British Journal of Pharmacology (1998) 124, 719–728
http://www.stockton-press.co.uk/bjp

Loss of endothelium-derived nitric oxide in rabbit aorta by oxidant stress: restoration by superoxide dismutase mimetics

Andrew MacKenzie & [1]William Martin

Clinical Research Initiative, Institute of Biomedical & Life Sciences, University of Glasgow, Glasgow G12 8QQ

1 Structurally distinct superoxide dismutase (SOD) mimetics were examined for their ability to protect nitric oxide (NO) from destruction by oxidant stress in rabbit aorta.

2 These were the spin traps, PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide), tempol (4-hydroxy 2,2,6,6,-tetramethylpiperidine-1-oxyl) and tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid), the metal salts, $CuSO_4$ and $MnCl_2$, and the metal-based agents CuDIPS (Cu (II)-[diisopropylsalicylate]$_2$) and MnTMPyP (Mn (III) tetrakis [1-methyl-4-pyridyl]porphyrin).

3 Oxidant stress was generated in isolated aortic rings by inactivating endogenous Cu/Zn SOD with diethyldithiocarbamate (DETCA; 60 min) either alone at 3 mM or at 0.3 mM in combination with superoxide generation using xanthine oxidase (XO; 4.8 mu ml^{-1}) and hypoxanthine (HX; 0.1 mM).

4 Acetylcholine (ACh)-induced relaxation was inhibited by DETCA (3 mM, 60 min) and was not restored by exogenous SOD (250 u ml^{-1}), suggesting the oxidant stress was intracellular. MnTMPyP (600 μM and 1 mM) and $MnCl_2$ (100 μM) were the only agents to reverse the blockade of ACh-induced relaxation.

5 Addition of XO/HX to DETCA (0.3 mM)-treated tissues powerfully impaired ACh-induced relaxation and exogneous SOD (250 u ml^{-1}) fully reversed the blockade, suggesting the oxidant stress was extracellular. CuDIPS (0.1–3 μM), $CuSO_4$ (0.3–3 μM), $MnCl_2$ (1–100 μM) and MnTMPyP (100–600 μM) also reversed blockade powerfully, tempol (30 μM–1 mM) and tiron (0.3–10 mM) reversed blockade weakly and PTIYO (10–300 μM) enhanced the blockade.

6 Thus, MnTMPyP was the only SOD mimetic to restore NO-dependent relaxation in conditions of both extracellular and intracellular oxidant stress. This agent may, therefore, provide a lead in the development of SOD mimetics for the treatment of pathologies associated with oxidant stress.

Keywords: Nitric oxide; oxidant stress; superoxide anion; superoxide dismutase; superoxide dismutase mimetics; rabbit aorta, endothelium

Introduction

Endothelium-derived nitric oxide (NO) is of vital importance in the regulation of vascular tone and blood pressure (for review see Moncada *et al*., 1991). However, NO is destroyed by the reduced species of molecular oxygen, superoxide anion (Gryglewski *et al*., 1986). In eukaryotic cells, 3 isoforms of superoxide dismutase (SOD) have evolved to cope with the stress induced by the oxygen-rich environment: an intracellular Cu/Zn-containing form, an extracellular Cu/Zn-containing form, and a Mn-containing form found in the mitochondria, and all dismutate superoxide at equivalent rates (Fridovich, 1983). Endogenous levels of Cu/Zn SOD have been shown to be critically important in protecting NO from destruction by superoxide in a number of isolated blood vessels including the aorta of the rabbit (Mügge *et al*., 1991) and rat (Mian & Martin, 1995) and the bovine pulmonary (Cherry *et al*., 1990) and coronary arteries (Omar *et al*., 1991).

The loss of vasodilator function following the interaction of NO with superoxide has led to the suggestion that this process is involved in a number of cardiovascular pathologies. For example, hypertension (Nakazono *et al*., 1991; Grunfeld *et al*., 1995; Bouloumie *et al*., 1997), atherosclerosis (Sharma *et al*., 1992; Ohara *et al*., 1993), ischaemia-reperfusion injury (Downey, 1990), diabetes (Hattori *et al*., 1991; Kamata & Kobayashi, 1996) and heart failure (McMurray *et al*., 1990; Katz *et al*., 1993) are all associated with increased free radical production and reduced NO-dependent relaxation. Endogenous levels of SOD may therefore be of critical importance in the aetiology of vascular pathologies associated with oxidant stress. Stable elevation of the activity of SOD might, therefore, form the basis of a rational strategy for therapeutic intervention in these cardiovascular pathologies. However, it is likely that therapeutic treatment with Cu/Zn SOD itself will be of limited effectiveness. This is because SOD is not orally active, is rapidly cleared from the circulation and, in view of its large size, is unable to pentrate cell membranes to protect NO from an intracellular oxidant stress. However, greater therapeutic potential may lie with low molecular weight, membrane-permeant compounds that exhibit SOD-like activity. A diverse range of these has been described, including the metal-based compounds CuDIPS (Cu [II]-[diisopropylsalicylate]$_2$; Huber *et al*., 1987; Sorenson, 1995) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin; Faulkner *et al*., 1994; Gardner *et al*., 1996) which dismutate superoxide in a manner similar to that of authentic Cu/Zn SOD and Mn SOD, respectively. Simple metal salts of Cu and Mn also possess SOD-like activity (Huber *et al*., 1987; Beyer & Fridovich, 1990). Other compounds, such as the spin traps PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide), tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl) (Mitchell *et al*., 1990; Ewing & Janero, 1995) and tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid; Ledenev *et al*., 1986) are also known to relieve oxidant stress in a number of test systems.

The aim of this study was to investigate the potential ability of the above putative SOD mimetics to restore NO-dependent vasodilator function following its inhibition by oxidant stress

[1] Author for correspondence.

in the rabbit aorta. Conditions of oxidant stress were created in isolated aortic rings by the generation of superoxide with the xanthine oxidase/hypoxanthine (XO/HX) system and by inactivating endogenous Cu/Zn SOD with diethyldithiocarbamate (DETCA; Cocco *et al*., 1981; Mian & Martin, 1995). Preliminary reports of these findings have already been published (MacKenzie & Martin, 1997; 1998).

Methods

Preparation of tissues

Male New Zealand White rabbits (2.5–3.5 kg) were killed with an injection of sodium pentobarbitone (200 mg kg^{-1}) into the marginal ear vein. The thoracic aorta was then carefully removed and cleaned of fat and connective tissue. Care was taken not to damage inadvertently the intimal surface of the aorta. Some tissues were used that day while others were stored in oxygenated Krebs solution overnight at 4°C for use the following day. The aorta was cut into transverse rings (2.5 mm wide). Aortic rings were then mounted under 2 g resting tension on stainless steel hooks within 10 ml tissue baths and maintained at 37°C in Krebs solution (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24, glucose 11, gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically with Grass FTO3C transducers and responses displayed and recorded on a MacLab (E Series, AD Instruments) or on a Grass polygraph model 7D. Tissues were allowed to equilibrate for 60 min before experiments were carried out, during which time the resting tension was re-adjusted to 2 g, as required.

Experimental protocols

Agonist-stimulated activity of NO was determined by assessing acetylcholine (ACh)-induced relaxation. Specifically, cumulative concentration-response curves to ACh (10 nM–3 μM) were constructed on endothelium-containing rings following induction of submaximal (40–60% of maximal) phenylephrine (PE; 30–300 nM)-induced tone. Following completion of each concentration-response curve, the baths were repeatedly washed out and the tissues allowed to re-equilibrate for at least 30 min before further experimentation.

When the effects of authentic exogenous Cu/Zn SOD or the putative SOD mimetics were assessed on ACh-induced relaxation, each was given as a 20 min pretreatment before cumulative concentration-response curves to ACh were constructed. The SOD mimetics investigated were the spin traps PTIYO (4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide), tempol (4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl) (Mitchell *et al*., 1990; Ewing & Janero, 1995) and tiron (4,5-dihydroxy-1,3-benzene-disulphonic acid; Ledenev *et al*., 1986), the metal-based compounds CuDIPS (Cu [II]-[diisopropylsalicylate]$_2$; Huber *et al*., 1987; Sorenson, 1995) and MnTMPyP (Mn [III] tetrakis [1-methyl-4-pyridyl] porphyrin; Faulkner *et al*., 1994; Gardner *et al*., 1996), and the metal salts $CuSO_4$ and $MnCl_2$ (Huber *et al*., 1987; Beyer & Fridovich, 1990).

In certain experiments, the effects of irreversible inhibition of endogenous Cu/Zn superoxide dismutase (SOD) with the copper chelator, diethyldithiocarbamate (DETCA; Mian & Martin, 1995), were investigated on ACh-induced relaxation. In these experiments, aortic rings were incubated with DETCA at concentrations of 0.3, 3 or 10 mM for 60 min before being repeatedly washed out. The tissues were again contracted submaximally with PE and cumulative concentration-response curves to ACh obtained. In other experiments, the effects of exogenous generation of superoxide anion by xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM) were investigated on ACh-induced relaxation. In these experiments, XO was added to PE-contracted aortic rings for 20 min to allow it to permeate the tissue. HX was then added and cumulative concentration-response curves to ACh were constructed.

The effects of superoxide generation by XO/HX on ACh-induced relaxation were also examined in tissues that had been pretreated with DETCA (0.3 mM, 60 min, followed by washout). The ability of authentic exogenous SOD and the putative SOD mimetics to protect ACh-induced relaxation against the inhibitory effects of DETCA alone and in combination with XO/HX was also studied. Again, SOD and the SOD mimetics were given as a 20 min pretreatment before addition of HX. This 20 min incubation time was adopted since SOD which has the largest molecular weight and therefore the least diffusability of all the agents was maximally effective within this time.

All experiments involving DETCA or XO/HX were conducted in the presence of catalase (1000 u ml^{-1}) to guard against accumulation of hydrogen peroxide.

Drugs

Acetylcholine chloride, catalase (bovine liver), 4,5-dihydroxy-1,3-benzene-disulphonic acid (tiron), diethyldithiocarbamic acid (DETCA), 4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), phenylephrine hydrochloride, hypoxanthine, superoxide dismutase (Cu/Zn-containing enzyme from bovine erythocytes) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, U.K.). Cu (II)-[diisopropylsalicylate]$_2$ (CuDIPS) and 4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide (PTIYO) were obtained from Aldrich (Dorset, U.K.). Mn (III) tetrakis [1-methyl-4-pyridyl] porphyrin (MnTMPyP) was obtained from Alexis (Nottingham, U.K.), whilst $CuSO_4$ and $MnCl_2$ were obtained from Hopkin & Williams (Essex, U.K.). All drugs were dissolved in saline (0.9%), except for hypoxanthine (50 mM) and Cu (II)-[diisopropylsalicylate]$_2$ (3 mM) which were dissolved in 0.1% sodium hydroxide and in 50% ethanol/50% tris buffer (50 mM, pH 7.4), respectively. Control experiments demonstrated that the solvents, sodium hydroxide and ethanol/tris buffer, did not account for the effects observed with hypoxanthine and CuDIPS, respectively. All dilutions were made in saline (0.9%).

Analysis of data

Results are expressed as the mean ± s.e.mean of n separate experiments. Relaxant responses are expressed as a percentage (%) relaxation of PE-induced tone. Statistical comparisons were made by one-way analysis of variance followed by Bonferroni's post-test. A value of $P<0.05$ was considered significant.

Results

Effects of exogenous SOD and SOD mimetics on ACh-induced relaxation

Following induction of PE (30–300 nM)-induced tone in rings of rabbit aorta, treatment for 20 min with authentic SOD (250 u ml^{-1}), or with the SOD mimetics, tiron (1–30 mM),

MnTMPyP (10 μM – 1 mM), or $MnCl_2$ (1 – 100 μM) (Figures 1a, 1c, 2c and 2d, respectively) failed to affect ACh (10 nM – 3 μM)-induced relaxation. However, relaxation was impaired by treatment with the SOD mimetics, PTIYO at 300 μM but not at 10 or 100 μM (Figure 1b), tempol at 3 mM but not at 0.1 or 1 mM (Figure 1d), and by both CuDIPS and $CuSO_4$ at 10 and 100 μM but not at 1 μM (Figures 2a and b).

Effects of exogenous SOD and SOD mimetics on ACh-induced relaxation in DETCA-treated tissues

Treatment of endothelium-containing rings of rabbit aorta with DETCA (0.3 – 10 mM) for 60 min (followed by washout) to inactivate endogenous Cu/Zn SOD led to a concentration-dependent impairment of ACh-induced relaxation (Figure 3a).

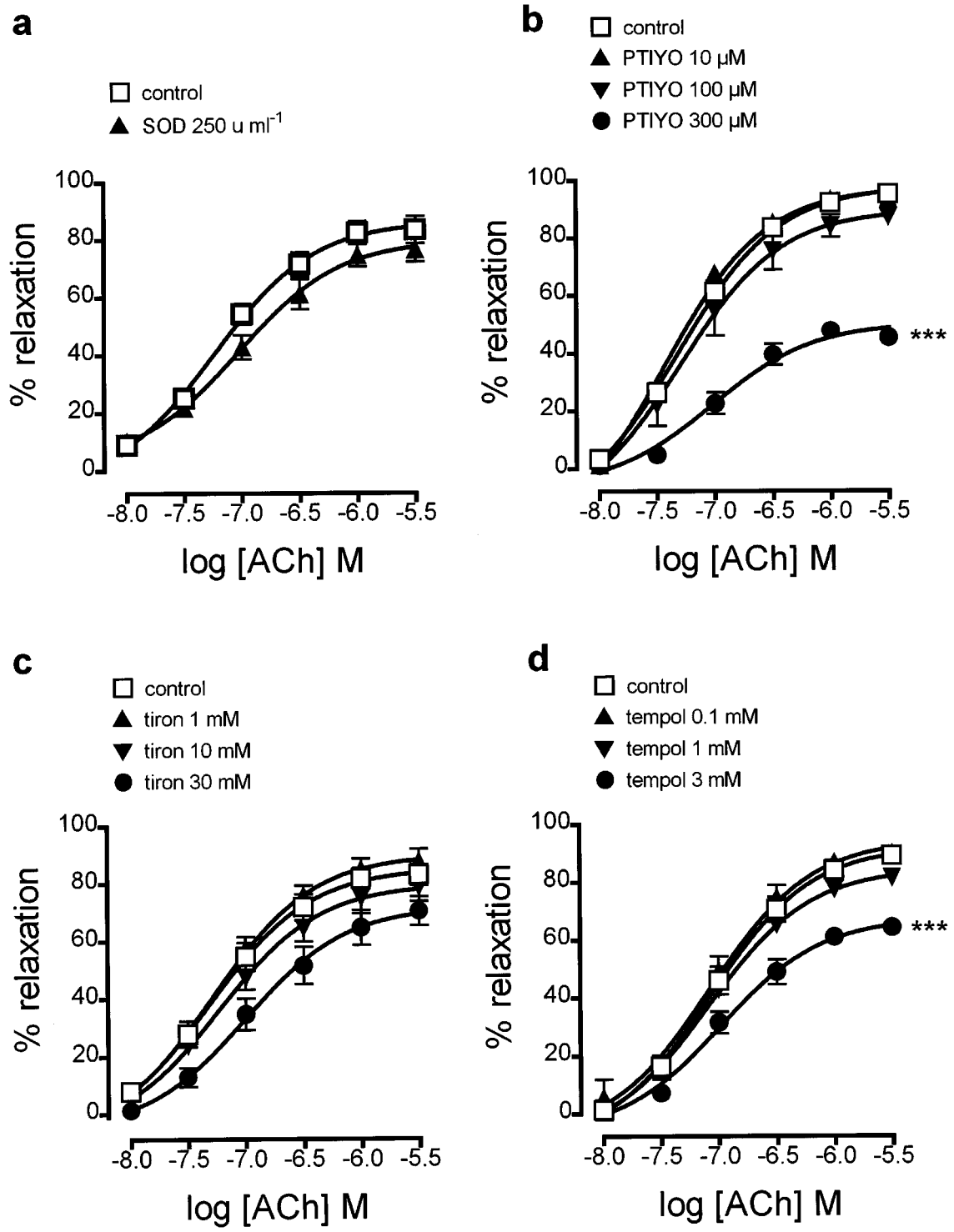

Figure 1 Concentration-response curves showing relaxation to acetylcholine (ACh) on phenylephrine (30 – 300 nM)-contracted endothelim-containing rings of rabbit aorta and the effects of superoxide dismutase (SOD) and SOD mimetics on this relaxation. (a) SOD (250 u ml^{-1}), (b) PTIYO (10, 100 and 300 μM), (c) tiron (1, 10 and 30 mM) and (d) tempol (0.1, 1 and 3 mM). Each point is the mean and vertical lines s.e.mean of $\geqslant 5$ observations. ***$P<0.001$ indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

The blockade induced by 3 mM DETCA was unaffected by treatment with exogenous SOD at 250 u ml^{-1} (Figure 4a) or at 750 u ml^{-1} (data not shown). Furthermore, no reversal of blockade was observed following treatment with PTIYO (10 μM), tiron (1 mM), or tempol (0.1 mM) (Figure 4b) or with CuDIPS or $CuSO_4$ (both at 1 μM, Figure 4c). In contrast, reversal of blockade was observed following treatment with MnTMPyP (600 μM and 1 mM) and with $MnCl_2$ (100 μM) (Figure 4d).

Ability of exogenous SOD and SOD mimetics to protect ACh-induced relaxation from blockade by XO/HX in DETCA-treated tissues

Treatment of endothelium-containing rings with XO (4.8 mu ml^{-1})/HX (0.1 mM), to generate superoxide, impaired ACh-induced relaxation (Figure 3b). Furthermore, in DETCA (0.3 mM)-treated tissues the ability of XO/HX to inhibit ACh-

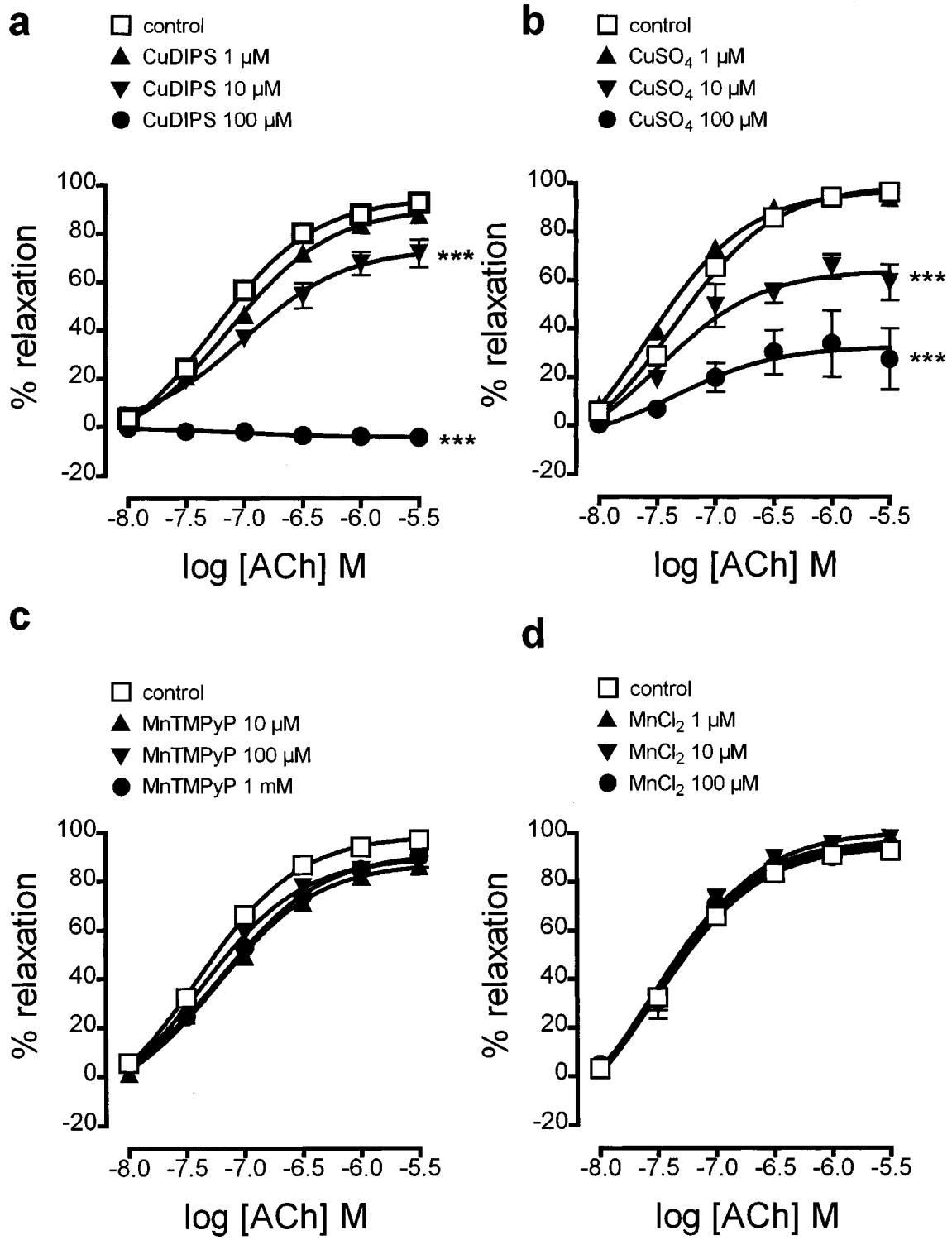

Figure 2 Concentration-response curves showing relaxation to acetylcholine (ACh) on phenylephrine (30–300 nM)-contracted endothelium-containing rings of rabbit aorta and the effects of superoxide dismutase mimetics on this relaxation. (a) CuDIPS (1, 10 and 100 μM), (b) $CuSO_4$ (1, 10 and 100 μM), (c) MnTMPyP (10 and 100 μM and 1 mM) and (d) $MnCl_2$ (1, 10 and 100 μM). Each point is the mean and vertical lines s.e.mean of $\geqslant$ 5 observations. ***$P<0.001$ indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

induced relaxation was powerfully potentiated (Figure 3b). This enhanced blockade was abolished following treatment with exogenous SOD (250 u ml^{-1}; Figure 5a) and reversed in a concentration-dependent manner by MnTMPyP (100–600 μM; Figure 6c) and $MnCl_2$ (1–100 μM; Figure 6c) and $MnCl_2$ (1–100 μM; Figure 6d). Substantial reversal of blockade was observed following treatment with CuDIPS at 1 and 3 μM but not at 0.1 mM (Figure 6a) and with $CuSO_4$ (0.3–3 μM; Figure 6b). However, there was a tendency for both of these at the highest concentration used (3 μM), to be less effective at concentrations of ACh of 1 μM and above. Weak reversal of blockade of ACh-induced relaxation was observed following treatment with tiron at 1 mM but none was seen at 0.3 or 10 mM (Figure 5c). Tempol produced weak reversal of blockade at 0.1 mM but none was seen at 30 μM or 1 mM (Figure 5d). However, no reversal of blockade was observed following treatment with PTIYO at 10 and 100 μM, and at 300 μM the blockade was intensified (Figure 5b).

Discussion

Treatment with high concentrations of DETCA inactivates both intracellular and extracellular isoforms of Cu/Zn SOD (Kelner *et al*., 1989), and in a number of blood vessels leads to increased levels of superoxide together with severe impairment of NO-dependent relaxation (Cherry *et al*., 1990; Mügge *et al*., 1991; Omar *et al*., 1991; Mian & Martin, 1995). As with these previous studies we found that treatment with DETCA alone produced a concentration-dependent blockade of acetylcholine (ACh)-induced relaxation in rabbit aortic rings. Furthermore, we found that the blockade of ACh-induced, NO-dependent relaxation produced by a high concentration of DETCA (3 mM) was not reversed by treatment with membrane-impermeant exogenous Cu/Zn SOD. Thus, the blockade results from either a non-selective action of DETCA or impairment of the intracellular isoform of Cu/Zn SOD. The latter explanation seems the more likely since the SOD mimetic MnTMPyP and the simple salt $MnCl_2$ produced reversal of this blockade. However, neither agent fully reversed this DETCA-induced inhibition; MnTMPyP produced no greater restoration when used at 1 mM than at 600 μM, while $MnCl_2$ precipitation in Krebs solution limited its use to concentrations of 100 μM or less. Thus, a major component of the actions of DETCA is likely to result from inhibition of intracellular Cu/Zn SOD, with a lesser component reflecting non-selective effects of DETCA, such as depletion of intracellular glutathione and generation of lipid peroxides (Kelner *et al*., 1989). In view of the membrane permeance of MnTMPyP (Faulkner *et al*., 1994; Gardner *et al*., 1996) it may not be surprising that this agent can reverse the effects of inhibition of intracellular Cu/Zn SOD. In contrast, Mn^{2+} ions resulting from addition of $MnCl_2$ would not be expected to cross cell membranes readily to relieve an oxidant stress. However, it is possible that this ion gained entry to cells through divalent cation channel. $CuSO_4$ has previously been shown to restore endothelium-dependent and nitrovasodilator-mediated relaxation following inhibition by DETCA in rat aorta (Plane *et al*., 1997). We found no such restoration in rabbit aorta, perhaps reflecting a reduced ability of Cu^{2+} ions to enter cells in this tissue.

We also found following treatment of rabbit aortic rings with a low concentration of DETCA (0.3 mM), which itself had little effect on ACh-induced relaxation, that the ability of superoxide, generated by xanthine oxidase (XO)/hypoxanthine (HX), to impair relaxation was potentiated. This confirms previous findings that endogenous Cu/Zn SOD is vital in protecting NO against destruction by superoxide (Cherry *et al*., 1990; Mügge *et al*., 1991; Omar *et al*., 1991; Mian & Martin, 1995). This enhanced blockade was completely

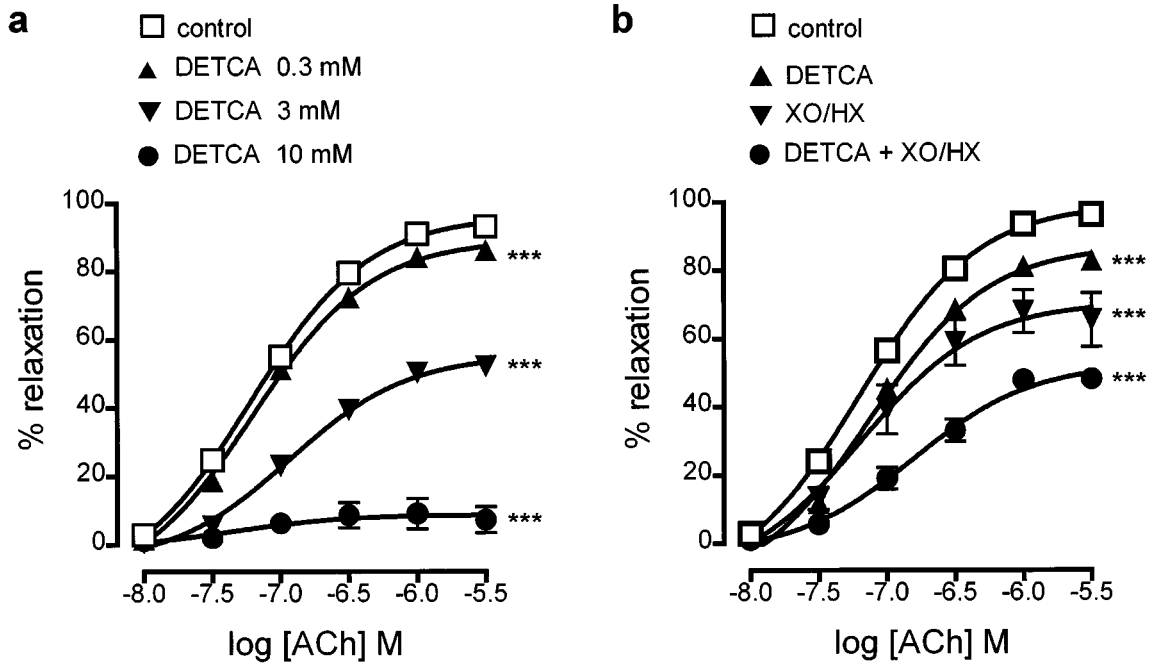

Figure 3 (a) Concentration-response curves showing relaxation to acetylcholine (ACh) on phenylephrine (30–300 nM)-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following treatment with diethyldithiocarbamate (DETCA; 0.3, 3 and 10 mM). (b) Concentration-response curves showing relaxation to ACh and the blockade of this relaxation following treatment with DETCA (0.3 mM) alone, xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM) alone, or the combination of these two treatments. Each point is the mean and vertical lines s.e.mean of $\geqslant 5$ observations. ***$P<0.001$ indicates a significant difference from the relaxation induced by the maximal concentration of ACh in untreated rings.

reversed by treatment with exogenous Cu/Zn SOD suggesting that destruction of NO by superoxide was localized to the extracellular space. The ability of DETCA to inhibit intracellular or extracellular isoforms of Cu/Zn SOD may, therefore, differ with the concentration used, i.e. low concentrations producing blockade which is reversed by membrane-impermeant SOD reflecting inhibition of extracellular SOD, and high concentrations producing blockade which is reversed by certain membrane permeant SOD mimetics but not by exogenous SOD reflecting inhibition of

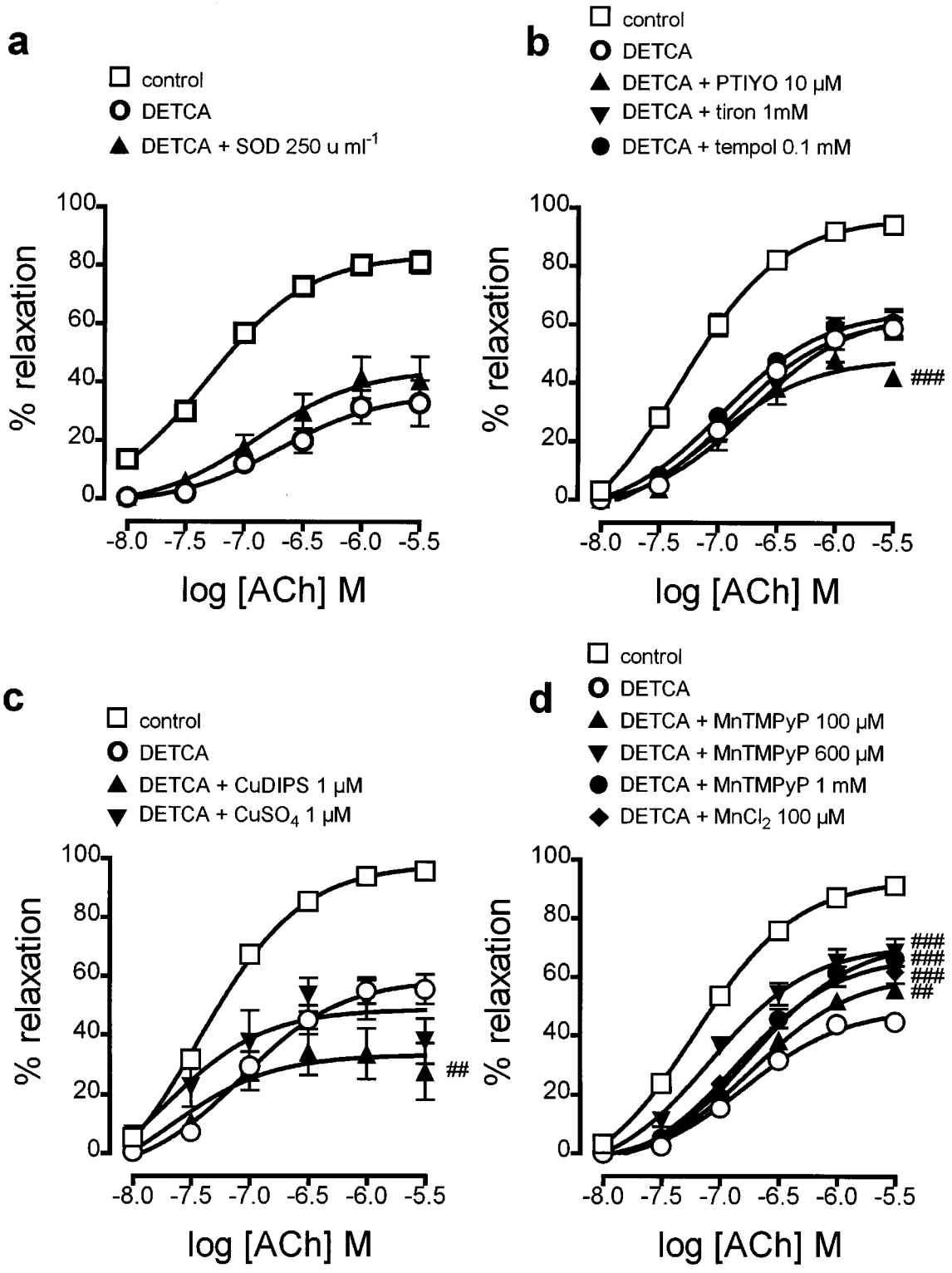

Figure 4 Concentration-response curves showing relaxation to acetylcholine (ACh) on phenylephrine (30–300 nM)-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following treatment with diethyldithiocarbamate (DETCA; 3 mM). The effects of superoxide dismutase (SOD) and SOD mimetics on this blockade are also shown. (a) SOD (250 u ml^{-1}), (b) PTIYO (10 μM), tiron (1 mM) and tempol (0.1 mM); (c) CuDIPS (1 μM) and $CuSO_4$ (1 μM); and (d) MnTMPyP (100 and 600 μM and 1 mM) and $MnCl_2$ (100 μM). Each point is the mean and vertical lines s.e.mean of $\geqslant 5$ observations. $\#\#P<0.01$ and $\#\#\#P<0.001$ indicate that the SOD mimetic had a significant effect on the maximal relaxation induced by ACh in diethyldithiocarbamate-treated rings.

intracellular SOD. Of the spin trap agents tested, tempol and tiron demonstrated modest ability to restore ACh-induced relaxation but this was lost at higher concentrations of the agents, while PTIYO failed to have any protective effect and intensified the blockade when used at high concentrations. The potential ability of these spin traps to restore ACh-induced relaxation by removing superoxide anion is likely to have been compromised by their additional ability to inhibit ACh-induced relaxation themselves; effects which we observed on control rings of rabbit aorta. The ability of stable nitroxide spin traps such as tempol and PTIYO to inhibit the activity of NO in blood vessels by a direct reaction has already been

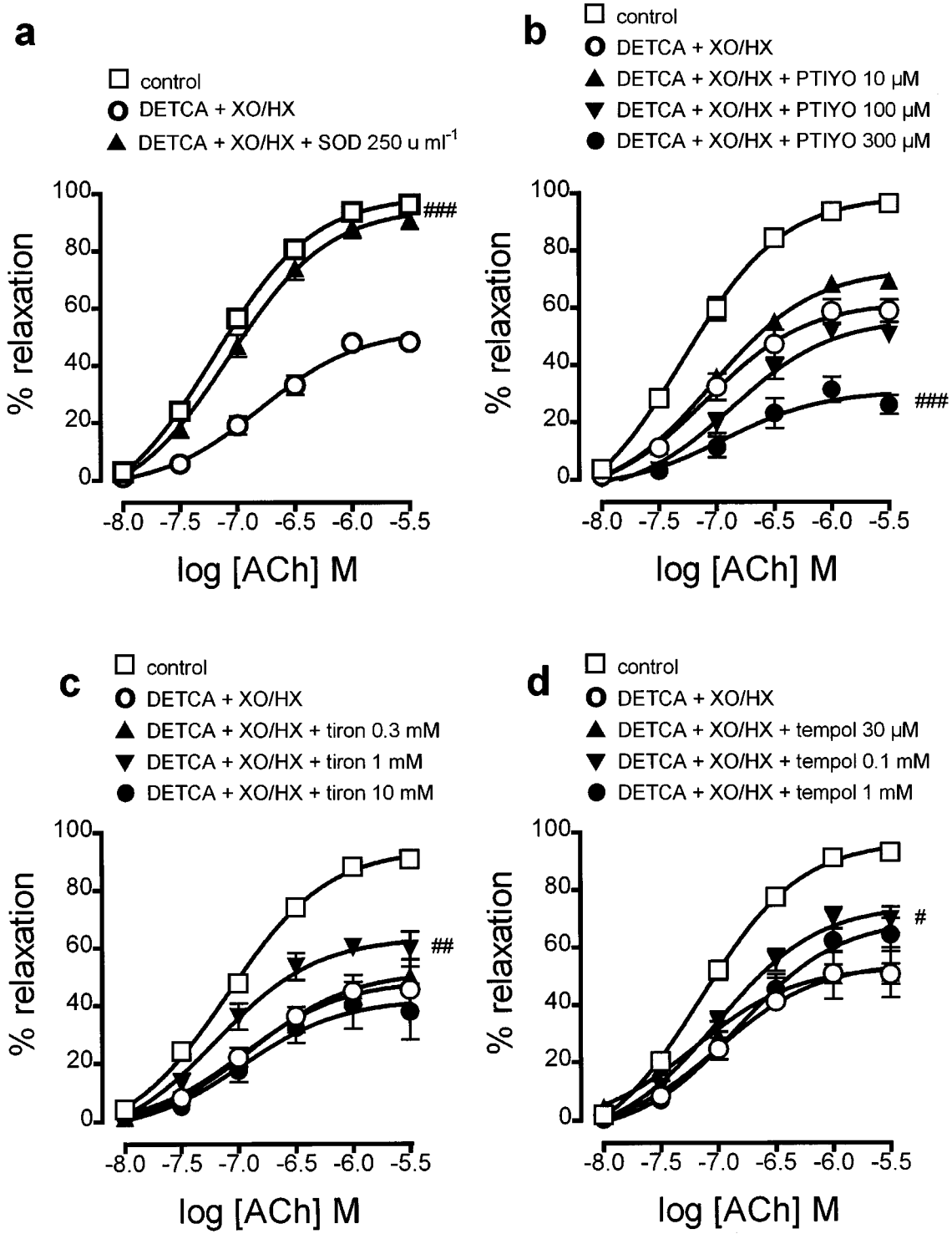

Figure 5 Concentration-response curves showing relaxation to acetylcholine (ACh) on phenylephrine (30–300 nM)-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following combined treatment with diethyldithiocarbamate (DETCA; 0.3 mM) and xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM). The effects of superoxide dismutase (SOD) and SOD mimetics on this blockade are also shown. (a) SOD (250 u ml^{-1}), (b) PTIYO (10, 100 and 300 μM), (c) tiron (0.3, 1 and 10 mM) and (d) tempol (30 μM and 0.1 and 1 mM). Each point is the mean and vertical lines s.e.mean of $\geqslant 5$ observations. $\#P<0.05$, $\#\#P<0.01$ and $\#\#\#P<0.001$ indicate that the SOD mimetic had a significant effect on the maximal relaxation induced by ACh in rings treated with both diethyldithiocarbamate and xanthine oxidase/hypoxanthine.

demonstrated (Akaike *et al.*, 1993). The metal-based agents demonstrated the greatest ability to restore ACh-induced relaxation following its inhibition by XO/HX in DETCA (0.3 mM)-treated rings of rabbit aorta. CuDIPS and the simple metal salt $CuSO_4$ both powerfully restored ACh-induced relaxation in a concentration-dependent manner. However, there was a tendency for both at the highest concentration used (3 μM) to be less effective at concentrations of ACh of 1 μM and above. This may be explained by our finding that both compounds produced a concentration-dependent impairment

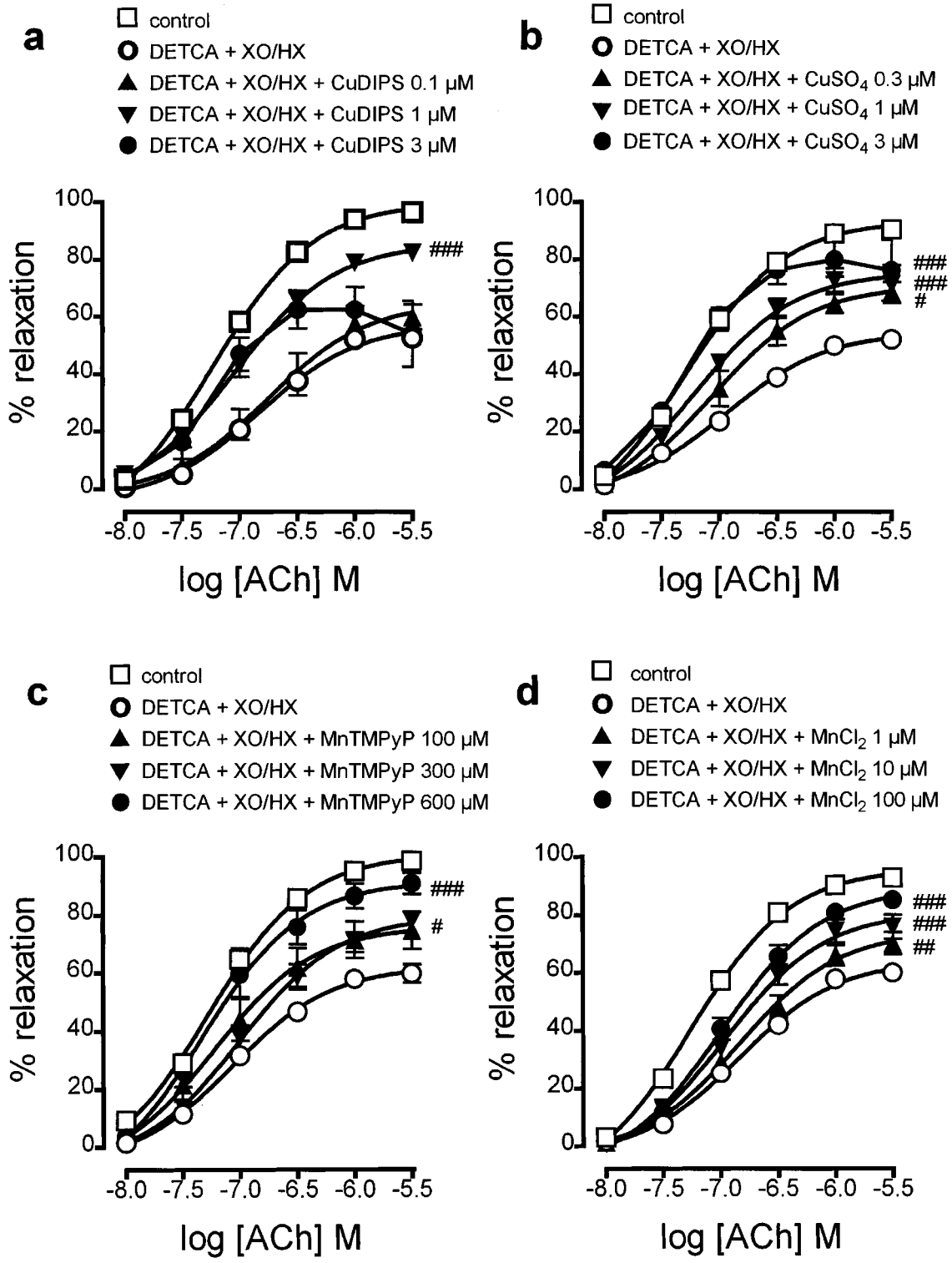

Figure 6 Concentration-response curves showing relaxation to acetylcholine (ACh) on phenylephrine (30–300 nM)-contracted endothelium-containing rings of rabbit aorta and the blockade of this relaxation following combined treatment with diethyldithiocarbamate (DETCA; 0.3 mM) and xanthine oxidase (XO; 4.8 mu ml^{-1})/hypoxanthine (HX; 0.1 mM). The effects of superoside dismutase (SOD) mimetics on this blockade are also shown. (a) CuDIPS (0.1, 1 and 3 μM), (b) $CuSO_4$ (0.3, 1 and 3 μM), (c) MnTMPyP (100, 300 and 600 μM) and (d) $MnCl_2$ (1, 10 and 100 μM). Each point is the mean and vertical lines s.e.mean of $\geqslant 5$ observations. #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ indicate that the SOD mimetic had a significant effect on the maximal relaxation induced by ACh in rings treated with both diethyldithiocarbamate and xanthine oxidase/hypoxanthine.

of ACh-induced relaxation in control tissues, perhaps reflecting the reported ability of CuDIPS to inactivate NO synthase (Baquial & Sorenson, 1995) or of Cu^{2+} ions to generate hydroxyl radicals by the Fenton reaction. MnTMPyP and $MnCl_2$ both fully restored ACh-induced relaxation, although the former was considerably less potent than the latter. Faulkner *et al*. (1994) showed that the rate constant for removal of superoxide anion by MnTMPyP was substantially different depending on the valency state of the metal i.e. the Mn(II) form was 100 fold more active than the native Mn(III) form. Reduction of the native state of Mn(III)TMPyP to the more active Mn(II)TMPyP form by the superoxide dismutation process is relatively slow but can proceed faster, at least in *E. coli*, by reduction at the hand of cellular elements such as NADPH and glutathione (Faulkner *et al*., 1994). This is likely to explain the reportedly greater superoxide removing potency of MnTMPyP in *E. coli* than in *in vitro* biochemical studies (Faulkner *et al*., 1994). The higher affinity of Mn(II) over Mn(III) for superoxide anion may, therefore, explain the greater potency of $Mn(II)Cl_2$ than of Mn(III)TMPyP in restoring ACh-induced relaxation following its inhibition by oxidant stress. SOD mimetics based on a Mn(II) metal centre may therefore have greater therapeutic potential than those based on the Mn(III) form.

In conclusion, we assessed the ability of a series of structurally distinct putative SOD mimetics to protect NO from destruction by oxidant stress in blood vessels. We found that compounds based on the metal centres of Cu/Zn and Mn SOD were substantially more potent than spin trapping agents. Other than the simple metal salts which are too toxic to be considered as potential therapeutic agents, MnTMPyP proved to be the most effective in restoring NO-dependent relaxations in conditions of extracellular and intracellular oxidant stress. This reflected our previous finding that MnTMPyP was the most effective of these agents in reversing the blockade of nitrergic transmission in conditions of oxidant stress in the bovine retractor penis muscle (Mok *et al*., 1998). The membrane-permeant nature of MnTMPyP may provide a lead in the development of SOD mimetics with greater therapeutic potential than membrane-impermeant SOD itself in the treatment of vascular pathologies associated with oxidant stress.

Abbreviations

CuDIPS, Cu(II)-[diisopropylsalicylate]$_2$; DETCA, diethyldithiocarbamate; HX, hypoxanthine; MnTMPyP, Mn(III) tetrakis [1-methyl-4-pyridyl] porphyrin; PTIYO, 4-phenyl-2,2,5,5-tetramethyl imidazolin-1-yloxy-5-oxide; SOD, superoxide dismutase; tempol, 4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl; tiron, 4,5-dihydroxy-1,3-benzene-disulphonic acid; XO, xanthine oxidase

A.M. is an MRC student.

References

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

BAQUIAL, J.G.L. & SORENSON, J.R.J. (1995). Down-regulation of NADPH-Diaphorase (nitric oxide synthase) may account for the pharmacological activities of $Cu(II)_2$(3,5-Diisopropylsalicylate)$_4$. *J. Inorg. Biochem.,* **60,** 133–148.

BEYER, W.F., JR. & FRIDOVICH, I. (1990). Superoxide dismutase mimic prepared from desferrioxamine and manganese dioxide. *Methods Enzymol.,* **186,** 242–249.

BOULOUMIE, A., BAUERSACHS, J., LINZ, W., SCHOLKENS, B.A., WIEMER, G., FLEMING, I. & BUSSE, R. (1997). Endothelial dysfunction coincides with an enhanced nitric oxide synthase expression and superoxide anion production. *Hypertension,* **30,** 934–941.

CHERRY, P.D., OMAR, H.A., FARRELL, K.A., STUART, J.S. & WOLIN, M.S. (1990). Superoxide anion inhibits cGMP-associated bovine pulmonary arterial relaxation. *Am. J. Physiol.,* **259,** H1056–H1062.

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

DOWNEY, J.M. (1990). Free radicals and their involvement during long-term myocardial ischemia and reperfusion. *Ann. Rev. Physiol.,* **52,** 487–504.

EWING, J.F. & JANERO, D.R. (1995). Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. *Anal. Biochem.,* **232,** 243–248.

FAULKNER, K.M., LIOCHEV, S.I. & FRIDOVICH, I. (1994). Stable Mn(III) porphyrins mimic superoxide dismutase in vitro and substitute for it in vivo. *J. Biol. Chem.,* **269,** 23471–23476.

FRIDOVICH, I. (1983). Superoxide radical: an endogenous toxicant. *Ann. Rev. Pharmacol. Toxicol.,* **23,** 239–257.

GARDNER, P.R., NGUYEN, D.D.H. & WHITE, C.W. (1996). Superoxide scavenging by Mn(II/III) tetrakis (1-methyl-4-pyridyl) porphyrin in mammalian cells. *Arch. Biochem. Biophys.,* **325,** 20–28.

GRUNFELD, S., HAMILTON, C.A., MESAROS, S., MCCLAIN, S.W., DOMINICZAK, A.F., BOHR, D.F. & MALINSKI, T. (1995). Role of superoxide in the depressed nitric oxide production by the endothelium of genetically hypertensive rats. *Hypertension,* **26,** 854–857.

GRYGLEWSKI, R.J., PALMER, R.M.J. & MONCADA, S. (1986). Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. *Nature,* **320,** 454–456.

HATTORI, Y., KAWASAKI, H., ABE, K. & KANNO, M. (1991). Superoxide dismutase recovers altered endothelium-dependent relaxation in diabetic rat aorta. *Am. J. Physiol.,* **261,** H1086–H1094.

HUBER, K.R., SRIDHAR, R., GRIFFITH, E.H., AMMA, E.L. & ROBERTS, J. (1987). Superoxide dismutase-like activities of copper(II) complexes tested in serum. *Biochim. Biophys. Acta,* **915,** 267–276.

KAMATA, K. & KOBAYASHI, T. (1996). Changes in superoxide dismutase mRNA expression by streptozotocin-induced diabetes. *Br. J. Pharmacol.,* **119,** 583–589.

KATZ, S.D., SCHWARZ, M., YUEN, J. & LEJEMTEL, T.H. (1993). Impaired acetylcholine-mediated vasodilation in patients with congestive heart failure: Role of endothelium-derived vasodilating and vasocontricting factors. *Circulation,* **88,** 55–61.

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.

LEDENEV, A.N., KONSTANTINOV, A.A., POPOVA, E. & RUUGE, E.K. (1986). A simple assay of the superoxide generation rate with tiron as an ESP-visible radical scavenger. *Biochem. Int.,* **13,** 391–396.

MACKENZIE, A. & MARTIN, W. (1997). Assessment of superoxide dismutase mimetic compounds in the rabbit aorta. *Br. J. Pharmacol.,* **122,** 195P.

MACKENZIE, A. & MARTIN, W. (1988). A superoxide dismutase mimetic protects acetylcholine-induced relaxation from oxidant stress in the rabbit aorta. *Br. J. Pharmacol.,* **123,** 10P.

MOK, J.S.L., PAISLEY, K. & MARTIN, W. (1998). Inhibition of nitrergic neurotransmission in the bovine retractor penis muscle by an oxidant stress: effects of superoxide dismutase mimetics. *Br. J. Pharmacol.,* (in press).

MCMURRAY, J., MCLAY, J., CHOPRA, M., BRIDGES, A. & BELCH, J.J.F. (1990). Evidence for enhanced free radical activity in chronic congestive heart failure secondary to coronary artery disease. *Am J. Cardiol.,* **65,** 1261–1262.

MIAN, K.B. & MARTIN, W. (1995). Differential sensitivity of basal and acetylcholine-stimulated activity of nitric oxide to destruction by superoxide anion in rat aorta. *Br. J. Pharmacol.,* **115,** 993–1000.

MITCHELL, J.B., SAMUNI, A., KRISHNA, M.C., DEGRAFF, W.G., AHN, M.S., SAMUNI, U. & RUSSO, A. (1990). Biologically active metal-independent superoxide dismutase mimics. *Biochemistry,* **29,** 2802–2807.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.,* **43,** 109–142.

MÜGGE, A., ELWELL, J.H., PETERSON, T.E. & HARRISON, D.G. (1991). Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. *Am. J. Physiol.,* **260,** C219–C225.

NAKAZONO, K., WATANABE, N., MATSUNO, K., SASAKI, J., SATO, T. & INOUE, M. (1991). Does superoxide underlie the pathogenesis of hypertension? *Proc. Natl. Acad. Sci. U.S.A.,* **88,** 10045–10048.

OHARA, Y., PETERSON, T.E. & HARRISON, D.G. (1993). Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.*, **91,** 2546–2551.

OMAR, H.A., CHERRY, P.D., MORTELLITI, M.P., BURKE-WOLIN, T. & WOLIN, M.S. (1991). Inhibition of coronary artery superoxide dismutase atenuates endothelium-dependent and-independent nitrovasodilator relaxation. *Circ. Res.,* **69,** 601–608.

PLANE, F., WIGMORE, S., ANGELINI, G.D. & JEREMY, J.Y. (1997). Effect of copper on nitric oxide synthase and guanylyl cyclase activity in the rat isolated aorta. *Br. J. Pharmacol.,* **121,** 345–350.

SHARMA, R.C., CRAWFORD, D.W., KRAMSCH, D.M., SEVANIAN, A. & JIAO, Q. (1992). Immunolocalization of native antioxidant scavenger enzymes in early hypertensive and atherosclerotic arteries: Role of oxygen free radicals. *Arterioscler. Thromb.,* **12,** 403–415.

SORENSON, J.R.J. (1995). Pharmacological activities of copper compounds. In *Handbook of Metal Ligand Interactions in Biological Fluids*. ed. Berthon, G. pp. 1128–1139. New York: Marcel Dekker Inc.

(Received February 26, 1998
Revised March 18, 1998
Accepted March 20, 1998)