



# Influence of bilirubin and other antioxidants on nitrenergic relaxation in the pig gastric fundus

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**1** The influence of several antioxidants (bilirubin, urate, ascorbate,  $\alpha$ -tocopherol, glutathione (GSH), Cu/Zn superoxide dismutase (SOD) and the manganese SOD mimic EUK-8) on nitrenergic relaxations induced by either exogenous nitric oxide (NO;  $10^{-5}$  M) or electrical field stimulation (4 Hz; 10 s and 3 min) was studied in the pig gastric fundus.

**2** Ascorbate ( $5 \times 10^{-4}$  M),  $\alpha$ -tocopherol ( $4 \times 10^{-4}$  M), SOD (300–1000 u ml<sup>-1</sup>) and EUK-8 ( $3 \times 10^{-4}$  M) did not influence the relaxations to exogenous NO. In the presence of GSH ( $5 \times 10^{-4}$  M), the short-lasting relaxation to NO became biphasic, potentiated and prolonged. Urate ( $4 \times 10^{-4}$  M) and bilirubin ( $2 \times 10^{-4}$  M) also potentiated the relaxant effect of NO. None of the antioxidants influenced the electrically evoked relaxations.

**3** 6-Anilino-5,8-quinolinedione (LY83583;  $10^{-5}$  M) had no influence on nitrenergic nerve stimulation but nearly abolished the relaxant response to exogenous NO. Urate and GSH completely prevented this inhibitory effect, while it was partially reversed by SOD and bilirubin. Ascorbate,  $\alpha$ -tocopherol and EUK-8 were without effect.

**4** Hydroquinone ( $10^{-4}$  M) did not affect the electrically induced nitrenergic relaxations, but markedly reduced NO-induced relaxations. The inhibition of exogenous NO by hydroquinone was completely prevented by urate and GSH. SOD and ascorbate afforded partial protection, while bilirubin, EUK-8 and  $\alpha$ -tocopherol were ineffective.

**5** Hydroxocobalamin ( $10^{-4}$  M) inhibited relaxations to NO by 50%, but not the electrically induced responses. Full protection versus this inhibitory effect was obtained with urate, GSH and  $\alpha$ -tocopherol.

**6** These results strengthen the hypothesis that several endogenous antioxidant defense mechanisms, enzymatic as well as non-enzymatic, might play a role in the nitrenergic neurotransmission process. *British Journal of Pharmacology* (2000) **129**, 1201–1211

**Keywords:** pig gastric fundus; LY83583; hydroquinone; hydroxocobalamin; bilirubin; urate; glutathione; superoxide dismutase; EUK-8; nitric oxide

**Abbreviations:** DETCA, diethyldithiocarbamate; EFS, electrical field stimulation; GSH, glutathione; 5-HT, 5-hydroxytryptamine; LY83583, 6-anilino-5,8-quinolinedione; NANC, non-adrenergic non-cholinergic; NO, nitric oxide; NO<sup>+</sup>, nitrosonium cation; NO<sup>-</sup>, nitroxyl anion; SNP, sodium nitroprusside; SOD, superoxide dismutase

## Introduction

Peripheral nitrenergic neurotransmission, mediating smooth muscle relaxation, is well established in the gastrointestinal, urogenital and respiratory tracts. The term 'nitrenergic' refers to nerves whose transmitter function depends on the release of NO (Moncada *et al.*, 1997). Although it is accepted that the neurotransmitter, released from nitrenergic nerves, is synthesized by neuronal NO-synthase in the nerves (Förstermann & Kleinert, 1995), there still remains doubt on the exact biochemical identity of the nitrenergic neurotransmitter (Rand & Li, 1995). This is based on observations that superoxide anion generators and NO-scavengers discriminate between exogenous NO and the nitrenergic neurotransmitter: they inhibit the relaxation by exogenous NO but not that by activation of nitrenergic nerves (Hobbs *et al.*, 1991; Barbier & Lefebvre, 1992).

It has been suggested that the endogenous neurotransmitter is not free NO but NO linked to a carrier before release into the neuroeffector region. In this region, the carrier would protect NO versus superoxide anion generators and scavengers and release NO upon interaction with the effector smooth muscle cells. S-nitrosothiols seemed the most probable candidates to act as NO carriers (Gibson *et al.*, 1992; Barbier

& Lefebvre, 1994) but the similarity between the pharmacological properties of the nitrenergic neurotransmitter and S-nitrosothiols was not complete in all tissues (Iversen *et al.*, 1994; De Man *et al.*, 1995). Another possibility is that the nitrenergic neurotransmitter is nitrogen monoxide but that it is synthesized as or converted to another redox form i.e. nitrosonium (NO<sup>+</sup>) or nitroxyl (NO<sup>-</sup>) before leaving the neuron (Gibson *et al.*, 1995). This hypothesis is difficult to investigate as one has to rely on the assumption that NO<sup>-</sup> or NO<sup>+</sup> donors indeed only act by dissociation of NO<sup>-</sup> or NO<sup>+</sup> and that none of the NO<sup>-</sup> or NO<sup>+</sup> is oxidized or reduced to free radical NO<sup>•</sup>. The available evidence does not favour NO<sup>+</sup> as nitrenergic neurotransmitter (Goyal & He, 1998; Li *et al.*, 1999). In the rat anococcygeus, NO<sup>-</sup> more closely resembles the nitrenergic neurotransmitter than NO<sup>•</sup> but still, it does not behave exactly like the neurotransmitter (Li *et al.*, 1999). Furthermore, NO<sup>-</sup> as neurotransmitter requires a mechanism for transport through the membrane, as this ionized redox form will not diffuse freely.

Upon irreversible inhibition of the Cu/Zn containing enzyme superoxide dismutase (Cu/Zn SOD) by the Cu-chelator diethyldithiocarbamate (DETCA) in the bovine retractor penis muscle, nitrenergic neurotransmission became sensitive to the superoxide generators pyrogallol and hypox-

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anthine/xanthine oxidase (Martin *et al.*, 1994). Similar results were obtained in the mouse anococcygeus with duroquinone (Lilley & Gibson, 1995) and in the rat gastric fundus with LY83583 (Lefebvre, 1996), suggesting that high levels of tissue Cu/Zn SOD afford protection of the nitergic transmitter from destruction by superoxide. This mechanism can however not explain the following observations: (1) DETCA-pretreatment did not alter the differential action of superoxide generated by hypoxanthine/xanthine oxidase and pyrogallol in respectively the rat gastric fundus (Lefebvre, 1996) and the rat anococcygeus muscle (La & Rand, 1999); (2) the discriminating effect between exogenous NO and the endogenous nitergic neurotransmitter of the NO-scavenging agents carboxy-PTIO in the mouse anococcygeus (Lilley & Gibson, 1996) and hydroxocobalamin in the rat gastric fundus (Lefebvre, 1996), the latter still differentiating even in the presence of DETCA. Besides Cu/Zn SOD, other antioxidant defences might act in concert within nitergically-innervated tissues to protect free radical NO (Gibson & Lilley, 1997). Indeed, reduced glutathione,  $\alpha$ -tocopherol and ascorbate were shown to protect NO from some or all of a set of direct NO-scavengers and superoxide anion generators in the mouse anococcygeus (Lilley & Gibson, 1996) and the release of ascorbate and urate upon depolarization was demonstrated in the rat anococcygeus (Lilley & Gibson, 1997).

The aim of this study was to investigate the influence of several antioxidants on nitergic relaxations in the pig gastric fundus. The antioxidants tested included bilirubin; this bile pigment has a pronounced antioxidant effect (Stocker *et al.*, 1987). It is formed by rapid reduction of biliverdin; the enzyme catalyzing the formation of biliverdin from haeme, haeme oxygenase-2, is colocalized with neuronal NO-synthase in 60–70% of myenteric neurons (Zakhary *et al.*, 1997).

## Methods

### Tissue preparation

Experiments were carried out on isolated circular smooth muscle strips of the porcine gastric fundus. The stomach was removed from healthy 6 month old male castrated pigs, slaughtered at a local abattoir, and transported to the laboratory in ice-chilled physiological salt solution. After the mucosa was removed, strips (15 × 3 mm) were cut from the fundus in the direction of the circular muscle layer. All tissues were used immediately. Strips were mounted vertically between two platinum plate electrodes under a load of 2 g in 5 or 20 ml organ baths, containing physiological salt solution at 37°C and gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The composition of the physiological salt solution was (mM): Na<sup>+</sup> 137, K<sup>+</sup> 5.9, Ca<sup>2+</sup> 2.5, Mg<sup>2+</sup> 1.2, Cl<sup>-</sup> 124.1, HCO<sub>3</sub><sup>-</sup> 25, H<sub>2</sub>PO<sub>4</sub><sup>-</sup> 1.2 and glucose 11.5 (Mandrek & Milenov, 1991). To obtain NANC conditions, atropine (10<sup>-6</sup> M) and guanethidine (4 × 10<sup>-6</sup> M) were continuously present in the medium. Changes in length were recorded isotonicly *via* Hugo Sachs B40 Lever transducers type 373 on a Graphtec Linearcorder 8 WR 3500 in the 5 ml baths and *via* Palmer Bioscience T3 transducers on a Graphtec Linearcorder WR 3701 F in the 20 ml organ baths. Electrical field stimulation (EFS) (40 V, 0.1 ms, 4 Hz) was applied by means of a Hugo Sachs Stimulator I type 215/I in the 5 ml baths and by a Grass S88 stimulator in the 20 ml baths. The tissues were equilibrated for 90 min with rinsing every 15 min before starting the experiment.

### Protocols

After the equilibration period, all strips were first contracted with 3 × 10<sup>-7</sup> M 5-hydroxytryptamine (5-HT) and subsequently relaxed by 10<sup>-5</sup> M sodium nitroprusside (SNP) before continuing the experimental protocol.

In a first set of investigations, the effect of antioxidants *per se* on electrical field stimulation (EFS; 40 V, 0.1 ms, 4 Hz, 10 s and 3 min) and on a NO-bolus (10<sup>-5</sup> M) was studied as follows. Tissues were rinsed for 1 h; tone was raised again with 3 × 10<sup>-7</sup> M 5-HT and when a stable plateau contraction was obtained, three relaxant stimuli were consecutively studied with a 5 min interval in between: EFS at 4 Hz for 10 s, 10<sup>-5</sup> M NO and EFS at 4 Hz for 3 min. After repetitive rinsing for 1 h, the preparations were again contracted with 3 × 10<sup>-7</sup> M 5-HT. When the contraction amplitude was stable, one of the antioxidants was added and after a 2 min incubation period the three relaxant stimuli were studied for the second time. The concentrations of the antioxidants were: SOD, 300 and 1000 u ml<sup>-1</sup>; ascorbate, 5 × 10<sup>-4</sup> M;  $\alpha$ -tocopherol, 4 × 10<sup>-4</sup> M; bilirubin, 2 × 10<sup>-4</sup> M; GSH, 5 × 10<sup>-4</sup> M; urate, 4 × 10<sup>-4</sup> M. The cell permeable manganese superoxide dismutase mimetic EUK-8 was also tested (3 × 10<sup>-4</sup> M). In parallel control tissues, the solvents of the antioxidants were applied.

To study the influence of 6-anilino-5,8-quinolinedione (LY83583; 10<sup>-5</sup> M), hydroxocobalamin (10<sup>-4</sup> M) and hydroquinone (10<sup>-4</sup> M) on the relaxant stimuli, these agents were added 15 min before the third 5-HT-induced contraction, where the relaxant stimuli were tested a second time. To investigate the influence of the antioxidants on the effect of LY83583, hydroxocobalamin and hydroquinone, the latter agents were added 15 min before the third 5-HT-induced contraction, and the antioxidants on top of this contraction 2 min before EFS at 4 Hz for 10 s was applied. The influence of LY83583, hydroxocobalamin and hydroquinone was also tested on sustained relaxation induced by either continuous EFS at 4 Hz or by infusion of NO. We used two different experimental protocols. In the first, continuous NO administration for 10 min was started at the top of the second 5-HT-induced contraction by infusing per 10 s the amount yielding 10<sup>-5</sup> M, when given in bolus, into the bath *via* a Braun infusion pump; LY83583 (10<sup>-5</sup> M), hydroxocobalamin (10<sup>-4</sup> M) and hydroquinone (10<sup>-4</sup> M) or their solvent were injected into the organ bath 15 min before the third 5-HT-induced contraction, at the top of which the NO-infusion for 10 min was repeated. In the second protocol, the above mentioned substances were only added exactly 5 min after start of relaxation induced by NO-infusion or by continuous EFS at 4 Hz applied at the top of a second 5-HT-induced contraction; both the NO-infusion and the EFS were subsequently sustained for another 10 min.

### Drugs

The following drugs were used (supplied by Sigma unless stated otherwise): 6-anilino-5,8-quinolinedione (LY83583; Calbiochem), L-ascorbic acid, atropine sulphate, bilirubin ditaurate (Calbiochem), EUK-8 (Calbiochem), glutathione, guanethidine sulphate, hydroquinone, hydroxocobalamin acetate, 5-hydroxytryptamine creatinine monosulphate (Janssen Chimica), sodium nitroprusside, Cu/Zn superoxide dismutase (from bovine erythrocytes),  $\alpha$ -tocopherol, uric acid. Drugs were dissolved in deionized water except LY83583, that was dissolved in 100% ethanol, uric acid that was dissolved in 0.1 M NaOH and  $\alpha$ -tocopherol, that was dissolved in

dimethylsulphoxide. Solvents themselves were without significant effect at the concentrations used in the experiments unless otherwise indicated. Stock solutions were made of LY83583 ( $10^{-2}$  M) and SOD (100,000 u ml $^{-1}$ ); other solutions were prepared on the day of the experiment. A saturated NO solution was prepared as described by Kelm & Schrader (1990), yielding a vial containing NO in a concentration taken to be  $2 \times 10^{-3}$  M.

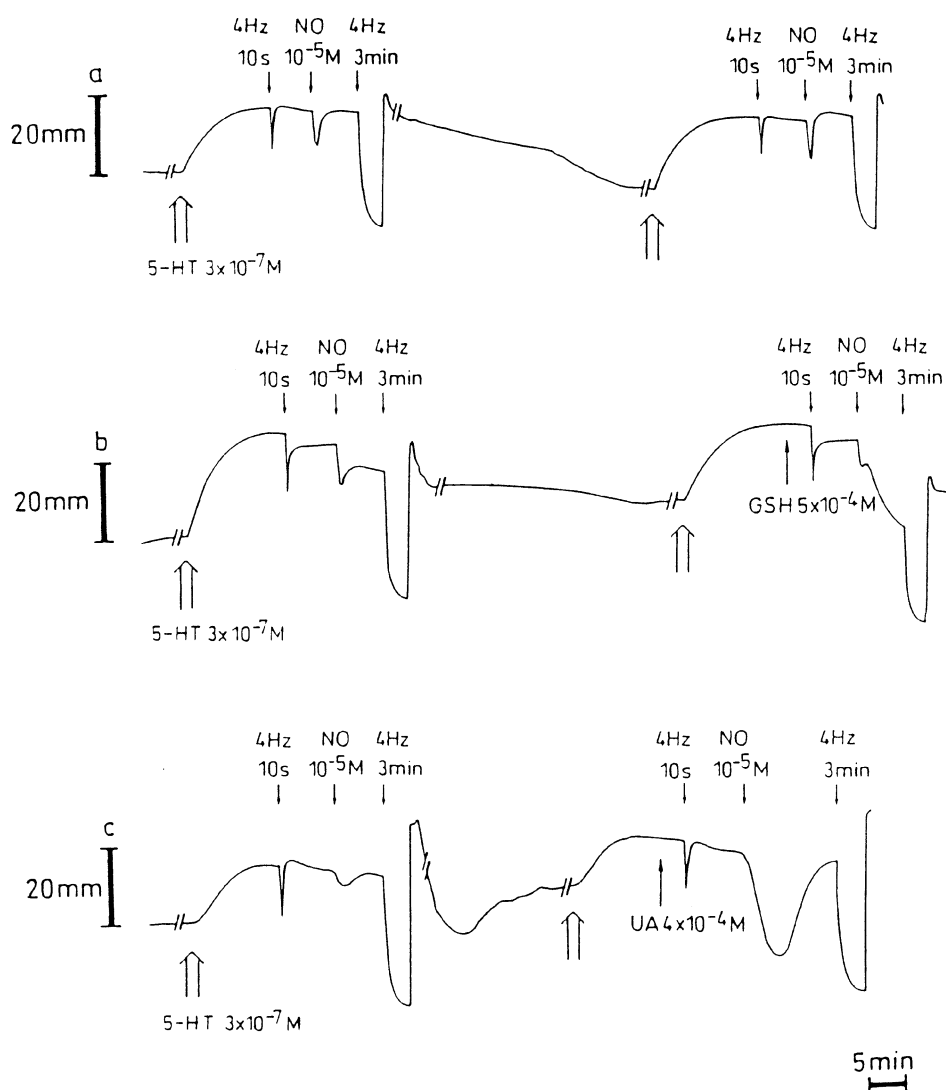
#### Data analysis

Relaxations induced by NO and EFS were expressed as percentage of the relaxation induced by  $10^{-5}$  M sodium nitroprusside at the beginning of the experiment. Responses in the presence of interfering drugs were related to those obtained before administration of these drugs. Experimental data are expressed as means  $\pm$  s.e.mean and  $n$  refers to the number of tissues from different animals. Results within tissues were compared by a paired  $t$ -test and results between tissues with an unpaired  $t$ -test; a probability value of  $P < 0.05$  was considered statistically significant.

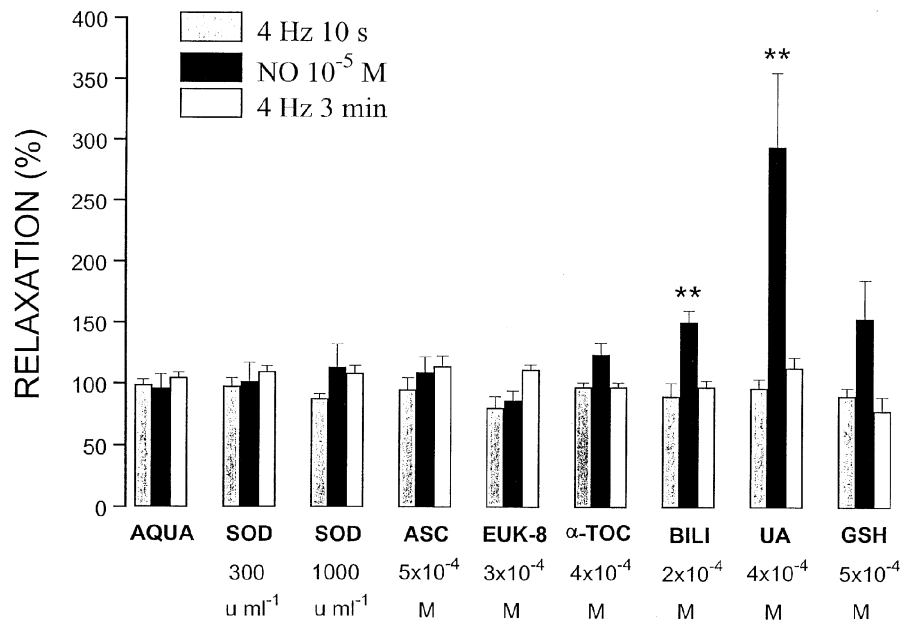
## Results

### Effect of the antioxidants per se

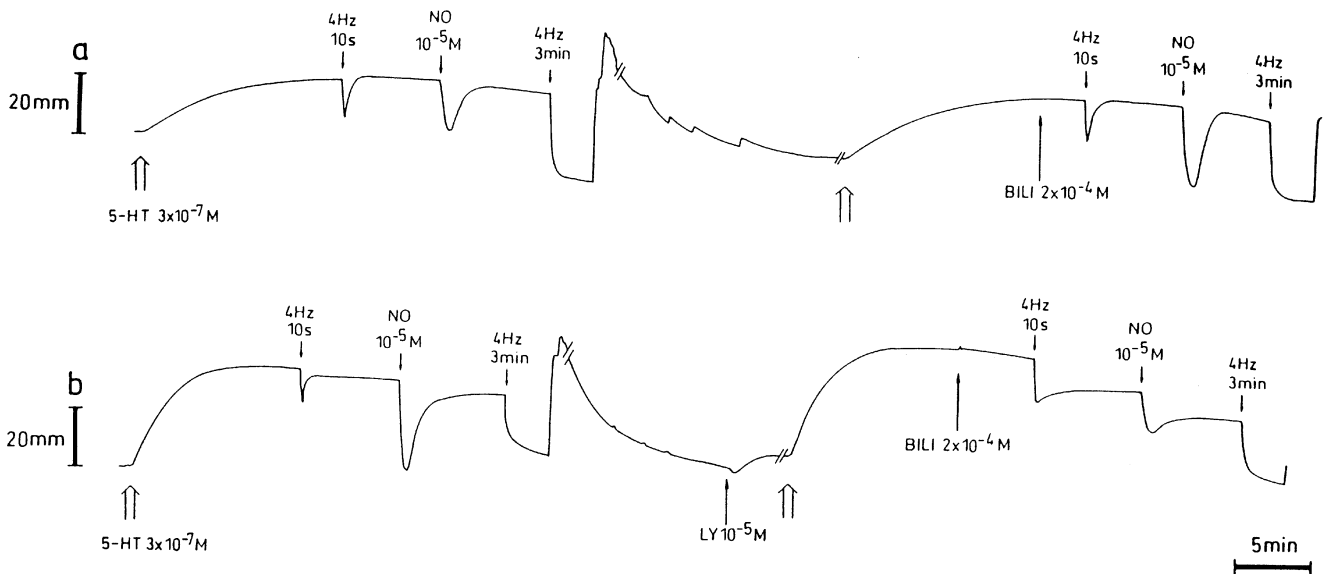
In control tissues, the relaxant responses to EFS at 4 Hz for 10 s, NO ( $10^{-5}$  M) and EFS at 4 Hz for 3 min were well maintained (respectively  $98.9 \pm 4.3\%$ ,  $96.2 \pm 11.3\%$  and  $105.0 \pm 3.9\%$ ;  $n=6$ ; Figures 1a and 2). SOD (300 and 1000 u ml $^{-1}$ ),  $\alpha$ -tocopherol ( $4 \times 10^{-4}$  M), ascorbate ( $5 \times 10^{-4}$  M) and EUK-8 ( $3 \times 10^{-4}$  M) did not influence the relaxation to exogenous NO (Figure 2). In the presence of GSH ( $5 \times 10^{-4}$  M), the short-lasting relaxation to an exogenous bolus of NO ( $10^{-5}$  M) became biphasic ( $n=6$ ; Figure 1b): the first phase yielded  $74.4 \pm 10.4\%$  ( $n=6$ ) of the response before GSH, the second phase (depicted in Figure 2)  $154.2 \pm 31.2\%$  ( $n=6$ ). Urate ( $4 \times 10^{-4}$  M) and bilirubin ( $2 \times 10^{-4}$  M) significantly potentiated the relaxant effect of NO to  $294.8 \pm 60.9\%$  and  $151.1 \pm 9.3\%$  ( $n=6$ ;  $P < 0.01$ ; Figures 1c, 2 and 3a). None of the antioxidants tested in this study *per se* affected the electrically evoked relaxations (Figure 2).



**Figure 1** Representative traces showing the responses to electrical field stimulation (40 V, 0.1 ms, 4 Hz, 10 s and 3 min) and to a bolus of exogenous NO ( $10^{-5}$  M) in a time control (a), before and in the presence of  $5 \times 10^{-4}$  M glutathione (GSH, b) or  $4 \times 10^{-4}$  M urate (UA, c). During intervals (//—//), the paper speed was reduced 5 fold.



**Figure 2** Relaxant responses to electrical field stimulation (40 V, 0.1 ms, 4 Hz, 10 s and 3 min) and exogenous NO ( $10^{-5}$  M) in the presence of antioxidants or aqua, the solvent of most antioxidants. The relaxations are expressed as a percentage of the response to the same stimulus before administration of the antioxidants. All results are the means  $\pm$  s.e. mean of six to eight strips. \*\* $P < 0.01$ : significantly different from the response before administration of the antioxidant (paired *t*-test). SOD = superoxide dismutase; ASC = ascorbate;  $\alpha$ -TOC =  $\alpha$ -tocopherol; BILI = bilirubin; UA = uric acid; GSH = glutathione.



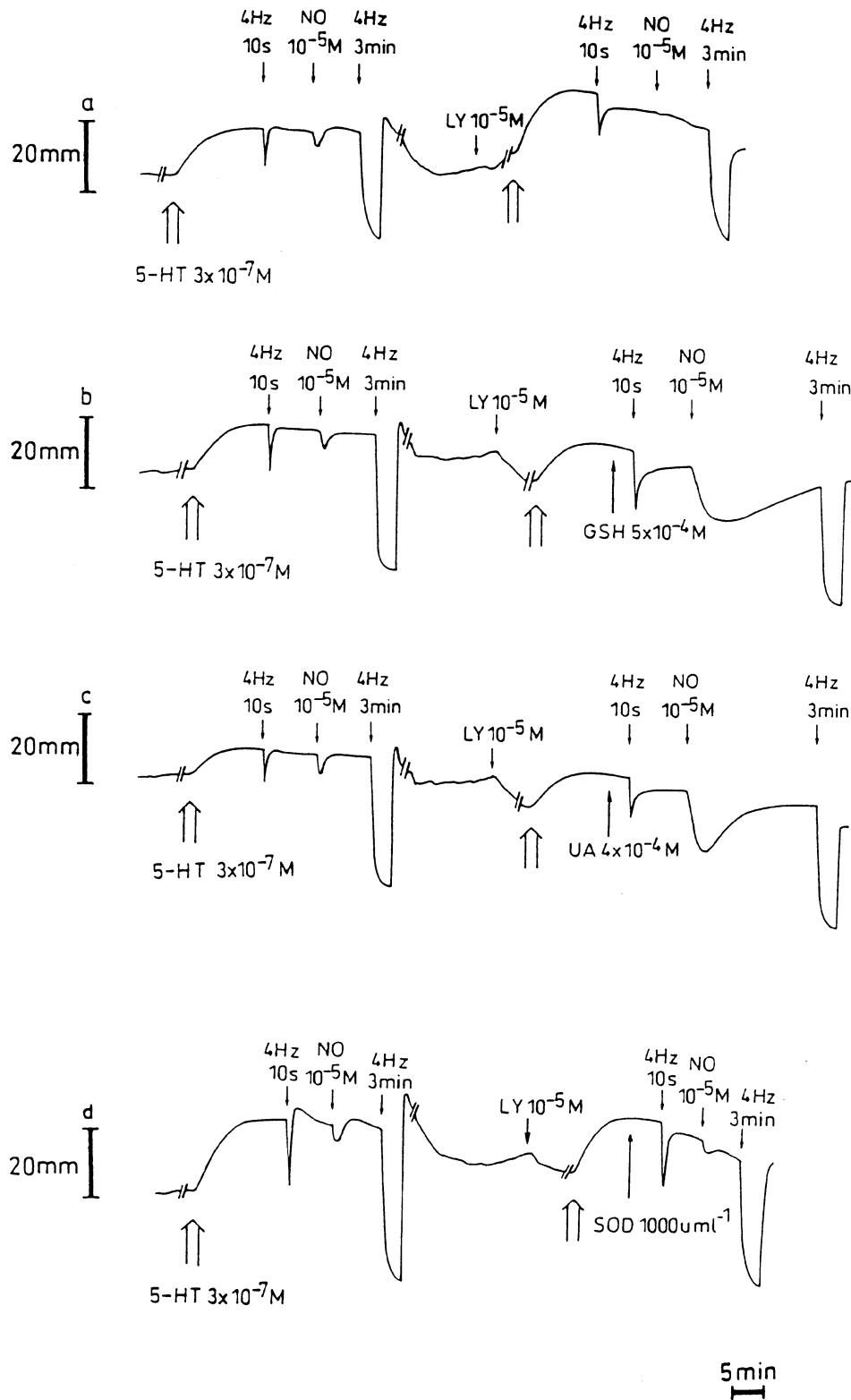
**Figure 3** Representative traces showing the effect of  $2 \times 10^{-4}$  M bilirubin (BILI) on the relaxations induced by electrical field stimulation (40 V, 0.1 ms, 4 Hz, 10 s and 3 min) and exogenous NO ( $10^{-5}$  M) in the absence (a) and in the presence (b) of  $10^{-5}$  M LY83583. During intervals (//—//), the paper speed was reduced 5 fold.

#### Effect of LY83583 and interaction with the antioxidants

Incubation with  $10^{-5}$  M LY83583 resulted in a slight decrease in tone in most strips but did not alter the amplitude of contraction induced by  $3 \times 10^{-7}$  M 5-HT. LY83583 ( $10^{-5}$  M) markedly reduced the relaxation elicited by  $10^{-5}$  M NO to  $8.7 \pm 4.4\%$  ( $n=7$ ) of the response before its administration; however, no inhibitory effect on the response to EFS at 4 Hz for 10 s and 3 min was observed (Figure 4a).

An NO-infusion induced a progressively developing relaxation of the tissues. When repeated in the presence of  $10^{-5}$  M LY83583, the relaxation was slowed down and the degree of relaxation at 1, 2, 3 and 5 min was significantly lower

than before administration of LY83583 (Figure 5b). In the presence of the solvent of LY83583, the response was also significantly decreased at 2 and 3 min of infusion (Figure 5a), but this decrease was significantly less pronounced than in the presence of LY83583 ( $P < 0.05$ , unpaired *t*-test). Addition of LY83583 ( $10^{-5}$  M) into the organ bath 5 min after the start of a continuous NO-infusion, instantly reversed the relaxation (Figure 6a), but the reversal was partial ( $55.6 \pm 8.0\%$ ;  $n=6$ ) and disappeared during the further course of the NO-infusion. The solvent of LY83583 had no influence. The relaxation induced by long-lasting EFS at 4 Hz on parallel strips was either not affected by LY83583 administered 5 min after the start of the stimulation (three strips out of six) or only reversed

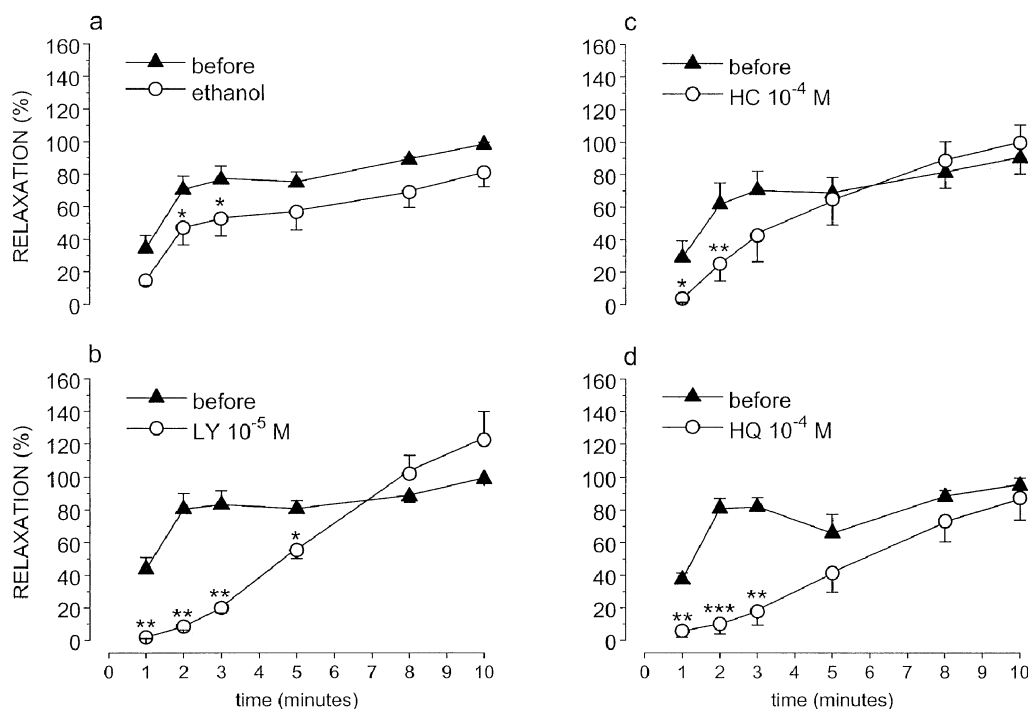


**Figure 4** Representative traces showing the influence of  $10^{-5}$  M LY83583 on the relaxations induced by electrical field stimulation (40 V, 0.1 ms, 4 Hz, 10 s and 3 min) and exogenous NO ( $10^{-5}$  M) (a), and the effect of  $5 \times 10^{-4}$  M glutathione (GSH, b),  $4 \times 10^{-4}$  M urate (UA, c) and  $1000 \text{ u ml}^{-1}$  SOD (d) on the inhibitory action of LY83583. During intervals (//—//), the paper speed was reduced 5 fold.

to a very small extent (three strips out of six) yielding a mean reversal of  $6.9 \pm 4.0\%$ ; the solvent of LY83583 was without effect.

The inhibitory effect of LY83583 ( $10^{-5}$  M) on the response to exogenous NO ( $10^{-5}$  M) was partially and concentration-dependently antagonized by 300 and  $1000 \text{ u ml}^{-1}$  SOD. The

response to NO increased from  $8.7 \pm 4.4\%$  ( $n=7$ ) in the presence of LY83583 alone to  $24.2 \pm 7.0\%$  ( $n=6$ ) and  $34.4 \pm 9.5\%$  ( $n=6$ ;  $P < 0.01$ ) in the combined presence of LY83583 and 300 and  $1000 \text{ u ml}^{-1}$  SOD respectively. GSH ( $5 \times 10^{-4}$  M) and urate ( $4 \times 10^{-4}$  M) completely prevented the blocking action of LY83583 versus NO; the response to NO



**Figure 5** Relaxations induced by exogenous NO infused for 10 min before and in the presence of  $10^{-5}$  M LY83583 (b) and its solvent ethanol (a),  $10^{-4}$  M hydroxocobalamin (HC, c) and  $10^{-4}$  M hydroquinone (HQ, d). The response measured at 1, 2, 3, 5, 8 and 10 min of infusion was expressed as a percentage of the maximum relaxation obtained during the NO-infusion before administration of the drugs. Means  $\pm$  s.e.mean of  $n=6$  are shown. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ : significantly different versus before.

was even significantly increased in comparison to that before administration of the drugs ( $285.8 \pm 47.0\%$  and  $264.7 \pm 30.7\%$ ;  $n=7$ ;  $P<0.001$ ; Figures 4b, c and 7); the biphasic relaxation in response to exogenous NO in the presence of GSH alone (see Figure 1b) changed in a monophasic answer in the presence of LY83583 and GSH (Figure 4b). In the presence of LY83583 plus GSH, the relaxation to EFS at 4 Hz for 3 min was significantly reduced ( $P<0.01$ ). This was the result of an incomplete recovery of tone observed after the foregoing very pronounced NO-induced relaxation in this series, as in an additional series (results not shown), the response to EFS at 4 Hz for 3 min was studied without foregoing NO exposure and was not influenced by LY83583 plus GSH. The addition of bilirubin ( $2 \times 10^{-4}$  M) provided partial protection of NO versus LY83583 (Figures 3b and 7), while ascorbate,  $\alpha$ -tocopherol and EUK-8 were without effect.

#### Effect of hydroxocobalamin and interaction with the antioxidants

Hydroxocobalamin ( $10^{-4}$  M) did not affect the basal tone or the 5-HT-induced contraction. Relaxant responses to EFS at 4 Hz for 10 s and 3 min were not inhibited by hydroxocobalamin, while the NO-induced relaxation was reduced to  $49.9 \pm 15.1\%$  ( $n=7$ ; Figure 7). The shape of the NO-induced response was changed: the transient, fast relaxation became a slower developing and more or less sustained relaxation. Incubation with hydroxocobalamin ( $10^{-4}$  M) significantly reduced the relaxation elicited by NO-infusion at min 1 and 2 (Figure 5c). When added during both continuous NO-infusion and long-lasting EFS at 4 Hz, no reversal of the relaxation was produced by hydroxocobalamin (Figure 6b).

The reduction by hydroxocobalamin ( $10^{-4}$  M) of the response to NO ( $10^{-5}$  M) was fully prevented by  $4 \times 10^{-4}$  M urate,  $5 \times 10^{-4}$  M GSH (with a monophasic answer to NO)

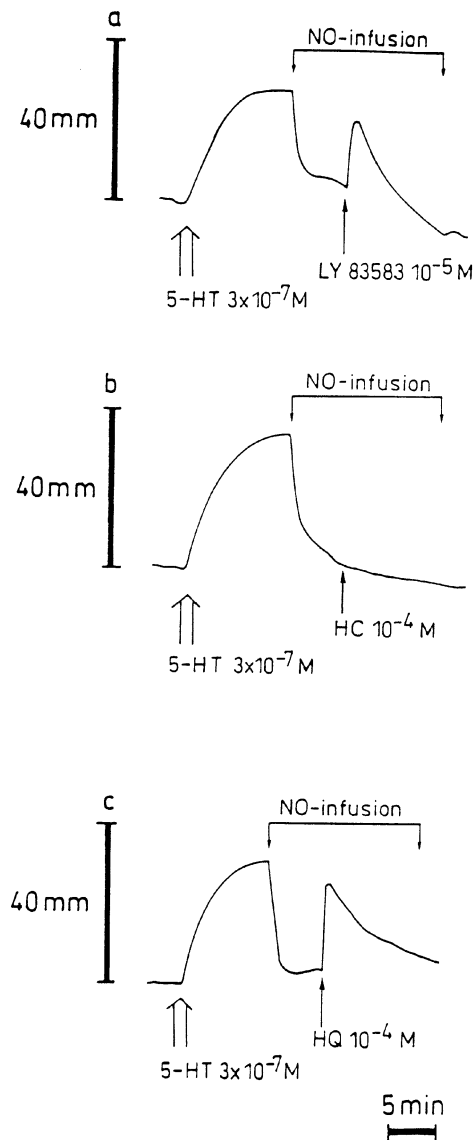
and  $4 \times 10^{-4}$  M  $\alpha$ -tocopherol (Figure 7). In contrast, SOD ( $1000 \text{ u ml}^{-1}$ ), ascorbate ( $5 \times 10^{-4}$  M), EUK-8 ( $3 \times 10^{-4}$  M) and bilirubin ( $2 \times 10^{-4}$  M) showed no protective action against the NO-scavenging capacity of hydroxocobalamin (Figure 7). The relaxation to EFS at 4 Hz for 3 min was significantly attenuated ( $P<0.001$ ) by the combination hydroxocobalamin ( $10^{-4}$  M) plus  $\alpha$ -tocopherol ( $4 \times 10^{-4}$  M). This effect was due to a non-recuperation of tone after application of the second relaxant stimulus (NO) in this series; in an additional series (results not shown), the response to EFS at 4 Hz for 3 min was studied without foregoing NO exposure and was not influenced by hydroxocobalamin plus  $\alpha$ -tocopherol.

#### Effect of hydroquinone and interaction with the antioxidants

Hydroquinone ( $10^{-4}$  M) had a moderate relaxant effect *per se* when administered on non-contracted tissues; the 5-HT-induced contraction however did not change in amplitude. Hydroquinone clearly differentiated between exogenous NO and nitergic nerve stimulation: the relaxant response to NO ( $10^{-5}$  M) was nearly abolished ( $7.8 \pm 2.6\%$  of the control value;  $n=8$ ), while responses to EFS at 4 Hz were unaffected (Figure 7).

Hydroquinone ( $10^{-4}$  M) markedly slowed down the relaxation induced by continuous NO-infusion at min 1, 2 and 3 of the infusion (Figure 5d). When hydroquinone was injected into the organ bath during NO-infusion, an instant partial reversal ( $61.4 \pm 16.7\%$ ;  $n=6$ ) of the relaxation occurred that waned off during the course of the infusion (Figure 6c); this effect was not seen when hydroquinone was given during long-lasting EFS at 4 Hz.

Ascorbate ( $5 \times 10^{-4}$  M) and SOD ( $1000 \text{ u ml}^{-1}$ ) partially reversed the inhibitory effect of  $10^{-4}$  M hydroquinone on

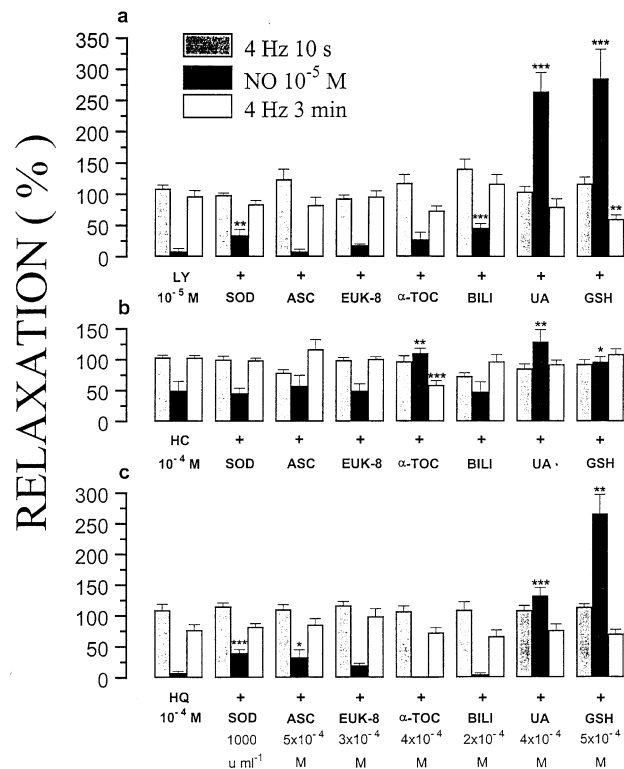


**Figure 6** Representative traces showing the influence of  $10^{-5}$  M LY83583 (a),  $10^{-4}$  M hydroxocobalamin (HC, b) and  $10^{-4}$  M hydroquinone (HQ, c) when added 5 min after starting a continuous NO-infusion. The NO-infusion was maintained for 15 min.

relaxations induced by  $10^{-5}$  M exogenous NO (Figure 7). Urate ( $4 \times 10^{-4}$  M) and GSH ( $5 \times 10^{-4}$  M) granted full protection of NO versus the action of hydroquinone (Figure 7). In five strips out of 7, the response to exogenous NO in the presence of hydroquinone and GSH had a biphasic course; only the value of the second phase was taken in account to show the mean response to NO for this series in Figure 7.  $\alpha$ -Tocopherol ( $4 \times 10^{-4}$  M), bilirubin ( $2 \times 10^{-4}$  M) and EUK-8 ( $3 \times 10^{-4}$  M) did not change the inhibitory effect of hydroquinone.

## Discussion

At the level of the proximal stomach, nitric oxide (NO) is thought to be involved in receptive relaxation as a non-adrenergic non-cholinergic inhibitory neurotransmitter (i.e. nitergic neurotransmission) (Lefebvre, 1993). In order to fulfil this neurotransmitter function, the reactive NO molecule has



**Figure 7** Relaxant responses to electrical field stimulation (40 V, 0.1 ms, 4 Hz, 10 s and 3 min) and exogenous NO ( $10^{-5}$  M), in the presence of  $10^{-5}$  M LY83583 (a),  $10^{-4}$  M hydroxocobalamin (b) or  $10^{-4}$  M hydroquinone (c) alone or plus one of the antioxidants indicated. The relaxations are expressed as a percentage of the response to the same stimulus before administration of the drugs under study. All results are the means  $\pm$  s.e. mean of six to eight strips. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ : significantly different from the response in the presence of  $10^{-5}$  M LY83583,  $10^{-4}$  M HC or  $10^{-4}$  M HQ alone (unpaired *t*-test). HC = hydroxocobalamin; HQ = hydroquinone; SOD = superoxide dismutase; ASC = ascorbate;  $\alpha$ -TOC =  $\alpha$ -tocopherol; BILI = bilirubin; UA = uric acid; GSH = glutathione.

to diffuse from its neuronal site of synthesis to its target enzyme soluble guanylate cyclase in the neighbouring effector smooth muscle cells, thereby encountering numerous molecules which can potentially decrease its bioavailability. Among these molecules, a prominent role is played by the superoxide anion; this reduced a chemically active form of oxygen is produced during mitochondrial respiration and other normal metabolic cell processes. When produced in proximity, the superoxide anion radical and NO can chemically interact at an almost diffusion-limited rate ( $6.7 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ) resulting in the abolition of the biological activity of NO and the generation of peroxynitrite (Huie & Padmaja, 1993).

It has been proposed that endogenous antioxidants protect the nitergic neurotransmitter from superoxide anions and even NO-scavenging molecules (Martin *et al.*, 1994; Lilley & Gibson, 1996; 1997). It was the aim of this study in the pig gastric fundus to investigate the potential influence of several antioxidants on nitergic relaxations induced by either electrical field stimulation or exogenous NO. To the antioxidants already studied before in this context (SOD, ascorbate,  $\alpha$ -tocopherol, urate and GSH) were added bilirubin and the synthetic agent EUK-8. The substances were tested on relaxations induced by exogenous NO and by EFS in NANC conditions. Previous experiments had indeed shown that the relaxant responses to EFS at 4 Hz for 10 s, 3 min or 15 min

are largely nitrenergic (Lefebvre *et al.*, 1995; Lefebvre & Vandekerckhove, 1998).

#### *Influence of the antioxidants per se*

Exogenously added Cu/Zn SOD did not enhance the NO-induced relaxations or the electrically induced NANC-relaxations in the pig gastric fundus, suggesting that the endogenous amount of SOD is sufficiently high to protect the nitrenergic neurotransmitter from superoxide mediated destruction; this is in accordance with previous results obtained in the rat anococcygeus (La & Rand, 1999), the rat gastric fundus (Lefebvre, 1996) and the bovine retractor penis (Liu *et al.*, 1994). In the presence of GSH, the short-lasting relaxation to an exogenous bolus of NO ( $10^{-5}$  M) became biphasic. Furthermore, the second part of this relaxation was potentiated and prolonged in comparison to the NO-induced relaxation in the absence of GSH. This effect is most likely due to the fact that our experimental conditions in the pig gastric fundus favour the formation of S-nitrosoglutathione from exogenous NO and GSH. This process of nitrosothiol formation (Kharitonov *et al.*, 1995) might serve to stabilize free radical NO, thereby prolonging its lifetime and potentiating its biological activity. A somewhat identical phenomenon was observed in the presence of urate: an exogenous bolus of NO produced a significantly potentiated and prolonged relaxation of the gastric fundus strips. Two hypotheses can be considered for this observation. First, although urate does not bind free radical NO (Becker, 1993), a nitrated uric acid derivative might be generated in conditions of urate abundance, slowly releasing NO and inducing relaxation of smooth muscle preparations. A similar nitrosation of uric acid, requiring peroxynitrite as nitrosating agent, has already been reported; the uric acid derivative resulted in endothelium-independent vasorelaxation of rat thoracic aorta and its activity was completely blocked by the NO-scavenger oxyhaemoglobin (Skinner *et al.*, 1998). A second hypothesis is provided by findings that antioxidants such as thiols or ascorbate enhance activation of soluble guanylate cyclase, presumably by maintaining the iron of the metalloporphyrin in a reduced state, thereby facilitating the reaction between NO and (ferrous) haem (Hobbs, 1997). Indeed, also urate with its inherent antioxidant potential might prevent oxidation of the haem group to a ferric state, the latter process resulting in loss of enzyme activity. However, as urate and GSH had no influence on relaxations elicited by EFS in our study, and as ascorbate was without effect on relaxations induced by both EFS and NO, this hypothesis can be rejected in the pig gastric fundus. The effect of GSH and urate on exogenous NO was not seen with the endogenous nitrenergic neurotransmitter, released by EFS. This might be related to the short distance the endogenous nitrenergic neurotransmitter has to travel to reach the smooth muscle cells in comparison to exogenous NO. The potentiating effect of bilirubin on exogenous NO might be due to its antioxidant effect, the non-effect on the endogenous nitrenergic neurotransmitter then being related to its optimal antioxidant protection.

#### *Influence of LY83583, hydroxocobalamin and hydroquinone*

LY83583, hydroxocobalamin and hydroquinone showed the discriminating effect between exogenous NO and the nitrenergic neurotransmitter mentioned in the introduction. None of the three agents affected EFS, performed both for short periods (10 s) and longer terms (3 and 10 min) of stimulation. While

LY83583 ( $10^{-5}$  M) and hydroquinone ( $10^{-4}$  M) nearly abolished the relaxant response to a bolus of exogenous NO ( $10^{-5}$  M), hydroxocobalamin at  $10^{-4}$  M reduced the amplitude of this NO-induced relaxation to about 50% and at the same time changed the fast and transient relaxation profile into a more or less sustained one. This marked increase in duration of the NO-induced response is quite similar to that described by Jenkinson *et al.* (1995), corroborating their hypothesis that hydroxocobalamin reversibly sequesters NO to form nitrosocobalamin. Our finding that hydroxocobalamin does not reverse the relaxation upon addition during continuous NO-infusion can be explained in two ways: either the formed nitrosocobalamin liberates its NO relatively fast, supporting the view of a reversible binding (Rochelle *et al.*, 1995), or the rate of NO-infusion is sufficiently high to overcome the scavenging effect of  $10^{-4}$  M hydroxocobalamin even if the binding is irreversible. In contrast with hydroxocobalamin, LY83583 and hydroquinone instantly reversed the relaxation elicited by continuous NO-infusion. This corroborates that their mode of action implies a mechanism other than direct NO-scavenging, most likely the generation of superoxide. Still, the reversal by LY83583 and hydroquinone was only temporary showing that continuous NO supply can overwhelm their effect.

#### *Interaction of the antioxidants with LY83583, hydroquinone and hydroxocobalamin*

Addition of exogenous Cu/Zn SOD resulted in a partial reversal of the inhibitory action of LY83583 and hydroquinone on a bolus of exogenous NO; this observed reversal supports the proposal that, at least under the experimental conditions used in the pig gastric fundus, these compounds act *via* production of superoxide anions. Indeed, the mechanism of action of hydroquinone may differ between tissues: NO-scavenging activity is suggested in the mouse anococcygeus (Lilley & Gibson, 1995) and in the rat gastric fundus (Lefebvre, 1996), while superoxide anion production is proposed in the bovine retractor penis muscle (Paisley & Martin, 1996). Cu/Zn SOD failed to reverse the inhibitory effect of hydroxocobalamin, indicating that direct free radical scavenging constitutes the NO-blocking action of this latter substance. As LY83583 and hydroquinone can generate superoxide anions extracellularly as well as intracellularly (Boersma *et al.*, 1994) and exogenous Cu/Zn SOD cannot enter cells, we examined the effect of EUK-8, a synthetic cell-permeable mimetic of the mitochondrial Mn SOD. Mn SOD is the mitochondrial isozyme that disposes of superoxide generated by respiratory chain activity. Mn SOD immunoreactivity is demonstrated in nerve cells and smooth muscle cells in the stomach wall of rats (Fang & Christensen, 1995) and in nerve cells in the myenteric plexus of the opossum oesophagus (Thomas *et al.*, 1996). The enzyme effectively catalyzes the dismutation of superoxide with a rate constant of  $1.8 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  (Forman & Fridovich, 1973). These findings together with the demonstration that it is colocalized with NADPH diaphorase (a marker for NO-synthase) in the gastrointestinal tract (Fang & Christensen, 1995), support the theory that Mn SOD might be involved in protecting the nitrenergic neurotransmission process in the stomach. However, in the pig gastric fundus, EUK-8 did not prevent the inhibitory effect of LY83583 and hydroquinone. This contrasts markedly with results obtained in rat aortic segments treated with DETCA to induce an endogenous flux of superoxide: EUK-8 fully restored endothelium-derived NO-mediated arterial relaxation in response to acetylcholine, while Cu/Zn SOD had only a partial effect (Jackson *et al.*, 1998).



Since DETCA is known to block both the Cu/Zn containing extracellular and intracellular isozyme of SOD but not the Mn requiring isozyme, the total recovery provided by EUK-8 is probably due to compensation for the loss of intracellular Cu/Zn SOD activity. Incomplete cellular access and/or poor sensitivity of the tissue may account for the complete lack of superoxide dismutation by EUK-8 in the pig gastric fundus. We therefore increased the experimental concentration of EUK-8 up to  $5 \times 10^{-4}$  M, but tissues responded with marked phasic activity interfering with the relaxant responses.

The bile pigment bilirubin partially reversed the inhibitory effect of LY83583 versus exogenous NO. The protective effect versus LY83583 is associated with the bilirubin moiety of bilirubin ditaurate since taurine alone is ineffective (results not shown). Bilirubin is formed in mammals *via* a pathway involving two enzymes. The microsomal haeme oxygenase catalyzes the cleavage of ferritroporphyrin IX (haeme) to form ferrous iron, carbon monoxide and biliverdin; subsequently, biliverdin is rapidly reduced by the cytosolic enzyme biliverdin reductase to bilirubin (Stocker *et al.*, 1990). This machinery is found integrally in myenteric plexus neurons: biliverdin reductase is present in large functional excess in all tissues and about 70% of neurons of the myenteric plexus contain for constitutive haeme oxygenase-2 and neuronal nitric oxide synthase (Zakhary *et al.*, 1996; 1997). Bilirubin can be added to the list of physiological antioxidants, proposed by Lilley & Gibson (1996), which have been demonstrated to preserve the biological actions of NO. Bilirubin interacts chemically with superoxide with a second-order rate constant estimated to be  $2.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$  (Robertson & Fridovich, 1982). This is  $10^5$  times less than the rate at which superoxide reacts with NO, suggesting that the concentration of bilirubin would have to exceed that of NO  $10^5$  times to become effective for the protection of NO versus superoxide. Still, the protecting capacity of antioxidants versus superoxide is not only determined by their intrinsic chemical reactivity toward radicals, but is equally influenced by the site of the antioxidant, the concentration and mobility at the microenvironment, and the interaction with other antioxidants (Niki *et al.*, 1995). Bilirubin will probably be located into neuronal membranes or alternatively, it can be a cytosolic antioxidant bound to glutathione S-transferases. Cytosolic glutathione S-transferases are present in various mammalian organ systems, including the nervous system (Johnson *et al.*, 1993), and participate in the intracellular binding and/or transport of a broad range of endogenous molecules such as hormones, neurotransmitters and lipophilic compounds including bilirubin (Abramovitz *et al.*, 1988). This potential presence of bilirubin in both lipophilic and hydrophilic cell compartments with the possibility of migrating between those two phases, enhances its antioxidant potency.

On the basis of the general hypothesis that products of degradation metabolic pathways may play a useful biological role (as also exemplified by bilirubin in this study), and as urate is released upon depolarization of nerve cells in the rat anococcygeus (Lilley & Gibson, 1997), the effect of uric acid, another degradation product, was tested versus the three

differentiating agents LY83583, hydroxocobalamin and hydroquinone. Urate (in the physiological pH range, about 99% of the total uric acid is present as the monovalent anion urate) displays powerful antioxidant activity, evidenced by its ability to scavenge reactive oxygen species (Ames *et al.*, 1981) and to chelate iron, therefore, preventing iron-catalyzed oxidation (Davies *et al.*, 1986). As high levels of urate are excreted *via* gastric and intestinal juices (Ames *et al.*, 1981), it was of interest to find that in the pig gastric fundus urate provided an overall protection of exogenous NO against both superoxide and scavenger mediated inactivation. The tripeptide thiol GSH, a major determinant of intracellular redox state and antioxidant defences (Meister & Anderson, 1983), exhibited the same protective profile as urate: in its presence, LY83583, hydroquinone and hydroxocobalamin could not reduce the NO-induced relaxation. Whether direct interaction of GSH and urate with noxious oxygen radicals and scavenging molecules or the chemical nitrosation process with formation of respectively S-nitrosoglutathione and a nitrated uric acid derivative, as described above, is responsible for these results, requires further research.

The antioxidant vitamins ascorbate and  $\alpha$ -tocopherol showed only a partial reversal of the NO-blocking effect of respectively hydroquinone and hydroxocobalamin. The limited effect of ascorbate contrasts with results in the mouse anococcygeus, where ascorbate was able to reverse the inhibitory effect versus exogenous NO of all superoxide anion generators and NO-scavengers tested (Lilley & Gibson, 1996). This suggests that the importance of the antioxidants involved in the protection of nitergic neurotransmission might differ from tissue to tissue. Regional differences in antioxidants with a concomitant different use of antioxidant mechanisms to withstand oxidative stress have also been reported in the cardiovascular system (Palace *et al.*, 1999).

### Conclusion

Our results in the pig gastric fundus indicate that several antioxidant defence systems can preserve the biological actions of exogenous NO when confronted with superoxide anions or scavenging molecules. Whether SOD as enzymatic and GSH, urate, bilirubin, ascorbate and  $\alpha$ -tocopherol as non-enzymatic antioxidants might contribute to the resistance of the endogenous nitergic neurotransmitter versus NO-inhibitors, requires further studies with tissues in which the antioxidants have been selectively depleted or inhibited, since the other possibilities described in the introduction can not yet be completely ruled out.

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