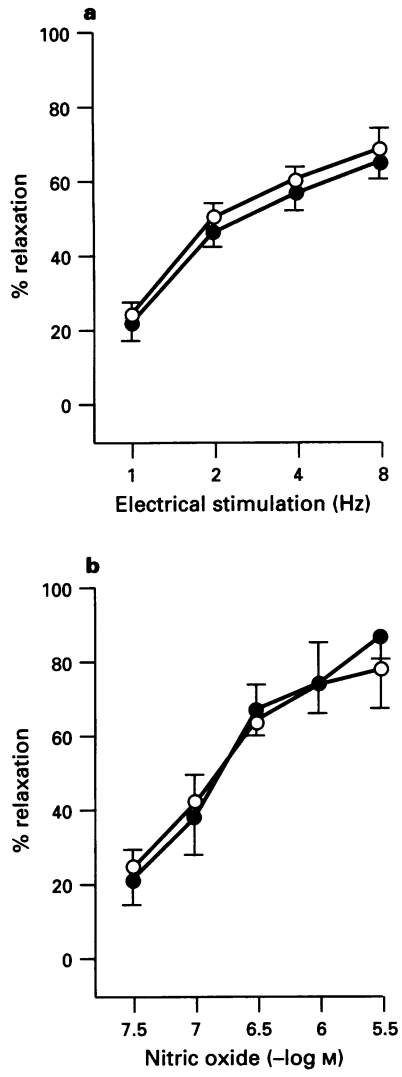

Figure 2 Frequency-response curves to (a) electrical stimulation (1–8 Hz, 1 ms) and concentration-response curves to nitric oxide (0.03–3 μM) (b) in control conditions (○) and after treatment with DETC (1 mM, ●). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n=7-9$ experiments.

nitrergic NANC neurotransmitter is not free NO but a superoxide-resistant, NO-containing compound, such as a nitrosothiol. In the present study, we have provided evidence that the nitrergic NANC neurotransmitter in the rat gastric fundus is sensitive to superoxides only after inhibition of Cu/Zn SOD. In addition, we have demonstrated that thiol modulators inhibit the thiol dependent relaxations to nitroglycerin but not the relaxations to nitrergic stimulation, suggesting that the nitrergic NANC neurotransmitter in the rat gastric fundus is not a nitrosothiol but more likely free nitric oxide.

The Cu^{2+}-chelators DETC and TETA inhibit Cu/Zn SOD (Misra, 1979; Cocco *et al.*, 1981; Abdalla & Will, 1995). In our study, these Cu/Zn SOD inhibitors did not affect the relaxations to nitrergic stimulation. Also the superoxide anion generators, pyrogallol or duroquinone, did not affect nitrergic relaxations confirming our results in the canine ileocolonic junction (Boeckxstaens *et al.*, 1994). However, these superoxide anion generators inhibited nitrergic relaxations in the rat gastric fundus after blockade of Cu/Zn SOD by DETC, suggesting that endogenous SOD protects the nitrergic neurotransmitter. Surprisingly, TETA treatment had no effect in our study. One possible explanation might be that the concentration of TETA was too low although this concentration was shown to be effective in pulmonary arteries (Abdalla & Will, 1995). We did not use higher concentrations as they decreased the $PGF_{2\alpha}$-induced contraction of the rat gastric fundus. Alternatively, the inhibition of Cu/Zn SOD activity is a slow process (Misra, 1979) and even in a cell-free system the enzyme is effectively inhibited only after a prolonged incubation period (Misra, 1979; Cocco *et al.*, 1981). Therefore, the incubation time of TETA might be too short to obtain an effect. Exogenously added SOD did not reverse the inhibitory effect of pyrogallol or duroquinone after DETC treatment which is in contrast to previous findings in the bovine retractor penis and in mouse anococcygeus muscle (Martin *et al.*, 1994; Lilley & Gibson, 1995; Paisley & Martin, 1996). However, when SOD was added to DETC-treated tissues before the superoxide anion generators, it prevented the inhibitory effect of the latter, suggesting that the observed effect resulted from superoxides. This finding also suggests that once the superoxides have penetrated the tissue, their effect cannot be neutralised by exogenous SOD, demonstrating that at least in the rat gastric fundus, exogenous SOD cannot adequately reach the neuromuscular junction to protect the endogenously released nitrergic neurotransmitter. This is in agreement with our previous findings in the rat gastric fundus: in organ bath experiments exogenous SOD does not enhance nitrergic relaxations whereas in bioassay experiments, SOD significantly enhances the biological activity of the nitrergic neurotransmitter (Boeckxstaens *et al.*, 1991). Alternatively, superoxides may inhibit the biological activity of the nitrergic neurotransmitter at an intracellular site which is inaccessible for exogenously added SOD. Finally, a non specific effect of pyrogallol after treatment with DETC should be considered as the contraction to $PGF_{2\alpha}$ and the NO-independent relaxation to ATP, which acts directly on the smooth muscle (Boeckxstaens *et al.*, 1991), were also reduced. However, duroquinone had no such non specific effect and the inhibitory effect of both superoxide anion generators was immediately reversed after washing the tissues with normal Krebs-Ringer solution suggesting that the superoxide anion generators did not induce irreversible cell damage.

After inhibition of Cu/Zn SOD, the superoxide anion generators still had a different potency in inhibiting relaxations to NANC nerve stimulation and relaxations to NO. Possibly, these NANC relaxations were mediated not only by NO but also by VIP, which is also an inhibitory NANC neurotransmitter in the rat gastric fundus (Boeckxstaens *et al.*, 1991), and which is likely to be insensitive to superoxides. However, we previously demonstrated that NANC relaxations induced by short periods of low frequency stimulation are almost completely inhibited by blockers of the NO biosynthesis (De Man *et al.*, 1995a), illustrating that these NANC relaxations are nitrergic in nature. More likely, the nitrergic neurotransmitter is protected not only by Cu/ZnSOD but also by other antioxidants such as manganese SOD. Manganese SOD, which is not inhibited by DETC, was recently shown to be partially colocalized with NO synthase in rat colon (Ceilley *et al.*, 1995) suggesting that this enzymic antioxidant may protect the biological activity of the nitrergic neurotransmitter. In addition, cells contain a variety of natural non-enzymic antioxidants which have an effective SOD-like activity (Kim *et al.*, 1995). Finally, Wood & Garthwaite (1994) suggested that the differential potency might result from the difference in diffusion pathway of the endogenously released nitrergic neurotransmitter and the exogenously applied NO. Altogether, this is in agreement with our previous finding in a bioassay set-up in which the nitrergic neurotransmitter of the canine ileocolonic junction behaved like free nitric oxide and not like a nitrosothiol (Boeckxstaens *et al.*, 1994; De Man *et al.*, 1995b).

Treatment of the rat gastric fundus with the thiol modulators ethacrynic acid, a non specific thiol alkylator, and buthionine sulphoximine, an inhibitor of γ-glutamylcysteine synthetase, an essential enzyme for the biosynthesis of glutathione, did not affect the relaxations to nitrergic stimulation or those to NO. In contrast, relaxations to nitroglycerin were

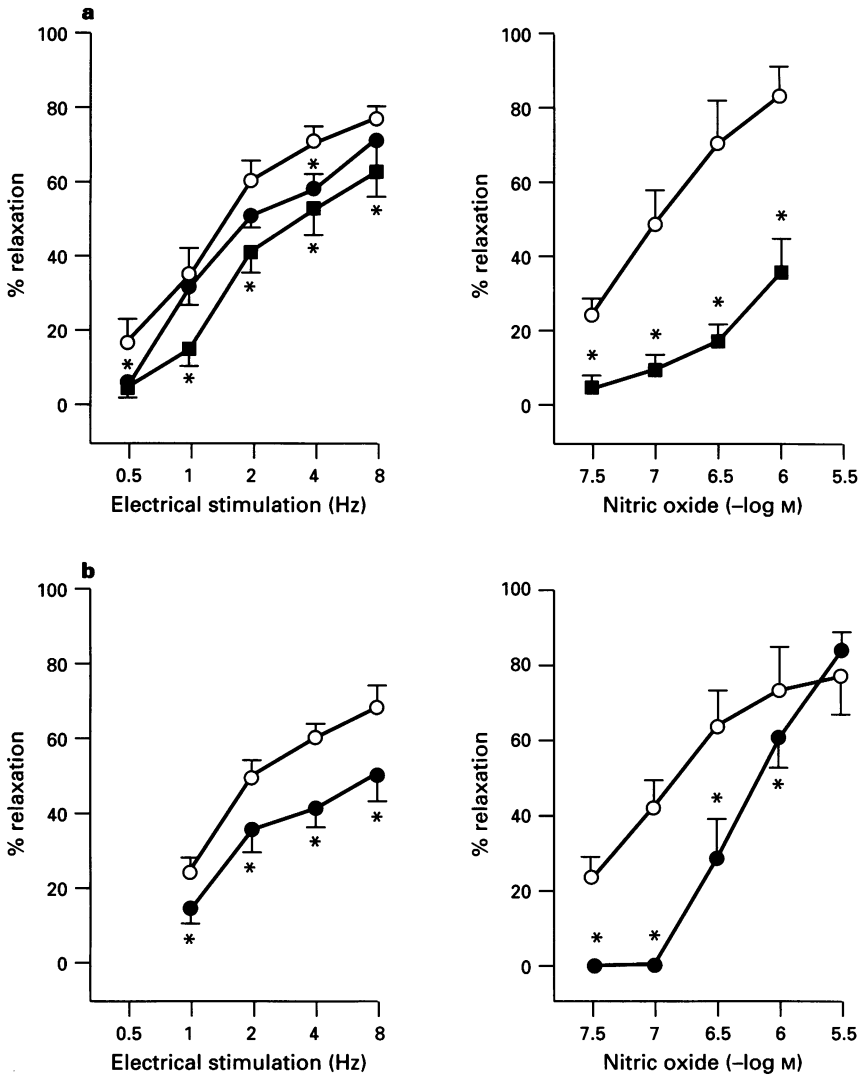

Figure 3 Frequency-response curves to electrical stimulation (0.5–8 Hz, 1 ms) and concentration-response curves to nitric oxide (0.03–3 μM) in control conditions (○) and after treatment with (a) 1 mM DETC plus 30 μM duroquinone (●) and 1 mM DETC plus 60 μM duroquinone (■) and after treatment with (b) 1 mM DETC plus 30 μM pyrogallol (●). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n=7-8$ experiments. $^*P<0.05$ is considered as significantly different from control; Student's t test for paired observations.

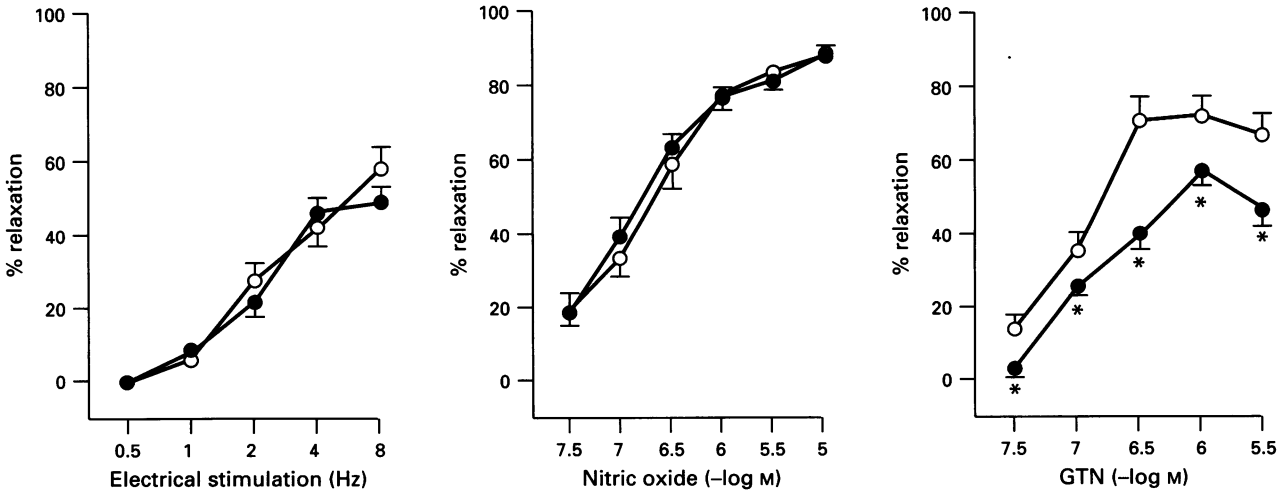

Figure 4 Frequency-response curve to electrical stimulation (0.5–8 Hz, 1 ms) and concentration-response curves to nitric oxide (0.03–10 μM) and nitroglycerin (GTN, 0.03–3 μM) in control conditions (○) and after treatment with buthionine sulphoximine (●, 1 mM). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n=6-8$ experiments. $^*P<0.05$ is considered as significantly different from control; Student's t test for paired observations.

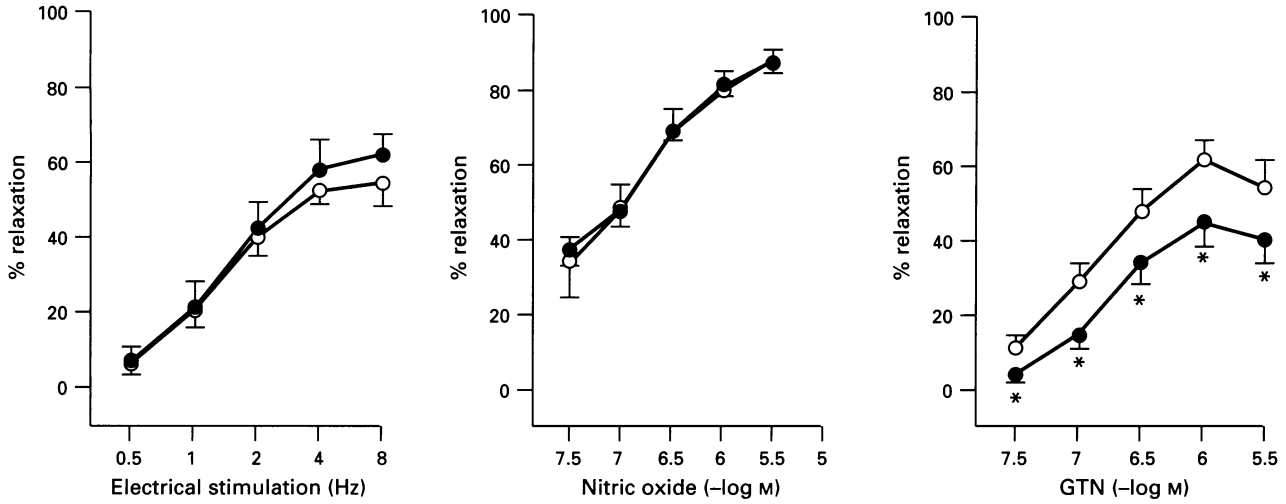

Figure 5 Frequency-response curve to electrical stimulation (0.5–8 Hz, 1 ms) and concentration-response curves to nitric oxide (0.03–10 μM) and nitroglycerin (GTN, 0.03–3 μM) in control conditions (○) and after treatment with ethacrynic acid (●, 30 μM). Results are expressed as percentage decrease of the prostaglandin $F_{2\alpha}$-induced contraction and shown as mean ± s.e.mean for $n = 6$–8 experiments. $^*P < 0.05$ is considered as significantly different from control; Student's t test for paired observations.

significantly inhibited by these thiol modulators. This inhibition most likely results from the thiol-dependency of nitroglycerin (Needleman *et al.*, 1973) with nitrosothiols as active intermediates (Ignarro *et al.*, 1981). These results indicate that relaxations to nitrergic stimulation or to NO are not thiol-dependent and suggest that the nitrergic neurotransmitter in the rat gastric fundus is not a nitrosothiol. Similarly, depletion of thiols in bovine and porcine aortic endothelial cells did not parallel the inhibition of the release of NO from these cells (Murphy *et al.*, 1991; Hecker *et al.*, 1992), arguing against thiols as intermediates in NO-mediated, endothelial-dependent responses in aortic smooth muscle. In our study, the thiol modulators, sulphobromophthalein and diamide, which were shown to be effective in the aorta (Yeates *et al.*, 1989; Siegle *et al.*, 1991), were without effect at least in the concentration used in our study. At higher concentrations, diamide inhibited the relaxation to ATP and the contraction to $PGF_{2\alpha}$ suggesting a non-specific effect of this thiol modulator. Our observation that ethacrynic acid inhibits responses to nitroglycerin is in agreement with findings in vascular smooth muscle (Needleman *et al.*, 1973) and also in the rat anococcygeus (Li *et al.*, 1994). In the rat anococcygeus, ethacrynic acid also inhibited the relaxations to nitrergic stimulation but also the relaxations to NO and the NO-independent relaxations to papaverine (Li *et al.*, 1994). In our study, ethacrynic acid and buthionine sulphoximine had no effect on the relaxations to NO or on the NO-independent relaxations to ATP. Therefore the differential effect of thiol modulators on relaxations to nitrergic stimulation and to GTN suggest that the nitrergic neurotransmission in the rat gastric fundus is not thiol-dependent. Finally, a recent report demonstrated that DETC also decomposes nitrosothiols (Arnelle *et al.*, 1995). In view of this finding, the observation in our study that DETC did not affect responses to nitrergic stimulation adds indirect evidence that the nitrergic neurotransmitter in the rat gastric fundus is not a nitrosothiol.

In summary, we have demonstrated that relaxations to nitrergic stimulation of the rat gastric fundus are inhibited by superoxide anion generators only after inhibition of Cu/Zn SOD. In addition, relaxations to nitroglycerin but not those to nitrergic stimulation are inhibited by thiol modulators. From these results we conclude that thiols are not involved in mediating nitrergic neurotransmission in the rat gastric fundus and that the nitrergic neurotransmitter in this tissue is not a nitrosothiol but more likely free NO which is effectively protected from breakdown by tissue SOD.

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