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Inhibition of relaxations to nitrergic stimulation of the mouse anococcygeus by duroquinone

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1 The role of copper/zinc superoxide dismutase (Cu/Zn SOD) in protection of nitrergic neurotransmission in the mouse anococcygeus was investigated by use of duroquinone (DQ), which generates superoxide anions within tissues via reduction by flavoprotein enzymes.

2 In control anococcygeus muscles, DQ (10-100 μM) produced concentration-related inhibition ($-\log IC_{40}=4.41$) of relaxations to exogenous nitric oxide (NO; 15 μM). Nitrergic relaxations induced by field stimulation (10 Hz; 10 s train) were much less affected, 100 μM DQ reducing nitrergic relaxations by only $14\pm6\%$.

3 Following incubation with the Cu/Zn SOD inhibitor, diethyldithiocarbamate (DETCA; 3 mM; 45 min incubation; 10 min washout), the inhibitory effects of DQ on relaxations to NO were potentiated ($-\log IC_{40}=5.22$), and clear, concentration-related inhibitions of nitrergic relaxations were now observed ($-\log IC_{40}=4.54$). In both cases, these inhibitions were partially reversed by Cu/Zn SOD (250 u ml^{-1}). In DETCA-treated tissues, DQ (100 μM) also reduced relaxations to sodium nitroprusside (1 μM) and S-nitroso-glutathione (30 μM), but potentiated those to 8-Br-cyclic GMP (100 μM).

4 Neither hydroquinone (HQ; 100 μM) nor 1,4-benzoquinone (BQ; 100 μM), both of which reduced responses to exogenous NO, inhibited relaxations induced by field stimulation in DETCA-treated tissues. Indeed, when added during DQ-induced inhibition of nitrergic relaxations, both HQ and BQ produced partial reversal of the block.

5 DQ had no effect on the detection of superoxide anions estimated via the xanthine: xanthine oxidase chemiluminescence assay, or of authentic NO as measured by a chemical microsensor. However, the detection of both superoxide anions and NO in these assays was inhibited by inclusion of either HQ or BQ.

6 The results support the proposal that nitrergic transmission in the peripheral nervous system is protected by Cu/Zn SOD activity in the region of the neuroeffector junction, and this may explain the lack of effect of superoxide anion generating drugs such as DQ. Such an explanation does not hold for either HQ or BQ, which appear to be acting directly as free radical scavengers in these experiments.

Keywords: Anococcygeus (mouse); 1,4-benzoquinone; diethyldithiocarbamate; duroquinone; hydroquinone; nitrergic neurotransmission; nitric oxide; superoxide anions; superoxide dismutase

Introduction

There is now substantial evidence that the L-arginine/NO pathway generates the non-adrenergic, non-cholinergic transmitter which mediates relaxation of smooth muscle in the respiratory, gastrointestinal and urogenital tracts (for reviews see Rand, 1992; Rand & Li, 1995). However, several aspects of this novel (nitrergic) neurotransmission system await full elucidation, including the observation that a number of drugs are potent inhibitors of relaxations induced by exogenous NO, but have little effect on relaxation due to nitrergic nerve stimulation; these drugs include hydroquinone (HQ, Hobbs *et al.*, 1991; Gibson *et al.*, 1992), hydroxocobalamin (Rand & Li, 1993), pyrogallol and LY83583 (Gillespie & Sheng, 1990; Barbier & Lefebvre, 1992; Knudsen *et al.*, 1992; Liu *et al.*, 1994). Such results have inspired a number of possible explanations (for reviews see Gibson *et al.*, 1995; Rand & Li, 1995): these are: (1) the NO produced in the nerve terminal may be released in a modified form, perhaps attached to a carrier molecule; (2) the rapid rate of diffusion of NO released from a point source (Wood & Garthwaite, 1994) may render it resistant to the actions of scavenger molecules, at least over short distances; and (3) NO may indeed be released as a free radical, but may be protected by other substances which preferentially interact with potential NO-scavengers. In relation to this last hypothesis, Martin *et al.*, (1994) have proposed that the resistance of nitrergic relaxations of the bovine retractor penis to superoxide anion generators (pyrogallol, hypoxanthine/xanthine oxidase, and LY83583) may be due to high levels of copper/zinc superoxide dismutase (Cu/Zn SOD) in the vicinity of the neuroeffector junction, since the drugs did block nitrergic relaxations following treatment with the Cu/Zn SOD inhibitor, diethyldithiocarbamate (DETCA). The object of the present study was to determine whether this explanation might also hold true in the mouse anococcygeus, and might explain the differential effect of HQ on NO- and nerve-induced relaxations of this tissue, since there is some uncertainty whether HQ acts as a superoxide anion generator or via some other 'free radical scavenger' mechanism (Hobbs *et al.*, 1991). To investigate the interaction of superoxide anions and Cu/Zn SOD with nitrergic relaxations in the anococcygeus, we used duroquinone (DQ, 2,3,5,6-tetramethyl-1,4-benzoquinone), a compound structurally related to HQ, but which is known to generate superoxide anions within tissues via chemical reduction by several flavoprotein enzymes (Powis & Appel, 1980; Boersma *et al.*, 1994). Preliminary accounts of some of the work contained in this paper have already been published (Gibson & Lilley, 1995a, b).

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

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glass organ baths containing Krebs bicarbonate buffer (mM: NaCl 118.1, KCl 4.7, KH_2PO_4 1.0, $MgSO_4$ 1.0, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 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 recorded with a Grass FT03 force-displacement transducer attached to a Graphtec pen-recorder (WR3101). A period of 45 min was allowed for tissue equilibration before the start of the experiment. Field stimulation was applied via two parallel platinum electrodes running down either side of the tissue; these were attached to Grass S48 stimulator (0.5 ms pulse width, 70 V). Sympathetic responses were prevented by including 1 μM phentolamine in the Krebs solution and by preincubating each muscle in 30 μM guanethidine for 15 min during the equilibration period. In all cases, muscle tone was raised with 50 μM carbachol and relaxations measured as percentage reductions of this carbachol-induced tone. $-\log IC_{40}$ values (negative $\log_{10}$ of the molar concentration of drug reducing relaxations to NO or field stimulation by 40%) were calculated by regression analysis of individual concentration-response curves. The effects of DQ, and other quinones, on responses to relaxant drugs were investigated by adding the quinone to the organ bath 10 min before testing the relaxant drug; the bath was washed with fresh Krebs solution once the relaxant response had been obtained. To determine the effect on relaxations to nitrergic stimulations, three control relaxations to field stimulation (10 Hz; 10 s train every 100 s) were established and the quinone was then added; the organ bath was washed out once the inhibition had stabilized, usually after 3–4 further stimulations.

Superoxide anion detection

Superoxide anions were detected by chemiluminescence (Hobbs *et al.*, 1991). Two ml of Krebs solution was placed in clear plastic test 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 readout, and in analogue form using a Maclab. Control responses were established with 70 mu ml^{-1} xanthine oxidase in the test tubes and, after 5 min, adding increasing concentrations of xanthine through an auto-injector (LKB 1250-104). Results were calculated as the area under the curve (mV s) obtained following addition of xanthine.

NO detection

Measurement of authentic NO was achieved with a NO microelectrode (Diamond General Development Corporation 2030). The electrode was immersed in Krebs solution (37°C; 95% O_2:5% CO_2) in a 1 ml glass organ bath and allowed to equilibrate for 1 h before the start of the experiment. To reduce electrical noise the electrode and organ bath assembly were enclosed within a Faraday cage. The NO signal (mV) was monitored by digital display (Chemical Microsensor, Diamond General Development Corporation 1201) and in analogue form with a Maclab. Authentic NO was rapidly injected into the organ bath to give concentrations equivalent to those used in the mechanical studies. When other drugs were used they were placed in the bath 5 min before addition of NO. Responses were recorded as the peak signal (mV) obtained after addition of NO. The signal was taken to represent NO since it was absent if the NO stock solution was opened to the air 5 min before addition of an aliquot to the organ bath, and was prolonged by reducing the temperature of the organ bath to 25°C or cutting off the gas supply.

Statistics

Results are expressed as mean ± s.e. mean. Statistical analysis was by Student's t test (unpaired); $P<0.05$ was taken as indicating significant differences.

Drugs

All drugs were dissolved in distilled water, except xanthine which was dissolved (stock 100 mM) in 0.1 M NaOH, and duroquinone or 1,4-benzoquinone which were dissolved in dimethylsulphoxide (each at 10 mM). Solvents themselves were without signficant effect in the concentrations used in the experiments. NO solutions were prepared as described previously (Gibson & Mirzazadeh, 1989). Drugs used were (from Sigma unless stated otherwise): 1,4-benzoquinone (Aldrich), 8-bromo-cyclic guanosine monophosphate, carbachol (BDH), diethyldithiocarbamic acid, duroquinone (Aldrich), guanethidine sulphate, hydroquinone, lucigenin (*bis*-methylacridinium nitrate), nitric oxide (99%, BDH), S-nitrosoglutathione (prepared as described previously, Gibson *et al.*, 1992), phentolamine HCl, sodium nitroprusside, superoxide dismutase (copper/zinc containing enzyme from bovine erythrocytes), xanthine, xanthine oxidase (from buttermilk).

Results

Field stimulation (10 s trains; 1–40 Hz) and NO (3–60 μM) produced graded relaxations of carbachol (50 μM)-induced tone; 10 Hz field stimulation reduced tone by 46 ± 9% ($n=11$) and 15 μM NO by 48 ± 5% ($n=11$), and these parameters were used in subsequent experiments to investigate the effects of DQ on relaxant responses to the two stimuli.

Effects of DQ in control muscles

In control anococcygeus muscles, DQ (10–100 μM) produced a concentration-dependent inhibition of relaxations to NO (Figure 1), giving a $-\log IC_{40}$ of 4.41 ± 0.01 ($n=6$), but it was much less effective against relaxations induced by field stimulation (Figure 1), producing an inhibition of only 14 ± 6% at 100 μM ($n=6$). In both cases, this inhibition was partially reversed by 250 u ml^{-1} Cu/Zn SOD (18 ± 2% reversal of the

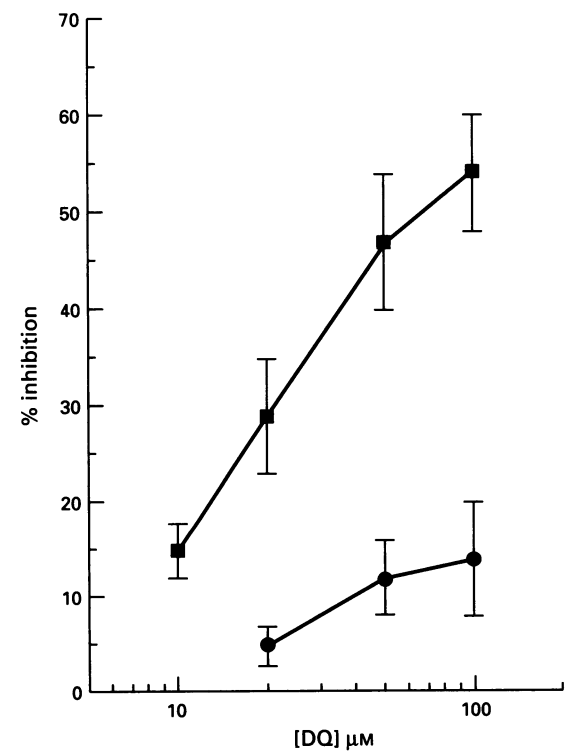


Figure 1 Concentration-response curves for the inhibitory effects of duroquinone (DQ) on relaxations of mouse anococcygeus muscles in response to nitric oxide (15 μM; ■) or nitrergic field stimulation (10 Hz, 10 s; ●). Each point is the mean ± s.e. mean from at least 5 individual muscle preparations.

effect of 100 μM DQ on NO, $n=7$; 89±11% reversal of the effect of 100 μM DQ on nitrergic stimulation, $n=5$). Cu/Zn SOD (250 u ml^{-1}), by itself, had no effect on carbachol-induced tone, or on relaxations to NO or field stimulation (data not given).

Effects of DQ in DETCA-treated muscles

In the bovine retractor penis, Martin *et al.* (1994) treated tissues for 60 min with 3 mM DETCA, followed by 10 min washout. In initial experiments with the mouse anococcygeus, 60 min incubation with DETCA was found to reduce markedly carbachol-induced tone. Decreasing time to 45 min followed by 10 min washout, resulted in only a slight inhibition of the ability of 50 μM carbachol to induce tone (460±26 mg tension in controls; 385±20 mg tension after DETCA; $n=6$ in both cases; $P<0.05$) and, therefore, this incubation pattern was used in subsequent experiments to determine the effects of DETCA treatment on the potency of DQ against NO and field stimulation.

Incubation with DETCA had no effect *per se* on relaxant responses to NO (Figure 2a), but produced a clear potentiation of the inhibitory effects of DQ on NO (Figure 2b). In the presence of Cu/Zn SOD (250 u ml^{-1}), this potentiation was partially reversed (Figure 2b; − log IC_{40} after DETCA, 5.22±0.19, $n=10$; after DETCA with Cu/Zn SOD, 4.71±0.13, $n=6$; $P<0.05$).

DETCA treatment also had no effect *per se* on relaxations induced by field stimulation (41±6% relaxation in controls, $n=6$; 42±6% relaxation in DETCA-treated tissues, $n=6$; $P>0.05$). However, again, the inhibitory effect of DQ on nitrergic relaxations was much greater after DETCA treatment, an effect that was partially reversed by Cu/Zn SOD (250 u ml^{-1}; Figure 3a,b; − log IC_{40} after DETCA, 4.54±0.06, $n=7$; after DETCA with Cu/Zn SOD, 4.20±0.01, $n=7$; $P<0.05$).

The selectivity of action of DQ was determined by investigating its effect on relaxations induced by two NO-donors, and by 8-Br-cyclic GMP. In DETCA-treated tissues, sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO) and 8-Br-cyclic GMP each produced concentration-dependent relaxations (Figure 4a); 100 μM DQ significantly reduced relaxations induced by SNP and GSNO, but enhanced those to 8-Br-cyclic GMP (Figure 4b).

Effects of HQ and 1,4-benzoquinone (BQ)

Having established that DETCA pretreatment greatly increased the potency of DQ against relaxations to field stimulation, it was of interest to determine whether such pretreatment would also render nitrergic relaxations sensitive to HQ, and to its 2-electron oxidised form, BQ.

Both HQ and BQ inhibited NO-induced relaxations of control muscles; 100 μM HQ reduced responses to NO by 52±10% ($n=8$) and 100 μM BQ by 60±5% ($n=10$). However, relaxations induced by field stimulation were unaffected

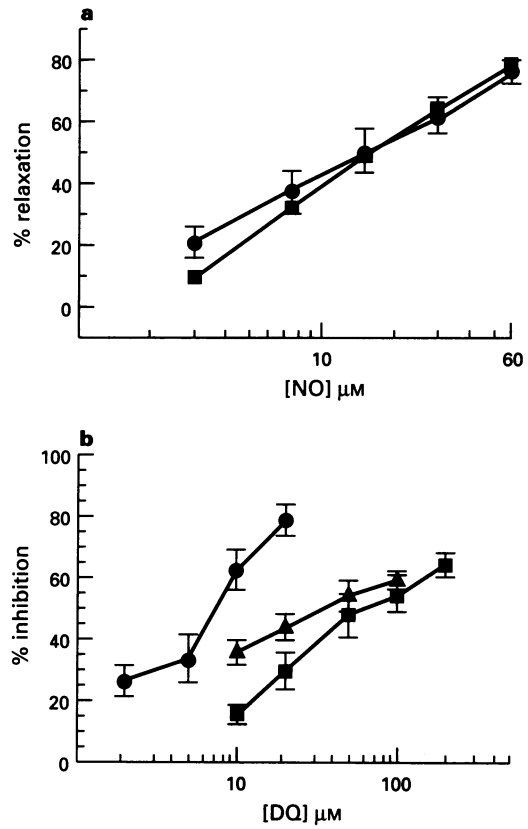


Figure 2 (**a**) Concentration-response curves for relaxations of mouse anococcygeus muscle to nitric oxide (NO) in control tissues (■) and following incubation with diethyldithiocarbamate (DETCA; 3 mM; 45 min incubation; 10 min washout; ●). (**b**) Concentration-response curves for the inhibitory effects of duriquinone (DQ) on relaxations of mouse anococcygeus muscles to NO (15 μM) in control tissues (■), following incubation with DETCA (as above, ●), or following incubation with DETCA and then in the presence of Cu/Zn superoxide dismutase (250 u ml^{-1}; ▲). In both (a) and (b), each point represents the mean±s.e. mean from at least 5 individual muscle preparations.

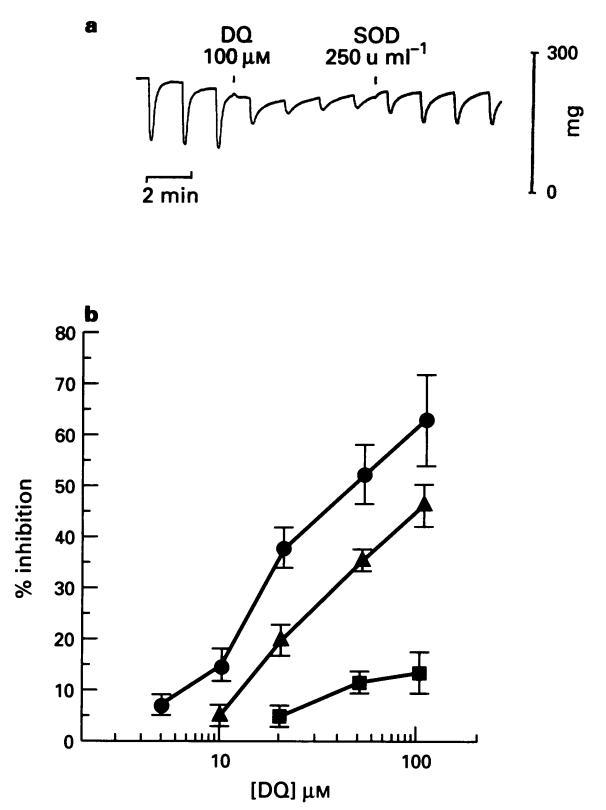


Figure 3 (**a**) Trace showing relaxations of a mouse anococcygeus muscle in response to nitrergic field stimulation (10 Hz, 10 s) following incubation with diethyldithiocarbamate (DETCA; 3 mM; 45 min incubation; 10 min washout), and the effect on these relaxations of duroquinone (DQ) and Cu/Zn superoxide dismutase (SOD). (**b**) Concentration-response curves for the inhibitory effects of duroquinone (DQ) on relaxations of mouse anococcygeus muscles to nitrergic field stimulation (10 Hz; 10 s) in control tissues (■), following incubation with DETCA (as above, ●), or following incubation with DETCA and then in the presence of SOD (250 u ml^{-1};▲). Each point in (**b**) is the mean ±s.e. mean from at least 5 individual muscle preparations.

by either HQ and BQ at this concentration (data not given), confirming previous observations with HQ (Hobbs *et al.*, 1991). After 45 min incubation with DETCA, followed by 10 min washout, nitrergic relaxations to field stimulation were still unaffected by 100 μM HQ (relaxations before HQ, 45±5%; after HQ, 46±5%, $n=5$ in both cases; $P>0.05$) or 100 μM BQ (relaxations before BQ 49±5%; after BQ 49±5%; $n=5$ in both cases; $P>0.05$). Indeed, both drugs, when added during DQ (50 μM)-induced inhibition of nitrergic relaxations, resulted in a partial reversal of the block (Figure 5; 44±8% reversal with 100 μM HQ, $n=6$; 27±6% reversal with 100 μM BQ, $n=3$).

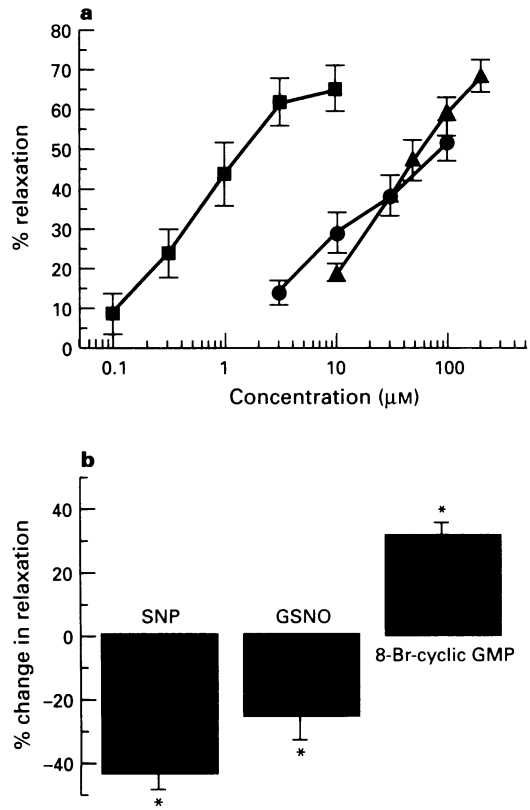


Figure 4 (**a**) Concentration-response curves for relaxations of mouse anococcygeus muscles to sodium nitroprusside (SNP; ■), S-nitrosoglutathione (GSNO; ●) and 8-Br-cyclic GMP (▲) in tissues incubated with diethyldithiocarbamate (DETCA; 3 mM; 45 min incubation; 10 min washout). Each point is the mean±s.e. mean from at least 5 individual muscle preparations. (**b**) Histograms showing the effect of duroquinone (100 μM) on relaxations of DETCA-treated muscles to SNP (1 μM), GSNO (30 μM) and 8-Br-cyclic GMP (100 μM). Each column represents the mean±s.e. mean from at least 5 individual muscle preparations. *$P<0.05$, significant change in relaxant response.

Effects of DQ, HQ and BQ on superoxide anions and authentic NO in Krebs solution

A number of experiments were carried out to determine whether DQ, HQ and BQ might act as direct free radical scavengers. In a first series, the effect of these drugs on superoxide

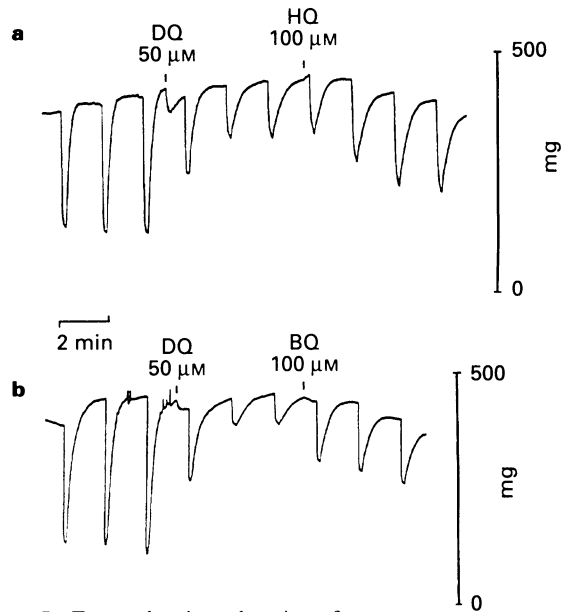
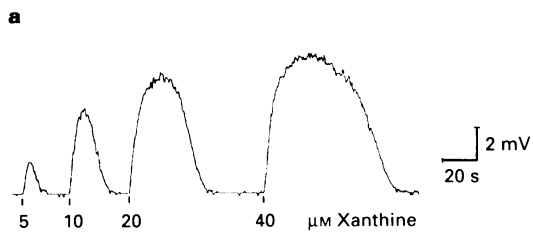


Figure 5 Traces showing relaxation of mouse anococcygeus muscles in response to nitrergic field stimulation (10 Hz, 10 s) following incubation with diethyldithiocarbamate (3 mM; 45 min incubation; 10 min washout) and the effect on these relaxations of duroquinone (DQ) followed by either hydroquinone (HQ, in **a**) or 1, 4-benzoquinone (BQ, in **b**).



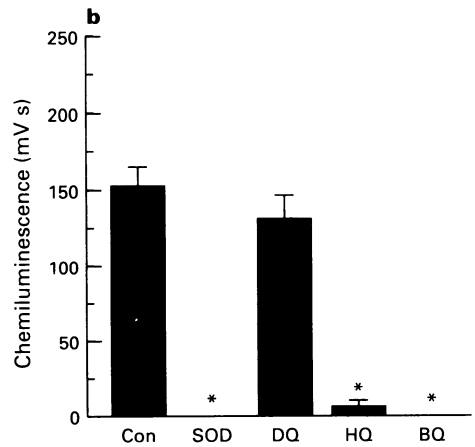


Figure 6 (**a**) Trace showing the concentration-related chemiluminescence signal produced by superoxide anions generated by addition of increasing amounts of xanthine to a solution containing xanthine oxidase (70 mu ml^{-1}) and lucigenin (250 μM). (**b**) Histogram showing the chemiluminescence signal (measured as the area under the curve) produced by addition of 10 μM xanthine to a solution containing xanthine oxidase (70 mu ml^{-1}) and lucigenin (250 μM) and the effect on this of Cu/Zn superoxide dismutase (SOD; 250 u ml^{-1}), duroquinone (DQ, 100 μM), hydroquinone (HQ; 100 μM), or 1,4-benzoquinone (100 μM). Each column represents the mean±s.e. mean from at least 5 individual assays. *$P<0.05$, significantly different from control (Con).

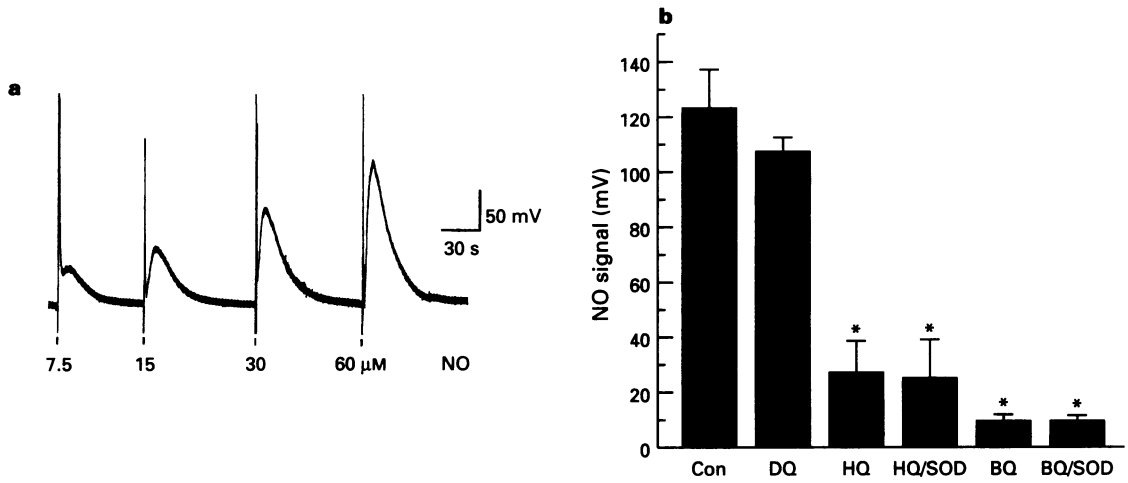


Figure 7 (**a**) Trace showing the concentration-related signal produced by the nitric oxide (NO)-electrode following addition of increasing amounts of authentic NO to Krebs solution in an organ bath. Note the large injection artefact which precedes the signal on each occasion. (**b**) Histogram showing the signal generated by addition of 15 μM NO (concentration calculated as in functional experiments) to the organ bath (measured as the peak increase) and the effect on this of duroquinone (DQ; 100 μM), hydroquinone (HQ; 100 μM), 1,4-benzoquinone (BQ, 100 μM), and HQ or BQ in the presence of Cu/Zn superoxide dismutase (SOD; 250 u ml^{-1}). *$P < 0.05$, significantly different from control (Con).

anion generation by xanthine:xanthine oxidase was investigated. Addition of increasing concentrations of xanthine (50–200 μM) to 70 mu ml^{-1} xanthine oxidase resulted in increasing generation of superoxide anions, as represented by the chemiluminescence signal (Figure 6a). The superoxide anion signal was greatly reduced by Cu/Zn SOD (250 u ml^{-1}), HQ (100 μM) and BQ (100 μM), but was unaffected by DQ (100 μM; Figure 6b).

In a second series of experiments, the effects of the drugs on NO were studied. Addition of authentic NO to the organ bath containing the microsensor resulted in a signal that was proportional to the amount of NO added (Figure 7a). The signal was greatly attenuated by HQ and BQ, but was unaffected by DQ (all at 100 μM; Figure 7b). The inhibition observed with HQ and BQ was not affected by Cu/Zn SOD (Figure 7b).

Discussion

The results obtained in this study support the proposal made by Martin *et al.* (1994), based on experiments with the bovine retractor penis, that nitrergic transmission is protected from the inhibitory effects of superoxide anion generating drugs by the actions of Cu/Zn SOD. Thus, in the mouse anococcygeus, the superoxide anion generating drug DQ (Powis & Appel, 1980; Boersma *et al.*, 1994) had little effect on field stiumlation-induced relaxations in concentrations that greatly reduced responses to exogenous NO; this differentiation parallels that observed with superoxide anion generating drugs in other tissues (Gillespie & Sheng, 1990; Barbier & Lefebvre, 1992; Knudsen *et al.*, 1992; Liu *et al.*, 1994). However, following treatment with the Cu/Zn SOD inhibitor, DETCA (Misra, 1979; Cocco *et al.*, 1981; Kelmer *et al.*, 1989), the nitrergic relaxations became much more sensitive to block by DQ. That this increase in the effect of DQ was related to inhibition of Cu/Zn SOD was supported by the partial reversal of DQ-induced block observed on addition of exogenous Cu/Zn SOD; presumably the reversal was only partial because DQ, like LY83583 (Martin *et al.*, 1994), can increase superoxide anion concentrations both intra- and extracellularly and only the latter would be metabolized by exogenous Cu/Zn SOD. One difference between the bovine retractor penis and the mouse anococcygeus was that DETCA treatment *per se* reduced the magnitude of nitrergic relaxations in the former (Martin *et al.*, 1994) while, in the latter, relaxations to NO and field stimulation were unaffected. This suggests that endogenous superoxide anion production in the mouse anococcygeus, in the absence of stimulating drugs, is less than that in the bovine retractor penis.

While the present results suggest that high levels of Cu/Zn SOD activity in the tissue might explain the lack of effect of some drugs on nitrergic relaxations, they do not conclude the debate on the nature of the substance actually released from the nerves (see Introduction). In DETCA-treated tissues, DQ inhibited relaxations not only to NO and field stimulation but also to the NO-donors SNP and GSNO. This would be expected since the superoxide anions generated by DQ within the smooth muscle cell would be able to attack the NO released from these donor drugs, or from any NO-containing transmitter substance, before it reached its target guanylyl cyclase. DQ did not inhibit relaxations to 8-Br cyclic GMP which activates the relaxant mechanism without the need for NO; why the responses to 8-Br cyclic GMP were, in fact, potentiated by DETCA pretreatment is as yet unclear. Further, although DETCA treatment potentiated the inhibitory effect of DQ on nitrergic stimulation, it also potentiated the inhibitory effect of DQ on exogenous NO; indeed, the potency of DQ against NO ($-\log IC_{40} = 5.22$) remained five times greater than against nitrergic stimulation ($-\log IC_{40} = 4.54$) following incubation with DETCA. This may be due to differences in the levels of Cu/Zn SOD in the region of the neuroeffector junction compared with the rest of the tissue. However, equally, the above results could reasonably be explained by the possibility that NO is released from the nerves in some modified form, and not as a free radical (Gibson *et al.*, 1995; Rand & Li, 1995).

One of the objects of the study was to compare the effects of DQ with those of HQ, which has been shown previously to differentiate between NO- and field stimulation-induced relaxations of the mouse anococcygeus (Hobbs *et al.*, 1991; Gibson *et al.*, 1992). Unlike DQ, HQ still failed to inhibit nitrergic relaxations after DETCA treatment. Indeed, when HQ, or its two electron oxidised form BQ, was added to the organ bath during DQ-induced inhibition of nitrergic relaxations then a partial reversal of the block was observed. If HQ, or BQ, was acting to generate superoxide anions then a potentiation of the block would have been expected; the observed reversal supports the previous proposal that, under the experimental conditions used, HQ acts by a direct free radical scavenging action, rather than via production of superoxide anions (Hobbs *et al.*, 1991). Confirmation of this was provided by the experiments with xanthine:xanthine oxidase and with the NO microelectrode, since both HQ and BQ, but not DQ,

attenuated the signals generated by superoxide anions and NO. DQ produces superoxide anions when it is reduced by flavoprotein enzymes such as NADPH cytochrome reductase (Boersma *et al.*, 1994). The first step in the reduction reaction is the formation of a semiquinone radical which donates an electron to molecular oxygen and therefore produces superoxide anions. However, the semiquinone radicals of simple, unsubstituted quinones (e.g., HQ/BQ) are much less liable to donate electrons to molecular oxygen and therefore have a much reduced potential for producing superoxide anions (Powis & Appel, 1980; Rao *et al.*, 1988; Boersma *et al.*, 1994), although this potential may vary under different experimental conditions (Boersma *et al.*, 1994). HQ can auto-oxidize in solution, and it is possible that the semiquinone radical soformed could interact directly with NO. However, both HQ and BQ in Krebs solution, in the absence of tissue, could scavenge superoxide anions and NO; HQ can auto-oxidize in solution but is less likely that BQ would be reduced. Alternatively, it may be that HQ and BQ interact directly with free radicals, possibly on the 2,3,5,6 positions on the quinone ring which are known to undergo addition reactions (Lau *et al.*, 1988; Rao *et al.*, 1988). Certainly DQ, which has methyl groups in these positions, did not interact directly with NO or superoxide anions. Thus, the differential effect of HQ on NO- and field stimulation-induced relaxations of the mouse anococcygeus, at least under the experimental conditions used here, does not appear to be related to Cu/Zn SOD activity within the tissue. In preliminary experiments, we have found that the inhibitory effects of HQ on relaxations of the mouse anococcygeus to exogenous NO can be prevented by glutathione (Brave *et al.*, 1993) raising the possibility that anti-oxidant mechanisms in addition to Cu/Zn SOD might be involved in protection of the nitrergic neurotransmission process from attack by drugs.

In conclusion, the results of this study have shown that nitrergic relaxations of the mouse anococcygeus muscle, like those in the bovine retractor penis (Martin *et al.*, 1994), are protected by Cu/Zn SOD from inhibition by superoxide anion generating drugs like DQ. Thus, anti-oxidant status may be very important in determining the function of the nitrergic neurotransmission process; reduced anti-oxidant activity, when associated with increased superoxide anion production, would lead to an inhibition of the physiological function of the nerves, and to toxicity as a result of increased peroxynitrite formation.

E.L. has an MRC studentship

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