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Differential sensitivity of basal and acetylcholine-stimulated activity of nitric oxide to destruction by superoxide anion in rat aorta

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1 In this study we compared the ability of superoxide anion to destroy the relaxant activity of basal and acetylcholine (ACh)-stimulated activity of NO in isolated rings of rat aorta.

2 Superoxide dismutase (SOD, 1-300 u ml^{-1}) induced a concentration-dependent relaxation of phenylephrine (PE)-induced tone in endothelium-containing rings which was blocked by N^G-nitro-L-arginine (L-NOARG, 30 μM), but had no effect on endothelium-denuded rings. It was likely therefore that the relaxant action of SOD resulted from protection of basally produced NO from destruction by superoxide anion, generated either within the tissue or in the oxygenated Krebs solution.

3 In contrast, a concentration of SOD (50 u ml^{-1}) which produced almost maximal enhancement of basal NO activity, had no effect on ACh (10 nM-3 μM)-induced relaxation.

4 In the presence of catalase (3000 u ml^{-1}) to prevent the actions of hydrogen peroxide, superoxide anion generation using hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) produced an augmentation of PE-induced tone in endothelium-containing but not endothelium-denuded rings. This was likely to have resulted from removal of the tonic vasodilator action of basally-produced NO by superoxide anion, since it was blocked in tissues treated with SOD (250 u ml^{-1}), N^G-monomethyl-L-arginine (L-NMMA, 30 μM) or L-NOARG (30 μM). Pyrogallol (0.1 mM) had a similar action to HX/XO, but produced an additional augmentation of tone by an endothelium-independent mechanism.

5 In contrast to their ability to destroy almost completely the basal activity of NO, HX (0.1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 mM) had no effect on ACh-induced relaxation at any concentration. An increase in the concentration of HX to 1 mM or pyrogallol to 0.3 mM did, however, lead to a profound decrease in the magnitude and time course of ACh-induced relaxation at all concentrations.

6 Treatment with diethyldithiocarbamate (DETCA, 0.1 mM, 1 h) to inhibit endogenous Cu-Zn SOD, augmented PE-induced tone in endothelium-containing rings and abolished the ability of HX (0.1 mM)/XO (16 mu ml^{-1}) and L-NMMA (30 μM) to augment tone. It was likely that DETCA had led to the destruction of basal NO activity by increasing superoxide anion levels since its actions were reversed by exogenous SOD (10-300 u ml^{-1}).

7 In contrast to its ability to destroy basal activity of NO completely, DETCA (0.1 mM) produced only a slight blockade of ACh-induced relaxation. However, if these tissues were subsequently treated with concentrations of HX (0.1 mM)/XO (16 mu ml^{-1}) or pyrogallol (0.1 mM), which had no effect by themselves on ACh-induced relaxation, a profound blockade was seen and this was reversed completely with SOD (250 u ml^{-1}).

8 The data suggest that basal activity of NO is more sensitive to inactivation by superoxide anion than ACh-stimulated activity and this probably results from differential protection by endogenous Cu-Zn SOD. It is possible therefore that endogenous SOD lowers superoxide anion levels to such an extent that only small amounts of NO, such as those produced under basal conditions, are destroyed. Following generation of superoxide anion with HX/XO or pyrogallol, or inhibition of Cu-Zn SOD with DETCA, levels of the free radical will increase such that greater amounts of NO, such as those produced following stimulation with ACh, will then be destroyed.

Keywords: Nitric oxide; superoxide anion; superoxide dismutase; diethyldithiocarbamate; vasodilatation; acetylcholine; EDRF

Introduction

Endothelium-derived relaxing factor (EDRF) is a potent vasodilator produced by vascular endothelial cells (Furchgott & Zawadzki, 1980) and has recently been characterized as nitric oxide (NO) or a chemically related species (Palmer *et al.*, 1987; Myers *et al.*, 1989; 1990). Recently, the interaction between NO and superoxide anion has received a great deal of attention. This interaction leads to destruction of the vasodilator actions of EDRF and authentic nitric oxide in cascade bioassay systems (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986a; Furchgott *et al.*, 1990), induction of endothelium-dependent vasoconstriction in arterial rings from loss of the dilator actions of basally produced nitric oxide (Katusic & Vanhoutte, 1989; Ohlstein & Nichols, 1989), and production of a cytotoxic oxidant, peroxynitrite (Beckman *et al.*, 1990). This widespread potential for loss of NO-induced vasodilatation and production of peroxynitrite following the reaction of NO with superoxide anion has led to the suggestion that this process contributes to a number of pathological situations such as hypertension (Wei *et al.*, 1985), ischaemia-reperfusion injury (Downey, 1990), hypercholesterolaemia/ atherosclerosis (Minor *et al.*, 1990), diabetes (Langenstroer & Pieper, 1992) and cytotoxic brain injury (Lipton *et al.*, 1993).

In vascular endothelium, the production of NO is subject to complex control; it is stimulated by a large number of biological mediators such as acetylcholine (ACh; Furchgott & Zawadzki, 1980) and by the physical shearing force of flowing

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blood (Rubanyi *et al.*, 1986). In addition, there is a basal production of NO which exerts a tonic vasodilator action on endothelium-containing arterial rings (Eglème *et al.*, 1984; Martin *et al.*, 1986; Martin, 1988). Recent reports have suggested differences in the effects of drugs on basal and agonist-stimulated activity of NO. For example, in the perfused vascular bed of the rabbit ear, N^G-nitro-L-arginine methyl ester (L-NAME) inhibits both basal and ACh-induced activity of NO, but only in the former case is blockade reversed by L-arginine (Randall & Griffith, 1991). Furthermore, in the isolated coronary artery of the greyhound, the inhibitor of acetyl-coA lysolecithin acyltransferase, thimerosal, has a complex action, transiently producing endothelium-dependent relaxation and then blocking agonist-stimulated but not basal production of NO (Crack & Cocks, 1992). Furthermore, in the rat aorta N^G-monomethyl-L-arginine (L-NMMA) selectively inhibits basal but not agonist-stimulated production of NO (Frew *et al.*, 1993). The ability of superoxide dismutase (SOD) to potentiate the effects of basal NO in a cascade bioassay or arterial rings (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986a; Ohlstein & Nichols, 1989; Langenstroer & Pieper, 1992), but to have no effect on the relaxant actions of ACh in arterial rings (Abrahamsson *et al.*, 1992), suggests differential actions of superoxide anion on basal and agonist-stimulated activity of NO.

In this study, we wished to determine if basal and ACh-stimulated activity of NO in rat aorta were equally sensitive to destruction by superoxide anion. The effects of superoxide anion were assessed either by generating the free radical by use of the hypoxanthine/xanthine oxidase (HX/XO) system or the drug, pyrogallol, or by increasing the background level of the free radical by inhibiting the endogenous Cu-Zn form of SOD with diethyldithiocarbamate (DETCA; Heikkila *et al.*, 1976; Cocco *et al.*, 1981).

Methods

Preparations of aortic rings

Female Wistar rats of approximately 200–250 g were killed by stunning and exsanguination. The thoracic aorta was removed and cut into 2.5 mm wide transverse rings with a razor blade slicing device. In some experiments, the endothelium was removed by locating the aortic ring between two stainless steel hooks, placing a 2 g weight on the bottom hook and gently rubbing the intimal surface with a moist matchstick for 10–20 s. Endothelial denudation was deemed successful if no relaxation took place in response to ACh (1 μM).

Tension recording

The aortic rings were mounted under 1 g resting tension on stainless steel hooks in 20 ml organ baths maintained at 37°C containing Krebs solution (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24, and glucose 11 and gassed with 95% O_2 and 5% CO_2. Tension was recorded isometrically by means of Grass FT03C transducers and responses were displayed on a Grass polygraph model 7. Tissues were allowed to equilibrate for 90 min before experiments were carried out, during which time the resting tension was re-adjusted to 1 g, as required.

Experimental protocols

Basal activity of nitric oxide (NO) was assessed indirectly by measuring the endothelium-dependent depression of phenylephrine (PE)-induced vasoconstriction (Martin *et al.*, 1986). The rationale for these experiments is that basal production of NO in endothelium-containing rings of rat aorta exerts a tonic vasodilator action opposing the effects of vasoconstrictor agents. Consequently, this endothelium-dependent depression of vasoconstriction will be blocked or enhanced by agents which block or enhance the activity of NO, respectively. In these experiments, endothelium-containing rings were contracted with PE (20 nM) and when the contraction had stabilized, the effects of the superoxide anion generators, hypoxanthine (HX, 0.1 mM)/xanthine oxidase (16 mu ml^{-1}) and pyrogallol (0.1 mM), the superoxide anion scavenger, superoxide dismutase (SOD, 250 u ml^{-1}), or the inhibitors of NO synthase, N^G-monomethyl-L-arginine (L-NMMA, 30 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM), were examined on tone. In the early experiments with HX/XO, it was clear that a delayed relaxant effect was produced resulting from the generation of hydrogen peroxide. Consequently, where indicated in the results, experiments were performed in the presence of catalase (3000 u ml^{-1}) to prevent the accumulation of hydrogen peroxide. In certain experiments, the effects of inhibiting endogenous Cu-Zn SOD with the copper chelator, diethyldithiocarbamate (DETCA; Heikkila *et al.*, 1976; Cocco *et al.*, 1981) were also investigated on PE-induced tone. In these experiments, aortic rings were incubated for 1 h with DETCA (0.1 mM) before the contractile actions of PE (20 nM) were examined. Each of the above experiments was also conducted in endothelium-denuded rings but the concentration of PE used was lowered to 1–3 nM so as to attain a similar degree of tone to that obtained in endothelium-containing rings.

Agonist-stimulated activity of NO was determined by assessing ACh-induced relaxation. Cumulative concentration-response curves to ACh (10 nM–3 μM) were constructed in endothelium-containing rings following induction of submaximal PE (30–100 nM)-induced tone. The baths were washed out, and the tissues allowed to re-equilibrate. In experiments in which the effects of HX (0.1 or 1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 or 0.3 mM) were to be studied on ACh-induced relaxation, we ensured that the level of tone prior to inducing relaxation was similar to that of untreated preparations. In order to achieve this, tissues were initially precontracted with lower concentrations of PE (2–30 nM). Subsequent addition of the free radical generators produced a further elevation of tone in endothelium-containing tissues by destroying basal NO activity, and when the contraction had stabilized, a further cumulative concentration-response curve to ACh was obtained. In studies involving inhibition of endogenous Cu-Zn SOD, the rings were incubated with DETCA for 1 h. Various concentrations of DETCA were tested, and 0.1 mM was chosen for most experiments since it produced a sub-maximal (25–30%) inhibition of ACh-induced relaxation by itself. Cumulative concentration-response curves to ACh (10 nM–3 μM) in the

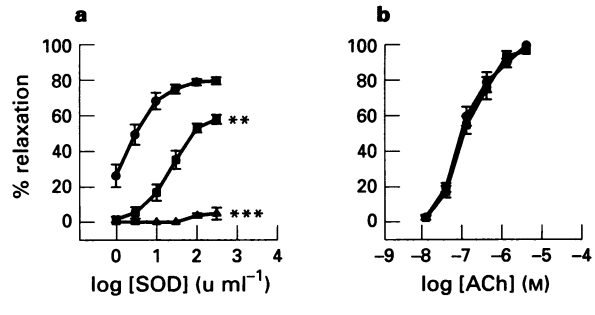

Figure 1 (a) Concentration-response curves showing relaxation to superoxide dismutase (SOD, 1–300 u ml^{-1}) on phenylephrine-contracted endothelium-containing (●) and endothelium-denuded (▲) rings of rat aorta and blockade of relaxation following inhibition of endogenous Cu-Zn SOD with diethyldithiocarbamate (DETCA, 0.1 mM, 1 h, ■). (b) Concentration-response curves showing relaxation to acetylcholine (ACh, ●) and the inability of SOD (50 u ml^{-1}, ■) to affect this relaxation. Each point is the mean ± s.e.mean of 6 observations. $^{**}P < 0.005$ and $^{***}P < 0.001$ indicate a significant difference from maximal relaxation in untreated endothelium-containing rings, respectively.

presence of HX (0.1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 mM) were also constructed in DETCA-treated rings. All experiments involving HX/XO, pyrogallol or DETCA were conducted in the presence of catalase (3000 u ml^{-1}), added as 5 min pretreatment to prevent accumulation of hydrogen peroxide. In some experiments, the ability of exogenously added SOD to protect against the inhibitory effects of HX/XO, pyrogallol or DETCA was studied and, in these, it was added as a 20 min pretreatment. Responses to ACh were expressed as a percentage (%) relaxation of the PE-induced tone.

Drugs

Acetylcholine chloride, catalase (bovine liver), diethyldithiocarbamate, N^G-nitro-L-arginine, phenylephrine hydrochloride, hypoxanthine, superoxide dismutase (bovine erythrocyte, Cu-Zn-containing) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, Dorset), whilst pyrogallol was obtained from BDH (Poole, Dorset). N^G-monomethyl-L-arginine was a gift from Wellcome Laboratories (Beckenham, Kent). All drugs were dissolved and dilutions made in saline (0.9%), except for hypoxanthine which was dissolved in 0.1% sodium hydroxide.

Statistical analysis

Results are expressed as the mean ± s.e.mean for n separate experiments and comparisons were made by one-way analysis of variance followed by Fisher's test. A probability of 0.05 or less was considered significant.

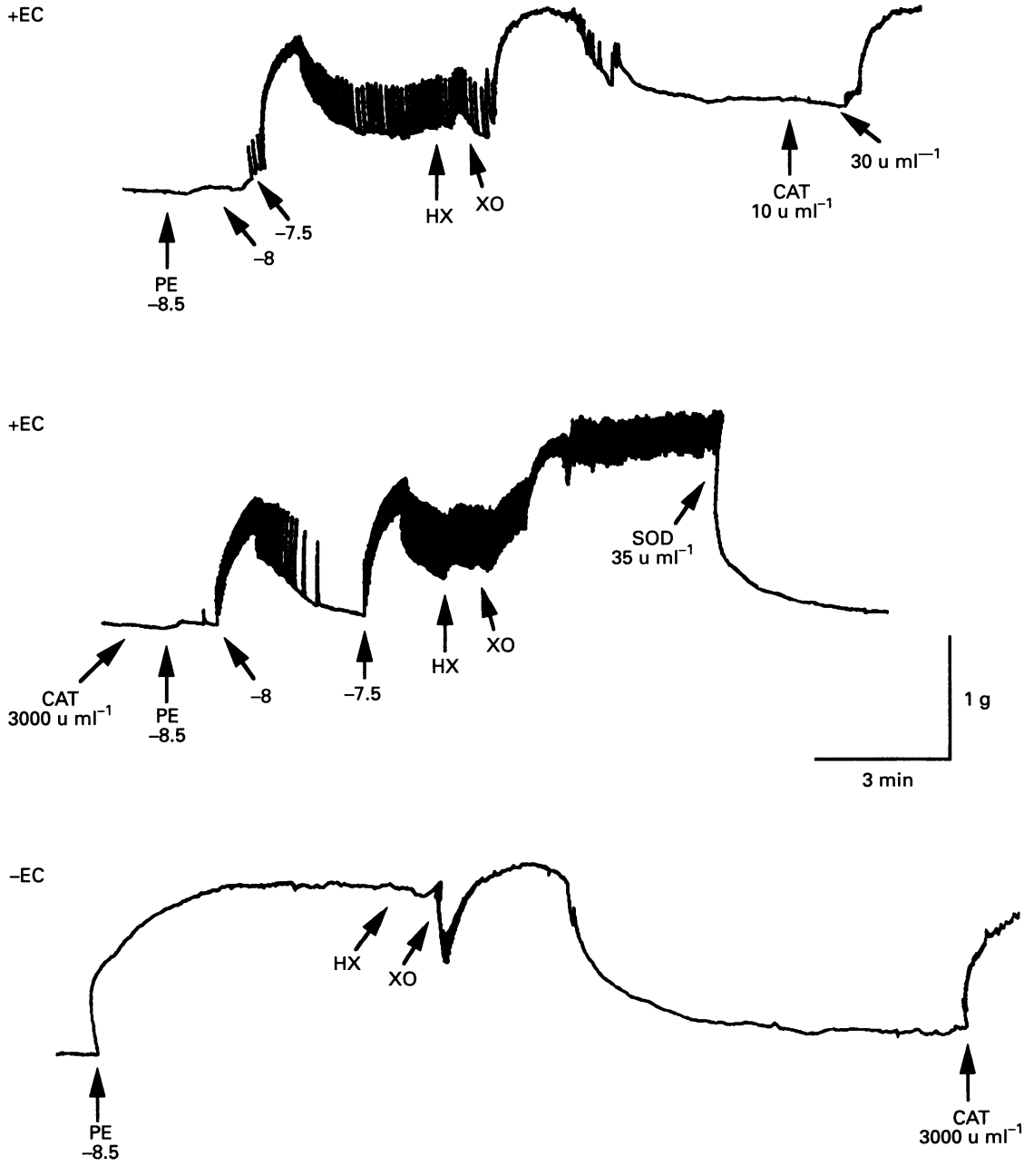

Figure 2 Individual experimental tracings showing two separate effects of hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) on rings of rat aorta; an immediate rise in phenylephrine (PE)-induced tone in endothelium-containing (+EC) but not endothelium-denuded (–EC) rings which was reversed by superoxide dismutase (SOD, 35 u ml^{-1}), and a delayed fall in tone in both endothelium-containing and endothelium-denuded rings which was inhibited by catalase (CAT, 30–3000 u ml^{-1}). Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}.

Results

Effects of superoxide dismutase on basal and ACh-stimulated activity of NO

Following induction of phenylephrine (PE, 30–100 nM)-induced tone (1.60±0.10 g, $n=8$) in endothelium-containing rings of rat aorta, superoxide dismutase (SOD, 1–300 u ml^{-1}) produced a powerful concentration-dependent relaxation (maximum relaxation 79.9±2.0%, Figure 1a, Figure 3a). In endothelium-denuded rings, lower concentrations of PE (3–10 nM) were required to induce a similar degree of tone (1.54±0.15 g, $n=6$) to endothelium-containing rings, but in these, SOD produced no relaxation. Pretreatment with L-NOARG (30 μM) for 10 min to inhibit basal NO synthesis blocked SOD-induced relaxation in endothelium-containing rings (data not shown). Treatment of endothelium-containing rings with DETCA (0.1 mM) for 1 h to inactivate endogenous Cu-Zn SOD led to a 30±1.4% reduction in the maximal relaxation induced by SOD (Figure 1a). In endothelium-containing rings pretreated with SOD (50 u ml^{-1}), higher concentrations of PE (100–300 nM) were required to induce a similar degree of tone (1.08±0.05 g, $n=6$) to untreated rings (1.12±0.08 g, $n=6$) contracted with PE (30–100 nM) but ACh (10 nM–3 μM)-induced relaxation was unaffected (Figure 1b).

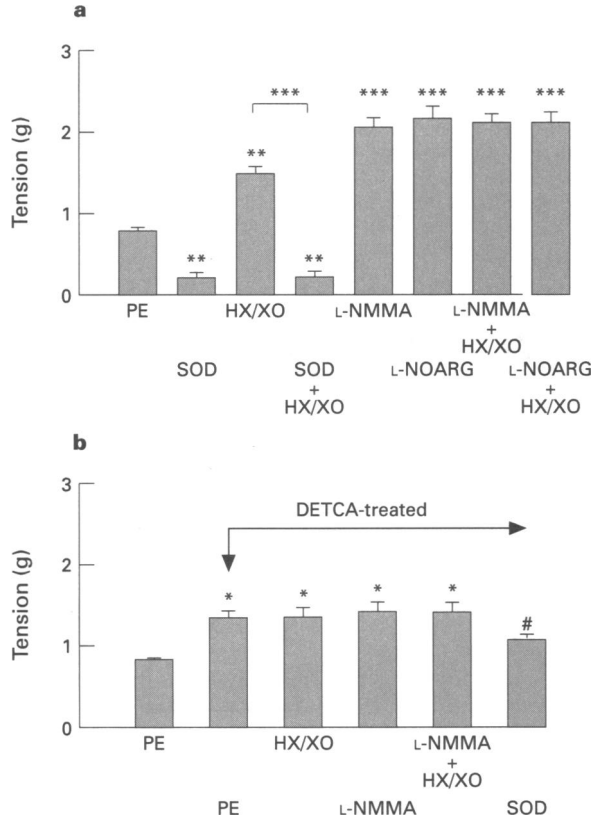

Figure 3 (a) Hypoxanthine (HX, 0.1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}), N^G-monomethyl-L-arginine (L-NMMA, 30 μM) and N^G-nitro-L-arginine (L-NOARG, 30 μM) all potentiated phenylephrine (PE, 20 nM)-induced tone in endothelium-containing rings of rat aorta. Superoxide dismutase (SOD, 250 u ml^{-1}) relaxed PE-induced tone and prevented the potentiation induced by HX/XO. Furthermore, following treatment with L-NMMA or L-NOARG, subsequent treatment with HX/XO fail to enhance tone further. (b) Diethyldithiocarbamate (DETCA, 0.1 mM, 1 h) enhanced PE (20 nM)-induced tone in endothelium-containing rings of rat aorta and prevented the ability of HX (0.1 mM)/XO (16 mu ml^{-1}) and L-NMMA (30 μM) to potentiate tone. SOD (250 u ml^{-1}) produced only a slight relaxation of PE-induced tone in DETCA-treated endothelium-containing rings. All experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the relaxant effects of HX/XO. Each column is the mean±s.e.mean of 6 observations. $^*P<0.05$ $^{**}P<0.005$ and $^{***}P<0.001$ indicate a significant difference from rings receiving PE only. $^\#P<0.05$ indicates a significant difference from DETCA-treated endothelium-containing rings receiving PE only.

Effects of hypoxanthine/xanthine oxidase (HX/XO) on basal and ACh-stimulated activity of NO

HX (0.1 mM)/XO (16 mu ml^{-1}) produced two effects on rings of rat aorta; an immediate rise in PE-induced tone in endothelium-containing but not endothelium-denuded rings which was blocked by SOD (Figure 2, Figure 3a), and a delayed fall in both endothelium-containing and endothelium-denuded rings which was blocked by catalase (30–3000 u ml^{-1}, Figure 2). All subsequent experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the relaxant actions of HX/XO. Treatment of endothelium-containing rings with L-NMMA (30 μM) or L-NOARG (30 μM) to block basal NO synthesis potentiated PE-induced tone and blocked the ability of HX/XO to enhance tone (Figure 3a). Furthermore, treatment for 1 h with DETCA (0.1 mM) enhanced PE-induced tone in endothelium-containing rings (Figure 3b) and prevented the ability of HX/XO or L-NMMA to potentiate tone (Figure 3b). Treatment with HX/XO, L-NMMA, L-NOARG or DETCA had no effect on PE-induced tone in endothelium-denuded rings of rat aorta (data not shown).

In the presence of HX (0.1 mM)/XO (16 mu ml^{-1}), lower concentrations of PE (10–30 nM) were required to induce a similar degree of tone (1.35±0.08 g) to that of control rings (1.42±0.10 g) contracted with PE (30–100 nM). However, HX/XO had no effect on relaxations produced by ACh at any concentration in endothelium-containing rings (Figure 4, Figure 5a). Increasing the concentration of HX to 1 mM did, however, lead to a profound blockade of ACh-induced relaxation (Figure 4, Figure 5a). HX (1 mM)/XO (16 mu ml^{-1}) also reduced the duration of relaxation (Figure 4). Furthermore, treatment with DETCA (0.1 mM, 1 h) to inhibit endogenous Cu-Zn SOD led to a partial inhibition of ACh-induced relaxation by itself and potentiated the ability of HX (0.1 mM)/XO (16 mu ml^{-1}) to block ACh-induced relaxation (Figure 5b). The blockade of ACh-induced relaxation by HX (1 mM)/XO (16 mu ml^{-1}) alone or by HX (0.1 mM)/XO (16 mu ml^{-1}) in DETCA-treated tissues was prevented by pretreatment with exogenous SOD (250 u ml^{-1}). The ability of DETCA alone to inhibit ACh-induced relaxation was, however, only partially reversed by exogenous SOD (250 u ml^{-1}, data not shown).

Effects of pyrogallol on basal and ACh-stimulated activity of NO

Pyrogallol (0.1 mM) produced an immediate rise in PE-induced tone in both endothelium-containing and endothelium-denuded rings but the rise was significantly greater in the former (Figure 6). Pretreatment with SOD (250 u ml^{-1}) partially blocked the rise in tone induced by pyrogallol in endothelium-containing but had no effect in endothelium-denuded rings (Figure 6). Pyrogallol, like HX/XO, also produced a delayed fall in tone in both endothelium-containing and endothelium-denuded rings which was blocked by catalase (100–3000 u ml^{-1}). Consequently, all experiments with this agent were conducted in the presence of catalase (3000 u ml^{-1}).

In the presence of pyrogallol (0.1 mM), lower concentrations of PE (2–20 nM) were required to induce a similar degree of tone (1.49±0.08 g) to that of control rings (1.56±0.07 g) contracted with PE (30–100 nM). Pyrogallol had no effect, however, on relaxations produced by ACh at any concentration in endothelium-containing rings (Figure 7a). Increasing

the concentration of pyrogallol to 0.3 mM did, however, lead to a profound blockade of ACh-induced relaxation (Figure 7a) and this also reduced the duration of relaxation (not shown). Furthermore, treatment with DETCA (0.1 mM, 1 h) potentiated the ability of pyrogallol (0.1 mM) to block ACh-induced relaxation (Figure 7b). The blockade by pyrogallol (0.3 mM) alone or by pyrogallol (0.1 mM) in DETCA-treated tissues was prevented by pretreatment with exogenous SOD (250 u ml^{-1}).

Discussion

It is well established that exogenously added SOD potentiates both basal and agonist-stimulated activity of NO in cascade bioassay systems (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986a). In isolated arterial rings SOD also potentiates basal activity (Ohlstein & Nichols, 1989; Langenstroer & Pieper, 1992; this study) but, surprisingly, it has no effect on agonist-stimulated activity of NO (Abrahamsson *et al.*, 1992;

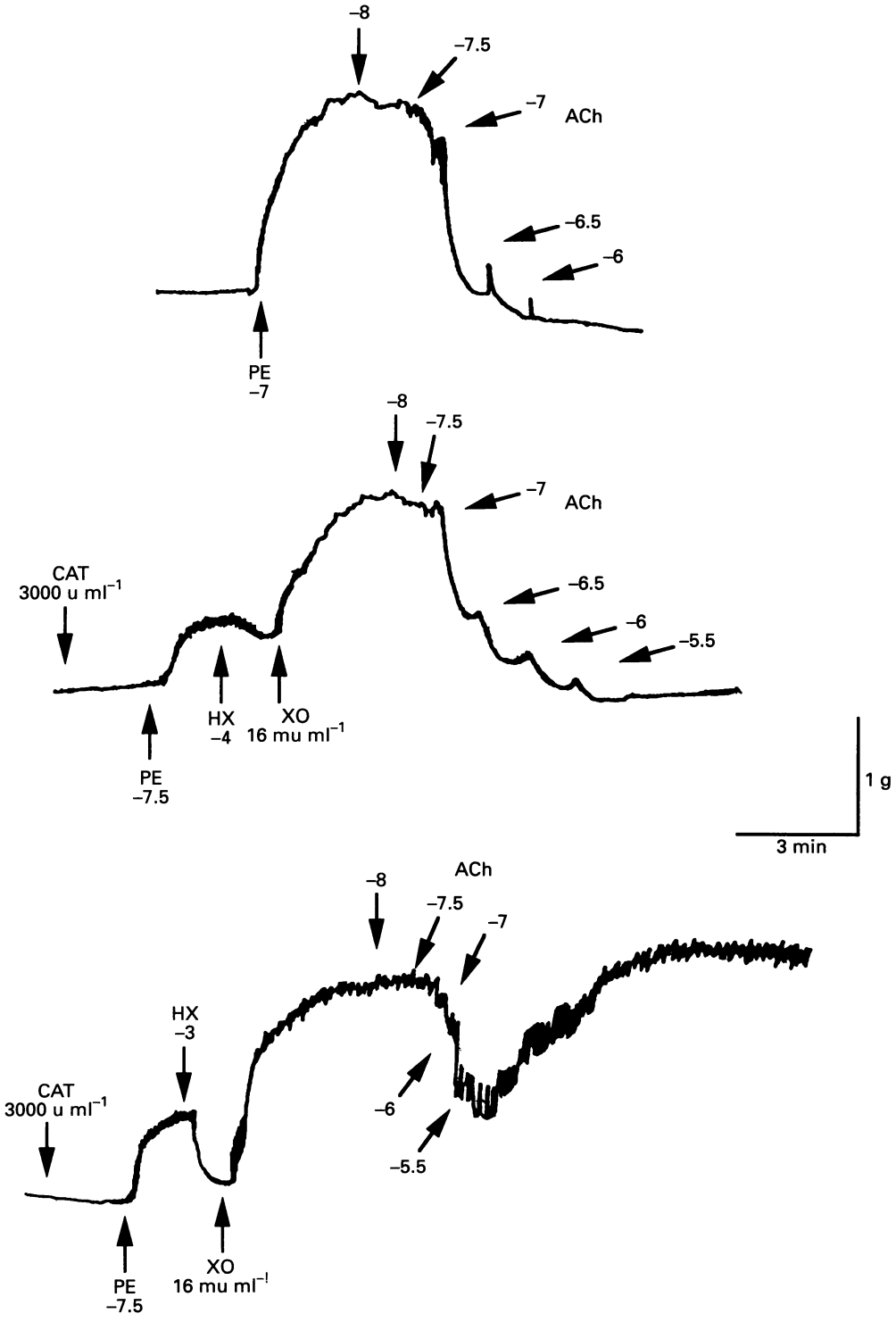

Figure 4 Individual experimental tracings showing the ability of hypoxanthine (HX, 0.1 mM and 1 mM)/xanthine oxidase (XO, 16 mu ml^{-1}) to inhibit acetylcholine (ACh)-induced relaxation in phenylephrine (PE)-contracted endothelium-containing rings of rat aorta. All experiments were conducted in the presence of catalase (CAT, 3000 u ml^{-1}) to prevent the relaxant effects of HX/XO. Drug concentrations are expressed in log molar units and enzyme concentrations are in units ml^{-1}.

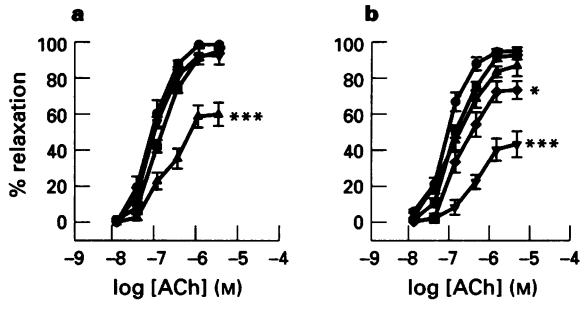

Figure 5 (a) Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta, blockade of relaxation by hypoxanthine (HX, 1 mM)/xanthine oxidase (XO, 16 u ml^{-1}, ▲) but no blockade by HX (0.1 mM)/XO (16 mu ml^{-1}, ■), and protection against blockade by the former by superoxide dismutase (250 u ml^{-1}, ▼). (b) Concentration-response curves to ACh (●) and the inability of HX (0.1 mM)/XO (16 m u ml^{-1}, ■) to block this relaxation. Following inhibition of endogenous Cu-Zn SOD with diethyldithiocarbamate (DETCA, 0.1 mM, 1 h, ◆) ACh-induced relaxation was partially inhibited and subsequent treatment with HX/XO (▼) now produced a profound blockade. Furthermore, this blockade was prevented by exogenous SOD (250 u ml^{-1}, ▲). All experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the relaxant effects of HX/XO. Each point is the mean ± s.e.mean of 5–12 observations. *$P<0.05$, and ***$P<0.001$ indicate a significant difference from maximal relaxation in untreated rings, respectively.

Figure 7 (a) Concentration-response curves showing relaxation to acetylcholine (ACh, ●) on phenylephrine (PE)-contracted endothelium-containing rings of rat aorta, blockade of relaxation by pyrogallol 0.3 mM (▲) but no blockade by 0.1 mM (■), and protection against blockade by the former by superoxide dismutase (250 u ml^{-1}, ▼). (b) Concentration-response curves to ACh (●) and the inability of pyrogallol (0.1 mM, ■) to block this relaxation. Following inhibition of endogenous Cu-Zn SOD with diethyldithiocarbamate (DETCA, 0.1 mM, 1 h, ▲) ACh-induced relaxation was partially inhibited and subsequent treatment with pyrogallol (▼) now produced a profound blockade. Furthermore, this blockade was prevented by exogenous SOD (250 u ml^{-1}, ◆). All experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the relaxant effects of pyrogallol. Each point is the mean ± s.e.mean of 6–12 observations. *$P<0.05$ and ***$P<0.001$ indicate a significant difference from maximal relaxation in untreated rings, respectively.

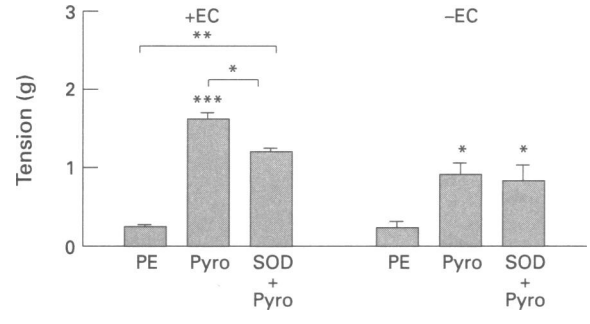

Figure 6 Augmentation of phenylephrine (PE)-induced tone by pyrogallol (Pyro, 0.1 mM) in endothelium-containing (+EC) and endothelium-denuded (–EC) rings of rat aorta and partial blockade of this in endothelium-containing but not endothelium-denuded rings by superoxide dismutase (SOD, 250 u ml^{-1}). All experiments were conducted in the presence of catalase (3000 u ml^{-1}) to prevent the relaxant effects of pyrogallol. The concentrations of PE in endothelium-containing (20 nM) and endothelium-denuded (1 nM) rings were chosen to produce equal levels of tone (0.26 ± 0.02 g and 0.26 ± 0.06 g, respectively). Each column is the mean ± s.e.mean of 6–9 observations. *$P<0.05$, **$P<0.005$ and ***$P<0.001$ indicate a significant difference from rings receiving PE only, or between groups joined by brackets.

this study). Clearly, the differential effects of SOD in cascade bioassay systems and arterial rings need to be explained. In the present study we attempted to determine if SOD and superoxide anion have differential effects on basal and agonist-stimulated activity of NO in rat aorta.

We found that SOD produced a powerful concentration-dependent relaxation of endothelium-containing rings of rat aorta but had no effect on endothelium-denuded rings. Relaxation appeared to be mediated by NO since it was blocked by L-NOARG, an inhibitor of NO synthesis (Moore *et al.*, 1990). The relaxant effects of SOD are therefore likely to have arisen from removal of superoxide anions, generated either within the tissue or in the oxygenated Krebs solution, which were destroying basally produced NO. Surprisingly, we found that a concentration of SOD (50 u ml^{-1}) which induced almost maximal potentiation of basal NO activity had absolutely no effect on ACh-induced relaxation. The background level of superoxide anion in the oxygenated tissue was therefore exerting a selective destructive action on basal but not agonist-stimulated activity of NO.

This selective action was investigated further by generating superoxide anion with HX/XO and pyrogallol. These experiments were complicated by the fact that superoxide anion-generating systems also give rise to other reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radical and peroxynitrite which can damage the endothelium and thereby impair endothelium-dependent relaxations (Kvietys *et al.*, 1989; Beckman *et al.*, 1990; Todoki *et al.*, 1992; Dowell *et al.*, 1993). We wished to examine specifically the interaction between NO and superoxide anion and, consequently, catalase (3000 u ml^{-1}) was included in all experiments in order to remove H_2O_2. The use of catalase successfully prevented the occurrence of any damage to the endothelium as seen by our ability to generate reproducible concentration-response curves to ACh in the presence of HX/XO and pyrogallol. We also found that the delayed relaxant action of superoxide anion generators resulting from the production of H_2O_2, which both increases the liberation of NO (Rubanyi & Vanhoutte, 1986b) and mediates direct vascular muscle relaxation (Burke & Wolin, 1987; Zembowicz *et al.*, 1993), was also abolished by catalase. In the presence of catalase, HX (0.1 mM)/XO (16 mu ml^{-1}) produced an immediate and sustained increase in PE-induced tone in endothelium-containing but not endothelium-denuded rings. The increase in PE-induced tone was not seen if the tissues had been pretreated with SOD or the inhibitors of NO synthase, L-NMMA or L-NOARG, and is consistent with the previously reported endothelium-dependent vasoconstrictor action of superoxide anion (Ohlstein & Nichols, 1989; Katusic & Vanhoutte, 1989) through destruction of basal NO. Pyrogallol (0.1 mM), however, enhanced PE-induced tone in both endothelium-containing and endothelium-denuded rings, although that obtained in the former was greater. The endothelium-dependent component, as with HX/XO, was absent in tissues pretreated with SOD and is therefore also likely to have been due to destruction of basal NO by superoxide anion. The origin of the endothelium-independent augmentation of tone by pyrogallol is unclear, but it may be unrelated to the ability of the drug to generate superoxide anion since it was unaffected by SOD.

A major novel finding of this study, however, was that despite almost completely blocking basal NO activity, as indicated by the enhancement of PE-induced tone which was only further enhanced slightly by L-NMMA, HX (0.1 mM)/XO (16 mu ml^{-1}) and pyrogallol (0.1 mM) had no effect on ACh-induced relaxation. Thus, these experiments together with those demonstrating SOD-induced relaxation, clearly highlighted a greater sensitivity of basal than agonist-stimulated activity of NO to destruction by superoxide anion. What was less clear at this stage, however, was whether the data suggested a chemical difference between basal and agonist-stimulated NO. Indeed, some workers have proposed that basal EDRF is free NO, while that released in response to agonists comes from a pre-formed store (Ignarro, 1991; Cocks & Angus, 1991) of a stable NO-releasing molecule such as an S-nitrosothiol (Myers *et al.*, 1990).

Although our study indicated a difference in the susceptibility to destruction by superoxide anion, the difference was one of degree rather than being absolute since generating higher levels of superoxide anion by employing higher concentrations now led to the magnitude and duration of ACh-induced relaxation being greatly inhibited. As in previous studies (Wei *et al.*, 1985; Rubanyi & Vanhoutte, 1986b; Abrahamsson *et al.*, 1992), the inhibition was likely to have occurred as a consequence of destruction of agonist-stimulated NO by superoxide anion since it was blocked by SOD. Thus, it appeared that higher concentrations of superoxide anion were required to destroy agonist-stimulated than basal NO.

It was possible that the greater sensitivity of basal than agonist-stimulated NO to destruction might reflect differential protection by endogenous SOD and this was tested by inhibiting the enzyme. There are two major forms of this enzyme, a Cu-Zn-containing form which is located both extracellularly and intracellularly and a Mn-containing form which resides mainly in mitochondria (Hassan, 1988). The copper chelator, DETCA, inhibits the Cu-Zn-containing form of the enzyme both intracellularly and extracellularly (Kelner *et al.*, 1989), leading to increased levels of superoxide anion, as detected by lucigenin-elicited chemiluminescence (Cherry *et al.*, 1990; Omar *et al.*, 1991). Previous studies have shown that treatment of cultured endothelial cells in a cascade bioassay with DETCA led to loss of NO activity and this was likely to have resulted from destruction rather than reduced synthesis of NO since the release of total nitrogen oxides, as measured by chemiluminescence, was unaffected (Mügge *et al.*, 1991). We found that treatment with DETCA (0.1 mM) for 1 h enhanced the sensitivity of endothelium-containing but not endothelium-denuded rings to PE. This augmentation is likely to have occurred from the complete loss of basal activity of NO since HX/XO and L-NMMA subsequently failed to enhance PE-induced tone in these tissues. The restoration of basal NO activity following addition of SOD to DETCA-treated tissues, albeit reduced in comparison to control tissues, indicated that the loss had occurred as a consequence of destruction by the greater steady-state levels of superoxide anion. Furthermore, in these experiments where treatment with DETCA had led to a complete loss of basal activity of NO, ACh-induced relaxation was blocked only slightly, further strengthening the view that basal and agonist-stimulated activity are differentially sensitive to destruction. Others have reported that increasing the concentration of DETCA can lead to almost complete loss of ACh-induced relaxation (Mügge *et al.*, 1991; Omar *et al.*, 1991) and we have confirmed this, but such inhibition is only partially reversed by SOD so may be mainly due to destruction intracellularly in endothelial cells where exogenous SOD cannot penetrate. Consistent with this proposed intracellular site of action is the ability of DETCA to inhibit relaxation to nitrovasodilators, an effect reported to occur through destruction of NO by superoxide anion inside smooth muscle cells (Omar *et al.*, 1991). In our experiments we found that following pretreatment with a low concentration of DETCA (0.1 mM), subsequent treatment with concentrations of HX (0.1 mM)/XO (16 mu ml^{-1}) or pyrogallol (0.1 mM) that had little effect on ACh-induced relaxation themselves now produced a profound blockade and this was completely reversed upon addition of exogenous SOD. Clearly, therefore, endogenous Cu-Zn SOD protects agonist-induced NO from destruction by superoxide anion and this can be abolished following treatment with DETCA. It is likely therefore that the greater sensitivity of basal than agonist-stimulated activity of NO to destruction by superoxide anion is due to differential protection by Cu-Zn SOD rather than to differences in their chemical nature. Our proposed explanation for this is that under normal circumstances, endogenous Cu-Zn SOD activity lowers levels of superoxide anion, which may originate from endothelial cells or from the oxygenated Krebs solution, to such an extent that the low levels of NO produced under basal conditions can be destroyed but high levels produced following stimulation by ACh cannot. However, if levels of superoxide anion are increased further, either by generation of the free radical or by reducing its breakdown by inhibiting Cu-Zn SOD with DETCA, the higher levels of NO produced following agonist stimulation can now be destroyed. This explanation may also account for the inability of exogenous SOD to potentiate agonist-stimulated activity of NO in arterial rings (Abrahamsson *et al.*, 1992; this study), since there is already sufficient endogenous SOD present. In contrast, SOD is probably able to potentiate the activity of NO in a cascade bioassay system (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986a; Furchgott *et al.*, 1990) because there is no SOD in the intervening space to protect against the destructive action of superoxide anion.

In conclusion, our findings suggest that both basal and ACh-stimulated activity of NO can be destroyed by superoxide anion. The apparently greater sensitivity of the former may not necessarily indicate a chemical difference from agonist-stimulated NO but may simply reflect differential protection by endogenous Cu-Zn SOD.

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