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Antioxidant protection of NO-induced relaxations of the mouse anococcygeus against inhibition by superoxide anions, hydroquinone and carboxy-PTIO

Elliot Lilley & [1]Alan Gibson

Pharmacology Group, Biomedical Sciences Division, King's College London, Manresa Road, London SW3 6LX

1 The potential protective effect of several antioxidants [Cu/Zn superoxide dismutase (Cu/Zn SOD), ascorbate, reduced glutathione (GSH), and α-tocopherol (α-TOC)] on relaxations of the mouse anococcygeus muscle to nitric oxide (NO; 15 μM) and, where appropriate, nitrergic field stimulation (10 Hz; 10 s trains) was investigated.

2 The superoxide anion generating drug duroquinone (100 μM) reduced relaxations to exogenous NO by 54±6%; this inhibition was partially reversed by Cu/Zn SOD (250 u ml^{-1}), and by ascorbate (500 μM). Following inhibition of endogenous Cu/Zn SOD activity with diethyldithiocarbamate (DETCA), duroquinone (50 μM) also reduced relaxations to nitrergic field stimulation (by 53±6%) and this effect was again reversed by Cu/Zn SOD and by ascorbate. Neither GSH (500 μM) nor α-TOC (400 μM) afforded any protection against duroquinone.

3 Xanthine (20 mu ml^{-1}):xanthine oxidase (100 μM) inhibited NO-induced relaxations by 73±14%, but had no effect on those to nitrergic field stimulation, even after DETCA treatment. The inhibition of exogenous NO was reduced by Cu/Zn SOD (250 u ml^{-1}) and ascorbate (400 μM), but was unaffected by GSH or α-TOC (both 400 μM).

4 Hydroquinone (100 μM) also inhibited relaxations to NO (by 52±10%), but not nitrergic stimulation. In this case, however, the inhibition was reversed by GSH (5-100 μM) and ascorbate (100-400 μM), although Cu/Zn SOD and α-TOC were ineffective.

5 2-(4-Carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO, 50 μM) inhibited NO-induced relaxations by 50±4%, but had no effect on nitrergic responses; the inhibition was reduced by ascorbate (2-200 μM) and α-TOC (10-200 μM), but not by Cu/Zn SOD or GSH.

6 Hydroxocobalamin (5-1000 μM) inhibited, equally, relaxations to both NO ($-\log IC_{40}$ 3.14±0.33) and nitrergic stimulation ($-\log IC_{40}$ 3.17±0.22).

7 Thus, a number of physiological antioxidants protected NO from superoxide anions, and from direct NO-scavengers. The possibility that the presence of these antioxidants within nitrergically-innervated tissues might explain the lack of effect of the NO inhibitors on nerve-induced relaxation, without the need to invoke a transmitter other than free radical NO, is discussed.

Keywords: Anococcygeus (mouse); ascorbate; carboxy-PTIO; glutathione; hydroquinone; hydroxocobalamin; nitric oxide; superoxide anions; superoxide dismutase; α-tocopherol

Introduction

There is now compelling evidence that the L-arginine/nitric oxide (NO) pathway generates the non-adrenergic, non-cholinergic transmitter which mediates smooth muscle relaxation in a variety of tissues (for reviews see Rand, 1992; Rand & Li, 1995a). However, one strange aspect of this novel nitrergic neurotransmission process is that certain drugs powerfully inhibit relaxations to exogenous NO, but have little or no effect on relaxations to electrical field stimulation; such drugs include the superoxide anion generators duroquinone (Lilley & Gibson, 1995), pyrogallol (Gillespie & Sheng, 1990), and xanthine:xanthine oxidase (Gibson *et al.*, 1994), the NO-scavengers hydroxocobalamin (Rajanayagam *et al.*, 1993) and 2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; Rand & Li, 1995b), and hydroquinone, which can act as a superoxide anion generator or as a NO-scavenger, depending on the experimental conditions (Hobbs *et al.*, 1991; Lilley & Gibson, 1995; Paisley & Martin, 1996).

A partial explanation of some of these findings was provided by recent observations that inhibition of Cu/Zn superoxide dismutase (Cu/Zn SOD) within the tissue renders the nitrergic transmitter in the bovine retractor penis (Martin *et al.*, 1994) and mouse anococcygeus (Lilley & Gibson, 1995) sensitive to inhibition by superoxide generating drugs such as pyrogallol, xanthine:xanthine oxidase, and duroquinone; thus, the presence of high levels of Cu/Zn SOD in the vicinity of the neuroeffector junction might protect the endogenous transmitter from attack by superoxide anions, while exogenous NO would still be vulnerable before it reaches the tissue. However, Cu/Zn SOD inhibition did not cause nitrergic relaxations of the mouse anococcygeus to become sensitive to hydroquinone and, in addition, application of exogenous Cu/Zn SOD only partially reversed the inhibitory effects of duroquinone (Lilley & Gibson, 1995). One possibility is that there may be other antioxidant mechanisms, apart from Cu/Zn SOD, which are present in the tissue to protect the reactive NO radical on its journey from the presynaptic nerve terminal to the target guanylate cyclase within the postsynaptic cell. In the present study, therefore, we have investigated the potential protective effects of several antioxidants [Cu/Zn SOD, ascorbate, reduced glutathione (GSH), and α-tocopherol (α-TOC)] on relaxations of the mouse anococcygeus produced by exogenous NO and, where appropriate, nitrergic field stimulation. A preliminary account of some of the work contained in this paper has been presented to the British Pharmacological Society (Lilley & Gibson, 1996).

[1] Author for correspondence.

Methods

Mouse anococcygeus muscle

Male mice (LACA; 25–35 g) were killed by stunning and exsanguination. The paired anococcygeus muscles were dissected, joined by the ventral bar, and set up in series in 2 ml glass organ baths containing Krebs bicarbonate buffer (mM; NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) which was maintained at 37°C and gassed continuously with 95% O_2: 5% CO_2. The tissues were set up with a resting tension of 300–400 mg and changes in tension were monitored by a Grass FTO3 force displacement transducer attached to a Graphtech pen-recorder (WR 3101). A period of 45 min was allowed before the start of the experiment, for tissue equilibration. Field stimulation was applied to the tissue via two parallel platinum electrodes running down either side of the tissue; these were attached to a Grass S48 stimulator (0.5 ms pulse width; 70V). Sympathetic responses were negated by including 1 μM phentolamine in the Krebs solution and by preincubating each muscle with 30 μM guanethidine for 15 min during the equilibration time. In all cases, tone was raised with 50 μM carbachol and relaxations measured as percentage reductions in this carbachol-induced tone. The effects of drugs used to inhibit responses to NO (15 μM; Lilley & Gibson, 1995) were investigated by addition of the drug to the bath 5 min before NO; the bath was washed with fresh Krebs solution when the relaxant response had been obtained. When the effect of antioxidants on the inhibitory potential of drugs against NO was being established, the antioxidant was added together with the inhibitory drug, 5 min before NO. To determine the effect on nitrergic relaxations, three control relaxations to field stimulation (10 Hz; 10 s train every 100 s; Lilley & Gibson, 1995) were obtained and the inhibitory drug was then added; the organ bath was washed out once any inhibition had stabilised, usually after 3–4 further stimulations. If the effect of antioxidants on the inhibitory potential of drugs against nitrergic relaxations was being tested, the antioxidant was added after the inhibitory response had stabilised, and the bath was washed out once any reversal had been established, again usually after 3–4 further stimulations. To determine the effect of xanthine:xanthine oxidase on relaxations to exogenous NO, xanthine oxidase was added to the bath 5 min before the tone was raised with carbachol, and xanthine added immediately (2–3 s) before addition of NO; against nitrergic relaxations, xanthine oxidase was added to the bath before the tone was raised with carbachol, and xanthine added after 3–4 control relaxations had been obtained and immediately before the next stimulation.

Chemiluminescence studies

Superoxide anions were detected by chemiluminescence (Hobbs *et al.*, 1991); 2 ml of Krebs solution was placed into clear plastic tubes, and to this was added 250 μM lucigenin. The tubes were placed in a LKB-Wallac 1250 luminometer in which they were maintained at 37°C and gassed continuously with 95% O_2: 5% CO_2. The chemiluminescence signal was monitored by digital read-out and in graphical form by Mac-lab. Responses were established with the required concentration of xanthine oxidase in the cuvette and, after an incubation period of 10 min, xanthine was added via an autoinjector (LKB 1250-104). Results were calculated as the area under the curve (mV.s) obtained following addition of xanthine. With the concentrations of xanthine and xanthine oxidase used in the present study (in order to produce a prolonged production of superoxide anions), a biphasic time-course of superoxide anion generation was obtained; however, as found previously (Lilley & Gibson, 1995), the chemiluminescence signal was abolished in the presence of Cu/Zn SOD (250 u ml^{-1}).

Statistics

Results are expressed as mean ± s.e.mean ($n \geq 5$). Statistical analysis was by Student's t test (unpaired); a probability value of $P < 0.05$ was taken to indicate statistical significance.

Drugs

All drugs were dissolved in distilled water, except xanthine which was dissolved (stock 100 mM) in 0.1 M NaOH and α-tocopherol, or duroquinone, which were dissolved in dimethylsulphoxide (each at 10 mM). Solvents themselves were without significant effect at the concentrations used in the experiments. NO solutions were prepared as described previously (Gibson & Mirzazadeh, 1989). Drugs used were (supplied by Sigma unless stated otherwise): ascorbic acid, carbachol (BDH), carboxy PTIO [2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide] (kindly donated by H. Maeda, Kumamato, Japan), diethyldithiocarbamic acid, duroquinone (Aldrich), glutathione, guanethidine sulphate, hydroquinone, hydroxocobalamin hydrochloride, lucigenin (bismethylacridinium nitrate), nitric oxide (99%,BDH), phentolamine HCl, Cu/Zn superoxide dismutase (from bovine erythrocytes), α-tocopherol, xanthine, xanthine oxidase (from buttermilk).

Results

In a series of control experiments, none of the antioxidants used in this study were found to affect, by themselves, relaxations of the mouse anococcygeus to either NO or nitrergic field stimulation (Table 1).

Effects of duroquinone

We have previously shown (Lilley & Gibson, 1995) that nitrergic relaxations of the mouse anococcygeus become much more sensitive to block by the superoxide anion generating drug duroquinone following treatment with the Cu/Zn SOD inhibitor diethyldithiocarbamate (DETCA; 3 mM; 45 min incubation with a 10 min washout). In the present study, following such DETCA treatment, duroquinone (50 μM) reduced relaxations to field stimulation by 53 ± 6%, and this inhibition was partially reversed by Cu/Zn SOD (250 u ml^{-1}; Figure 1). However, ascorbate (100–400 μM) also produced a concentration-related reversal of the inhibitory effect of duroquinone (Figure 1), while GSH (400 μM) and α-TOC (400 μM) were without effect (data not given). The reversal observed with ascorbate (400 μM; 48 ± 9% reversal) was not

Table 1 Effect of antioxidants on relaxations to NO and nitrergic field stimulation in the mouse anococcygeus

	% relaxation	
	NO (15 μM)	*Nitrergic stimulation* (10 Hz; 10 s trains)
Control	49 ± 4	37 ± 14
SOD	49 ± 4	46 ± 17
Control	58 ± 6	61 ± 9
Ascorbate	62 ± 9	69 ± 10
Control	36 ± 12	53 ± 10
GSH	38 ± 15	60 ± 9
Control	36 ± 12	62 ± 9
α-TOC	38 ± 10	66 ± 9

Responses are given as mean ± s.e.mean ($n \geq 5$) of % relaxation of carbachol-induced tone in controls, or in the presence of superoxide dismutase (SOD; 250 μml^{-1}), ascorbate (500 μM), reduced glutathione (GSH; 500 μM) or α-tocopherol (α-TOC; 400 μM)

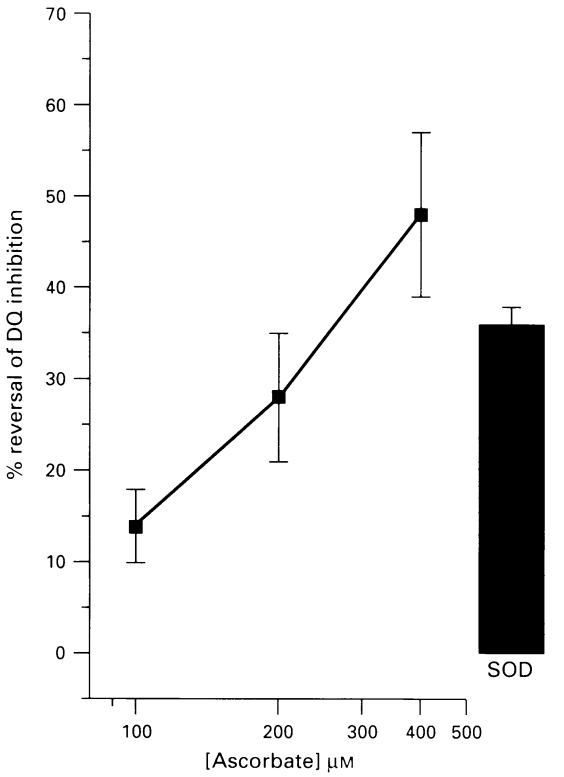

Figure 1 Concentration-response curve for ascorbate, and for superoxide dismutase (SOD; 250 u ml^{-1}; column), causing reversal of duroquinone (DQ; 50 μM)-induced inhibition of relaxations of mouse anococcygeus muscles in response to nitrergic field stimulation (10 Hz; 10 s trains). Each point (column) represents the mean of at least 5 individual muscle preparations; vertical lines show s.e.mean.

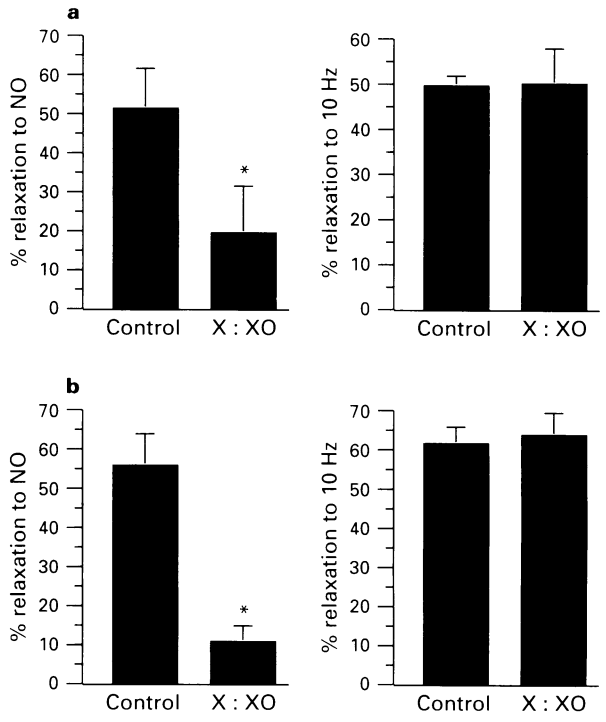

Figure 2 Histograms showing the effect of xanthine (X; 100 μM):xanthine oxidase (XO; 20 mu ml^{-1}) on relaxations of mouse anococcygeus muscles to exogenous nitric oxide (NO; 15 μM) and to nitrergic field stimulation (10 Hz; 10 s trains), in untreated tissues (a), and in tissues in which superoxide dismutase activity had been inhibited with diethyldithiocarbamate (3 mM; 45 min; 10 min washout; b). Each column represents the mean ± s.e.mean (vertical lines) of at least 5 individual muscle preparations. *Significantly different from adjacent control column.

increased when combined with Cu/Zn SOD (250 u ml^{-1}; 56 ± 9% reversal.

The effect of duroquinone on relaxations to NO was studied in muscles which were not pre-treated with DETCA; here, 100 μM duroquinone reduced responses to NO by 54 ± 6%. This inhibition was partially reversed by Cu/Zn SOD (250 u ml^{-1}; 47 ± 13% reversal) and ascorbate (500 μM; 50 ± 4% reversal) but was unaffected by either 500 μM GSH or 200 μM α-TOC (data not given).

Effects of xanthine:xanthine oxidase

In control anococcygeus muscles, 20 mu ml^{-1} xanthine oxidase plus 100 μM xanthine inhibited NO-induced relaxations by 73 ± 14%, but had no effect on relaxations induced by field stimulation (Figure 2); this inhibition was partially reversed by Cu/Zn SOD (250 u ml^{-1}; 51 ± 16% reversal). Unlike our observations with duroquinone, however, the differential effect of xanthine:xanthine oxidase on relaxations to NO and to field stimulation persisted, even after DETCA treatment (Figure 2). We considered the possibility that the combination of xanthine:xanthine oxidase used did not produce sufficient superoxide anions to interfere with the nitrergic transmitter; the time-course of superoxide anion generation is shown in Figure 3 (area under curve, 641 ± 35 mV.s). Increasing the concentration of both xanthine (to 1 mM) and xanthine oxidase (to 140 mu ml^{-1}) produced a much enhanced superoxide generation (3579 ± 421 mV.s; Figure 3). However, even with this higher concentration of xanthine:xanthine oxidase, in DETCA treated tissues, nitrergic relaxations were still unaffected (74 ± 6% relaxation without xanthine:xanthine oxidase; 85 ± 5% relaxation with xanthine:xanthine oxidase).

To determine the effects of antioxidants, we used the lower concentration of xanthine:xanthine oxidase in DETCA treated tissues. Relaxations to NO were reduced by 83 ± 6%, and this

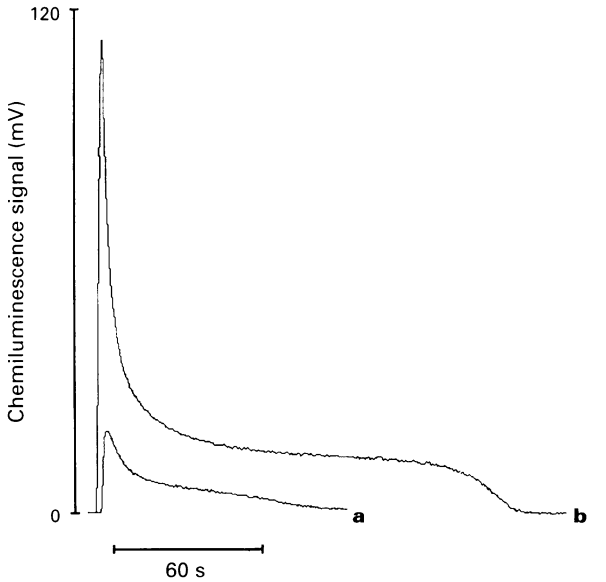

Figure 3 Traces showing the amplitude/time-course of the chemiluminesence signal produced by superoxide anions generated by two different concentrations of xanthine (100 μM in (a); 1 mM in (b)): xanthine oxidase (20 mu ml^{-1} in (a); 140 mu ml^{-1} in (b)).

inhibition was partially reversed by Cu/Zn SOD (250 u ml^{-1}; 23 ± 7% reversal) and ascorbate (400 μM; 65 ± 5% reversal), but was unaffected by 400 μM GSH or 400 μM α-TOC (data not given).

Effects of hydroquinone

As found previously (Hobbs *et al.*, 1991; Lilley & Gibson, 1995), 100 μM hydroquinone clearly differentiated between relaxations induced by NO and by field stimulation, inhibiting the former by $52 \pm 10\%$ while having no effect on the latter (either in control or DETCA treated tissues). In the present study, this inhibitory effect of hydroquinone on NO-induced relaxations was reversed, in a concentration-related manner, by GSH (5–100 μM; Figure 4) and ascorbate (100–400 μM; Figure 4), but was unaffected by 250 u ml^{-1} SOD or 400 μM α-TOC (data not given).

Effects of carboxy-PTIO

Carboxy-PTIO (2–100 μM) produced a concentration-related inhibition of relaxations to NO (Figure 5a). Relaxations to nitrergic field stimulation were, however, unaffected by carboxy-PTIO (500 μM; data not shown). At concentrations higher than 500 μM, carboxy-PTIO, by itself, caused marked relaxations of tone, making any estimation of its effect on nitrergic relaxations impossible. Carboxy-PTIO (50 μM) inhibited relaxations to NO by $50 \pm 4\%$, and this inhibition was reversed by ascorbate (2–200 μM) and α-TOC (10–200 μM; Figure 5b), but was unaffected by either 250 u ml^{-1} Cu/Zn SOD or 400 μM GSH (data not given).

Effects of hydroxocobalamin

Hydroxocobalamin (50–1000 μM) produced concentration-related inhibition of relaxations to NO (Figure 6). However, relaxations to nitrergic field stimulation were also inhibited to a similar degree (Figure 6), there being no significant difference between the $-\log IC_{40}$ value against NO (3.14 ± 0.33) and against the nitrergic transmitter (3.17 ± 0.22).

Discussion

The observations that a variety of drugs inhibit relaxations to exogenous NO, without affecting those to nitrergic field stimulation in several tissues, have been the subject of considerable debate (for reviews see Rand & Li, 1995a; Gibson *et al.*, 1995), since they questioned the role of free radical NO as the transmitter released from the nitrergic nerves. One proposed explanation for these findings was that certain substances may be present in the region of the neuroeffector junction which act

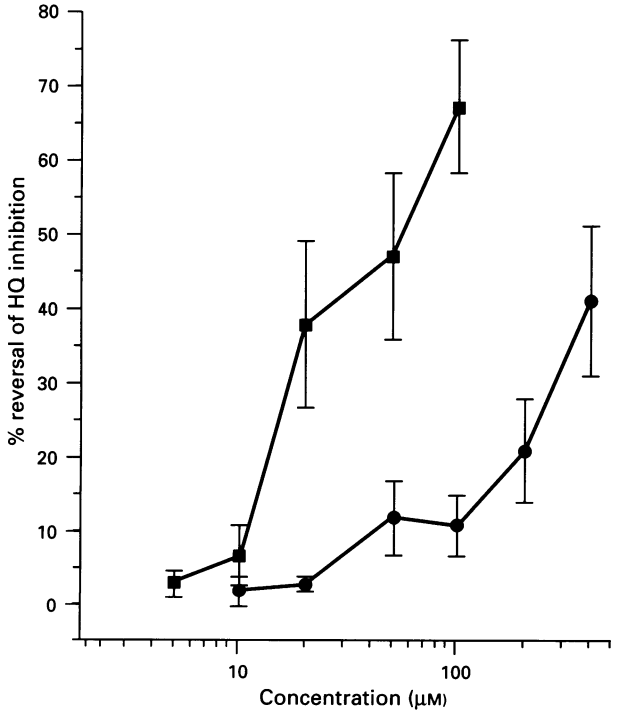

Figure 4 Concentration-response curves for reduced glutathione (■) and ascorbate (●) causing reversal of hydroquinone (HQ; 100 μM)-induced inhibition of relaxations of mouse anococcygeus muscles to exogenous nitric oxide (NO; 15 μM). Each point represents mean of at least 5 individual muscle preparations; vertical lines show s.e.mean.

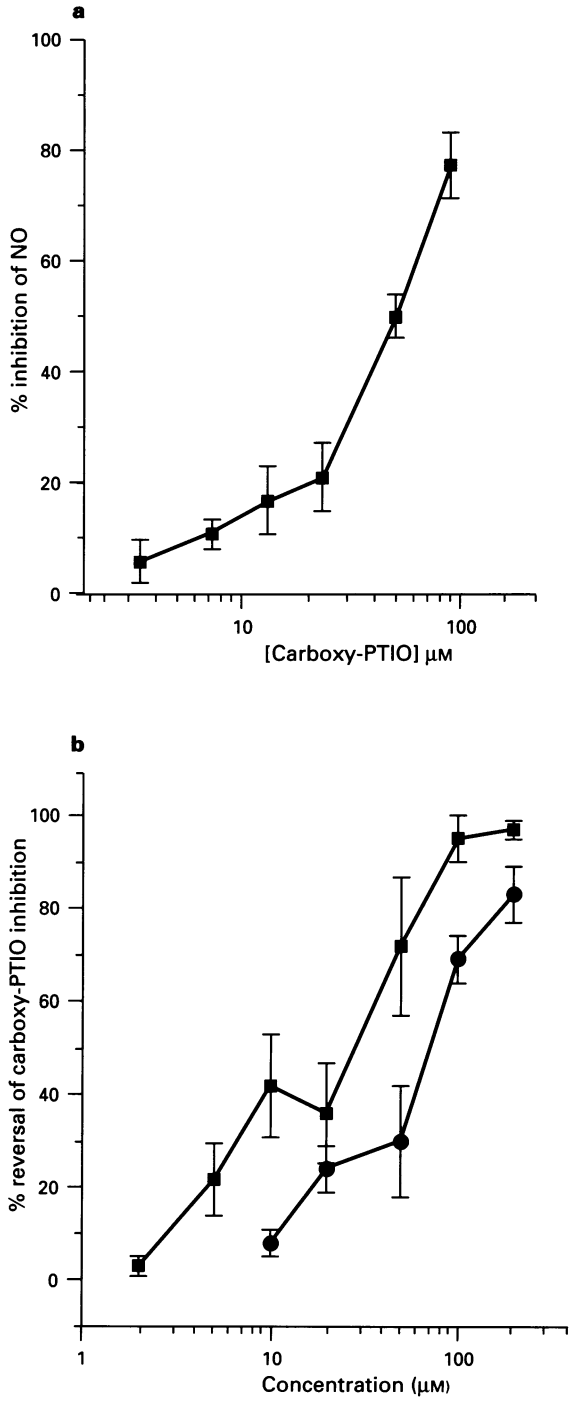

Figure 5 Concentration-response curves for (a) carboxy-PTIO-induced inhibition of relaxations of mouse anococcygeus muscles to exogenous nitric oxide (NO; 15 μM), and (b) the reversal of this inhibitory effect of 50 μM carboxy-PTIO by ascorbate (■) and α-tocopherol (●). Each point represents mean of at least 5 individual muscle preparations; vertical lines show s.e.mean.

to protect the reactive NO from attack by scavenging molecules (Brave *et al.*, 1993; Martin *et al.*, 1994; Lilley & Gibson, 1995). Evidence in favour of this was provided by experiments showing that, following inhibition of Cu/Zn SOD, nitrergic relaxations became sensitive to the superoxide anion generating compounds pyrogallol, LY83583 and xanthine:xanthine oxidase in the bovine retractor penis (Martin *et al.*, 1994), and to duroquinone in the mouse anococcygeus (Lilley & Gibson, 1995). In the latter tissue, however, re-addition of exogenous Cu/Zn SOD only partially reversed the inhibitory effect of duroquinone, and hydroquinone still failed to reduce nitrergic relaxations after Cu/Zn SOD inhibition, although it powerfully reduced relaxations to exogenous NO. These results suggested that protection of the nitrergic transmitter by Cu/Zn SOD partly explains the lack of effect of superoxide anion generators in the mouse anococcygeus, although it did not provide an explanation for the effects of hydroquinone, which acts as a NO-scavenger in our experiments (Hobbs *et al.*, 1991; Lilley & Gibson, 1995). The important new findings of the present study are that other physiological antioxidants, in addition to SOD, can protect NO from attack both from superoxide anion generators and from direct NO-scavengers. A summary of the main findings is given in Table 2.

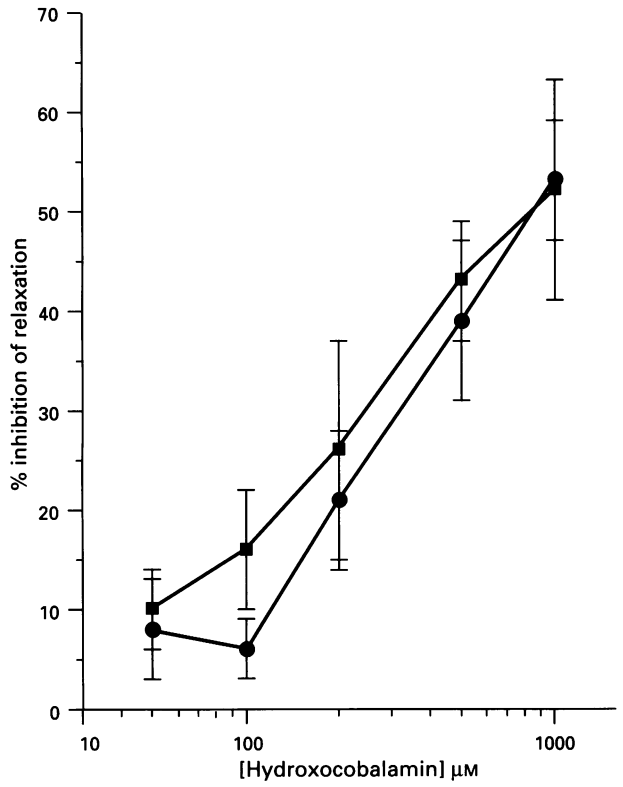

Figure 6 Concentration-response curves showing hydroxocobalamin-induced inhibition of relaxations of mouse anococcygeus muscles to exogenous nitric oxide (NO; 15 μM; ■) and nitrergic field stimulation (10 Hz; 10 s trains; ●). Each point represents mean of at least 5 individual muscle preparations; vertical lines show s.e.mean.

The results confirm that duroquinone reduces nitrergic relaxations of the mouse anococcygeus after Cu/Zn SOD inhibition, and this effect can be partially overcome on re-addition of Cu/Zn SOD (Lilley & Gibson, 1995). However, they show additionally that ascorbate can also reverse the inhibitory effect of duroquinone, although GSH and α-TOC cannot. The partial nature of the reversal observed with SOD probably occurs because exogenous SOD only restored enzyme activity extracellularly and not inside the cell; it is not clear why the reversal observed with ascorbate was only partial. This pattern of antioxidant protection was also shown with duroquinone against exogenous NO (Table 2). In addition, both Cu/Zn SOD and ascorbate protected NO against another superoxide anion generating system, xanthine: xanthine oxidase. However, the results with xanthine:xanthine oxidase against nitrergic field stimulation were different from those observed with duroquinone since xanthine:xanthine oxidase, even in high concentrations and after Cu/Zn SOD inhibition, failed to inhibit nerve-induced relaxations. The reason for the difference may lie in the mechanisms by which duroquinone and xanthine:xanthine oxidase generate superoxide anions. Duroquinone requires conversion to the semiquinone radical via the action of flavoprotein enzymes (Boersma *et al.*, 1994), and therefore the majority of the superoxide anions will be produced inside the cell; however, the ability of exogenous SOD partially to protect NO does indicate that duroquinone also causes increased superoxide anion concentrations in the extracellular fluid. On the other hand, xanthine:xanthine oxidase will act mainly extracellularly. The relative importance of Cu/Zn SOD and ascorbate as antioxidants in the intra- and extra-cellular compartments may therefore influence the sensitivity of the nitrergic transmitter to different superoxide anion generators. It should be noted, however, that Martin *et al.* (1994) found that inhibition of nitrergic relaxations of the bovine retractor penis by xanthine:xanthine oxidase was completely reversed by re-addition of Cu/Zn SOD; this may indicate a difference between this tissue and the mouse anococcygeus. Despite these uncertainties, the results clearly show that Cu/Zn SOD, and ascorbate, can protect NO from the two superoxide anion generating systems used in this study.

The effects of three direct NO-scavengers were also investigated. Two of these, hydroquinone and carboxy-PTIO, clearly differentiated between relaxations induced by NO and by field stimulation, while the third, hydroxocobalamin, did not. The results with hydroxocobalamin are similar to those observed in the bovine retractor penis (Paisley & Martin, 1996), although, in the rat anococcygeus, Rajanayagam *et al.* (1993) did find that hydroxocobalamin was much more potent against NO than against nitrergic stimulation. Since no such differentiation was found in the mouse anococcygeus, the interaction of hydroxocobalamin with the antioxidants was not pursued. Hydroquinone may act in some tissues as a superoxide anion generator (Paisley & Martin, 1996), but in our hands several pieces of evidence indicate that it acts as a free radical scavenger: first, hydroquinone does not generate su-

Table 2 Summary of the protective effect of antioxidants against various NO inhibitors in the mouse anococcygeus

	Antioxidant effect on NO relaxation			
NO inhibitor	*SOD*	*GSH*	*Ascorbate*	*α-TOC*
Duroquinone	Protects	No effect	Protects	No effect
X:XO	Protects	No effect	Protects	No effect
Hydroquinone	No effect	Protects	Protects	No effect
Carboxy-PTIO	No effect	No effect	Protects	Protects

SOD (superoxide dismutase); GSH (reduced glutathione); α-TOC (α-tocopherol); X:XO (xanthine: xanthine oxidase)

peroxide anions as detected by chemiluminescence, but rather it reduces the chemiluminescence signal generated by xanthine:xanthine oxidase (Hobbs *et al.*, 1991; Lilley & Gibson, 1995); second, hydroquinone inhibits the signal produced by a NO-microelectrode on addition of NO to an organ bath containing Krebs solution (Lilley & Gibson, 1995), an effect not reversed by Cu/Zn SOD; and third, as shown in the present study, the inhibitory effect of hydroquinone on relaxations to exogenous NO is not reversed by Cu/Zn SOD. Thus, Cu/Zn SOD activity in the region of the neuroeffector junction is unlikely to explain the resistance of nitrergic relaxations to hydroquinone. However, the important new finding of the present study is that both GSH and ascorbate can protect NO from hydroquinone. Carboxy-PTIO is a stable radical compound which interacts with NO (Akaike *et al.*, 1993) and which has been shown to inhibit relaxations of the rat anococcygeus to exogenous NO without affecting nitrergic relaxations (Rand & Li, 1995b). Carboxy-PTIO had a similar effect in the mouse anococcygeus, and the inhibitory effect on NO was reversed by ascorbate and α-TOC, but not GSH or SOD. Again, the bovine retractor penis appears to differ from the mouse anococcygeus, since carboxy-PTIO did inhibit nitrergic relaxations of the former (Paisley & Martin, 1996).

In conclusion, our results indicate that physiological antioxidants can protect NO from a variety of agents which have been shown to differentiate between relaxations induced by exogenous NO and nitrergic field stimulation, although the effectiveness of the antioxidants depends on the agent under investigation. The presence of these antioxidants in the neuroeffector region may therefore provide an explanation for the lack of effect of the NO inhibitors on nitrergic responses, without the need to invoke a transmitter other than free radical NO. Thus, NO released from nitrergic nerves would be protected from superoxide anions, and from other NO scavengers such as carboxy-PTIO, by the redox environment of the tissue, whereas exogenous NO would be vulnerable to attack in the organ bath, before reaching the protection of the tissue. Conclusive evidence would require investigation of tissues in which the antioxidants had been inhibited or depleted, and such evidence is available in the case of Cu/Zn SOD (Martin *et al.*, 1994; Lilley & Gibson, 1995; Paisley & Martin, 1996; this study). Experiments are in progress to address this question with the other three antioxidants, particularly with ascorbate since it could protect NO from all of the inhibitors tested. Certainly, ascorbate is present in high concentrations in some tissues (e.g. 6 μmol g^{-1} in brain; Martensson & Meister, 1991); it is considered to be an important extracellular antioxidant and, in the brain, has extracellular concentrations similar to those used in the present study (200–400 μM; Miele *et al.*, 1994). GSH is also found in high (millimolar) concentrations in tissues, although in this case the highest levels are found intracellularly, extracellular concentrations being in the range 0.3–15 μM (Frei, 1994); α-TOC is mainly localised to membranes and lipoproteins (Frei, 1994). It will clearly be of interest to measure levels of ascorbate, and of the other antioxidants, in nitrergically-innervated tissues. However, depletion experiments will be complicated since ascorbate, GSH and α-TOC are interlinked as antioxidants, with induced changes in one leading to compensatory changes in the others (Jain *et al.*, 1992; Martensson & Meister, 1992; Meister, 1994) and, in addition, while ascorbate is a vitamin in certain species, it is synthesised in adult rats and mice (Meister, 1994). It is possible that such variation in antioxidant function may explain some of the species differences alluded to earlier. Certainly, further investigation of the interrelationship between the nitrergic transmitter and endogenous antioxidant systems is of fundamental importance for a full understanding of the physiological and pathophysiological significance of this novel neurotransmission process.

E.L. is an MRC student.

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