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Blockade of nitrergic transmission by hydroquinone, hydroxocobalamin and carboxy-PTIO in bovine retractor penis: role of superoxide anion

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1 The effects of inhibiting endogenous Cu/Zn superoxide dismutase (SOD) with diethyldithiocarbamate (DETCA) were examined on the ability of hydroquinone, hydroxocobalamin and carboxy-PTIO to block nitrergic relaxation in the bovine retractor penis (BRP) muscle.

2 Incubation of strips of BRP with DETCA (3 mM) for 2 h reduced SOD activity from 73.1 ± 15.7 to 8.2 ± 1.9 units mg^{-1} protein.

3 Hydroquinone (10 μM – 1 mM) produced weak inhibition of nitrergic (4 Hz, 10 s) relaxation in control strips of BRP, but powerful inhibition in strips treated with DETCA (3 mM, 2 h). Exogenous SOD (250 units ml^{-1}) produced a partial blockade of the ability of hydroquinone to inhibit nitrergic relaxation in DETCA-treated strips.

4 In an assay of SOD-inhibitable reduction of cytochrome C, hypoxanthine (0.1 mM)/xanthine oxidase (16 munits ml^{-1}) and pyrogallol (10 μM), led to the rapid generation of superoxide anion. Hydroquinone (10 μM) also led to the generation of the free radical, although the rate of generation was slower.

5 Two NO-scavenging agents, hydroxocobalamin (0.1 μM – 1 mM) and carboxy-PTIO (0.1 – 1 mM), produced concentration-dependent blockade of nitrergic relaxation of the BRP. The magnitude of the blockade induced by these agents was unaffected following treatment with DETCA or SOD.

6 The findings with hydroquinone support our previous proposal that endogenous Cu/Zn SOD plays a vital role in protecting nitrergic neurotransmission from inactivation by superoxide anion. Results with hydroxocobalamin and carboxy-PTIO are consistent with the known ability of these agents to scavenge NO. The nitrergic neurotransmitter in the BRP thus appears to have the properties of NO.

Keywords: Nitrergic nerves; NANC; nitric oxide; superoxide dismutase; superoxide anion; hydroquinone; hydroxocobalamin; carboxy-PTIO; diethyldithiocarbamate

Introduction

Although first proposed for the anococcygeus (Gillespie *et al.*, 1989; Li & Rand, 1989) and bovine retractor penis (BRP) muscles (Liu *et al.*, 1991), it is now widely accepted that the L-arginine-nitric oxide pathway mediates non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmission in a wide variety of tissues (see Martin *et al.*, 1994a; Rand & Li, 1995a for reviews). Debate still continues, however, as to whether the neurotransmitter released by these 'nitrergic nerves' is free nitric oxide (NO) or a related molecule. This doubt has arisen as a consequence of findings in a number of laboratories that certain drugs have differential effects on the relaxant actions of authentic NO and nitrergic nerves. For example, in the bovine retractor penis (BRP, Gillespie & Sheng, 1990; Liu *et al.*, 1994), mouse anococcygeus (Gibson *et al.*, 1994) and the rat gastric fundus (Barbier & Lefebvre, 1992), the superoxide anion generating agents, pyrogallol, xanthine/xanthine oxidase and LY 83583, respectively destroy the activity of NO without affecting nitrergic transmission. In addition, hydroquinone, which has variously been described as a superoxide anion generator (Moncada *et al.*, 1986; Liu *et al.*, 1994) or free radical scavenger (Hobbs *et al.*, 1991), blocks the actions of NO but not of nitrergic nerve stimulation in the BRP, mouse anococcygeus, rat gastric fundus, and guinea-pig trachea (Gillespie & Sheng, 1990; Hobbs *et al.*, 1991). Furthermore, the putative NO-scavenging agents, hydroxocobalamin (Kaczka *et al.*, 1951) and carboxy-PTIO (Akaike *et al.*, 1993), inhibit relaxation induced by NO in rat anococcygeus muscle but not that induced by nitrergic nerve stimulation (Rajanayagam *et al.*, 1993; Rand & Li, 1995b). These actions contrast with those of another NO-binding substance, haemoglobin, which powerfully inhibits the actions of nitrergic nerves (Bowman *et al.*, 1982).

Investigators have proposed a number of possible explanations to account for the contrasting actions of the above drugs on authentic NO and nitrergic neurotransmission. Some have considered the possibility that the neurotransmitter is an NO-releasing molecule such as an S-nitrosothiol (Gibson *et al.*, 1992; Barbier & Lefebvre, 1994; Rand & Li, 1995a). Others have suggested the neurotransmitter is indeed NO but that, on theoretical grounds, the long diffusion pathway of exogenously added NO compared with endogenously released NO, renders the former more susceptible to inactivation (Wood & Garthwaite, 1994). We have recently established in the BRP that the ineffectiveness of the superoxide anion generators, pyrogallol, LY 83583 and hypoxanthine/xanthine oxidase, in blocking nitrergic relaxation is due to protection by endogenous Cu/Zn superoxide dismutase (SOD) activity (Martin *et al.*, 1994b). This was based upon our finding that inhibition of this enzyme with the copper chelator, diethyldithiocarbamate (DETCA; Cocco *et al.*, 1981; Kelner *et al.*, 1989), led to powerful blockade of nitrergic transmission by these same agents. Thus, NO released from nitrergic nerves within the tissue appears to be protected by Cu/Zn SOD, whereas NO added to the bathing solution in a tissue bath, where no SOD is present, is rapidly destroyed by superoxide anion generators.

In this study we have inhibited endogenous Cu/Zn SOD in the BRP with DETCA and have then examined whether this has any effect on the ability of hydroquinone, hydroxo-

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cobalamin and carboxy-PTIO to block nitrergic relaxation. A preliminary account of these findings has already been given (Martin & Paisley, 1995).

Methods

Preparation of tissues

BRP muscles were obtained from a local abattoir and transported to the laboratory. Some tissues were used that day, but others were stored at 4°C in Krebs solution for use the following day. BRP muscle strips 2–3 mm wide and 1 cm long were cut for tension recording and mounted under 2 g resting tension within Ag–AgCl ring electrodes in 12 ml organ chambers and bathed at 37°C in Krebs bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, and gassed with 95% O_2 and 5% CO_2. Tension was measured with Grass FTO3C isometric transducers and displayed on a Grass Polygraph. In all experiments, adrenergic motor responses were blocked and the tone raised in BRP muscle strips with guanethidine (30 μM). Electrical field stimulation (4 Hz, 10 s) was delivered from a Grass S88 stimulator at a pulse width of 0.5 ms and at supramaximal voltage.

Inhibition of endogenous Cu/Zn-superoxide dismutase with diethyldithiocarbamate

The inhibitor of Cu/Zn SOD, DETCA (Cocco *et al.*, 1981; Kelner *et al.*, 1989), was added to BRP muscle strips for 2 h at a concentration of 3 mM. At the end of this period, DETCA was washed from the tissue baths, leaving tissue Cu/Zn SOD essentially irreversibly inhibited. This protocol had the advantage of permitting the application of exogenous SOD in an attempt to block the effects of DETCA without the possibility of inhibiting this added enzyme. Tone was then induced with guanethidine (30 μM) and nitrergic relaxation (4 Hz, 10 s) was elicited in these tissues. When at least three reproducible responses had been obtained, hydroquinone (10 μM–1 mM), hydroxocobalamin (0.1 μM–1 mM) or carboxy-PTIO (0.1–1 mM) was added cumulatively, and their effects on relaxation compared with those obtained on control BRP muscle strips. In some experiments following treatment of strips with DETCA, exogenous Cu/Zn SOD (250 units ml^{-1}, from bovine erythrocytes) was given as a 5 min treatment to determine if it could prevent the blockade of nitrergic relaxation induced by subsequent addition of hydroquinone, hydroxocobalamin or carboxy-PTIO. Furthermore, in other experiments, it was added once blockade of nitrergic relaxation had been established by hydroquinone, hydroxocobalamin or carboxy-PTIO in control and DETCA-treated strips, to determine if blockade could be reversed.

Measurement of superoxide dismutase activity of BRP muscle

Strips of BRP muscle (~100 mg wet weight) were incubated in Krebs bicarbonate solution, as described above for relaxation studies, with or without DETCA (3 mM) for 2 h. They were subsequently blotted dry on filter paper, weighed, frozen in liquid nitrogen and pulverized in a cooled stainless steel mortar and pestle. The homogenized muscle was then extracted into 500 μl of HEPES (5 mM)-buffered Krebs solution, sonicated for 20 min, and spun at 40,000 *g* for 10 min at 4°C. The supernatant was then placed in an Amicon CF25 ultrafiltration membrane cone and spun at 40,000 *g* for 20 min at 4°C to obtain a concentration of 3–5 fold. The protein content of the concentrate was measured by the method of Bradford (1976) and the SOD activity was measured by a modification of the method of Marklund & Marklund (1974), based upon the superoxide anion-dependent auto-oxidation of pyrogallol. Briefly, the ability of concentrated homogenates to inhibit the auto-oxidation of pyrogallol (200 μM) in 50 mM Tris (2-amino-2-hydroxymethyl-1-3-propanediol)-HCl buffer (pH 8.2) containing the heavy metal chelator, diethylenetriaminepentaacetic acid (1 mM), was assessed following a 15 min incubation at 20°C in 96 well plates by measuring the increase in absorbance at 420 nm with a Dynatech plate reader. A standard curve was prepared with bovine erythrocyte SOD (0.1–100 units ml^{-1}) and the SOD activity of concentrates was expressed as units mg^{-1} protein.

Measurement of superoxide anion generation

Superoxide anion generation was assessed by measuring the SOD-inhibitable increase in absorbance at 550 nm associated with reduction of oxidized cytochrome C (Fridovich, 1970). Specifically, the ability of hypoxanthine (0.1 mM)/xanthine oxidase (16 munits ml^{-1}), pyrogallol (10 μM) and hydroquinone (10 μM) to increase the absorbance of solutions of oxidized cytochrome C (10 μM) in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, 5 mM)-buffered Krebs solution in 96 well plates was measured with a Dynatech programmable plate reader. The role of superoxide anion in any reduction seen was determined by the additional presence of SOD (2500 units ml^{-1}). Catalase (100 units ml^{-1}) was present throughout to prevent re-oxidation of cytochrome C by any hydrogen peroxide generated from superoxide anion.

Drugs

Pyrogallol was obtained from BDH Ltd. (Poole, U.K.), carboxy-PTIO (2-[4-carboxyphenyl]-4,4,5,5-tetraethylimidazoline-1-oxyl-3-oxide) was obtained from Alexis Biochemicals (Nottingham U.K.) and catalase (bovine liver), cytochrome C (oxidized), diethyldithiocarbamate, guanethidine sulphate, hydroquinone, hypoxanthine, hydroxocobalamin acetate, phenylephrine hydrochloride, superoxide dismutase (Cu/Zn-containing enzyme from bovine erythrocytes) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, U.K.). All drugs were dissolved in saline (0.9%) except for hypoxanthine which was made as a 10 mM stock in 0.2 M NaOH, with all subsequent dilutions being made in saline (0.9%).

Data analysis

The magnitude of the blockade of nitrergic relaxation induced by drugs was expressed as percentage inhibition (mean ± s.e.mean) of the control relaxant response obtained before addition of the drug. Statistical analysis was carried out with either Student's *t* test or one-way analysis of variance followed by Fisher's test, as appropriate. A value of $P<0.05$ was considered significant.

Results

Inhibition of endogenous Cu/Zn SOD by DETCA

The SOD activity of control strips of BRP muscle was 73.1 ± 15.7 units mg^{-1} protein ($n=8$). Following incubation with DETCA (3 mM) for 2 h, SOD activity was reduced significantly to an average of 8.2 ± 1.9 units mg^{-1} protein ($P<0.001$) in three strips and fell below detectable levels (~5 units mg^{-1} protein) in a further seven.

DETCA-enhanced blockade of nitrergic relaxation by hydroquinone

In control strips of BRP muscle, hydroquinone (10 μM–1 mM) induced a weak, concentration-dependent inhibition of nitrergic relaxation induced by electrical field stimulation (4 Hz, 10 s, Figures 1a and 2): the maximum blockade obtained at a concentration of 1 mM was 23.3 ± 3.8%, ($n=9$). The sensitivity of nitrergic relaxation to blockade by hydroquinone was en-

hanced greatly following treatment for 2 h with DETCA (3 mM). Figure 2 shows that the blockade of nitrergic relaxation induced by 100 μM hydroquinone in DETCA-treated tissues was rapidly reversed upon addition of exogenous SOD (250 units ml^{-1}). Furthermore, if SOD (250 units ml^{-1}) was added to DETCA-treated tissues, the blockade of nitrergic relaxation produced upon subsequent addition of hydroquinone was greatly inhibited.

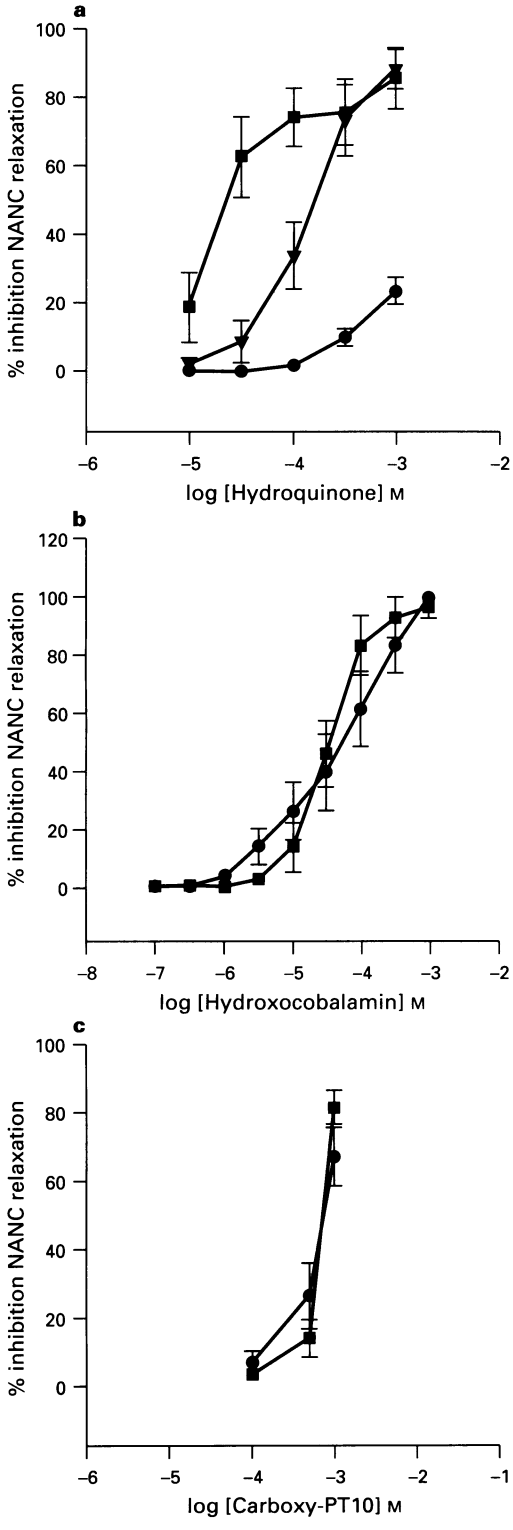

Figure 1 The ability of (a) hydroquinone, (b) hydroxocobalamin and (c) carboxy-PTIO to block nitrergic neurotransmission (4 Hz, 10 s) in control strips of BRP muscle (●) and in strips in which endogenous Cu/Zn superoxide dismutase (SOD) was inhibited (■) by treatment for 2 h with diethyldithiocarbamate (DETCA, 3 mM). In (a), following treatment with DETCA, exogenous SOD (250 units ml^{-1}) was added to determine if it could block the actions of hydroquinone added subsequently (▼). Each point is the mean ± s.e.mean of 6–16 observations.

Measurement of superoxide anion generation

We found that two well-characterized superoxide anion generating systems, hypoxanthine (0.1 mM)/xanthine oxidase (16 munits ml^{-1}) and pyrogallol (10 μM), each produced SOD (2500 units ml^{-1})-inhibitable reduction of cytochrome C during a 3 min incubation (Figure 3a). Hydroquinone (10 μM) also induced reduction of cytochrome C; this was slight at 3 min, substantial at 30 min, and was inhibited powerfully by SOD (2500 units ml^{-1}; Figure 3b).

Effects of hydroxocobalamin and carboxy-PTIO on nitrergic relaxation

Treatment of control strips of BRP with hydroxocobalamin (0.1 μM–1 mM, Figures 1b and 4) or carboxy-PTIO (0.1–1 mM, Figures 1c and 5) produced concentration-dependent inhibition of nitrergic relaxation (4 Hz, 10 s); the inhibition obtained with 1 mM of each agent was 99.3 ± 0.7% (n = 6), and 66.9 ± 8·3% (n = 7), respectively. Treatment for 2 h with DETCA (3 mM) had no effect on the ability of hydroxocobalamin or carboxy-PTIO to block nitrergic relaxation. Furthermore, SOD (250 units ml^{-1}) had no effect on the blockade of nitrergic relaxation obtained when added either before or after hydroxocobalamin or carboxy-PTIO.

Discussion

In a previous paper (Martin *et al.*, 1994b), we proposed that the ability of a number of superoxide anion generating agents, including hypoxanthine/xanthine oxidase, pyrogallol and LY 83583, to block the relaxant actions of authentic NO but not of nitrergic nerve stimulation in the BRP resulted from high levels of Cu/Zn SOD in the tissue. This proposal was based on our observation that the ability of each of these agents to block nitrergic relaxation of the tissue was enhanced greatly following inhibition of endogenous Cu/Zn SOD with the copper chelator, diethyldithiocarbamate (DETCA; Cocco *et al.*, 1981; Kelner *et al.*, 1989). The aim of this study was to determine if inhibition of Cu/Zn SOD had any effect on the ability of hydroquinone, hydroxocobalamin or carboxy-PTIO to block neurotransmission in the BRP.

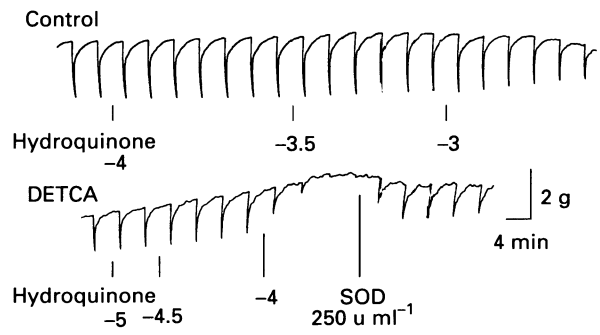

Figure 2 Individual tracings showing that hydroquinone produced only a slight inhibition of nitrergic relaxation (4 Hz, 10 s) of control strips of BRP. In contrast, following pretreatment with diethyldithiocarbamate (DETCA, 3 mM) for 2 h followed by washout of the drug, addition of hydroquinone produced powerful inhibition of nitrergic relaxation and this was reversed partially by exogenous superoxide dismutase (SOD, 250 units ml^{-1}). The concentrations of hydroquinone are given in log molar units.

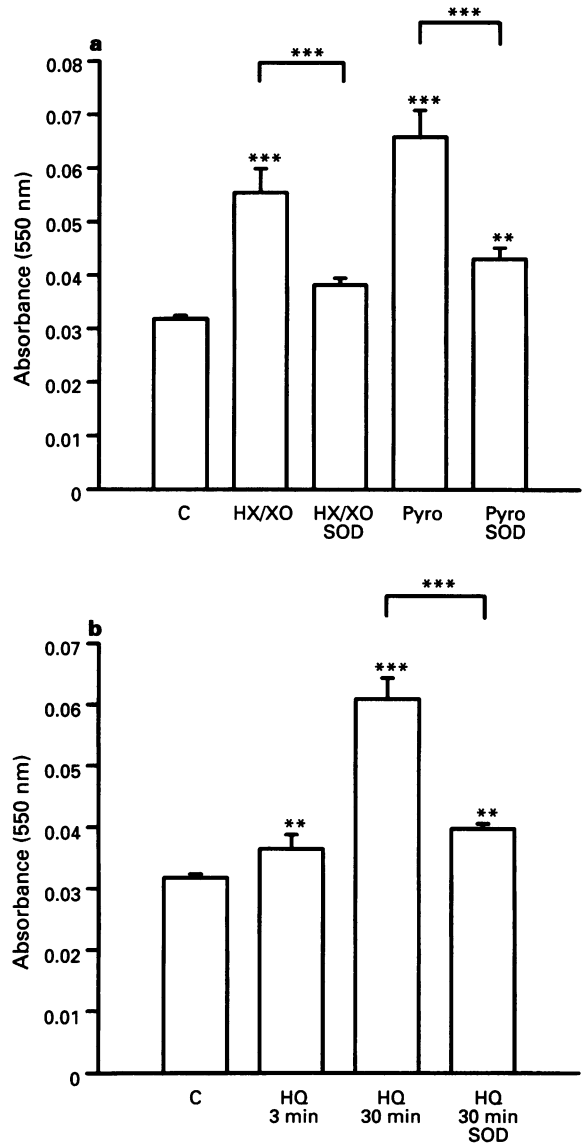

Figure 3 The generation of superoxide anion by (a) hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 munits ml^{-1}) and pyrogallol (Pyro, 10 μM) and (b) hydroquinone (HQ, 10 μM) as assessed by the superoxide dismutase (SOD, 2500 units ml^{-1})-inhibitable increase in absorbance of cytochrome C at 550 nm. HX/XO and Pyro were incubated with cytochrome C for 3 min and HQ was incubated for 3 min or 30 min. Data are given as the mean ± s.e.mean of 4–20 observations. $^{**}P<0.005$ and $^{***}P<0.001$ indicate significant differences from control (C) or between groups joined by a bracket.

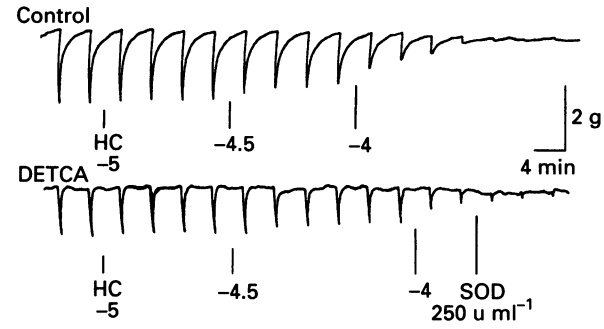

Figure 4 Individual tracings showing that hydroxocobalamin (HC) produced a concentration-dependent blockade of nitrergic relaxation (4 Hz, 10 s) in control strips of BRP. The magnitude of the inhibitory action of HC was unaffected following incubation of strips of BRP for 2 h with diethyldithiocarbamate (DETCA, 3 mM). Superoxide dismutase (SOD, 250 units ml^{-1}) did not reverse the actions of HC in DETCA-treated tissues. The concentrations of HC are given in log molar units.

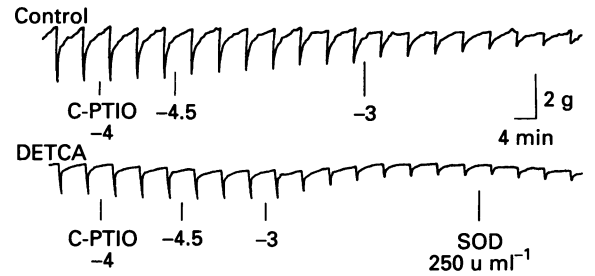

Figure 5 Individual tracings showing that carboxy-PTIO (C-PTIO) produced a concentration-dependent blockade of nitrergic (4 Hz, 10 s) relaxation in control strips of BRP. The magnitude of the inhibitory action was unaffected following incubation of strips of BRP for 2 h with diethyldithiocarbamate (DETCA, 3 mM). Superoxide dismutase (SOD, 250 units ml^{-1}) did not reverse the actions of C-PTIO in DETCA-treated tissues. The concentrations of C-PTIO are given in log molar units.

In this study, we measured a total SOD activity in the supernatant fraction of homogenates of BRP muscle of 73.1 ± 15.7 units mg^{-1} protein, which is comparable to that observed for vascular smooth muscle (120 ± 32 units mg^{-1} protein; Mügge *et al.*, 1991). Furthermore, this activity was reduced towards undetectable levels (~5 units mg^{-1} protein) following incubation for 2 h with DETCA, thus establishing the effectiveness of this agent.

In keeping with previous findings on control strips of BRP (Gillespie & Sheng, 1990), we found that hydroquinone had little inhibitory effect on nitrergic relaxation; a maximum blockade of around 20% was found at a concentration of 1 mM. Hydroquinone has also been reported to have no effect on nitrergic relaxation in the mouse anococcygeus, rat gastric fundus and guinea-pig trachea (Hobbs *et al.*, 1991). In contrast, hydroquinone produced powerful blockade of nitrergic relaxation in strips of BRP when Cu/Zn SOD was inhibited following treatment with DETCA. This blockade almost certainly arose as a consequence of the generation of superoxide anion since exogenously added Cu/Zn SOD inhibited this action when added either before or after hydroquinone. In the BRP, therefore, hydroquinone behaved like the superoxide anion generating agents, hypoxanthine/xanthine oxidase, pyrogallol and LY 83583 (Martin *et al.*, 1994b). In fact, using the SOD-inhibitable reduction of cytochrome C as an assay system (Fridovich, 1970), hydroquinone was indeed observed to produce superoxide anion, although it did so at a slower rate than hypoxanthine/xanthine oxidase or pyrogallol.

Our findings with hydroquinone in the BRP clearly differ from those obtained in the mouse anococcygeus (Hobbs *et al.*, 1991). In this latter tissue, hydroquinone and its two electron oxidized derivative, benzoquinone, failed to block nitrergic transmission even in tissues treated with DETCA (Gibson & Lilley, 1995; Lilley & Gibson, 1995). Both, however, blocked the actions of authentic NO, although this was attributed to the radical scavenging actions of the agents and not to the generation of superoxide anion. In contrast, in the same study, the related compound, duroquinone, did behave in a similar manner to hydroquinone in the BRP, i.e. it produced powerful blockade of nitrergic transmission in the mouse anococcygeus, but only following treatment with DETCA. Furthermore this blocking action was inhibited by exogenous application of SOD showing clearly that it was mediated by superoxide anion. What is far from clear, however, is why after treatment

with DETCA, hydroquinone blocks nitrergic transmission in the BRP but not the mouse anococcygeus. The explanation may perhaps lie in differences in the redox environments within the two tissues. Specifically, it is known that hydroquinones have the capacity to auto-oxidise (Marklund & Marklund, 1974), and quinones can be reduced by a variety of flavoprotein enzymes (Boersma *et al.*, 1994). Both reactions lead to the intermediate generation of semi-quinone radicals which can donate an electron to molecular oxygen, thereby generating superoxide anion. Accordingly, although the redox milieu with the BRP may permit the auto-oxidation of hydroquinone leading to the generation of destructive superoxide anions, that of the mouse anococcygeus may not. Further work will be required to test this hypothesis. What is evident, however, is that the findings with duroquinone in the mouse anococcygeus together with our own findings in the BRP suggest that in both tissues, endogenous Cu/Zn SOD is vitally important in protecting nitrergic neurotransmission from the destructive action of superoxide anion.

Experiments with NO-scavenging agents have also yielded variable effects on nitrergic transmission in different tissues. It is generally accepted that haemoglobin uniformly blocks nitrergic relaxation in all tissues tested, including the anococcygeus muscle of the rat and the BRP (Bowman & Gillespie, 1982; Bowman *et al.*, 1982). In contrast, two other NO-scavenging agents, hydroxocobalamin and carboxy-PTIO, inhibit the relaxant actions of authentic NO but not those of nitrergic nerves in the rat anococcygeus (Rajanayagam *et al.*, 1993; Rand & Li, 1995b). Our own findings with these two agents in the BRP were strikingly different; hydroxocobalamin produced a powerful, concentration-dependent blockade of nitrergic transmission over the concentration range 1–1000 μM, which is similar to the range over which it blocks the direct relaxant actions of NO (Rajanayagam *et al.*, 1993). Carboxy-PTIO also produced powerful blockade of nitrergic transmission but this took place at higher concentrations (0.1–1 mM) than have been reported to block the relaxant actions of NO (10–300 μM; Rand & Li, 1995b). We can only speculate that the shorter diffusion pathway of the nitrergic transmitter when compared with that of exogenously added NO may provide less opportunity to scavenge the former, thus accounting for the need for higher concentrations of carboxy-PTIO.

Our new findings have a direct bearing on the debate concerning the nature of the nitrergic neurotransmitter. Specifically, they show that nitrergic neurotransmission in the BRP can be blocked by agents that either destroy (superoxide anion generating systems) or scavenge (hydroxocobalamin and carboxy-PTIO) NO. Consequently, although it is perhaps possible that the neurotransmitter in this tissue is an NO-releasing substance (Gibson *et al.*, 1992; Barbier & Lefebvre, 1994; Rand & Li, 1995a), we have thus far no reason to suspect that it is anything other than NO *per se*. Whether the release of an NO-adduct as neurotransmitter in the rat anococcygeus accounts for the inability of hydroxocobalamin (Rajanayagam *et al.*, 1993) and carboxy-PTIO (Rand & Li, 1995b) to block nitrergic transmission in this tissue remains to be determined.

In conclusion, our data show that in the BRP, hydroquinone powerfully inhibits nitrergic neurotransmission following treatment with diethyldithiocarbamate by an action involving destruction of the nitrergic neurotransmitter by superoxide anion. These findings thus support the proposal that endogenous Cu/Zn SOD has a vital role in protecting nitrergic transmission from interruption by this reactive oxygen species (Martin *et al.*, 1994b; Gibson & Lilley, 1995; Lilley & Gibson, 1995). These findings, taken together with those demonstrating blockade by hydroxocobalamin and carboxy-PTIO are consistent with NO acting as neurotransmitter of the nitrergic nerves in the BRP.

References

AKAIKE, T., YOSHIDA, M., MIYAMOTO, Y., SATO, K., KOHNO, M., SASAMOTO, K., MIYAZAKI, K. & MAEDA, H. (1993). Antagonistic action of imidazolineoxy N-oxides against endothelium-derived relaxing factor/NO through a radical action. *Biochemistry*, **32**, 827–832.

BARBIER, A.J.M. & LEFEBVRE, R.A. (1992). Effect of LY 83583 on relaxation induced by non-adrenergic, non-cholinergic nerve stimulation and exogenous nitric oxide in the rat gastric fundus. *Eur. J. Pharmacol.*, **219**, 331–334.

BARBIER, A.M.J. & LEFEBVRE, R.A. (1994). Influence of S-nitrosothiols and nitrate tolerance in the rat gastric fundus. *Br. J. Pharmacol.*, **111**, 1280–1286.

BOERSMA, M.G., BALVERS, W.G., VERVOORT, J. & RIETJENS, I.M.C.M. (1994). NADPH cytochrome reductase catalysed redox cycling of 1,4-benzoquinone; hampered at physiological conditions, initiated at increased pH values. *Biochem. Pharmacol.*, **47**, 1949–1955.

BOWMAN, A. & GILLESPIE, J.S. (1982). Blockade of some non-adrenergic inhibitory responses of smooth muscle by a substance from haemolysed erythrocytes. *J. Physiol.*, **328**, 11–25.

BOWMAN, A., GILLESPIE, J.S. & POLLOCK, D. (1982). Oxyhaemoglobin blocks non-adrenergic inhibition in the bovine retractor penis muscle. *Eur. J. Pharmacol.*, **85**, 221–224.

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of nanogram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

COCCO, D., CALABRESE, L., RIGO, A., ARGESE, E. & ROTILO, G. (1981). Re-examination of the reaction of diethyldithiocarbamate with the copper of superoxide dismutase. *J. Biol. Chem.*, **256**, 8983–8986.

FRIDOVICH, I. (1970). Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J. Biol. Chem.*, **245**, 4053–4057.

GIBSON, A., BABBEDGE, R., BRAVE, S.R., HART, S.L., HOBBS, A.J., TUCKER, J.F., WALLACE, P. & MOORE, P.K. (1992). An investigation of some S-nitrosothiols, and of hydroxy-arginine, on the mouse anococcygeus. *Br. J. Pharmacol.*, **107**, 715–721.

GIBSON, A., BRAVE, S.R. & TUCKER, J.F. (1994). Differential effect of xanthine: xanthine oxidase on NANC- and NO-induced relaxations of the mouse anococcygeus. *Can. J. Physiol. Pharmacol.*, **72** (Suppl. 1), P14.3

GIBSON, A. & LILLEY, E. (1995). Duroquinone, but not hydroquinone, inhibits nitrergic relaxations of the mouse anococcygeus after inhibition of superoxide dismutase. *Br. J. Pharmacol.*, **115**, 148P.

GILLESPIE, J.S., LIU, X. & MARTIN, W. (1989). The effects of L-arginine and N^G-monomethyl- L-arginine on the response of the rat anococcygeus to NANC nerve stimulation. *Br. J. Pharmacol.*, **98**, 1080–1082.

GILLESPIE, J.S. & SHENG, H. (1990). The effects of pyrogallol and hydroquinone on the response to NANC nerve stimulation in the rat anococcygeus and bovine retractor penis muscles. *Br. J. Pharmacol.*, **99**, 194–196.

HOBBS, A.J., TUCKER, J.F. & GIBSON, A. (1991). Differentiation by hydroquinone of relaxations induced by exogenous and endogenous nitrates in non-vascular smooth muscle. *Br. J. Pharmacol.*, **104**, 645–650.

KACZKA, E.A., WOLF, D.E., KUEHL, F.A.J. & FOLKERS, K. (1951). Vitamin B_{12}. Modifications of cyano-cobalamin. *J. Am. Chem. Soc.*, **73**, 3569–3572.

KELNER, M.J., BAGNELL, R., HALE, B. & ALEXANDER, N.M. (1989). Inactivation of intracellular copper-zinc superoxide dismutase by copper chelating agents without glutathione depletion and methemoglobin formation. *Free Radical. Biol. Med.*, **6**, 355–360

LI, C.G. & RAND, M.J. (1989). Evidence for a role of nitric oxide in the neurotransmitter system mediating relaxation of the rat anococcygeus muscle. *Clin. Exp. Pharmacol. Physiol.* **16,** 933-938.

LILLEY, E. & GIBSON, A. (1995). Inhibition of relaxations to nitrergic stimulation of the mouse anococcygeus by duroquinone. *Br. J. Pharmacol.*, **116,** 3231–3236.

LIU, X., GILLESPIE, J.S., GIBSON, I.F. & MARTIN, W. (1991). Effects of N^G-substituted analogues of L-arginine on NANC relaxation of the rat anococcygeus and bovine retractor penis muscles and the bovine penile artery. *Br. J. Pharmacol.*, **104,** 53–58.

LIU, X., GILLESPIE, J.S. & MARTIN, W. (1994). Non-adrenergic, non-cholinergic relaxation of the bovine retractor penis muscle: role of S-nitrosothiols. *Br. J. Pharmacol.*, **111,** 1287–1295.

MARKLUND, S. & MARKLUND, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.*, **47,** 469–474.

MARTIN, W., GILLESPIE, J.S. & LIU, X. (1994a). Nitric oxide as an inhibitory neurotransmitter in the autonomic nervous system. In *Nitric Oxide: Roles in Neuronal Communication and Neurotoxicity*. ed. Takagi, H. pp.65–69. Tokyo: Japan Scientific Societies Press.

MARTIN, W., MCALLISTER, H.M. & PAISLEY, K. (1994b). NANC neurotransmission in the bovine retractor penis muscle is blocked by superoxide anion following inhibition of superoxide dismutase with diethyldithiocarbamate. *Neuropharmacology*, **33,** 1293–1301.

MARTIN, W. & PAISLEY, K. (1995). Blockade of nitrergic relaxation in bovine retractor penis muscle by hydroquinone but not hydroxocobalamin is enhanced by diethyldithiocarbamate. *Br. J. Pharmacol.*, **116,** 323P.

MONCADA, S., PALMER, R.M.J. & GRYGLEWSKI, R.J. (1986). Mechanism of action of some inhibitors of endothelium-derived relaxing factor. *Proc. Natl. Acad. Sci. U.S.A.*, **83,** 9164–9168.

MÜGGE, A., ELWELL, J.H., PETERSON, T.E., HOFMEYER, T.G., HEISTAD, D.D. & HARRISON, D.G. (1991). Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. *Circ. Res.*, **69,** 1293–1300.

RAJANAYAGAM, M.A.S., LI, C.G. & RAND, M.J. (1993). Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle. *Br. J. Pharmacol.*, **108,** 3–5.

RAND, M.J. & LI, C.G. (1995a). Nitric oxide as a neurotransmitter in peripheral nerves: nature of transmitter and mechanism of transmission. *Annu. Rev. Physiol.*, **57,** 659–682.

RAND, M.J. & LI, C.G. (1995b). Discrimination by the NO-trapping agent, carboxy-PTIO, between NO and the nitrergic transmitter but not between NO and EDRF. *Br. J. Pharmacol.*, **116,** 1906–1910.

WOOD, J. & GARTHWAITE, J. (1994). Models of the diffusional spread of nitric oxide (NO): Implications for neural NO signalling and its pharmacological properties. *Neuropharmacology*, **33,** 1235–1244.

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