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# Influence of superoxide dismutase inhibition on the discrimination between NO and the nitrergic neurotransmitter in the rat gastric fundus

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**1** The influence of diethyldithiocarbamate (DETCA), that irreversibly inhibits Cu/Zn-containing superoxide dismutase, on the inability of 6-anilino-5,8-quinolinedione (LY83583), hypoxanthine/xanthine oxidase, hydroquinone and hydroxocobalamin to reduce electrically-induced NANC relaxations in the rat gastric fundus was investigated.

**2** Longitudinal muscle strips of the rat gastric fundus were mounted for auxotonic recording in the presence of atropine and guanethidine and tone was raised by administration of prostaglandin $F_{2\alpha}$. DETCA ($3 \times 10^{-3}$ M) slightly reduced the short-lasting relaxations induced by $10^{-5}$ M exogenous nitric oxide (NO) and transmural electrical stimulation for 10 s at 4 Hz but this effect was not influenced by 1000 u $ml^{-1}$ superoxide dismutase (SOD).

**3** DETCA ($3 \times 10^{-5} - 3 \times 10^{-3}$ M) concentration-dependently potentiated the inhibitory effect of LY83583 upon the electrically-induced relaxations, although this was less pronounced than the inhibition of the NO-induced relaxations. The inhibition of the electrically-induced non-adrenergic non-cholinergic (NANC) relaxations was not reversed by SOD while that of the NO-induced relaxations was partially reversed.

**4** The inhibitory effect of hypoxanthine/xanthine oxidase, hydroquinone and hydroxocobalamin on the electrically-induced NANC relaxations in the presence of DETCA ($3 \times 10^{-3}$ M) was not different from the inhibitory effect of DETCA alone.

**5** It was concluded that the differentiating effect of LY83583 between exogenous NO and the endogenous nitrergic neurotransmitter is partially related to protection of the endogenous nitrergic neurotransmitter by high levels of intracellular superoxide dismutase. This mechanism does not hold for hydroquinone and hydroxocobalamin, as they still discriminate between exogenous NO and the endogenous nitrergic neurotransmitter in the presence of DETCA. The possibility that the endogenous nitrergic neurotransmitter is not free NO in the rat gastric fundus therefore remains open.

**Keywords:** Rat gastric fundus; NANC relaxation; diethyldithiocarbamate; superoxide dismutase; LY83583; hypoxanthine/xanthine oxidase; hydroquinone; hydroxocobalamin

## Introduction

This study investigates the influence of irreversible inhibition of the Cu/Zn-containing superoxide dismutase with diethyldithiocarbamate (DETCA) in the rat gastric fundus on the effect of compounds such as 6-anilino-5,8-quinolinedione (LY83583) and hydroquinone, that inhibit nitric oxide (NO)-induced but not electrically induced relaxations in this tissue. The evidence that NO is a non-adrenergic non-cholinergic (NANC) neurotransmitter in peripheral nerves is accumulating fast (Lefebvre, 1995; Rand & Li, 1995). Inhibitory NANC neurones are involved in the receptive and adaptive relaxation of the proximal stomach (Abrahamsson, 1986) and NO is involved in NANC neurotransmission at this level (Lefebvre, 1993). With regard to the rat proximal stomach, nitric oxide synthase-positive neurones were found in the myenteric plexus, both by using nicotinamide adenine dinucleotide phosphate-diaphorase activity as a marker for neuronal nitric oxide synthase (Aimi *et al.*, 1993; Forster & Southam, 1993) or by immunocytochemistry with antibodies against neuronal nitric oxide synthase (Ekblad *et al.*, 1994). *In vitro* studies with longitudinal muscle strips of the rat gastric fundus revealed that NO mediates short-lasting relaxations and the initial phase of more sustained relaxations induced by transmural electrical stimulation (Li & Rand, 1990; Boeckxstaens *et al.*, 1991; 1992; D'Amato *et al.*, 1992). Correspondingly, the initial rapid relaxation induced by vagal stimulation in the vascularly isolated perfused stomach of the rat or *in vivo*, by measurement of intragastric pressure, was antagonized by nitric oxide synthase inhibitors (Lefebvre *et al.*, 1992; Takahashi & Owyang, 1995).

Some doubt has been raised about the precise nature of the nitrergic neurotransmitter in the rat gastric fundus, as the free radical scavenger and/or superoxide anion generator hydroquinone, the superoxide anion generator LY83583 and the nitric oxide-binding substances hydroxocobalamin and haemoglobin inhibit the relaxation induced by nitric oxide but not that induced by electrical stimulation of the NANC nerves (Hobbs *et al.*, 1991; Barbier & Lefebvre, 1992; Jenkinson *et al.*, 1995). Similar observations were obtained in other tissues such as the guinea-pig trachea, the bovine retractor penis, and the mouse and rat anococcygeus (Gillespie & Sheng, 1990; Hobbs *et al.*, 1991; Rajanayagam *et al.*, 1993). It was suggested that the endogenous neurotransmitter is not free NO but NO linked to a carrier, protecting it from scavengers and superoxide radical generators, S-nitrosothiols being candidates (Gibson *et al.*, 1992; Rand & Li, 1993; Barbier & Lefebvre, 1994). In the canine ileocolonic junction, pyrogallol, hydroquinone and hydroxocobalamin inhibit NO-induced relaxations but to a lesser extent or not at all the relaxations induced by stimulation of the NANC neurones when studied in organ baths; however, they do inhibit the relaxations induced by the transferable nitrergic factor, released by NANC nerve stimulation in the canine ileocolonic junction in a superfusion bioassay. As the relaxant effect of nitrosothiols was not sensitive to the scavengers and/or superoxide anion generators in both setups, it was suggested that free NO rather than a nitrosothiol is the nitrergic neurotransmitter in the canine ileocolonic junction (Boeckxstaens *et al.*, 1994; De Man *et al.*, 1995). Still, the hypothesis that an endogenous nitrosothiol,

released from the ileocolonic junction, liberates its NO during diffusion from the tissue nerve endings into the superfusion stream via a mechanism not interfering with exogenous nitrosothiols cannot be excluded.

Recently, Martin *et al.* (1994) suggested that the discrimination between NO and the nitrergic neurotransmitter in the bovine retractor penis might be related to protection of the nitrergic neurotransmitter by high levels of superoxide dismutase. Indeed, pyrogallol, LY83583 and hypoxanthine/xanthine oxidase, having little or no effect on NANC relaxations in control conditions, clearly inhibited them after inhibition of Cu/Zn dismutase with diethyldithiocarbamate. We, therefore, studied the influence of diethyldithiocarbamate on the discriminating effects of LY83583, hydroquinone, hydroxocobalamin and hypoxanthine/xanthine oxidase between NO and the nitrergic neurotransmitter in the rat gastric fundus.

## Methods

### *Tissue preparation and general methodology*

Wistar rats of either sex (170–450 g) were killed by a blow on the head and bleeding after 24 h of fasting with free access to water. Two longitudinal muscle strips (approximately 15 mm long × 3 mm wide) were prepared from the gastric fundus and mounted under a load of 1 g in 7.5 ml organ baths containing Krebs solution at 37°C (composition in mM : NaCl 118.5, KCl 4.8, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1). The Krebs solution always contained $10^{-6}$ M atropine and $4 \times 10^{-6}$ M guanethidine to block cholinergic and noradrenergic responses, respectively, and was bubbled with 95% $O_2$/5% $CO_2$. Tension was recorded auxotonically (Grass force displacement transducer FT03 coupled in series with a 1 g $cm^{-1}$ spring) on a Graphtec linearcorder F WR3701. Transmural electrical stimulation (40 V, 1 ms, 4 Hz for 10 s) was performed via 2 platinum plate electrodes (22 × 7 mm, distance in between 6 mm) by a Grass S88 stimulator with a constant voltage unit. The tissues were equilibrated for 1 h with rinsing every 15 min.

### *Protocols*

The influence of diethyldithiocarbamate (DETCA) was studied as follows. After the equilibration, the tissues were contracted by administration of $10^{-6}$ M prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Two min after a stable plateau contraction was reached, relaxation was successively induced by $10^{-5}$ M NO, electrical stimulation at 4 Hz for 10 s and $10^{-8}$ M isoprenaline at 2 to 3 min intervals. The tissues were then repetitively rinsed for 30 min and then incubated with DETCA ($3 \times 10^{-5}$ M–$3 \times 10^{-3}$ M) or its solvent for 1 h; the compounds were then washed from the tissue baths. Ten min later, contraction was again induced with $10^{-6}$ M $PGF_{2\alpha}$. Immediately a stable plateau contraction had been reached, LY83583 ($10^{-5}$ M), hydroxocobalamin ($10^{-4}$ M), hydroquinone ($10^{-4}$ or $3 \times 10^{-4}$ M) or hypoxanthine ($3 \times 10^{-4}$ M) plus xanthine oxidase (64 mu $ml^{-1}$) was administered. Two min later, the 3 relaxant stimuli were again applied. Time controls and tissues, where only DETCA or one of the scavenging or superoxide anion-generating substances was administered, were studied in parallel.

To study the influence of exogenous superoxide dismutase (SOD) on the effect of the combination of DETCA ($3 \times 10^{-3}$ M) and LY83583 ($10^{-5}$ M), it was administered in a concentration of 300 or 1000 u $ml^{-1}$ 1 min after LY83583 (thus 1 min before NO) during the second contraction with $PGF_{2\alpha}$. The influence of SOD was also tested versus DETCA alone. To investigate the influence of SOD on the effect of hydroquinone and hypoxanthine plus xanthine oxidase against NO, tissues were also contracted twice with $10^{-6}$ M $PGF_{2\alpha}$. During both contractions, $10^{-5}$ M NO was administered 3 times with an interval of 3 min. Immediately after a stable plateau contraction was reached during the second $PGF_{2\alpha}$ cycle, i.e. 2 min before the first administration of NO, hydroquinone ($10^{-4}$ M) or hypoxanthine ($3 \times 10^{-4}$ M) plus xanthine oxidase (64 mu $ml^{-1}$) was administered. In some tissues, SOD (1000 u $ml^{-1}$) was then added when tone had recovered from the first NO-induced relaxation; time controls were studied in parallel. The influence of SOD *per se* on the electrically and NO-induced relaxations was also studied.

### *Drugs*

The following drugs were used: 6-anilino-5,8-quinolinedione (LY83583; Calbiochem, La Jolla, U.S.A.), atropine sulphate (Sigma, St. Louis, U.S.A.), diethyldithiocarbamic acid sodium salt (DETCA; Sigma), guanethidine sulphate (Sigma), hydroquinone (Sigma-Aldrich, Deisenhofen, Germany), hydroxocobalamin acetate (Sigma), hypoxanthine (Sigma), isopropylnoradrenaline hydrochloride (Sanofi-Winthrop, Brussels, Belgium), prostaglandin $F_{2\alpha}$ (Sigma), superoxide dismutase from bovine erythrocytes (SOD; Sigma), xanthine oxidase from buttermilk (Sigma). Drugs were dissolved in deionized water except DETCA and xanthine oxidase, that were dissolved in physiological salt solution, LY83583, that was dissolved in 100% ethanol, and hypoxanthine, that was dissolved in 0.2 M NaOH. Stock solutions were made of $PGF_{2\alpha}$ ($10^{-3}$ M), LY83583 ($10^{-2}$ M), SOD (100,000 u $ml^{-1}$) and xanthine oxidase (6400 mu $ml^{-1}$); other solutions were prepared on the day of the experiment. A saturated NO solution was prepared as described by Kelm & Schrader (1990), by bubbling argon gas and then NO gas through 3 consecutive in-line connected gas-tight vials, the first 2 containing KOH solutions, the latter deionized water. The concentration of NO in the saturated solution in vial 3 was taken as $2 \times 10^{-3}$ M; a concentration of $10^{-5}$ M NO in the organ bath was obtained by injecting 37.5 $\mu$l from the solution with a Hamilton microsyringe. The solution was not used for longer than 2 h.

### *Data analysis*

Relaxations are expressed as percentage reduction of the $PGF_{2\alpha}$-induced tone. Responses in the presence of interfering drugs were related to those obtained before administration of these drugs. Data are given as means ± s.e.mean, $n$ referring to strips from different animals. Results within tissues were compared by a paired $t$ test and results between tissues with an unpaired $t$ test. When more than 2 groups had to be compared, analysis of variance (ANOVA) was performed; if statistical significance was reached ($P<0.05$), comparison per 2 groups was performed by a $t$ test, corrected for multiple comparisons (Bonferroni correction; Ludbrook, 1991). A difference was considered statistically significant at $P<0.05$.

## Results

### *Effect of DETCA*

Preliminary experiments showed that incubation with DETCA ($3 \times 10^{-5}$–$3 \times 10^{-3}$ M) for 1 h did not significantly influence the tone of the tissues and the contraction induced by $10^{-6}$ M $PGF_{2\alpha}$ ($n=4$). DETCA ($3 \times 10^{-3}$ M) moderately reduced the relaxation induced by $10^{-5}$ M NO and electrical stimulation at 4 Hz for 10 s to 81.4 ± 6.1 and 78.9 ± 8.7% ($n=7$) of the responses before its administration although this did not reach significance. The responses were well maintained in parallel time controls (94.5 ± 3.9 and 96.9 ± 12.4%, $n=8$). When DETCA was not rinsed out and left in contact with the tissue during the second $PGF_{2\alpha}$ cycle, a similar result was obtained (80.5 ± 4.9 and 71.5 ± 7.2%, $n=8$). The effect of DETCA on the relaxations induced by NO and electrical stimulation was not significantly affected by 1000 u $ml^{-1}$ SOD ($n=6$; data not shown).

### *Influence of DETCA on the effects of LY83583*

Previous experiments had shown that administration of $10^{-5}$ M LY83583 before $PGF_{2\alpha}$ did not influence the amplitude of the $PGF_{2\alpha}$-induced contraction (Barbier & Lefebvre, 1992). However, when in preliminary experiments $10^{-5}$ M LY83583 was incubated for 10 min after rinsing out $3\times10^{-3}$ M DETCA, but before the second administration of $PGF_{2\alpha}$, a marked (more than 50%) reduction of the $PGF_{2\alpha}$-induced contraction was observed ($n=5$). LY83583 was therefore administered once the $PGF_{2\alpha}$-induced contraction reached a plateau. The inhibitory effect on the relaxations induced by NO remained the same and the reduction of $PGF_{2\alpha}$-induced tone was still present but was less pronounced (see below).

NO ($10^{-5}$ M), electrical stimulation at 4 Hz for 10 s and isoprenaline ($10^{-8}$ M) reduced the $PGF_{2\alpha}$-induced tone by $76.7\pm5.5$, $49.4\pm5.1$ and $38.1\pm4.2\%$ ($n=8$), respectively. LY83583 ($10^{-5}$ M) markedly reduced the relaxation by NO but had a clearly less pronounced effect on the electrically-induced relaxation (Figures 1 and 2). The isoprenaline-induced relaxation was reduced by $38.3\pm5.2\%$ ($n=8$). In this series, the $PGF_{2\alpha}$-induced tone in the presence of LY83583 declined progressively to $80.5\pm4.0\%$ ($n=8$) of its original value. DETCA ($3\times10^{-5}$–$3\times10^{-3}$ M) concentration-dependently increased the inhibitory effect of LY83583 upon the electrically-induced relaxations and NO-induced relaxations, while the isoprenaline-induced relaxations were not affected (Figure 2). The $PGF_{2\alpha}$-induced tone declined progressively in the presence of LY83583 to $61.5\pm4.6$, $69.3\pm3.8$ and $56.9\pm3.8\%$ ($n=8$ each) after incubation with $3\times10^{-5}$, $3\times10^{-4}$ and $3\times10^{-3}$ M DETCA, respectively. In an additional series, the influence of $3\times10^{-3}$ M DETCA, when left in contact with the tissue during the second $PGF_{2\alpha}$ cycle, on the effect of LY83583 was studied. It was similar whether or not it was rinsed out (the relaxations induced by $10^{-5}$ M NO and electrical stimulation at 4 Hz in the presence of $3\times10^{-3}$ M DETCA plus $10^{-5}$ M LY83583 were $6.2\pm2.4$ and $58.9\pm5.7\%$ of the responses before their administration, $n=8$, $P<0.01$; when $3\times10^{-3}$ M DETCA had been rinsed out, these values were $3.4\pm1.1$ and $48.0\pm4.0\%$, $n=8$, $P<0.01$, see Figure 2).

The inhibitory effect of the combination DETCA ($3\times10^{-3}$ M) and LY83583 ($10^{-5}$ M) on the NO-induced relaxations ($94.1\pm3.6\%$, $n=11$) was reduced to $72.0\pm3.0\%$ ($n=7$) and $53.8\pm3.7\%$ ($n=7$) by 300 and 1000 u $ml^{-1}$ SOD, respectively ($P<0.01$ for both concentrations of SOD). However, their inhibitory effect on the electrically-induced relaxations was not significantly influenced ($26.1\pm3.6\%$ in the absence of SOD, $n=11$; $17.2\pm5.0\%$ in the presence of 300 u $ml^{-1}$ SOD, $n=7$; $30.7\pm7.0\%$ in the presence of 1000 u $ml^{-1}$ SOD, $n=7$). The latter was not due to the effect of NO being obtained before that of electrical stimulation as SOD also did not influence the inhibitory effect of DETCA plus LY83583 on the electrically-induced relaxations when the addition of NO was omitted from the protocol ($n=3$ for SOD 300 and 1000 u $ml^{-1}$ each; data not shown).

### *Influence of DETCA on the effects of hydroquinone, hydroxocobalamin and hypoxanthine plus xanthine oxidase*

Preliminary experiments showed that the inhibitory effects of $10^{-4}$ M hydroquinone and $10^{-4}$ M hydroxocobalamin on the NO-induced relaxations were the same when administered either 10 min before the second $PGF_{2\alpha}$ cycle or 2 min before addition of NO on top of the $PGF_{2\alpha}$-induced contraction ($n=4$ for each substance). Hydroquinone and hydroxocobalamin did not affect the $PGF_{2\alpha}$-induced contraction but hydroquinone had a moderate contractile effect *per se* when administered on non-contracted tissues; the hydroquinone-induced tone was added to that induced by $PGF_{2\alpha}$ in order to calculate the relaxant responses. In all subsequent experiments, these substances were administered once the $PGF_{2\alpha}$-induced contraction had reached a plateau. The combination of hypoxanthine and xanthine oxidase was only studied on top of the $PGF_{2\alpha}$-induced contraction to avoid substrate exhaustion during longer incubation times; the $PGF_{2\alpha}$-induced tone was not affected.

The results with DETCA are summarized in Table 1. In the control groups for these experiments, the relaxant responses to NO, electrical stimulation and isoprenaline were well main-

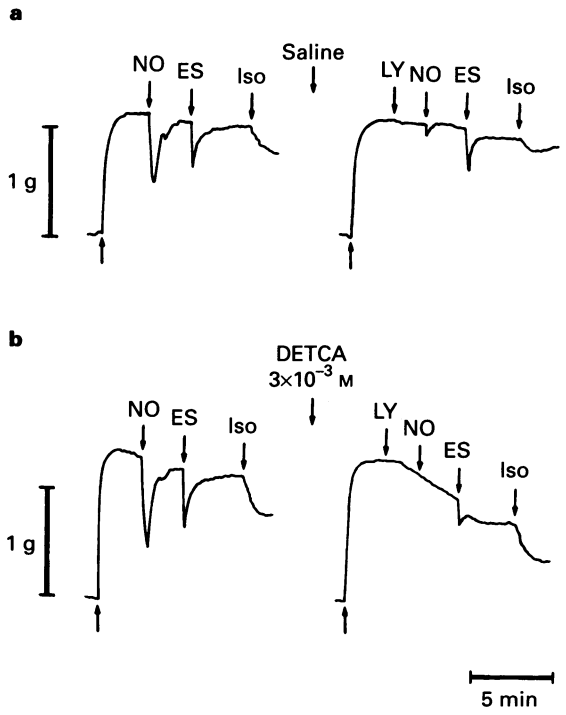


**Figure 1** Representative traces showing the influence of $10^{-5}$M LY83583 on the relaxant responses to $10^{-5}$M NO, electrical stimulation for 10 s at 4 Hz (ES) and $10^{-8}$M isoprenaline (Iso) after incubation of the tissue for 1 h with saline (a) or with $3\times10^{-3}$M diethyldithiocarbamate (DETCA) (b). The unmarked arrows indicate the administration of $10^{-6}$M prostaglandin $F_{2\alpha}$.

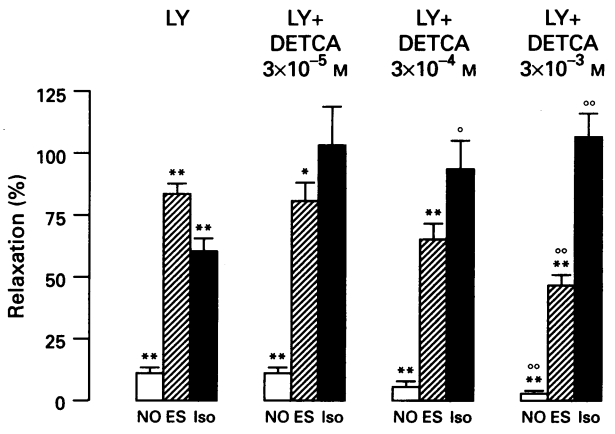


**Figure 2** Influence of diethyldithiocarbamate (DETCA) on the effect of LY83583. Relaxant responses induced by $10^{-5}$M NO, electrical stimulation for 10 s at 4 Hz (ES) and $10^{-8}$M isoprenaline (Iso) after administration of $10^{-5}$M LY83583 or DETCA ($3\times10^{-5}$M, $3\times10^{-4}$M or $3\times10^{-3}$M) and $10^{-5}$M LY83583, expressed as percentage of the responses to these stimuli obtained before administration of LY83583 and DETCA. Means ± s.e.mean of $n=8$ are shown. $^{*}P<0.05$; $^{**}P<0.01$: significantly different from the response before addition of LY83583 or DETCA and LY83583 (paired $t$ test); $^{\circ}P<0.05$; $^{\circ\circ}P<0.01$: significantly different from the responses after addition of LY83583 alone (ANOVA followed by Bonferroni multiple comparison $t$ test).

**Table 1** Influence of diethyldithiocarbamate (DETCA) on the effect of hydroquinone, hydroxocobalamin and hypoxanthine plus xanthine oxidase

| Hydroquinone $10^{-4}$ M | *Control* ($n=6$) | *HQ* ($n=6$) | *DETCA* ($n=5$) | *DETCA + HQ* ($n=6$) |
|---|---|---|---|---|
| NO | 81.0±4.0 | 26.5±9.4** | 85.3±8.0 | 18.1±4.8**,°° |
| ES | 68.9±6.3 | 101.4±9.9 | 78.7±10.0 | 88.8±10.5 |
| Iso | 74.8±6.3 | 94.4±6.7 | 125.4±15.8 | 97.2±3.1 |
| **Hydroquinone $3\times10^{-4}$ M** | *Control* ($n=6$) | *HQ* ($n=6$) | *DETCA* ($n=6$) | *DETCA + HQ* ($n=6$) |
| NO | 84.7±5.0 | 7.9±5.6** | 75.2±3.2 | 4.3±3.2**,°° |
| ES | 94.0±3.4 | 114.8±7.4 | 59.3±2.9** | 67.9±4.2**,△△ |
| Iso | 106.3±9.4 | 116.1±10.5 | 125.5±7.9 | 108.8±25.7 |
| **Hydroxocobalamin $10^{-4}$ M** | *Control* ($n=6$) | *HC* ($n=8$) | *DETCA* ($n=6$) | *DETCA + HC* ($n=7$) |
| NO | 92.5±3.5 | 78.6±6.1 | 85.3±7.2 | 9.7±1.7**,△△,°° |
| ES | 92.5±12.7 | 88.0±7.5 | 75.9±6.5 | 68.0±3.1 |
| Iso | 92.3±6.4 | 74.5±6.8 | 111.8±8.0 | 110.1±8.6△ |
| **Hypoxanthine $3\times10^{-4}$ M plus xanthine oxidase 64 mu $ml^{-1}$** | *Control* ($n=8$) | *HX/XO* ($n=8$) | *DETCA* ($n=8$) | *DETCA + HX/XO* ($n=8$) |
| NO | 94.3±5.6 | 16.9±2.8** | 72.0±3.7* | 8.4±2.0**,°° |
| ES | 104.3±6.6 | 116.1±8.9 | 81.8±7.2 | 74.4±5.0*,△△ |
| Iso | 102.9±8.6 | 69.8±17.5 | 136.6±15.3 | 77.6±23.3 |

In four parallel tissues, the relaxant responses to $10^{-5}$ M NO, electrical stimulation at 4 Hz for 10 s (ES) and $10^{-8}$ M isoprenaline (Iso) were studied before and after administration of $3\times10^{-3}$ M DETCA, hydroquinone (HQ), hydroxocobalamin (HC) or hypoxanthine/xanthine oxidase (HX/XO), the combination or the solvents (control). The relaxations to NO, ES and Iso after administration of these drugs are given, expressed as percentage of the responses to these stimuli before the administration; means±s.e.mean. *$P<0.05$; **$P<0.01$: significantly different versus control; △$P<0.05$; △△$P<0.01$: significantly different versus HQ or HC or HX/XO; °°$P<0.01$: significantly different versus DETCA (ANOVA followed by Bonferroni multiple comparison $t$ test).

tained, except in the series where $10^{-4}$ M hydroquinone was studied. Confirming results obtained previously, DETCA *per se* tended to decrease the relaxations induced by NO and electrical stimulation but the decrease of the NO-induced relaxation was only significant in the series where hypoxanthine plus xanthine oxidase was studied, and that of the electrically-induced relaxation in the series where $3\times10^{-4}$ M hydroquinone was studied.

Hydroquinone ($10^{-4}$ and $3\times10^{-4}$ M) concentration-dependently inhibited the NO-induced relaxation without influencing the electrically-induced relaxation (Table 1). After the tissues had been incubated for 1 h in $3\times10^{-3}$ M DETCA, the inhibitory effect of hydroquinone on NO-induced relaxations was not significantly changed. After incubation with DETCA, the electrically-induced relaxation in the presence of $3\times10^{-4}$ M hydroquinone (67.9±4.2%, $n=6$) was significantly smaller than when hydroquinone was administered alone (114.8±7.4%, $n=6$); however, this response was not significantly different from that after incubation with DETCA alone (59.3±2.9%, $n=6$). Responses to isoprenaline were not influenced by hydroquinone or DETCA + hydroquinone.

While $10^{-4}$ M hydroxocobalamin reduced the relaxation induced by NO to 65% of that before its administration in the preliminary experiments, it now only reduced this response to 78.6±6.1% ($n=8$, Table 1). After incubation with DETCA, the inhibitory effect of hydroxocobalamin versus NO was greatly enhanced but its effect on the electrically-induced relaxation was not significantly changed (Figure 3 and Table 1). Responses to isoprenaline were not inhibited by hydroxocobalamin, alone or after incubation with DETCA.

The combination of hypoxanthine ($3\times10^{-4}$ M) plus xanthine oxidase (64 mu $ml^{-1}$) clearly reduced the NO-induced relaxation without influencing those to electrical stimulation and to isoprenaline (Table 1). In the presence of hypoxanthine/xanthine oxidase, NO either induced a small fast relaxation (4 strips out of 8) or it induced a small slowly developing relaxation (4 strips out of 8). When DETCA was added, the relaxant effect of NO in the presence of hypoxanthine/xanthine oxidase (8.4±2.0%, $n=8$) did not significantly differ from that when hypoxanthine/xanthine oxidase was added alone (16.9±2.8%, $n=8$), but the electrically-induced relaxation (74.4±5.0%, $n=8$) was sig-

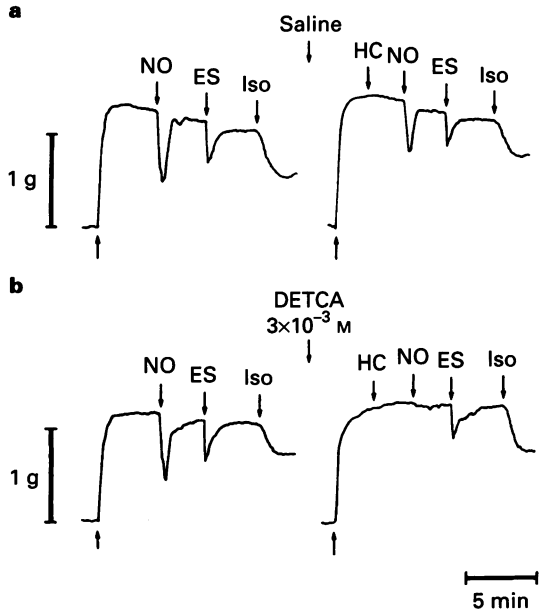


**Figure 3** Representative traces showing the influence of $10^{-4}$ M hydroxocobalamin (HC) on the relaxant responses to $10^{-5}$ M NO, electrical stimulation for 10 s at 4 Hz (ES) and $10^{-8}$ M isoprenaline (Iso) after incubation of the tissue for 1 h with saline (a) or with $3\times10^{-3}$ M diethyldithiocarbamate (DETCA) (b). The unmarked arrows indicate the administration of $10^{-6}$ M prostaglandin $F_{2\alpha}$.

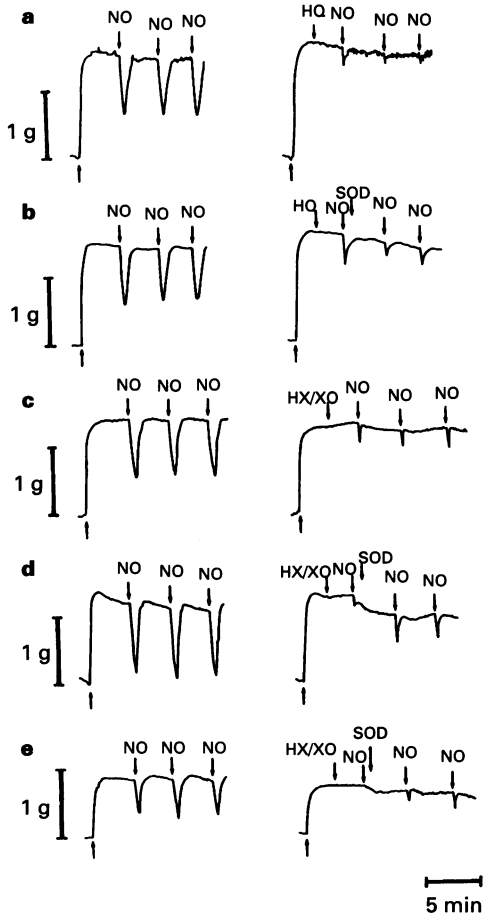


**Figure 4** Representative traces showing the influence of $10^{-4}$ M hydroquinone (HQ, a and b) and $3 \times 10^{-4}$ M hypoxanthine plus 64 mu ml$^{-1}$ xanthine oxidase (HX/XO, c, d and e) on the relaxant responses to $10^{-5}$ M NO. In (b, d and e) 1000 u ml$^{-1}$ superoxide dismutase (SOD) was added after the first response to NO in the presence of HQ or HX/XO had been obtained. The unmarked arrows indicate the administration of $10^{-6}$ M prostaglandin $F_{2\alpha}$.

nificantly smaller than when hypoxanthine/xanthine oxidase was added alone (116.1±8.9%, $n=8$). However, the response was not significantly different from that after addition of DETCA alone (81.8±7.2%, $n=8$).

### *Influence of SOD on the effects of hydroquinone and hypoxanthine plus xanthine oxidase on NO-induced relaxations*

In control tissues, the relaxant response to $10^{-5}$ M NO, administered 3 times with an interval of 3 min, was reproducible during the first and second $PGF_{2\alpha}$ cycle. SOD (1000 u ml$^{-1}$) did not reverse the inhibitory effect of hydroquinone ($10^{-4}$ M) on NO-induced relaxations. In the presence of SOD, the inhibitory effect was even somewhat increased (Figure 4b, $n=8$) but the same was observed in tissues where SOD was not added (Figure 4a, $n=8$). The inhibitory effect of hypoxanthine ($3 \times 10^{-4}$ M) plus xanthine oxidase (64 mu ml$^{-1}$) on NO-induced relaxations was well maintained (Figure 4c and 5) for 3 consecutive administrations of NO. SOD clearly reversed the inhibitory effect of hypoxanthine plus xanthine oxidase (Figure 5). Furthermore, the shape of the NO-induced relaxation returned to its original one (Figure 4d and e).

SOD *per se* did not significantly influence either the relaxations induced by $10^{-5}$ M NO or those induced by electrical stimulation at 4 Hz. Expressed as a percentage of the responses before solvent or SOD, the NO-induced relaxations were 82.0±2.0% in the presence of the solvent and 88.9±8.0% in the presence of 1000 u ml$^{-1}$ SOD ($n=4$); the electrically-induced relaxations in the presence of solvent and 1000 u ml$^{-1}$ SOD were 75.4±4.6% and 77.6±6.2%, respectively ($n=4$).

## Discussion

The aim of this study was to investigate whether irreversible inhibition of the Cu/Zn-containing superoxide dismutase with DETCA (Kelner *et al.*, 1989) in the rat gastric fundus influences the ability of some compounds to inhibit NO-induced relaxations while not affecting electrically-induced NANC relaxations. In the bovine retractor penis muscle, superoxide anion-generating agents inhibited NANC relaxations after treatment with DETCA, while having little effect in control conditions, suggesting that the endogenous nitrergic neurotransmitter is protected from interaction with superoxide anions by high levels of endogenous superoxide dismutase (Martin *et al.*, 1994). In the rat gastric fundus, as in the bovine retractor penis (Liu *et al.*, 1994), the electrically-induced NANC relaxations as well as the NO-induced relaxations were not potentiated by exogenous addition of superoxide dismutase. This suggests that the extracellular levels of endogenous superoxide dismutase are sufficiently high to protect the endogenous nitrergic neurotransmitter against extracellularly formed superoxide anions. As exogenous superoxide dismutase cannot enter cells, the possibility of degradation of the endogenous nitrergic neurotransmitter by intracellularly formed superoxide anions cannot be excluded.

In the rat gastric fundus, the influence of DETCA was tested versus both superoxide anion-generating compounds and substances, acting via another mechanism. LY83583 is often proposed as an inhibitor of soluble guanylate cyclase (Mülsch *et al.*, 1988; Kawada *et al.*, 1994) but Furchgott & Jothianandan (1991) suggested that LY83583 generates superoxide anions, rapidly inactivating the unstable NO molecule. Our previous results in the rat gastric fundus suggest that it inhibits NO-induced relaxations in this tissue by superoxide anion generation (Barbier & Lefebvre, 1992). Hydroquinone has been suggested to inhibit the relaxant effect of EDRF by generation of superoxide anions (Moncada *et al.*, 1986) but its inhibitory effect on NO-induced relaxations in the mouse anococcygeus was not reversed by superoxide dismutase and it failed to generate superoxide anions, as analysed by chemiluminescence, suggesting that it acts through free radical scavenging (Hobbs *et al.*, 1991). As the inhibitory effect of hydroquinone versus NO in the rat gastric fundus was not influenced by addition of superoxide dismutase, SOD probably acts as a free radical scavenger in this tissue too. The superoxide anion-generating capacity of the substrate hypoxanthine/enzyme xanthine oxidase system (Hobbs *et al.*, 1991; Berman & Martin, 1993) was confirmed in the rat gastric fundus as its inhibitory effect on NO-induced relaxation was reversed by superoxide dismutase. In contrast to the other inhibitors, hypoxanthine/xanthine oxidase also changed the shape of the NO-induced relaxations in some of the tissues. The moderate inhibitory effect of the NO scavenger hydroxocobalamin on NO-induced relaxations in the rat gastric fundus was confirmed in this study but we did not observe the increase in duration of the NO-induced response described by Jenkinson *et al.* (1995). Non-specific interference of the drugs and drug combinations tested in the rat gastric fundus with the relaxant mechanisms in the smooth muscle cells seems to be excluded as they did not influence the relaxant effect of isoprenaline, which acts by stimulating $\beta$-receptors and activation of adenylate cyclase (Lefebvre *et al.*, 1984; Bülbring & Tomita, 1987). A reduction of isoprenaline-induced relaxation by $10^{-5}$ M LY83583 was, indeed, not found in our previous study (Barbier & Lefebvre, 1992) and was also not present in the 3 series of experiments where LY83583 was studied after treatment with DETCA.

DETCA was used as described by Martin *et al.* (1994), i.e. it was incubated with the tissues for 1 h and then washed out. In

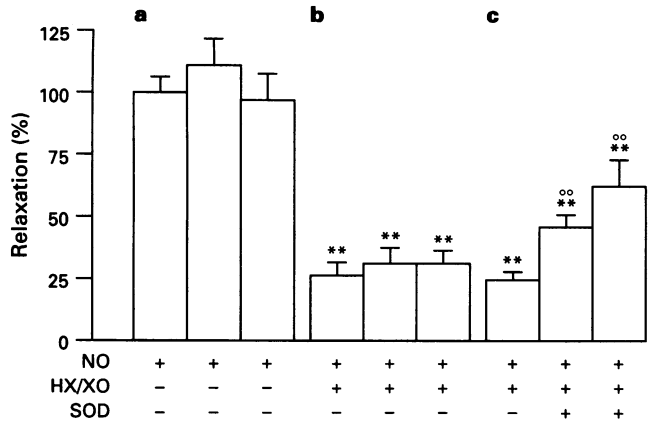


**Figure 5** Influence of superoxide dismutase (SOD) on the inhibitory effect of hypoxanthine plus xanthine oxidase (HX/XO) versus NO. Relaxant responses to 3 consecutive administrations of $10^{-5}$ M NO are shown (a) in control tissues, receiving the solvents of HX/XO, (b) in the presence of $3 \times 10^{-4}$ M HX plus 64 mu ml$^{-1}$ XO and (c) after addition of 1000 u ml$^{-1}$ SOD in the presence of HX/XO. The relaxations were expressed as percentage of the first NO-induced relaxation before the administration of HX/XO or the solvents. Means ± s.e.mean of $n=8$ are shown. $^{**}P<0.01$: significantly different from the first NO-induced relaxation; $^{\circ\circ}P<0.01$: significantly different from the response before addition of SOD (paired $t$ test).

view of the irreversible inhibition of superoxide dismutase by DETCA, one can expect that its effect is maintained and we, indeed, observed that its effect *per se* on the relaxations and its influence versus the effect of LY83583 was not increased when left in contact with the tissue after the 1 h incubation. DETCA *per se* moderately reduced the electrically-induced NANC relaxation but, in contrast to results obtained in the bovine retractor penis (Martin *et al.*, 1994), this inhibitory effect was not reversed by exogenous superoxide dismutase. As exogenous superoxide dismutase cannot enter cells, the inhibitory effect of DETCA might result from inhibition of intracellular superoxide dismutase; alternatively, it might be a non-specific effect. DETCA concentration-dependently increased the inhibitory effect of $10^{-5}$ M LY83583 versus the electrically-induced NANC relaxations, although it never became as pronounced as the inhibition of the NO-induced relaxations. The $PGF_{2\alpha}$-induced tone declined progressively in the presence of LY83583 after incubation with DETCA but this does not explain the decreased amplitude of the NANC relaxations, as the decrease of tone was similar for DETCA $3 \times 10^{-5}$ to $3 \times 10^{-3}$ M, while the amplitude of the NANC relaxations concentration-dependently decreased. The more pronounced effect of DETCA plus LY83583 on relaxations induced by exogenous NO than on those induced by electrical stimulation might be related to the shorter diffusion path of endogenous NO versus exogenous NO (Wood & Garthwaite, 1994). The inhibitory effect of DETCA plus LY83583 on the electrically-induced NANC relaxations was not reversed by exogenous superoxide dismutase, while the effect on NO-induced relaxations was partially reversed. As exogenous superoxide dismutase cannot enter cells, the latter observation illustrates that the interaction of exogenous NO with superoxide anions, at least in part, takes place extracellularly. DETCA blocks Cu/Zn superoxide dismutase both at the extracellular and intracellular levels (Kelner *et al.*, 1989) and LY83583 generates superoxide anions extra- and intracellularly (Mülsch *et al.*, 1988). The inability of exogenous superoxide dismutase to reverse the inhibition of the NANC relaxations by DETCA plus LY83583 might thus suggest that the interaction of the endogenous nitrergic neurotransmitter with superoxide anions mainly takes place intracellularly. This is corroborated by the observation that DETCA did not have an inhibitory influence on the effect of the extracellularly superoxide anion-generating system hypoxanthine/xanthine oxidase on electrically-induced relaxations. This contrasts with results obtained in the bovine retractor penis muscle, but in this tissue the interaction of the endogenous nitrergic neurotransmitter with superoxide anions seems to occur mainly at the extracellular level (Martin *et al.*, 1994). Our results thus suggest that the differentiating effects of LY83583 on relaxations induced by electrical stimulation and NO might be partially due to protection of the endogenous nitrergic neurotransmitter from destruction by superoxide anions afforded by high levels of intracellular superoxide dismutase. Still, the possibility that the endogenous nitrergic neurotransmitter is not free NO cannot be completely ruled out as DETCA did not influence the differentiating effect of the free radical scavenger hydroquinone and the NO-binding substance hydroxocobalamin in the rat gastric fundus, even when a concentration of hydroquinone that nearly abolished the relaxation by exogenous NO was used. In the mouse anococcygeus, DETCA also did not affect the inhibitory effect of hydroquinone on electrically-induced NANC relaxations, although it did affect that of the structurally related duroquinone; this difference was explained by the more pronounced liability of duroquinone to generate superoxide anions than hydroquinone (Lilley & Gibson, 1995). Surprisingly, DETCA markedly potentiated the inhibitory effect of hydroxocobalamin on the relaxation by exogenous NO. We have no obvious explanation why DETCA potentiates the formation of nitrosocobalamin from hydroxocobalamin.

In conclusion, the differentiating effect of LY83583 between exogenous NO and the endogenous nitrergic neurotransmitter in the rat gastric fundus is partially related to protection of the endogenous neurotransmitter by high levels of intracellular superoxide dismutase. DETCA did not abolish the discrimination between exogenous NO and the endogenous nitrergic neurotransmitter by hydroquinone and hydroxocobalamin. This leaves open the possibility that in the rat gastric fundus the endogenous nitrergic neurotransmitter is not free NO in contrast to the bovine retractor penis. The nitrergic neurotransmitter might, indeed, not be identical in all tissues (Rand & Li, 1995).

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