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Recovery by ascorbate of impaired nitric oxide-dependent relaxation resulting from oxidant stress in rat aorta

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1 In this study we investigated the ability of ascorbate to protect nitric oxide from destruction by superoxide anion.

2 Ascorbate produced concentration-dependent relaxation of rings of rat aorta, comprising two components: the first, seen at 1–300 μM, reached a maximum of 45.3±2.8%, and was abolished by endothelial removal or treatment with L-NAME (100 μM), demonstrating involvement of nitric oxide. The second occurred at concentrations of 1 mM and above and was associated with falls in the pH of the bathing fluid.

3 Pretreatment with ascorbate at concentrations up to 3 mM had no effect on the relaxation to acetylcholine (10 nM–10 μM) on endothelium-containing rings or adenosine (0.1 μM–3 mM) on endothelium-denuded rings.

4 An oxidant stress was applied to aortic rings, comprising inhibition of endogenous Cu/Zn superoxide dismutase by diethyldithiocarbamate (0.1 mM) followed by generation of superoxide anion by hypoxanthine (0.1 mM/xanthine oxidase (16 u ml^{-1}). This reduced maximal acetylcholine-induced relaxation from 96.7±1.3% to 42.4±3.5% ($P<0.001$). Treatment with ascorbate (30 μM–3 mM) reversed this blockade in a concentration-dependent manner.

5 Our findings show that ascorbate has the ability to protect nitric oxide from destruction by superoxide anion. This action is seen with ascorbate at levels normally present in plasma, suggesting that this antioxidant may exert a tonic protective effect on nitric oxide within the vasculature.

Keywords: Nitric oxide; endothelium; superoxide anion; superoxide dismutase; superoxide dismutase mimetic; oxidant stress; antioxidant; ascorbate.

Introduction

Nitric oxide reacts rapidly with superoxide anion leading to loss of its vasodilator activity (Gryglewski *et al*., 1986; Rubanyi & Vanhoutte, 1986) and formation of the damaging oxidant, peroxynitrite (Beckman *et al*., 1990). Findings following the use of the copper chelator, diethyldithiocarbamate (DETCA), which irreversibly inhibits Cu/Zn superoxide dismutase (SOD; Cocco *et al*., 1981; Kelner *et al*., 1989) suggest that both the extracellular and intracellular isoforms of this enzyme exert a vital role in protecting nitric oxide produced by the vascular endothelium (Omar *et al*., 1991; Mügge *et al*., 1991; Abrahamsson *et al*., 1992; Mian & Martin, 1995) and nitrergic nerves (Martin *et al*., 1994; Lilley & Gibson, 1995). This endogenous protective role makes it likely that SOD and SOD-like agents could have therapeutic potential in pathological states associated with oxidant stress, including hypertension (Nakazono *et al*., 1991; Bouloumie *et al*., 1997), atherosclerosis (Ohara *et al*., 1993) and diabetes (Hattori *et al*., 1991). In fact, authentic SOD (Ohlstein & Nichols, 1989; Mian & Martin, 1995) and certain Mn-based SOD mimetics (Kasten *et al*., 1994, 1995) are known to produce endothelium-dependent relaxation by protecting basal nitric oxide from destruction by endogenously produced superoxide. In addition, they protect nitric oxide from destruction in a variety of vascular (Kilgore *et al*., 1994; Day *et al*., 1995; Mian & Martin, 1995; MacKenzie & Martin, 1998) and neural (Mok *et al*., 1998) models of oxidant stress.

Ascorbate is another powerful scavenger of superoxide anion (Som *et al*., 1983; Gotoh & Niki, 1992) with the potential to protect nitric oxide. This antioxidant protects nitric oxide released from nitrergic nerves in the mouse and rat anococcygeus muscles from destruction by superoxide and, indeed, appears to be released from these nerves for this purpose (Lilley & Gibson 1996, 1997). Furthermore, treatment with ascorbate leads to significant recovery from the impairment of nitric oxide-mediated vasodilatation seen in patients with diabetes mellitus (Ting *et al*., 1996) and chronic heart failure (Hornig *et al*., 1998), two pathological states associated with oxidant stress.

The aim of this study was to examine the ability of ascorbate to protect endothelium-derived nitric oxide from destruction by superoxide anion in rat aorta. This was attempted by determining if ascorbate produces endothelium-dependent relaxation, and restores nitric oxide-mediated relaxation following inhibition by an applied oxidant stress.

Methods

Preparation of tissues

Female Wistar rats (200–250 g) were killed by stunning and exsanguination. The thoracic aorta was then carefully removed, cleaned of fat and connective tissue, and cut into transverse rings (2.5 mm wide). In some experiments, the endothelium was removed by gentle abrasion of the intimal surface using a moist wooden stick. Aortic rings were then

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mounted under 1 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 displayed on a MacLab (E Series, AD Instruments) or 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 1 g, as required.

Experimental protocols

All experiments involving relaxation on control, endothelium-containing aortic rings were conducted following induction of 40–60% (0.66±0.02 g) of maximal phenylephrine (PE)-induced tone (1.33±0.02 g, $n=15$): this level of tone was achieved with PE at 0.1–0.3 μM. A number of the experimental procedures employed, however, affected the level of tone induced by PE. Specifically, endothelial denudation or treatment with N^G-nitro-L-arginine methyl ester (L-NAME), diethyldithiocarbamate (DETCA), or hypoxanthine/xanthine oxidase (HX/XO) led to loss of basal nitric oxide activity and, consequently, enhanced PE-induced contraction (see Mian & Martin, 1995). Conversely, treatment with ascorbate led to a fall in vascular tone, with a consequent reduction in PE-induced contraction. In order to take account of these changes in sensitivity, the concentration of PE employed in each individual experiment was adjusted to ensure that the level of tone achieved was 40–60% of the maximum seen on control, endothelium-containing rings. No data were used from tissues which did not meet this strict criterion. This procedure ensured that physiological antagonism, resulting from differences in levels of tone, did not contribute to the results obtained.

Cumulative concentration-response curves for relaxation to ascorbate (1 μM–3 mM) were constructed on endothelium-containing rings following induction of PE-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. The effects of inhibition of nitric oxide synthase with L-NAME (100 μM, 30 min) and of endothelial denudation were also examined on relaxation to ascorbate. Furthermore, the effects of 20 min pretreatment with ascorbate were also examined on relaxation to acetylcholine (ACh, 10 nM–10 μM) on endothelium-containing rings and adenosine (0.1 μM–3 mM) on endothelium-denuded rings.

In certain experiments, aortic rings were subjected to an oxidant stress which was previously shown to inhibit ACh-induced relaxation by approximately 50% (Mian & Martin, 1995). Specifically, the aortic rings were treated with DETCA (0.1 mM for 90 min) followed by washout, in order to inhibit irreversibly endogenous Cu/Zn SOD (Cocco *et al.*, 1981; Kelner *et al.*, 1989). Thereafter, the tissues were exposed to the superoxide anion generating system, HX/XO, and effects on ACh-induced relaxation investigated. In these experiments, the tissues were incubated with XO (16 u ml^{-1}) for 30 min to allow it to permeate the tissues. Tone was then induced with PE, HX (0.1 mM) was added, and effects on ACh-induced relaxation observed within 5 min. The ability of ascorbate (10 μM–3 mM) to protect ACh-induced relaxation against inhibition by HX/XO in DETCA-treated tissues was also studied. In these experiments, ascorbate was given as a 20 min pretreatment before addition of PE and HX.

All experiments involving the use of DETCA or HX/XO were conducted in the presence of catalase (1000 u ml^{-1}) to guard against accumulation of hydrogen peroxide (Mian & Martin, 1995).

Drugs

Acetylcholine chloride, adenosine hemisulfate, catalase (bovine liver), diethyldithiocarbamic acid, hypoxanthine, N^G-nitro-L-arginine methyl ester, phenylephrine hydrochloride, superoxide dismutase (Cu/Zn-containing enzyme from bovine erythrocytes) and xanthine oxidase (buttermilk) were obtained from Sigma (Poole, U.K.). Ascorbate (L-ascorbic acid) was obtained from Koch-Light (Haverhill, U.K.). All drugs were dissolved and subsequent dilutions made in saline (0.9%), except for hypoxanthine (50 mM stock) which was dissolved in 0.1% sodium hydroxide. Control experiments were conducted to ensure that sodium hydroxide had no effects on the aortic rings.

Analysis of data

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

Results

Effects of ascorbate on rat aorta

Following induction of submaximal tone with phenylephrine (PE, 0.1-0.3 μM) in endothelium-containing rings of rat aorta, ascorbate produced concentration-dependent relaxation (Figures 1 and 2). This comprised two components: an initial component starting at 1 μM and peaking at 100–300 μM (45.3±2.8% relaxation, $n=17$), which was present on endothelium-containing but not endothelium-denuded rings and was abolished by pretreatment with L-NAME (100 μM). The second, more powerful component of relaxation was seen at 1 mM and above and was unaffected by endothelial removal or treatment with L-NAME.

In a separate group of independently-controlled experiments on endothelium-denuded rings, ascorbate at concentrations of 0.3 mM and below had no effect on the pH of the bathing solution (pH 7.55±0.03, $n=8$), but significant falls in pH were seen at concentrations of 1 mM (pH 7.43±0.01,

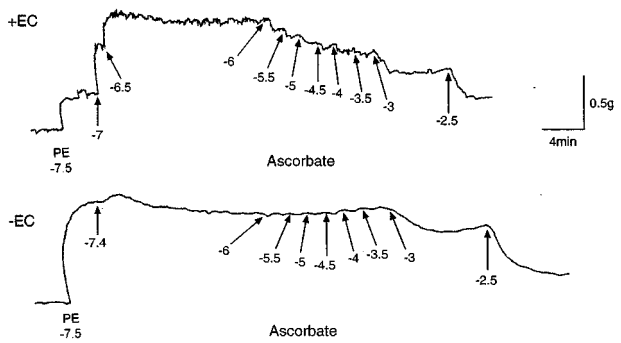

Figure 1 Individual experimental tracings showing that following induction of tone with phenylephrine (PE) in endothelium-containing (+EC) and endothelium-denuded (−EC) rings of rat aorta, addition of ascorbate produces concentration-dependent relaxation. Concentrations are given in log molar units.

$n=8$, $P<0.01$) and 3 mM (pH 7.26 ± 0.03, $n=8$, $P<0.001$). These concentrations produced relaxation of $6.6\pm2.9\%$ and $41.3\pm4.5\%$, respectively. Similar falls in bath pH (pH 7.34 ± 0.02 and pH 7.09 ± 0.03, both $n=8$, $P<0.001$) and vascular relaxations ($9.1\pm3.5\%$ and $57.9\pm3.4\%$) were seen when HCl was added at 1 and 3 mM, respectively.

Addition of acetylcholine (10 nM – 10 μM) to endothelium-containing rings of rat aorta resulted in concentration-dependent relaxation (Figure 3A). Pretreatment for 20 min with ascorbate (0.1 – 3 mM) had no effect on this relaxation. Pretreatment with ascorbate at 3 mM also had no effect on the relaxation induced by adenosine (0.1 μM – 3 mM) on endothelium-denuded rings (Figure 3B).

Effects of ascorbate on blockade of acetylcholine-induced relaxation by oxidant stress in rat aorta

Application of an oxidant stress, comprising inhibition of endogenous Cu/Zn SOD with diethyldithiocarbamate (DETCA, 0.1 mM, 90 min) followed by treatment with the superoxide anion generating system hypoxanthine (HX, 0.1 mM)/ xanthine oxidase (XO, 16 u ml^{-1}), in rat aortic rings led to significant blockade of acetylcholine-induced relaxation (Figure 4): maximum relaxation fell from $96.7\pm1.3\%$ to $42.5\pm3.5\%$ ($n=8$, $P<0.001$). Pretreatment for 20 min with ascorbate produced concentration-dependent reversal of this blockade of acetylcholine-induced relaxation: the lowest

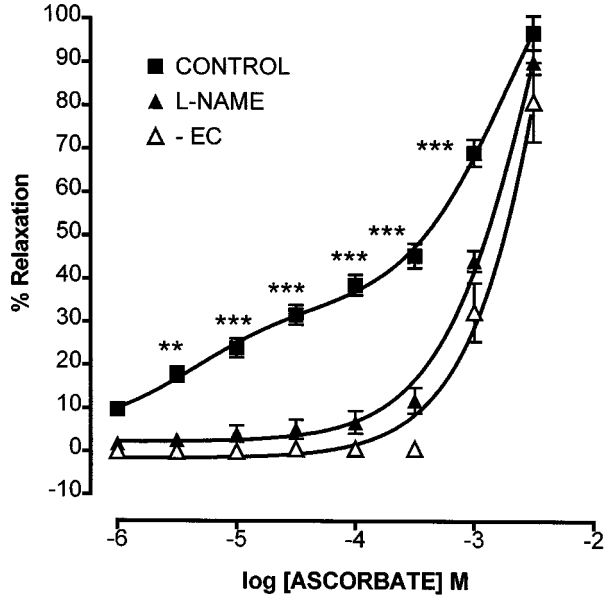

Figure 2 Concentration-response curves showing relaxation to ascorbate on phenylephrine-contracted, endothelium-containing rings of rat aorta (CONTROL) and the effects of endothelial removal (−EC) or treatment with L-NAME (100 μM) on these relaxations. Each point is the mean ± s.e.mean of 6 – 17 observations. $^{**}P<0.005$ and $^{***}P<0.001$ indicate a significant difference in maximal relaxation from L-NAME and −EC.

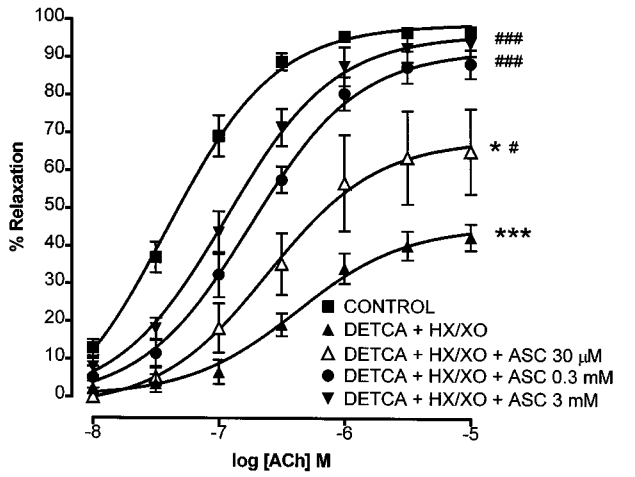

Figure 4 Concentration-response curves showing relaxation to acetylcholine (ACh, CONTROL) on phenylephrine-contracted, endothelium-containing rings of rat aorta, and the blockade of these relaxations following combined treatment with diethyldithiocarbamate (DETCA) and hypoxanthine (HX)/ xanthine oxidase (XO). The effects of pretreatment for 20 min with ascorbate (ASC) at 30 μM – 3 mM on the blockade of relaxation are also shown. Each point is the mean ± s.e.mean of 7 – 14 observations. $^{*}P<0.05$ and $^{***}P<0.001$ indicate a significant difference in maximal ACh-induced relaxation from CONTROL. $\#P<0.05$ and $\#\#\#P<0.001$ indicate a significant reversal of the blockade of maximal ACh-induced relaxation in tissues treated with DETCA and HX/XO.

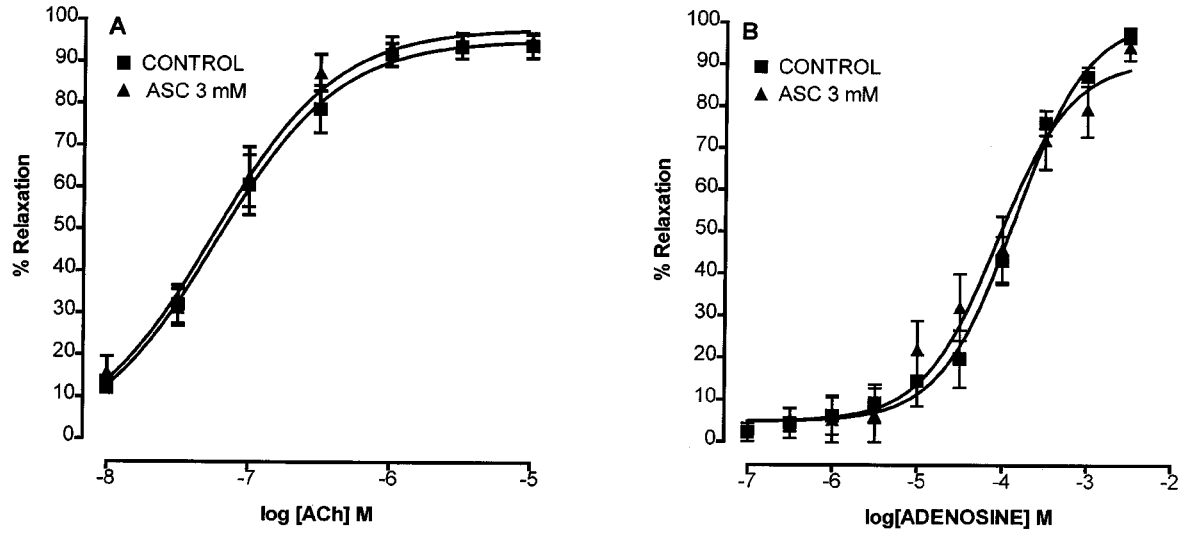

Figure 3 Concentration-response curves showing relaxation to (A) acetylcholine on endothelium-containing and (B) adenosine on endothelium-denuded rings of rat aorta (CONTROL), and the effects of 20 min pretreatment with ascorbate (ASC) 3 mM on these relaxations. Each point is the mean ± s.e.mean of six to nine observations.

concentration to produce significant reversal was 30 μM, and maximal reversal was seen at 0.3 – 3 mM.

Discussion

Ascorbate is a highly effective scavenger of superoxide anion (Som *et al*., 1983; Gotoh & Niki, 1992) and thus has the potential to protect nitric oxide from destruction in conditions of oxidant stress. Our first strategy to test this directly was to determine if this antioxidant produced endothelium-dependent relaxation. Agents which remove superoxide anion, such as authentic superoxide dismutase (SOD; Mian & Martin, 1995) and low molecular weight Mn-based compounds possessing SOD-like activity (Kasten *et al*., 1994, 1995), have previously been shown to produce endothelium-dependent relaxation by protecting basal nitric oxide from destruction by endogenously-produced superoxide anion. We indeed found that ascorbate at concentrations ranging from 1 to 300 μM produced powerful relaxation of endothelium-containing rings of rat aorta. This relaxation, like that induced by authentic SOD (Mian & Martin, 1995) or SOD mimetics (Kasten *et al*., 1994, 1995), was abolished by endothelial denudation or treatment with an inhibitor of nitric oxide synthase, demonstrating that it was mediated by nitric oxide. Concentrations of ascorbate of 1 mM or greater produced a second component of relaxation which was unaffected by endothelial removal or treatment with the nitric oxide synthase inhibitor, L-NAME, and which appeared to be due to a fall in bath pH since it was mimicked by HCl.

The magnitude of the maximal endothelium-dependent component of ascorbate-induced relaxation found in this study ($45.3 \pm 2.8\%$) is smaller than previously found for SOD ($79.9 \pm 2.0\%$; Mian & Martin, 1995). This difference may perhaps be explained by the lower efficiency of ascorbate than of SOD to scavenge superoxide (rate constants 5.3×10^6 $M^{-1}s^{-1}$ and 2.0×10^9 $M^{-1}s^{-1}$, respectively; Som *et al*., 1983; Huie & Padmaja, 1993). Interestingly, the concentration range over which ascorbate produced endothelium-dependent relaxation (1 – 300 μM) lies almost entirely within normal plasma limits for humans (50 – 200 μM; Halliwell & Gutteridge, 1989). It is therefore conceivable that ascorbate, together with Cu/Zn SOD (Omar *et al*., 1991; Mügge *et al*., 1991; Abrahamsson *et al*., 1992; Mian & Martin, 1995), is a physiologically important protector of nitric oxide in the vasculature. Indeed, it has already been proposed that ascorbate protects nitric oxide released by nitrergic nerves (Lilley & Gibson, 1996, 1997).

The ability of ascorbate to improve the impaired endothelium-dependent vasodilatation observed in patients with diabetes mellitus (Ting *et al*., 1996) and chronic heart failure (Hornig, *et al*., 1998) prompted us to examine the protective effects of this antioxidant in an established model of oxidant stress. We have previously shown that protection by endogenous Cu/Zn SOD ensures that superoxide generation by the hypoxanthine (HX)/xanthine oxidase (XO) system has only a minor effect on acetylcholine-induced relaxation in rat (Mian & Martin, 1995) and rabbit (MacKenzie & Martin, 1998) aorta. Following inhibition of endogenous Cu/Zn SOD with DETCA, however, the addition of HX/XO produces powerful inhibition of acetylcholine-induced relaxation. Using this protocol in rat aorta, we established an oxidant stress which reduced maximal acetylcholine-induced relaxation by approximately 50%. We have previously shown that this blockade can be almost completely reversed by authentic SOD (250 u ml^{-1}) in both rat (Main & Martin, 1995) and rabbit (MacKenzie & Martin, 1998) aorta. Similarly, we found that ascorbate produced significant recovery of acetylcholine-induced relaxation in rat aorta: the lowest effective concentration (30 μM) is well within the normal range for plasma, but the concentrations giving maximal recovery (0.3 – 3 mM) would not be expected under normal circumstances. It should be noted, however, that 2 g of ascorbate per day for 4 weeks was required to restore endothelial function in patients with chronic heart failure (Hornig *et al*., 1998). Since the EU recommended daily intake for ascorbate is 60 mg, it is likely that loading with higher levels of this antioxidant can provide additional protection for nitric oxide than can be achieved on a normal diet. The recovery of impaired acetylcholine-induced relaxation seen even with concentrations of ascorbate up to 3 mM in our study did, however, appear to be due to selective scavenging of superoxide anion, since similar concentrations failed to affect relaxation to acetylcholine in control preparations. Furthermore, 3 mM ascorbate was also without effect on relaxation to adenosine, which occurs independently of the endothelium.

In conclusion, our study shows that ascorbate elicits relaxation of rat aortic rings which, by analogy with SOD, probably results from protection of basal nitric oxide from destruction by endogenously-produced superoxide anion. This action occurs with concentrations of ascorbate normally present in plasma, suggesting that this antioxidant may exert a tonic protective effect on nitric oxide in the vasculature. This view is supported by our finding that plasma levels also provide a significant degree of protection of nitric oxide against an applied oxidant stress. Supplementation with ascorbate above normal levels, as has proved successful in patients with endothelial dysfunction associated with diabetes (Ting *et al*., 1996) and heart failure (Hornig *et al*., 1998), is likely, however, to be required to achieve maximal protection of nitric oxide. The additional possibility that ascorbate is co-released with nitric oxide from endothelial cells in a manner analogous to nitrergic nerves (Lilley & Gibson, 1996, 1997) also warrants consideration.

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