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Inhibition of nitrergic neurotransmission in the bovine retractor penis muscle by an oxidant stress: effects of superoxide dismutase mimetics

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1 A number of superoxide dismutase (SOD) mimetics were examined both biochemically for their ability to inhibit the superoxide-catalyzed reduction of cytochrome c and nitro blue tetrazolium, and functionally for their ability to mimic authentic Cu/Zn SOD in restoring nitrergic neurotransmission in bovine retractor penis (BRP) muscle following its inhibition by oxidant stress.

2 The SOD mimetics investigated were $CuSO_4$, $MnCl_2$, CuDIPS (copper [II] [diisopropylsalicylate]$_2$), MnTBAP (manganese [III] tetrakis 4-benzoic acid porphyrin), MnTMPyP (manganese [III] tetrakis 1-methyl-4-pyridyl porphyrin pentachloride), tiron (4,5-dihydroxy-1,3-benzene disulphonic acid), PTIYO (4-phenyl,2,2,5,5,-tetramethyl-3-imidazolin-1-yloxy-3-oxide) and tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-N-oxyl).

3 The rank order of potency in inhibiting the reduction of cytochrome c was: $CuSO_4 \geqslant MnCl_2 \geqslant$ CuDIPS $\geqslant$ MnTMPyP > MnTBAP > tempol $\geqslant$ tiron > PTIYO.

4 The requirement for EDTA (0.1 mM) prevented assessment of the activity of $CuSO_4$, $MnCl_2$ and CuDIPS in the assay involving inhibition of reduction of nitro blue tetrazolium. However, the rank order of potency for those agents which could be examined (MnTMPyP > MnTBAP > tiron $\geqslant$ tempol > PTIYO) was essentially similar to that seen in the cytochrome c assay.

5 Inhibition of endogenous Cu/Zn SOD with diethyldithiocarbamate (DETCA, 3 mM, 120 min) in BRP muscle strips, followed by addition of the superoxide anion generator, LY 83583 (1 μM), resulted in almost complete abolition of nitrergic relaxation (4 Hz, 10 s).

6 Authentic Cu/Zn SOD (1–300 u ml^{-1}), $CuSO_4$ (0.1–300 μM), $MnCl_2$ (0.1–100 μM) and MnTMPyP (10–300 μM) each restored nitrergic transmission by around 50%. However, CuDIPS (0.1–30 μM), MnTBAP (0.1–100 μM), tempol (10 μM–3 mM), PTIYO (1–300 μM) and tiron (10 μM–10 mM) all failed to restore nitrergic transmission.

7 The ability of MnTMPyP to restore nitrergic neurotransmission may therefore provide a lead in the development of SOD mimetics as therapeutic agents in the treatment of neuropathies associated with oxidant stress.

Keywords: Nitric oxide; nitrergic neurotransmission; oxidant stress; superoxide anion; superoxide dismutase; superoxide dismutase mimetic; bovine retractor penis

Introduction

A number of superoxide anion generating agents, including pyrogallol, hypoxanthine/xanthine oxidase, hydroquinone, and LY 83583, produce powerful inhibition of the relaxant actions of authentic nitric oxide (NO), but have little or no effect on nitrergic relaxation in a number of tissues, including the bovine retractor penis (BRP; Martin *et al*., 1994), the mouse anococcygeus (Gibson *et al*., 1994), the rat gastric fundus (Hobbs *et al*., 1991; Barbier & Lefebvre, 1992), and the guinea-pig trachea (Hobbs *et al*., 1991). Such findings have helped fuel speculation that the nitrergic neurotransmitter is not NO *per se*, but a stable NO-releasing molecule (see Gibson *et al*., 1995; Rand & Li, 1995, for reviews). However, more recent findings, obtained with the copper chelator diethyldithiocarbamate (DETCA), which produces selective inhibition of the endogenous isoform of Cu/Zn superoxide dismutase (SOD; Cocco *et al*., 1981; Kelner *et al*., 1989), have provided an explanation for the differential blockade by superoxide anion generators of relaxations to NO and nitrergic nerve stimulation, which precludes the need to propose an alternative transmitter candidate. Specifically, these studies demonstrate that following inhibition of endogenous Cu/Zn SOD in the BRP (Martin *et al*., 1994; Paisley & Martin, 1996), mouse (Lilley & Gibson, 1995) and rat (Liu *et al*., 1997) anococcygeus, rat gastric fundus (De Man *et al*., 1996; Lefebvre, 1996), and opossum oesophagus (Thomas *et al*., 1996), the normally resistant nitrergic transmission process becomes susceptible to inhibition by the superoxide anion generators. Quite apart from removing a major obstacle to acceptance of NO as the nitrergic transmitter, these studies demonstrate a vital role for Cu/Zn SOD in protecting nitrergic neurotransmission from inactivation by superoxide anion in a wide variety of tissues. Indeed, co-localization of Cu/Zn SOD with NO synthase has been demonstrated in nitrergic nerves of the rat anococcygeus (Liu *et al*., 1997), and Cu/Zn SOD and the other antioxidant enzymes, catalase and Mn SOD, are present in enteric nerves of the opossum oesophagus (Thomas *et al*., 1996). An additional antioxidant mechanism involving ascorbic acid may provide yet further protection of the nitrergic transmitter from interference by oxidant stress in the mouse and rat anococcygeus muscles (Lilley & Gibson, 1996; 1997).

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In view of the importance of these antioxidant mechanisms, it is possible to conceive of pathological situations in which this defence is overwhelmed by an oxidant stress leading to impairment of nitrergic transmission. For example, diabetes is associated with oxidant stress and loss of plasma and vascular Cu/Zn SOD protein and mRNA (Kamata & Kobayashi, 1996; Mohan & Das, 1997). Furthermore, blood vessels from diabetic animals exhibit impaired NO-dependent vasodilatation resulting from excess activity of superoxide anion and this can be reversed in part by exogenous application of SOD (Pieper & Gross, 1988; Hattori *et al*., 1991; Kamata & Kobayashi, 1996). Diabetic neuropathies, including the impaired nitrergic neurotransmission of diabetes (de Tejada *et al*., 1989; Jenkinson & Reid, 1995; Way & Reid, 1996) may also involve an oxidant stress (Van Dam & Bravenboer, 1997). A case therefore exists for augmenting existing antioxidant defence mechanisms in the hope of restoring nitrergic neurotransmission in situations of diabetes and other neuropathies associated with oxidant stress. Superoxide dismutase itself is unlikely to be of therapeutic benefit under such circumstances because its large size prevents it from entering cells to protect NO from an intracellular oxidant stress. However, interest is growing in the development of low molecular weight, membrane permeant SOD mimetics as therapeutic agents in the treatment of a vast array of pathologies associated with oxidant stress (Sorenson, 1995). A number of different classes of SOD mimetic have been described, including the superoxide scavenger tiron (Ledenev *et al*., 1986), spin trap nitroxides such as 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (tempol) and 4-phenyl-2,2,5,5-tetramethyl-3-imidazolin-1-yloxy-3-oxide (PTIYO) (Mitchell *et al*., 1990; Krishna *et al*., 1996), and metal based agents such as copper [II][diispropylsalicylate]$_2$ (CuDIPS) (Sorenson, 1995) and manganese[III]tetrakis 1-methyl-4-pyridyl porphyrin pentachloride (MnTMPyP) and manganese[III]tetrakis 4-benzoic acid porphyrin (MnTBAP) (Faulkner *et al*., 1994; Day *et al*., 1995; Gardner *et al*., 1996) which mimic the catalytic site of Cu/Zn SOD and Mn SOD, respectively.

The aims of this study were two fold: firstly, to rank the activity of each of the above putative SOD mimetics by use of established biochemical assays of SOD-like activity; and secondly, to determine if these agents share the ability of authentic exogenous Cu/Zn SOD to restore nitrergic neurotransmission in the BRP muscle following its inhibition by an oxidant stress. Preliminary accounts of these findings have already been published (Martin & Mok, 1997; Martin *et al*., 1998).

Methods

Biochemical assessment of SOD-like activity

SOD-like activity was assessed by the use of two separate assays which measured the ability of authentic Cu/Zn SOD and the putative SOD mimetics, $CuSO_4$, CuDIPS, $MnCl_2$, MnTMPyP, MnTBAP, tempol, PTIYO and tiron, to inhibit superoxide anion-catalyzed reactions. Both assays were conducted in final volumes of 0.3 ml in 96-well plates incubated at 20°C on an orbital shaker. Following incubation, changes in absorbance were measured with a microplate reader (Dynex Ltd.). The first of these assays was based on the ability of the superoxide anion generating system hypoxanthine (0.1 mM)/xanthine oxidase (3 mu ml^{-1}), to reduce cytochrome c (30 μM; McCord & Fridovich, 1968; Laight *et al*., 1997). The reaction was conducted in Tris buffer (tris[hydroxymethyl]-aminomethane hydrochloride, 50 mM pH 7.6), containing catalase (100 u ml^{-1}) to prevent the re-oxidation of cytochrome c by the build up of hydrogen peroxide. Following incubation for 30 min the reduction of cytochrome c was measured at 550 nm. The second assay was based on the ability of the superoxide anion generating system, NADH (78 μM) and phenazine methosulphate (3.3 μM), to reduce nitro blue tetrazolium (50 μM) to blue formazan dye (Ewing & Janero, 1995). The reaction was conducted in phosphate buffer (50 mM, pH 7.4), containing ethylenediaminetetraacetic acid (EDTA, 0.1 μM) in order to chelate contaminating heavy metals which interfere with the reduction of nitro blue tetrazolium. Following incubation for 5 min the formation of formazan dye was measured at 550 nm.

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 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 and 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 on BRP muscle strips by use of guanethidine (30 μM). In some experiments tone was augmented by the further addition of phenylephrine (0.1–1 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

Endogenous Cu/Zn SOD was inhibited in BRP strips as previously described (Martin *et al*., 1994; Paisley & Martin, 1996). Briefly, DETCA, which inhibits Cu/Zn SOD by chelating copper at its active site (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. Tone was then induced with guanethidine (30 μM) and nitrergic relaxation elicited by electrical field stimulation (4 Hz, 10 s). When at least three reproducible responses had been obtained, the superoxide generating agent, LY 83583 (1 μM), was added and its ability to block nitrergic relaxation assessed. The ability of authentic Cu/Zn SOD and the putative SOD mimetics, $CuSO_4$, CuDIPS, $MnCl_2$, MnTMPyP, MnTBAP, tempol, PTIYO and tiron, to reverse the blockade of nitrergic relaxation induced by LY 83583 in DETCA-treated strips was then assessed. In each case, SOD and the SOD mimetics were added to the tissue baths in a cumulative manner. The ability of each of the SOD mimetics to influence nitrergic relaxation (4 Hz, 10 s) in control BRP muscle strips was also assessed.

Drugs

CuDIPS (copper [II] [diisopropylsalicylate]$_2$) and PTIYO (4-phenyl-2,2,5,5-tetramethyl-3-imidazolin-1-yloxy-3-oxide) were obtained from Aldrich (Poole, U.K.), LY 83583 (6-anilino-5,8-quinolinedione) was obtained from Calbiochem (Nottingham,

U.K.), MnTBAP (manganese [III] tetrakis 4-benzoic acid porphyrin) and MnTMPyP (manganese [III] tetrakis 1-methyl-4-pyridyl porphyrin pentachloride) were obtained from Alexis (Nottingham U.K.), pyrogallol was obtained from BDH Ltd. (Poole, U.K.), and catalase (bovine liver), cytochrome c, diethyldithiocarbamate, guanethidine sulphate, hypoxanthine, NADH (β-nicotinamide adenine dinucleotide, reduced form), nitro blue tetrazolium, phenazine methosulphate, phenylephrine hydrochloride, superoxide dismutase (Cu/Zn-containing enzyme from bovine erythrocytes), tiron (4,5-dihydroxy-1,3-benzene disulphonic acid), tempol (4-hydroxy-tempo; 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, U.K.). All drugs were dissolved in saline (0.9%), except for: hypoxanthine which was prepared as a 10 mM stock in 0.2 M NaOH; LY 83583 which was prepared as a 20 mM stock in ethanol, CuDIPS which was prepared as a 3 mM stock in 50% ethanol/50% Tris buffer (50 mM, pH 7.6); and MnTBAP which was prepared as a 25 mM stock in ethanol, with all subsequent dilutions of these being made in saline. Control experiments were performed to ensure that the solvents had no effects by themselves on the tissues.

Data analysis

The magnitude of nitrergic relaxation is expressed as % relaxation (mean ± s.e.mean, for *n* separate experiments) of the guanethidine-induced tone which was present immediately before each stimulation. Statistical analysis was carried out by ANOVA followed by the Bonferroni *post hoc* test. A value of $P<0.05$ was considered significant. pEC_{50} values were calculated by use of a computer-based program (GraphPad, Prism).

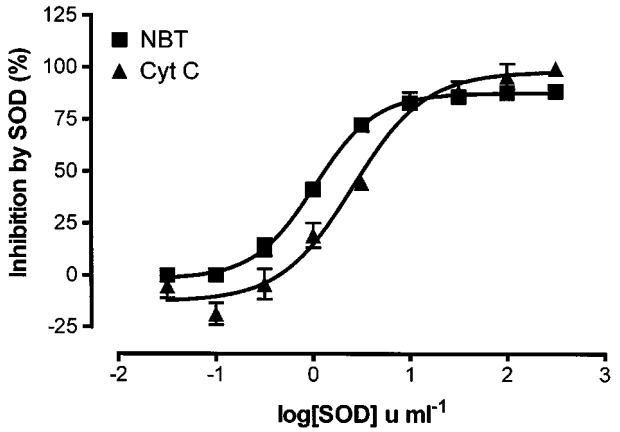

Figure 1 The ability of authentic Cu/Zn SOD to inhibit in a concentration-dependent manner the reduction both of cytochrome c (Cyt C) by hypoxanthine/xanthine oxidase and of nitro blue tetrazolium (NBT) by NADH and phenazine methosulphate. Each point is the mean, and vertical lines show s.e.mean, of 8 observations.

Results

Biochemical assays of SOD-like activity

Authentic Cu/Zn SOD (0.03 – 300 u ml^{-1}) produced concentration-dependent inhibition of reduction both of cytochrome c by hypoxanthine/xanthine oxidase and of nitro blue tetrazolium by NADH and phenazine methosulphate (Figure 1). Maximum inhibitions and pEC_{50} values obtained are given in Table 1.

All the putative SOD mimetics tested produced concentration-dependent inhibition of reduction of cytochrome c, but the range of effective concentrations and the maximum inhibitions obtained varied widely (Figure 2; Table 1). The simple metal salts, $CuSO_4$ and $MnCl_2$, and the metal-centred SOD mimetics, CuDIPS, MnTMPyP and MnTBAP, were the most potent, but of these, only $MnCl_2$ produced near complete inhibition of reduction. The metal-free SOD mimetics, tempol, PTIYO and tiron, were less potent. However, of these, only tiron failed to reach near complete inhibition of reduction, since at high concentrations it promoted reduction of cytochrome c by itself (data not shown), thus compromising the assay. The metal-centred SOD mimetics, MnTMPyP and MnTBAP, were strikingly more potent as inhibitors of the reduction of nitro blue tetrazolium than of cytochrome c (Figure 3; Table 1). In contrast, in view of the necessary presence of the chelating agent, EDTA (0.1 mM), in the incubation mixture, $CuSO_4$ and $MnCl_2$ were much less potent, and CuDIPS had no inhibitory activity at the highest concentration tested (10 μM). Tempol, PTIYO and tiron were also more potent in inhibiting the reduction of nitro blue tetrazolium and, as with the cytochrome c assay, only tiron failed to achieve near maximal inhibition.

Inhibition of nitrergic neurotransmission in the BRP in oxidant stress

Following treatment with guanethidine (30 μM) to block adrenergic responses and raise the tone, electrical field stimulation (4 Hz, 10 s) of strips of BRP muscle produced powerful nitrergic relaxation (96.8 ± 1.5%, $n=10$). As pre-

Table 1 The ability of authentic Cu/Zn SOD and SOD mimetics to inhibit the reduction of cytochrome c and nitro blue tetrazolium (NBT)

	Cytochrome c reduction		*NBT reduction*		
Compound	*pEC_{50}*	*Maximum inhibition* (%)	*pEC_{50}*	*Maximum inhibition* (%)	*Potency ratio* (cyt c/NBT)[#]
Cu/Zn SOD	–0.410 ± 0.086	99.1 ± 3.5	–0.016 ± 0.018	88.2 ± 1.3	2.5
Tempol	3.36 ± 0.13	103.9 ± 4.7	4.27 ± 0.03	100.5 ± 1.4	8.2
PTIYO	2.59 ± 0.23	88.2 ± 5.2	3.88 ± 0.09	97.3 ± 1.2	19.2
Tiron	3.11 ± 0.10	51.3 ± 5.1***	4.57 ± 0.16	62.5 ± 0.7**	28.8
$CuSO_4$	6.70 ± 0.04	75.7 ± 4.8	4.04 ± 0.01	81.4 ± 10.7	0.0022
CuDIPS	6.27 ± 0.11	69.7 ± 1.6*	ND	ND	ND
$MnCl_2$	6.46 ± 0.21	93.3 ± 4.0	4.28 ± 0.04	74.7 ± 2.4	0.0067
MnTMPyP	6.05 ± 0.17	40.0 ± 13.9***	7.87 ± 0.01	105.0 ± 2.6	65.1
MnTBAP	4.36 ± 0.01	60.0 ± 7.9***	6.03 ± 0.01	83.2 ± 1.3	47.3

Data are mean ± s.e.mean of 8 observations. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate a significant difference from maximal inhibition by Cu/Zn SOD. pEC_{50} values are expressed in molar units except for Cu/Zn SOD which are in u ml^{-1}. ND indicates not determined. [#]Potency ratios calculated from ED_{50} values.

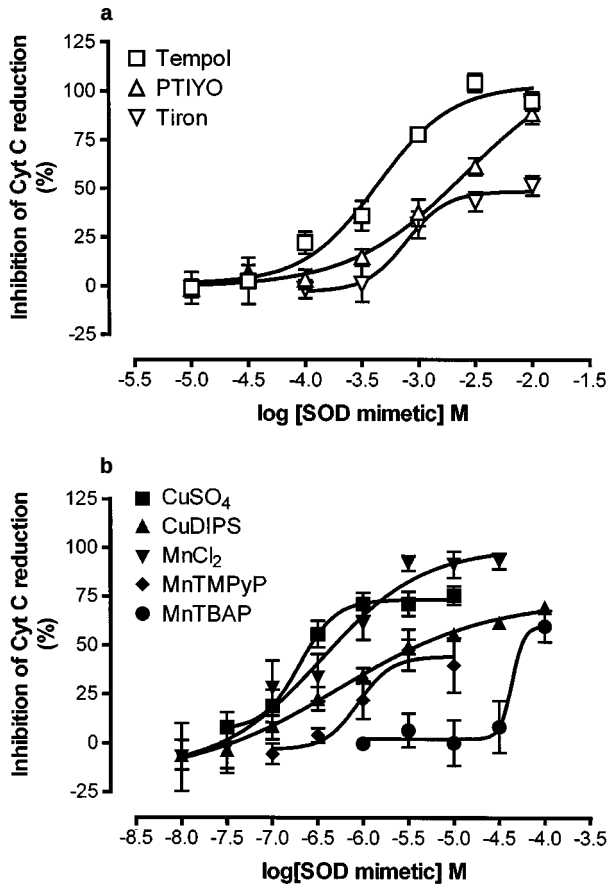

Figure 2 The ability of (a) tempol, PTIYO and tiron and (b) $CuSO_4$, CuDIPS, $MnCl_2$, MnTMPyP and MnTBAP to inhibit in a concentration-dependent manner the reduction of cytochrome c (Cyt C) by the hypoxanthine/xanthine oxidase superoxide anion generating system. Each point is the mean and vertical lines s.e.mean of 8 observations.

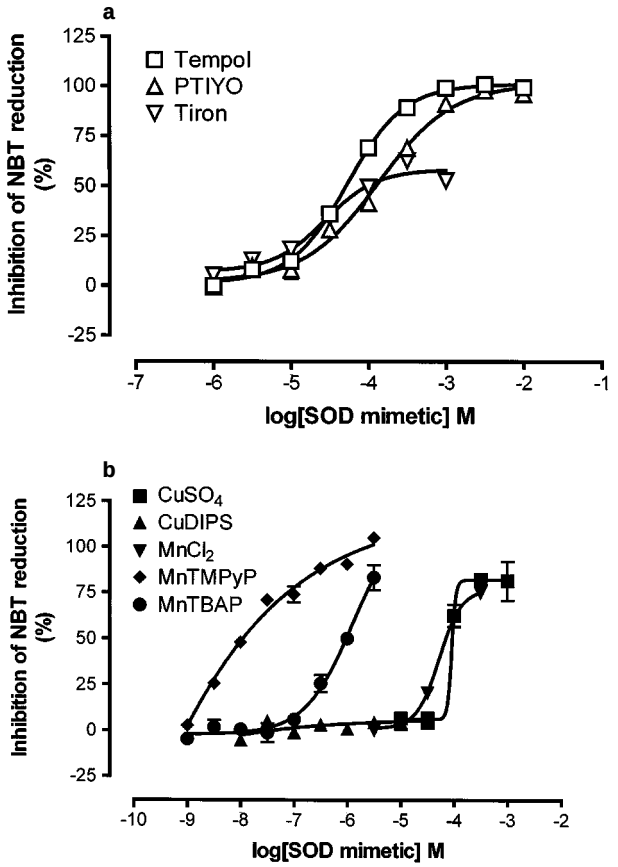

Figure 3 The ability of (a) tempol, PTIYO and tiron and (b) $CuSO_4$, CuDIPS, $MnCl_2$, MnTMPyP and MnTBAP to inhibit in a concentration-dependent manner the reduction of nitro blue tetrazolium (NBT) by the NADH/phenazine methosulphate superoxide anion generating system. Each point is the mean and vertical lines s.e.mean of 8 observations.

viously observed (Martin *et al.*, 1994), application of an oxidant stress consisting of blockade of endogenous Cu/Zn SOD by treatment with DETCA (3 mM, 120 min, followed by 10 min washout), followed by addition the superoxide generating agent, LY 83583 (1 μM), resulted in profound inhibition of nitrergic relaxation (Table 2).

Effects of SOD and SOD mimetics on the inhibition of nitrergic transmission produced by oxidant stress

Exogenous application of Cu/Zn SOD (0.1 – 300 u ml^{-1}) produced a concentration-dependent restoration of nitrergic relaxation (4 Hz, 10 s) following its inhibition by the combined treatment with DETCA and LY 83583; the maximum relaxation obtained was $46.9 \pm 6.4\%$, $n = 14$, $P < 0.001$ (Figure 4; Table 2).

$CuSO_4$ (0.1 – 300 μM), $MnCl_2$ (0.1 – 100 μM), and MnTMPyP (10 – 300 μM), each produced concentration-dependent restoration of nitrergic transmission following its inhibition by the combined treatment with DETCA and LY 83583; in each case the maximal restoration achieved was similar to that obtained with authentic Cu/Zn SOD (Figure 4; Table 2). However, CuDIPS (0.1 – 30 μM) and MnTBAP (0.1 – 100 μM) failed to restore nitrergic transmission. The metal-free SOD mimetics, tempol (10 μM – 3 mM), PTIYO (1 – 300 μM) and tiron (10 μM – 10 mM) also all failed to restore nitrergic transmission following its inhibition by the combined treatment with DETCA and LY 83583.

Table 2 The ability of Cu/Zn SOD and SOD mimetics to restore nitrergic neurotransmission following its inhibition by LY 83583 in DETCA-treated strips of BRP muscle

Compound	*Maximal nitrergic relaxation* (%)	n
None	3.9 ± 3.0	10
Cu/Zn SOD (300 u ml^{-1})	46.9 ± 6.4***	10
Tempol (3 mM)	3.2 ± 2.0	7
PTIYO (0.3 nM)	3.1 ± 1.7	12
Tiron (10 mM)	10.8 ± 3.5	7
$CuSO_4$ (0.3 mM)	47.7 ± 7.1*	5
CuDIPS (0.03 mM)	0.7 ± 0.7	8
$MnCl_2$ (0.1 mM)	49.8 ± 7.4***	6
MnTMPyP (0.3 mM)	57.1 ± 10.7***	8
MnTBAP (0.1 mM)	0.0 ± 0.0	6

Data represent the maximum relaxation (mean $\pm$ s.e.mean) obtained to electrical field stimulation (4 Hz, 10 s) when the highest concentrations of Cu/Zu SOD and each of the SOD mimetics were added following inhibition of nitrergic transmission by LY 83583 in DETCA-treated strips of BRP muscle. *$P < 0.05$ and ***$P < 0.001$, indicate a significant restoration of nitrergic transmission when compared to relaxation obtained in the absence of SOD or a SOD mimetic (None).

On control strips of BRP, $MnCl_2$ (0.1 – 100 μM), MnTMPyP (10 – 300 μM), MnTBAP (0.1 – 100 μM), tempol (10 μM – 3 mM) and tiron (10 μM – 3 mM), each had no significant effect on nitrergic relaxation (4 Hz, 10 s). $CuSO_4$

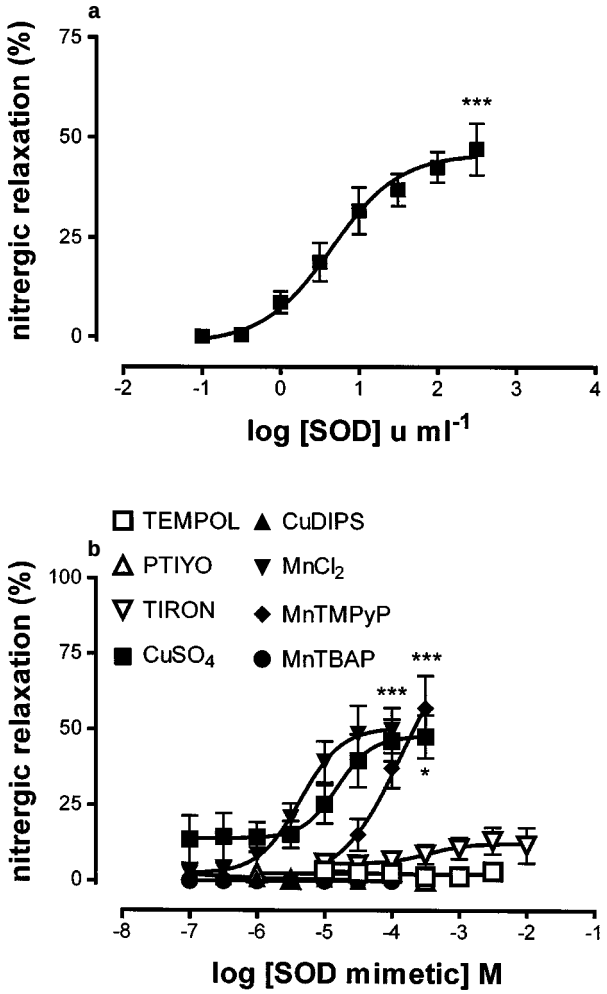

Figure 4 The blockade of nitrergic relxation (4 Hz, 10 s) in strips of BRP muscle resulting from inhibition of endogenous Cu/Zn SOD by DETCA (3 mM, 120 min) and generation of superoxide anion by LY 83583 (1 μM) is restored in part by (a) exogenous SOD and (b) $CuSO_4$, $MnCl_2$, and MnTMPyP but not by CuDIPS, MnTBAP, tempol, PTIYO or tiron. Each point is the mean and vertical lines s.e.mean of 6–10 observations. $*P<0.05$ and $***P<0.001$ indicate a significant restoration of nitrergic neurotransmission.

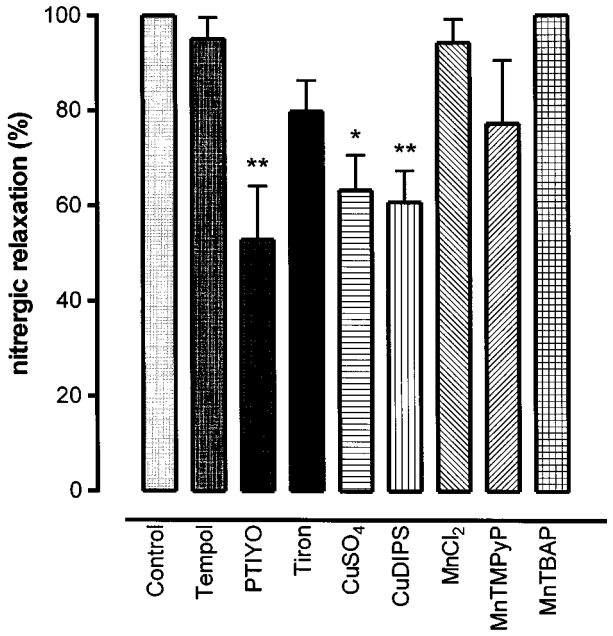

Figure 5 In control strips of BPR muscle nitrergic relaxation (4 Hz, 10 s) was unaffected by tempol (3 mM), tiron (3 mM), $MnCl_2$ (100 μM), MnTMPyP (300 μM), MnTBAP (100 μM) but blocked by PTIYO (300 μM), $CuSO_4$ (300 μM) and CuDIPS (30 μM). Each value is the mean and vertical lines s.e.mean of 6–12 observations. $*P<0.05$ and $**P<0.005$ indicate a significant blockade of nitrergic neurotransmission.

had no effect at concentrations up to 100 μM, but inhibited relaxation at 300 μM. In contrast, CuDIPS, (0.1–30 μM) and PTIYO (1–300 μM) each produced concentration-dependent inhibition of nitrergic relaxation. The effects of the highest concentration of each SOD mimetic on nitrergic relaxation are shown on Figure 5).

Discussion

A number of assays are available with which to measure SOD-like activity, and the majority are based on the ability of authentic SOD to inhibit superoxide anion-catalyzed colorimetric reactions. The two employed in this study, involving reduction of cytochrome c by the hypoxanthine/xanthine oxidase system (McCord & Fridovich 1968; Laight *et al*., 1997) and reduction of nitro blue tetrazolium by NADH and phenazine methosulphate (Ewing & Janero, 1995), were fully validated for this purpose by our finding that authentic Cu/Zn SOD produced virtually complete inhibition in each case.

The assay involving inhibition of reduction of cytochrome c proved to be the more versatile, since it permitted measurement of the activity of all of the putative SOD mimetics tested. The rank order of potency was: $CuSO_4 \geqslant MnCl_2 \geqslant$ CuDIPS $\geqslant$ MnTMPyP > MnTBAP > tempol $\geqslant$ tiron > PTIYO. Of the simple metal salts and the metal-based SOD mimetics, only $Mn(II)Cl_2$ produced near maximal inhibition of reduction of cytochrome c. The others, in which the transition metal is in its higher valency state, i.e. $Cu(II)SO_4$, Cu(II)DIPS, Mn(III)TMPyP and Mn(III)TBAP, produced smaller maximal inhibitions of around 50–75%. These lower maxima probably result from an additional superoxide-independent component of reduction of cytochrome c. Specifically, during the catalytic cycle of dismutation of superoxide, these metal centres are reduced to Cu(I) and Mn(II), respectively (de Alvare *et al*., 1976; Huber *et al*., 1987; Faulkner *et al*., 1994; Day *et al*., 1995). The electrochemical series dictates that these will then participate in redox reactions in which they are re-oxidized at the expense of reduction of the iron of cytochrome c, thus impairing the maximum inhibition of reduction of cytochrome c that can be achieved with the SOD mimetics. In contrast, the spin trap agents, tempol and PTIYO, which contain no metal produced virtually complete inhibition of reduction of cytochrome c. On the other hand, tiron produced only a maximal inhibition of around 50%, because at the higher concentrations used (3 and 10 mM), it too produced reduction of cytochrome c by a direct chemical reaction.

It is essential to conduct the assay involving the superoxide-catalyzed reduction of nitro blue tetrazolium in the presence of 0.1 mM EDTA (Ewing & Janero, 1995) in order to chelate contaminating heavy metal ions such as Cu^{2+} and Fe^{3+}, which would dismutase superoxide and so interfere with the measurement of activity of SOD or SOD mimetics. Consequently, assessment of the SOD-like activity of the simple metal salts, $CuSO_4$ and $MnCl_2$, and of CuDIPS, which exists as a weak co-ordination complex (Huber *et al*., 1987), was compromised in this assay: the two metal salts did exhibit

powerful SOD-like activity, but only at concentrations exceeding the binding capacity of the chelating agent, and CuDIPS displayed no activity because its active centre was removed. In fact, the insolubility of CuDIPS reduced the maximum usable concentration to 10 μM, and this clearly could not exceed the chelating capacity of 0.1 mM EDTA. In contrast, MnTMPyP and MnTBAP, in which the metal centre is firmly bound to the porphyrin ring and not removed by the chelator (Faulkner *et al*., 1994; Day *et al*., 1995), both displayed SOD-like activity in this assay.

Encouragingly, for those agents which could be employed in the nitro blue tetrazolium assay, the rank order of potency (MnTMPyP > MnTBAP > tiron $\geqslant$ tempol > PTIYO) was essentially similar to that found with the cytochrome c assay, thus providing further validation of these techniques for screening potential SOD mimetics. Our data also showed that each of the compounds, including authentic SOD itself, was more potent in the nitro blue tetrazolium assay than in the cytochrome c assay, suggesting that in the former, less superoxide is generated. However, for MnTMPyP and MnTBAP the difference in activity was strikingly greater (65 fold and 47 fold, respectively) than for the other agents, and can most likely be explained by the vastly different abilities of the Mn(II) and Mn(III) forms of these agents to remove superoxide anion. Specifically, the rate constants for removal of superoxide by Mn(II)TMPyP and Mn(III)TMPyP have been found to be 4×10^9 M^{-1} s^{-1} and 3.9×10^7 M^{-1} s^{-1} (Faulkner *et al*., 1994). The rate constant for Mn(III)TBAP is yet lower at 6.3×10^6 M^{-1} s^{-1} (Day *et al*., 1995) and that for Mn(II)TBAP has not been obtained but is liable to be substantially higher. Clearly, therefore, the optimum valency state for removal of superoxide by these compounds is the Mn(II) form. Although this will certainly be formed during the redox dismutation of superoxide by the Mn(II) form, this is a slow step. However, data have been presented showing that metabolic reduction by enzymes or by NADPH or glutathione results in formation of the Mn(II) form of MnTMPyP, with consequently greater superoxide scavenging activity *in vivo* than expected on the basis of the activity of the Mn(III)TMPyP in a biochemical assay of SOD-like activity with xanthine/xanthine oxidase as a superoxide generating system (Faulkner *et al*., 1994; Gardner *et al*., 1996). However, the nitro blue tetrazolium assay uses the reducing mixture of NADH and phenazine methosulphate to generate superoxide anion (Ewing & Janero, 1995). This mixture is therefore liable to convert at least some of the Mn(III) form of the metalloporphyrins to the more active Mn(II) state, thus accounting for their vastly greater SOD-like activity than in the cytochrome c assay.

However, more important than the activities of the various SOD mimetics in the biochemical assays, is the potential utility of these agents to recover impaired nitrergic transmission in disease states involving oxidant stress, such as diabetic neuropathy (Van Dam & Bravenboer, 1997). We have shown previously in the BRP muscle that generation of superoxide anion by agents such as LY 83583, pyrogallol and hypoxanthine/xanthine oxidase has little inhibitory effect on nitrergic neurotransmission unless the protective role of endogenous Cu/Zn SOD is first neutralized by DETCA (Martin *et al*., 1994; Paisley & Martin, 1996). These findings have essentially been confirmed in other tissues, including the rat and mouse anococcygeus, rat gastric fundus and opossum oesophagus (Lilley & Gibson, 1995; De Man *et al*., 1996; Lefebvre, 1996; Liu *et al*., 1997). The blockade of nitrergic transmission by LY 83583 in DETCA-treated strips of BRP is at least in part due to destruction of the nitrergic transmitter (NO) by superoxide, since authentic Cu/Zn SOD was able to restore transmission by about 50%. An additional action of inhibition of nNOS by LY 83583 (Luo *et al*., 1995) may account for the inability of SOD to restore transmission fully.

Despite possessing SOD-like activity both in the cytochrome c and nitro blue tetrazolium assays and their ability to protect mammalian cells from oxidant stress (Mitchell *et al*., 1990; Krishna *et al*., 1996), the spin traps, tempol and PTIYO, failed to mimic the ability of authentic Cu/Zn SOD to restore nitrergic neurotransmission following its inhibition by LY 83583 in DETCA-treated strips of BRP. Members of this class of stable nitroxide agent are known to scavenge nitric oxide (Akaike *et al*., 1993) with different degrees of potency and, indeed, we found that PTIYO but not tempol inhibited neurotransmission in control strips of BRP. It is therefore unlikely that this class of SOD mimetic could be developed as therapeutic agents with which to restore nitrergic transmission in pathologies associated with oxidant stress. The superoxide scavenger, tiron (Ledenev *et al*., 1986), also failed to restore nitrergic transmission in the BRP muscle, even when used in concentrations up to 10 mM.

However, the metal-based agents did display greater potential to restore nitrergic transmission following its inhibition by LY 83583 in DETCA-treated strips of BRP. We found that the simple metal salts, $CuSO_4$ and $MnCl_2$, restored neurotransmission, and the magnitude of this ($\sim$50%) was similar to that achieved with authentic Cu/Zn SOD. It is likely, therefore, that the restoration by these salts results from their SOD-like activity (de Alvare *et al*., 1976; Huber *et al*., 1987; Beyer & Fridovich, 1990). Surprisingly, CuDIPS, which provides antioxidant protection in a number of systems under oxidant stress (Burdon *et al*., 1995; Sorenson, 1995) failed to restore nitrergic transmission in the BRP, but the insolubility of this compound restricted its use to a maximum concentration of 10 μM. In fact, this agent actually produced concentration-dependent blockade of nitrergic neurotransmission in control strips of BRP, perhaps as a result of its additional ability to inhibit nNOS (Baquial & Sorenson, 1995).

The metalloporphyrins, MnTMPyP and MnTBAP, also provide antioxidant protection in a number of systems (Faulkner *et al*., 1994; Gardner *et al*., 1996), but we found that only the former, which possesses the greater superoxide scavenging activity in our two biochemical assays, produced restoration of nitrergic neurotransmission following its inhibition by LY 83583 in DETCA-treated strips of BRP muscle. The maximum restoration of transmission produced by MnTMPyP was around 50%, which was similar to that obtained with authentic Cu/Zn SOD, $CuSO_4$ and $MnCl_2$. Although effective in mimicking the ability of authentic SOD to restore nitrergic neurotransmission in the BRP following its inhibition by oxidant stress, this was achieved at concentrations of Mn TMPyP which were vastly greater (10–300 μM) than those required to scavenge superoxide (IC_{50} $\sim$14 nM in the nitro blue tetrazolium assay). It is likely, therefore, that metabolic reduction of the Mn(III) forms of the metalloporphyrins to their more active Mn(II) forms occurs to a very limited extent in the BRP muscle. The strong binding of the metal centre to the porphyrin ring in MnTMPyP (Faulkner *et al*., 1994; Day *et al*., 1995), together with the inability of 0.1 mM EDTA to affect the restoration of nitrergic transmission by MnTMPyP (unpublished observations), indicate that free Mn in solution cannot account for the activity of this compound.

In conclusion, we have used two biochemical assays involving the superoxide-catalyzed reduction of cytochrome c or nitro blue tetrazolium to obtain a rank order of potency

for a number of structurally distinct classes of SOD mimetic. Both assays demonstrate that the metal-centred SOD mimetics are substantially more potent that the spin trap agents. The most potent agent tested, MnTMPyP, shared the ability of authentic Cu/Zn SOD to restore nitrergic neurotransmission in the BRP muscle following its inhibition by an oxidant stress. This compound may therefore provide a lead in the development of SOD mimetics as potential therapeutic agents in the treatment of neuropathies associated with oxidant stress.

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