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Interaction between superoxide anion and nitric oxide in the regulation of vascular endothelial function

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1 Nitric oxide (NO)-mediated, endothelium-dependent vasodilator function in rat aortic smooth muscle was investigated in an *in vitro* model of endogenous vascular superoxide anion stress, generated by pretreatment with the Cu/Zn superoxide dismutase (SOD, EC 1.15.1.1) inhibitor, diethyldithiocarbamate (DETCA).

2 Contraction to noradrenaline (NA, 1 nM – 1 μM) in endothelium-intact vessels was augmented after a 30 min pretreatment with DETCA (10 mM) followed by 30 min washout. This effect was abolished by N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM) and removal of the endothelium and partially reversed by exogenous Cu/Zn SOD (200 u ml^{-1}).

3 Endothelium- and basal NO-dependent vasorelaxation to the phosphodiesterase (PDE) type V inhibitor ONO-1505 (4-[2-(2-hydroxyethoxy)ethylamino]-2-(1*H*-imidazol-1-yl)-6-methoxyquinazoline methanesulphonate) (0.1 – 10 μM) was inhibited after DETCA (10 mM) pretreatment. In addition, the ability of L-NAME (0.3 mM) to enhance established contractile tone was effectively absent.

4 In contrast, DETCA pretreatment did not significantly affect vasorelaxation to acetylcholine (ACh, 1 nM – 3 μM) or S-nitroso-N-acetyl penicillamine (SNAP, 0.03 – 30 μM). However, L-NAME (0.3 mM) unmasked an inhibitory effect of DETCA pretreatment on vasorelaxation to SNAP in endothelium-intact vessels while markedly potentiating vasorelaxation to SNAP in control tissue.

5 L-NAME (0.3 mM)- and exogenous catalase (200 u ml^{-1})-sensitive vasorelaxation to exogenous Cu/Zn SOD (200 u ml^{-1}) was greater after DETCA (10 mM) pretreatment in endothelium-intact aortic rings. This difference was abolished by catalase (200 u ml^{-1}).

6 In conclusion, tissue Cu/Zn SOD inhibition elicited a selective lesion in basal endothelial function in rat isolated aortic smooth muscle, consistent with the inactivation of basal NO by superoxide anion. The resulting leftward shift in nitrovasodilator reactivity, due to the loss of the tonic depression by basal NO, is likely to mask the inhibitory effect of superoxide anion on agonist-stimulated endothelial function and nitrovasodilator-derived NO, thereby accounting for the differential pattern of endothelial dysfunction after DETCA pretreatment.

Keywords: Endothelium; nitric oxide; superoxide dismutase; superoxide anion; rat aorta

Introduction

The endogenous nitrovasodilator, nitric oxide (NO; Furchgott & Zawadzki, 1980; Palmer *et al*., 1987), formed by endothelial nitric oxide synthase (eNOS) (Palmer *et al*., 1988; Knowles & Moncada, 1994), plays a major physiological role in vascular biology (see Moncada *et al*., 1991; Ignarro, 1993). Impaired endothelium-dependent vasodilator responsiveness, associated with reactive oxygen species (ROS), especially the superoxide anion (Mügge *et al*., 1991b; Grunfeld *et al*., 1995), features in many conditions including diabetes mellitus, atherosclerosis, hypertension, ischaemia/reperfusion injury (for reviews, see Maxwell, 1995; Halliwell, 1993; Matthys & Bult, 1997; Mohan & Das, 1997) and organic nitrate tolerance (Münzel *et al*., 1995). In these pathologies, the balance of NO and superoxide anion in the vascular wall is likely to be perturbed in favour of NO inactivation (see Gryglewski *et al*., 1986) and reduced NO functional availability (Darley-Usmar *et al*., 1995; Katusic, 1996; Stamler, 1996; Darley-Usmar & White, 1997; Münzel *et al*., 1997).

The major tissue defence against superoxide anion is superoxide dismutase (SOD), which exists in two major forms: Cu/Zn SOD, found both in the cytosol (as a dimer) and extracellularly (as a secretory tetramer), potentially bound via heparan sulphate proteoglycans to endothelium and connective tissue matrix (EC-SOD C); and Mn SOD, found in mitochondria (McCord & Fridovich, 1969; Crapo *et al*., 1978; Hassan, 1988; Marklund & Karlsson, 1989; Abrahamsson *et al*., 1992). Other physiologically relevant superoxide scavengers include ascorbate (see Halliwell & Gutteridge, 1990; Halliwell, 1996) and plausibly NO itself (Kanner *et al*., 1991; Heim *et al*., 1991; Rubanyi *et al*., 1991), since it can react at an almost diffusion-controlled rate with superoxide anion (Huie & Padmaya, 1993).

Several studies *in vitro* have demonstrated the ability of SOD to protect NO from inactivation by exogenous superoxide anion generating systems in vascular smooth muscle (Rubanyi & Vanhoutte, 1986a,b; Abrahamsson *et al*., 1992; Mian & Martin, 1995a,b). Moreover, studies with the intracellular copper chelator, diethyldithiocarbamate (DETCA; Misra, 1979; Cocco *et al*., 1981), have provided evidence that tissue Cu/Zn SOD activity protects nitrergic transmission from superoxide anion stress in visceral smooth muscle (Lilley & Gibson, 1995; 1996; Paisley & Martin, 1996; De Man *et al*., 1996; Lefebvre, 1996) and preserves agonist-stimulated endothelial function and to a lesser extent, vasorelaxation to exogenous nitrovasodilators, in vascular smooth muscle *in vitro* (Mügge *et al*., 1991a; Omar *et al*., 1991).

Mian & Martin (1995a) have recently demonstrated a differential protection of basal and agonist-stimulated NO by

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tissue Cu/Zn SOD in rat aorta, based on findings that basal endothelial function is more sensitive to inhibition by endogenously generated superoxide anion. Given the findings that endothelial NO production reduces the detection of superoxide anion in rabbit isolated aorta (Heim *et al*., 1991) and authentic NO inactivates superoxide anion produced by activated leukocytes (Rubanyi *et al*., 1991), the aim of the present study in rat isolated aortic smooth muscle, was to investigate a possible antioxidant role for basal NO in the preservation of agonist-stimulated, vascular endothelial function in experimental oxidant stress. In this scenario, while basal endothelial function is impaired by superoxide anion, agonist-stimulated and exogenous NO-mediated relaxation would be protected from inhibition by the scavenging activity of basal NO (see Rubanyi *et al*., 1991; Gumusel *et al*., 1996).

Methods

General

Male Wistar rats (250–300 g) were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p.), and the thoracic aorta carefully excised after a thorectomy. Aortic rings, approximately 2 mm in length, were mounted under isometric conditions in Krebs-Henseleit solution (KHS) (composition in mM: NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52 and D-glucose 7.8) containing indomethacin (10 μM), gassed with carbogen and warmed to 37°C. Stabilization was allowed for 1 h during which time the KHS was changed every 15 min. Rings were endothelium-intact unless otherwise stated.

Generation of oxidant stress in vitro

The generation of vascular oxidant stress was essentially as previously described in canine isolated arterial vascular smooth muscle, where superoxide anion generation was confirmed by chemiluminescence (Omar *et al*., 1991). Briefly, stabilized rings were treated with the Cu/Zn SOD inhibitor, DETCA (Misra, 1979; Cocco *et al*., 1981) (10 mM) for 30 min followed by 30 min washout while untreated, matched rings served as temporal, paired controls.

Vascular reactivity studies

In order to examine the effect of tissue Cu/Zn SOD inhibition on the ability of basal NO to modulate vasoconstrictor reactivity, contraction to noradrenaline (NA, 1 nM–1 μM) in DETCA-pretreated and control rings was assessed with and without a 10 min prior incubation with either the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM) or exogenous Cu/Zn SOD (200 u ml^{-1}). The concentration of SOD employed was previously determined to abolish effectively the reduction of ferricytochrome c by a xanthine oxidase/hypoxanthine system *in vitro* (Laight *et al*., 1997). Subsequently, DETCA-pretreated and control rings were equivalently precontracted with NA to assess endothelium-dependent vasorelaxation to either the PDE type V inhibitor ONO-1505 (0.1–10 μM) or ACh (1 nM–3 μM) or exogenous NO-dependent vasorelaxation to SNAP (0.03–30 μM). ONO-1505 ($\leqslant$10 μM) has previously been shown to elicit vasodilatation which is dependent on basal, endothelium-derived NO (Laight *et al*., 1996a;b). In further experiments in which the balance between superoxide anion and endothelial function was examined, NO-dependent vasorelaxation to exogenous Cu/Zn SOD (200 u ml^{-1}) was assessed with or without DETCA pretreatment. Since Cu/Zn SOD activity generates H_2O_2, a vasoactive ROS, we additionally performed these experiments in the presence or absence of exogenous catalase (200 u ml^{-1}, added 5 min before SOD in NA-precontracted rings) (see Zembowicz *et al*., 1993) which removes H_2O_2.

Drugs

Diethyldithiocarbamate sodium salt, (−)-noradrenaline bitartrate, N^G-nitro-L-arginine methyl ester, acetylcholine hydrochloride, S-nitroso-N-acetyl penicillamine (SNAP), indomethacin, bovine superoxide dismutase and bovine catalase were obtained from Sigma Chemical Co. (Poole, Dorset). ONO-1505 (4-[2-(2-hydroxyethoxy)ethylamino]-2-(1*H*-imidazol-1-yl)-6-methoxyquinazoline methanesulphonate) was a generous gift from ONO Pharmaceutical Co., Ltd. (Osaka, Japan).

Statistics

Data are expressed as mean ± s.e.mean. Differences between paired means were assessed by Student's two-tailed, paired *t* test while a multiple comparison of unpaired means was conducted by one way ANOVA followed by Bonferroni's or Dunnett's test. Significance was accepted at the 5% level.

Results

Effects of DETCA on contraction

Contraction to NA was enhanced by DETCA pretreatment (Figure 1a and Table 1). L-NAME (0.3 mM) reversed this effect and furthermore, unmasked an inhibitory action of DETCA on contractility (Figure 1b). Similar results were obtained after removal of the endothelium (data not shown, $n=5$).

Table 1 Effect of pretreatment with diethyldithiocarbamate (DETCA, 10 mM for 30 min followed by 30 min washout) in rat isolated aorta, on contraction to noradrenaline (1 nM–1 μM) in the absence and presence of N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM) or exogenous Cu/Zn superoxide dismutase (SOD, 200 u ml^{-1})

		Control			*DETCA*		
Intervention	pD_2	*AUC*	$E_{max}(g)$	pD_2	*AUC*	$E_{max}(g)$	n
None	7.68 ± 0.08	2.3 ± 0.2	1.4 ± 0.1	7.97 ± 0.10*	3.1 ± 0.3*	1.7 ± 0.1*	4
L-NAME	8.41 ± 0.08††	4.9 ± 0.5††	2.1 ± 0.2†	8.34 ± 0.09*†	4.1 ± 0.4*	1.8 ± 0.2	5
SOD	7.35 ± 0.07†	1.8 ± 0.1	1.3 ± 0.1	7.52 ± 0.05*††	2.2 ± 0.2	1.5 ± 0.1	5

AUC = area under concentration-response curve. Values are mean ± s.e.mean. *$P<0.05$ with respect to corresponding control group; †$P<0.05$, ††$P<0.01$ with respect to no intervention within control or DETCA group.

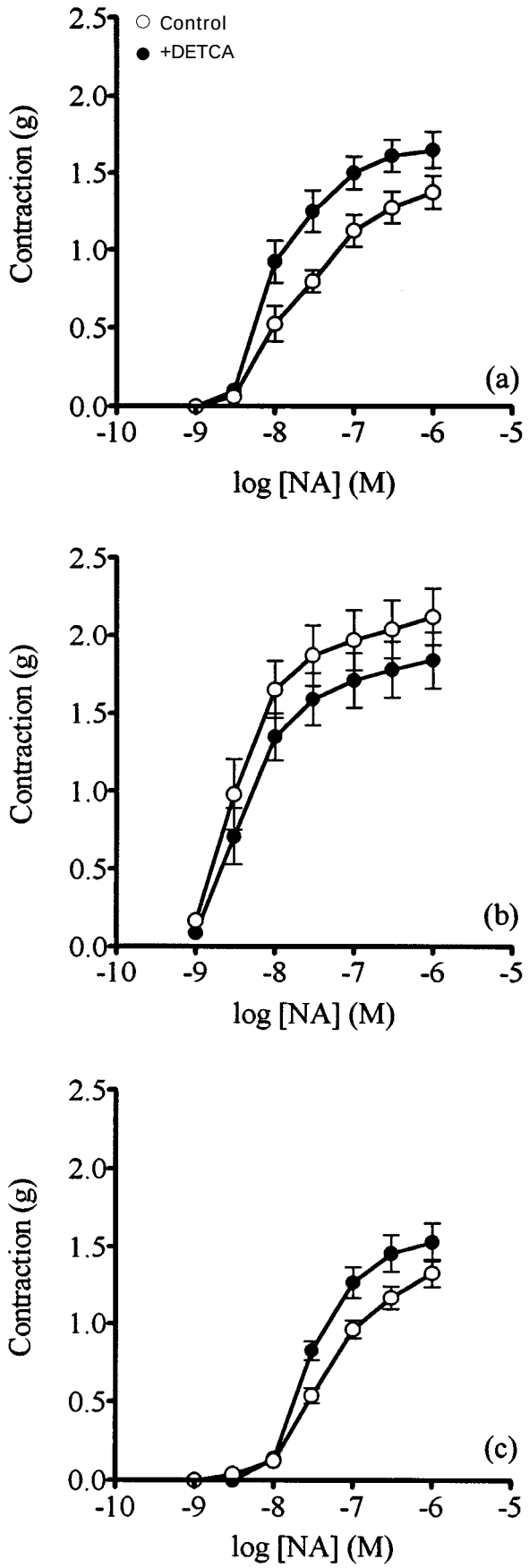

Figure 1 Effect of pretreatment with diethyldithiocarbamate (DETCA) (10 mM for 30 min followed by 30 min washout) in rat isolated aorta, on contraction to noradrenaline (NA) in the absence ($n=4$) (a) and presence of L-NAME (0.3 mM, $n=5$) (b) or exogenous Cu/Zn SOD (200 u ml^{-1}, $n=5$) (c). Values are mean and vertical lines show s.e.mean.

Exogenous SOD (200 u ml^{-1}) mitigated the DETCA-mediated enhancement in contraction to NA (1 nM – 1 μM) (Figure 1c and Table 1). In separate experiments, DETCA pretreatment prevented the action of L-NAME (0.3 mM) to augment equivalent, NA-established contractile tone (Figure 2).

In subsequent vasorelaxation studies, care was taken to obtain comparable levels of precontraction in matched control and DETCA-pretreated rings. NA elicited a contraction of 1.6 ± 0.1 g (at 30 nM) and 1.5 ± 0.1 g (at 100 nM) with and without DETCA, respectively, in the absence of L-NAME ($P>0.05$, $n=24$). Similarly, in the presence of L-NAME (0.3 mM), NA elicited a contraction of 1.3 ± 0.2 g (at 6 nM) and 1.4 ± 0.1 g (at 3.5 nM) with and without DETCA pretreatment, respectively ($P>0.05$, $n=5$).

Effects of DETCA on vasorelaxation

Vasorelaxation to ACh (Figure 3a) and SNAP (Figure 4a) was unaffected by DETCA pretreatment (Table 2), while, in contrast, vasorelaxation to ONO-1505 was depressed (Figure 3b). However, inhibition by DETCA pretreatment of vasorelaxation to SNAP became evident in the presence of L-NAME (0.3 mM) (Figure 4b and Table 2). In addition, L-NAME (0.3 mM) markedly potentiated vasorelaxation to SNAP in control rings (Figure 4). Furthermore, vasorelaxation to exogenous Cu/Zn SOD (200 u ml^{-1}), which was abolished by L-NAME (0.3 mM) (data not shown, $n=3$), was enhanced after DETCA pretreatment (Figure 5). This effect of DETCA was abolished by exogenous catalase (200 u ml^{-1}) (Figure 5), which did not exhibit vasoactivity *per se*. In additional studies further examining a role for H_2O_2 in control vasoactivity to SOD, catalase (200 u ml^{-1}) reduced vasorelaxation to SOD from $18.6\pm3.8\%$ to $8.5\pm1.4\%$ ($P=0.05$, $n=3$) in paired aortic rings.

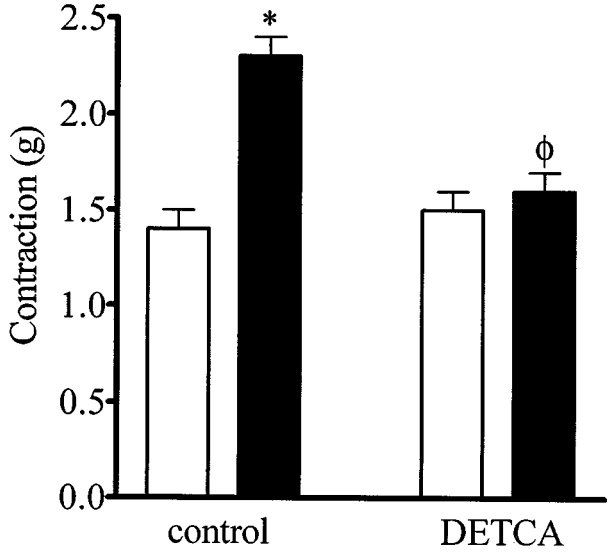

Figure 2 Effect of pretreatment with diethyldithiocarbamate (DETCA, 10 mM for 30 min followed by 30 min washout), on supplementary contraction to L-NAME (0.3 mM) (solid columns) in rat isolated aorta. DETCA-pretreated and control rings were equivalently precontracted with 30 and 100 nM noradrenaline, respectively. Values are mean$\pm$s.e.mean ($n=5$). *$P<0.05$ with respect to initial contraction (open columns) in control group; $\phi P<0.05$ with respect to L-NAME contraction in control group.

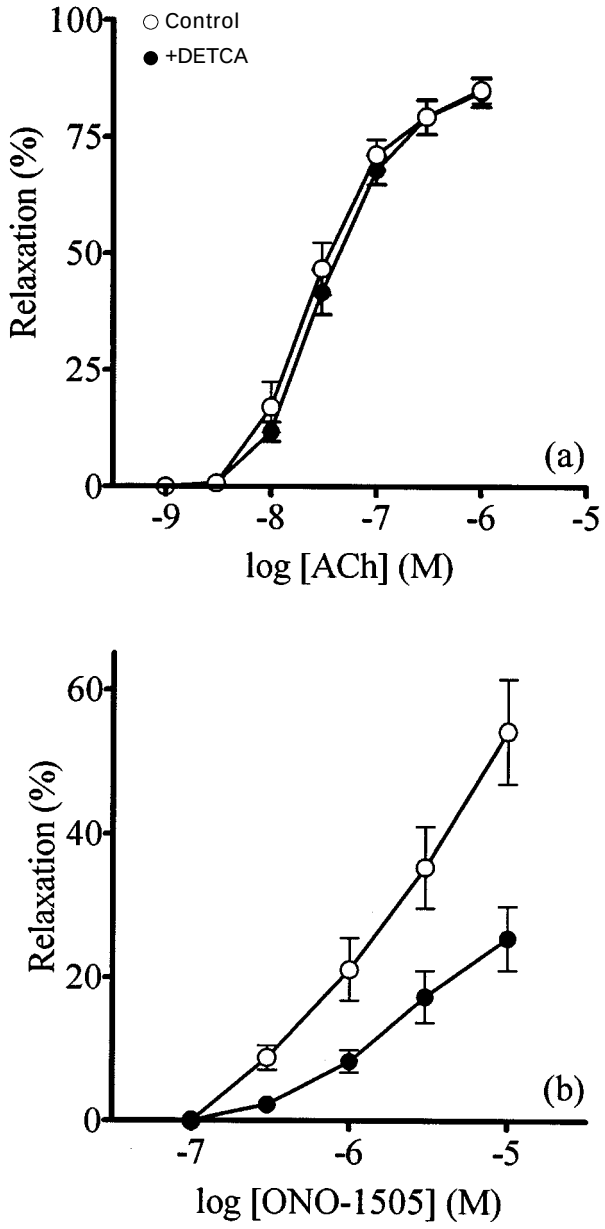

Figure 3 Effect of pretreatment with diethyldithiocarbamate (DETCA) (10 mM for 30 min followed by 30 min washout), on vasorelaxation to acetylcholine (ACh, $n=5$) (a) and ONO-1505 ($n=5$) (b) in noradrenaline-precontracted isolated aorta of the rat. Values are mean and vertical lines show s.e.mean.

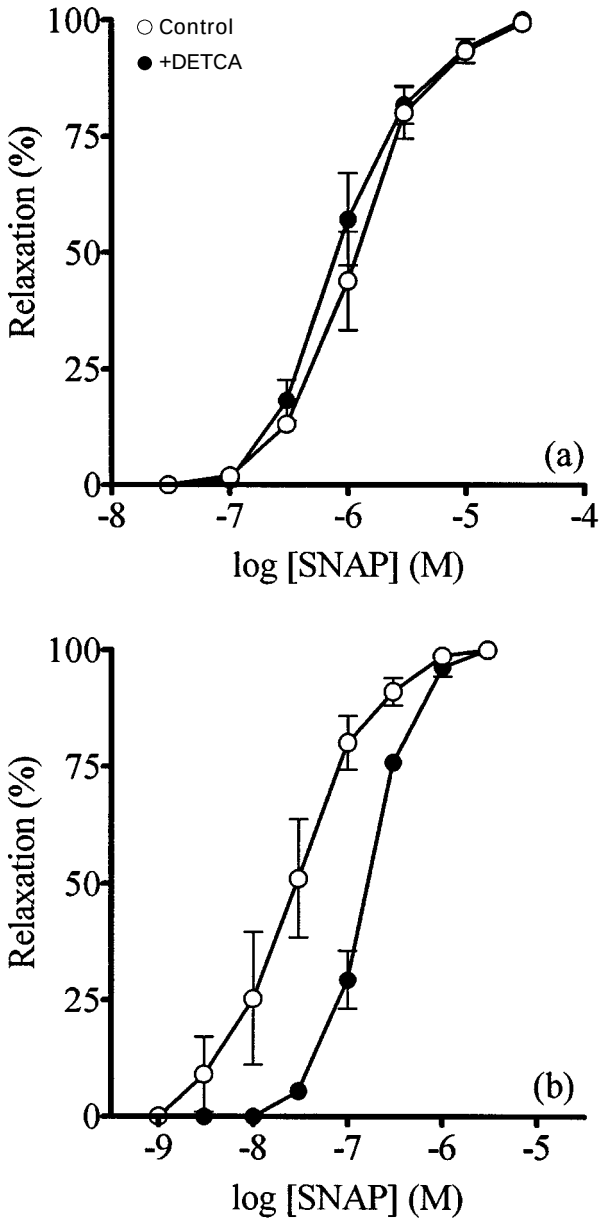

Figure 4 Effect of pretreatment with diethyldithiocarbamate (DETCA) (10 mM for 30 min followed by 30 min washout), on vasorelaxation to S-nitroso-N-acetyl penicillamine (SNAP) in the absence ($n=5$) (a) and presence of L-NAME (0.3 mM, $n=4$) (b), in noradrenaline-precontracted isolated aorta of the rat. Values are mean and vertical lines show s.e.mean.

Table 2 Effect of pretreatment with diethyldithiocarbamate (DETCA, 10 mM for 30 min followed by 30 min washout) in rat isolated aorta, on vasorelaxation to acetylcholine (ACh, 1 nM – 1 μM), ONO-1505 (0.1 – 10 μM) or S-nitroso-N-acetyl penicillamine (SNAP, 0.03 – 30 μM) in the absence or presence of N^G-nitro-L-arginine methyl ester (L-NAME, 0.3 mM)

		Control			*DETCA*		
Agent	pD_2	*AUC*	$E_{max}(\%)$	pD_2	*AUC*	$E_{max}(\%)$	n
ACh	7.44 ± 0.05	129.7 ± 7.7	90.6 ± 3.1	7.56 ± 0.10	122.8 ± 7.1	89.4 ± 3.5	5
SNAP	6.06 ± 0.10	140.0 ± 6.9	99.4 ± 0.6	5.93 ± 0.08	150.1 ± 9.4	100	5
ONO-1505	5.77 ± 0.04	46.8 ± 7.3	54.3 ± 7.3	5.83 ± 0.06	20.6 ± 4.0*	25.5 ± 4.4*	5
SNAP + L-NAME	7.37 ± 0.11††	182.4 ± 10.1††	100	6.79 ± 0.03**††	127.5 ± 3.2**	100	4

AUC = area under concentration-response curve. Values are mean ± s.e.mean. *$P<0.05$, **$P<0.01$ with respect to corresponding control group; †$P<0.05$, ††$P<0.01$ with respect to corresponding SNAP data without L-NAME.

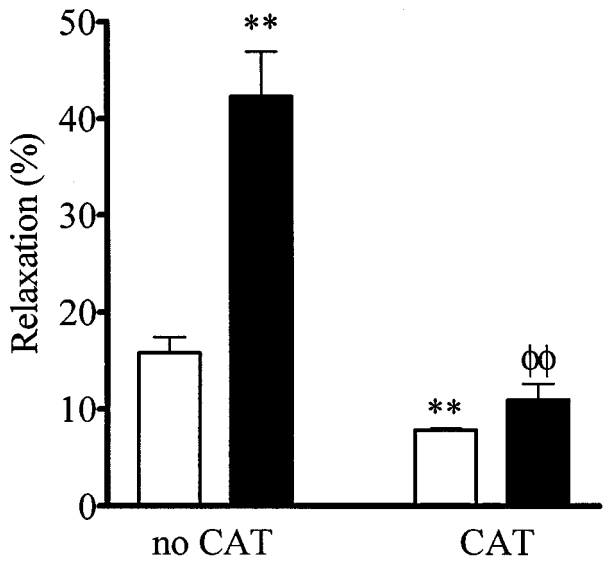

Figure 5 Effect of pretreatment with diethyldithiocarbamate (DETCA; solid columns) (10 mM for 30 min followed by 30 min washout), on vasorelaxation to exogenous Cu/Zn superoxide dismutase (200 u ml^{-1}) in the absence and presence of exogenous catalase (CAT, 200 u ml^{-1}) in noradrenaline-precontracted isolated aorta. of the rat Values are mean ± s.e.mean ($n=3-6$). $^{**}P<0.01$ with respect to control in absence of CAT; $\phi\phi P<0.01$ with respect to DETCA in absence of CAT.

Discussion

The experimental use of DETCA to generate endogenous superoxide anion stress, is well established in both visceral and vascular smooth muscle. In the present study, the L-NAME-reversible and exogenous Cu/Zn SOD-sensitive ability of DETCA to augment contraction to NA in rat aorta, indicating basal endothelial dysfunction, is consistent with the inactivation of endothelium-derived NO by enhanced endogenous superoxide anion levels, due to the inhibition of Cu/Zn SOD (Gryglewski *et al*., 1986; Rubanyi & Vanhoutte, 1986a,b; Omar *et al*., 1991; Mügge *et al*., 1991a; Mian & Martin, 1995a). The attenuation of the contraction to NA, observed after DETCA pretreatment in the presence of L-NAME, suggests that the true enhancement of vasoconstrictor reactivity due to Cu/Zu SOD inhibition is partially masked by a spasmolytic effect of DETCA. The failure of exogenous Cu/Zn SOD to reverse completely DETCA-induced effects has been noted by others (Mian & Martin, 1995a; Lilley & Gibson, 1996), and probably reflects its inability to permeate membranes and dismute intracellular superoxide.

A defect in basal NO vascular function after tissue Cu/Zn SOD inhibition was substantiated by both the limited ability of L-NAME pretreatment to augment subsequent contraction to NA and the failure of L-NAME to enhance significantly NA-established arterial tone (see Laight *et al*., 1996c). This latter finding was reflected in the reduced capacity of L-NAME to modulate responsiveness to higher concentrations of NA (see Figure 1a and b), demonstrating that the deficit in the regulation of vasoconstrictor reactivity by basal NO became more acute as the contractile stimulus increased. The residual endothelium- and basal NO-dependent vasorelaxation to ONO-1505 in the absence of a vasoconstrictor effect of L-NAME suggests that the nature of the superoxide-mediated lesion concerns a diminution in the functional availability of basal NO (Darley-Usmar *et al*., 1995; Darley-Usmar & White, 1997; Münzel *et al*., 1997), which may be partially rectified by enhancing NO-stimulated intracellular levels of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) following phosphodiesterase (PDE) type V inhibition (see Nicholson *et al*., 1991; Lugnier & Komas, 1993). The regulation of basal endothelial function and arterial tone by superoxide anion (see Lawson *et al*., 1990; Katusic, 1996) was implied by the ability of exogenous Cu/Zn SOD to both reduce contractility to NA and reverse established contractile tone in an NO-dependent manner. However, the attenuation of vasorelaxation to SOD by catalase indicates that a significant part of the vasoactivity of this enzymatic superoxide anion scavenger is due to a concomitant generation of H_2O_2, a ROS which has been shown to elicit NO-dependent vasorelaxation *per se* (Zembowicz *et al*., 1993; Mian & Martin, 1995b). Moreover, the greater vasorelaxation to SOD seen after DETCA pretreatment, which was completely reversed by exogenous catalase, is consistent with the enhanced extracellular generation of vasodilator H_2O_2 resulting from the dismutation of raised vascular levels of superoxide anion.

While increasing exposure to DETCA may lead to non-specific impairments in nitrovasodilator reactivity due to the generation of superoxide anion (Omar *et al*., 1991; Mügge *et al*., 1991a; Grunfeld *et al*., 1995; Plane *et al*., 1997), our experimental conditions resulted in a selective endothelial injury, i.e. basal NO dysfunction. This was confirmed in the present study by the DETCA-induced abrogation in vasorelaxtion to a PDE type V inhibitor, but not ACh. A differential sensitivity of basal and agonist-stimulated NO relaxation to inhibition by endogenous superoxide anion has also been documented in rat aorta by Mian & Martin (1995a). We hypothesized that this phenomenon might be explained by an antioxidant action of basal NO (see Kanner *et al*., 1991) to regulate superoxide anion levels following the inhibition of tissue Cu/Zn SOD, thereby protecting agonist-stimulated endothelial function. This notion was supported by the demonstration that DETCA pretreatment could depress vasorelaxation to SNAP once basal NO was removed by NOS inhibition. However, an ancillary finding with L-NAME was that vasorelaxation to SNAP was markedly enhanced in control vessels. This reflects a pronounced, tonic inhibition of nitrovasodilator reactivity by the endothelium, which is thought to be mediated by the desensitization of soluble guanylyl cyclase by basal NO (Shirasaki & Su, 1985; Busse *et al*., 1989; Flavahan & Vanhoutte, 1989; Kojda *et al*., 1994). While not excluding an antioxidant role of basal NO, this observation raises the possibility that a leftward shift in nitrovasodilator reactivity, arising as a consequence of the inactivation of basal NO by superoxide anion, could functionally mask a lesion in agonist-stimulated endothelial function.

In summary, the inhibition of tissue Cu/Zn activity in rat isolated aortic smooth muscle elicited a selective lesion in basal endothelial function, consistent with the inactivation of basal NO by superoxide anion. The partial reversal of DETCA effects by exogenous Cu/Zn SOD may involve not only the protection of basal NO, but also the extracellular generation of an NO-dependent vasodilator agonist, H_2O_2. Finally, the apparent insensitivity of agonist-stimulated endothelial function to disruption by superoxide anion stress may result, at least in part, from a compensatory leftward shift in nitrovasodilator reactivity due to a superoxide-mediated mitigation of tonic inhibition by basal NO. Our data therefore

suggest that the interaction between superoxide anion and basal NO modulates the endothelial regulation of both vasoconstrictor and nitrovasodilator reactivity; a phenomenon which may contribute to a relative preservation of agonist-stimulated endothelial function in diseases associated with vascular oxidant stress.

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