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Oxidation by trace Cu^{2+} ions underlies the ability of ascorbate to induce vascular dysfunction in the rat perfused mesentery

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ABSTRACT

Ascorbate has both antioxidant and pro-oxidant activities. We have previously shown that plasma levels of ascorbate induce constriction and blockade of dilatation mediated by endothelium-derived hyperpolarizing factor (EDHF). In this study we sought to determine if these detrimental actions were mediated by a pro-oxidant action of ascorbate. Since trace levels of transition metal ions including, Cu^{2+} and Fe^{3+}, promote oxidation of ascorbate, we examined the effects of the chelating agents, cuprizone and deferoxamine, and of $CuSO_4$ and $FeCl_3$ on ascorbate-induced constriction and blockade of EDHF in the perfused rat mesentery. Cuprizone abolished and Cu^{2+} but not Fe^{3+} ions enhanced both ascorbate (50 μM)-induced constriction and blockade of EDHF. The blockade of EDHF produced by ascorbate in the presence of $CuSO_4$ (0.5 μM) was abolished by the hydrogen peroxide scavenger, catalase, but unaffected by the scavengers of hydroxyl radical or superoxide anion, mannitol and superoxide dismutase (SOD), respectively. Consistent with these observations, the oxidation of ascorbate by $CuSO_4$ led to the rapid production of hydrogen peroxide. Catalase, mannitol and SOD had no effect on ascorbate-induced constriction. Thus, in the rat perfused mesentery, both the constrictor and EDHF-blocking actions of ascorbate arise from its oxidation by trace Cu^{2+} ions. The blockade of EDHF results from the consequent generation of hydrogen peroxide, but the factor producing constriction remains unidentified. These detrimental actions of ascorbate may help explain the disappointing outcome of clinical trials investigating dietary supplementation with the vitamin on cardiovascular health.

1. Introduction

Ascorbate (or vitamin C) plays a vital role in a wide variety of biological functions, including the synthesis of collagen, carnitine and amine transmitters, and is the most important water-soluble antioxidant in humans (Frei, 1994). In the cardiovascular system, the effects of ascorbate on nitric oxide-mediated vasodilatation are well studied (Dudgeon et al., 1998; Fontana et al., 1999; Carr et al., 2000; Fontana et al., 1999). These reports demonstrate that ascorbate is able to restore nitric oxide-dependent vasodilatation following its impairment by oxidant stress. Whether this protective action results from the ability of ascorbate to scavenge superoxide anion and so prevent it from destroying nitric oxide (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986) or from some other mechanism (Jackson et al., 1998) remains unresolved. Nevertheless, there are many reports where acute treatment with ascorbate improves the impaired nitric oxide-mediated dilatation in patients with a variety of cardiovascular pathologies, including essential hypertension (Taddei et al., 1998; Natali et al., 2000), atherosclerosis (Levine et al., 1996; Ting et al., 1997) and heart failure (Ellis et al., 2001; Hornig et al., 1998). Despite this, the outcome of clinical trials studying the effects of dietary supplementation with ascorbate on a range of cardiovascular endpoints has been disappointing (Collins et al., 2002; Duarte and Lunec, 2005). It is possible, therefore, that any benefit arising from the ability of ascorbate to enhance the activity of nitric oxide is offset by a detrimental pro-oxidant action (Duarte and Lunec, 2005).

We have previously shown in the rat perfused mesentery and bovine perfused ciliary artery that plasma levels of ascorbate (10–150 μM) produce two potentially detrimental actions: a constrictor action, rapid in the former but slowly developing in the latter, together with a time-dependent blockade (2–3 h max) of dilatation mediated by endothelium-derived hyperpolarizing factor (EDHF) in both tissues (Stirrat et al., 2006; Stirrat et al., 2008; McNeish et al., 2002). Furthermore, the inhibition of EDHF induced by ascorbate appears highly selective, since the vasodilator actions of endothelium-derived nitric oxide, the nitrovasodilator, glyceryl trinitrate, and the K_{ATP} channel opener, levcromakalim, remain entirely unaffected. We and others have shown, however, that very high concentrations of ascorbate (3–10 mM) impair acetylcholine-induced, nitric oxide-mediated dilatation in the rabbit aorta (de Saram et al., 2002; McNeish et al., 2003).

The aim of this study was to determine if the constrictor and EDHF-blocking actions seen in the rat perfused mesentery by plasma levels of ascorbate could be explained by a pro-oxidant action. Indeed, the findings show that oxidation of ascorbate by trace Cu^{2+} ions is responsible for both detrimental vascular actions.

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2. Materials and methods

2.1. Preparation of the rat perfused mesentery for pressure recording

All animal procedures were conducted according to the ethical guidelines of the University of Glasgow under Schedule 1 of the Animals (Scientific Procedures) Act 1986. Male Wistar rats (150–180 g) were killed by concussion followed by exsanguination. The superior mesenteric artery was cannulated and the mesenteric arterial vasculature dissected from the intestines and suspended in a heated organ bath as previously described (McNeish et al., 2002; Stirrat et al., 2008). Mesenteries were then perfused at 37 °C using a peristaltic pump (Minipuls 3, Gilson) at a flow rate of 15 ml/min with Krebs solution containing (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5; and gassed with 95% O_2/5% CO_2. Tissues were allowed to equilibrate for at least 30 min before the beginning of each experiment. Perfusion pressure was measured using Gould Statham P32 ID transducers via a side arm located immediately proximal to the inflow cannula and displayed on a PowerLab data acquisition system (AD Instruments, Hastings, U.K.).

2.2. Experimental protocols with the rat perfused mesentery

In order to observe EDHF-mediated dilator responses to acetylcholine, the perfusion pressure was raised to ~120 mm Hg using the thromboxane-mimetic, U46619 (10–100 nM). The effects of nitric oxide and cyclooxygenase products were routinely blocked using N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) and indomethacin (3 μM), respectively. Vasodilator responses (~50% max) to acetylcholine (10 nmol) were elicited at 15 min intervals during a 3 h study period by injecting into the perfusion fluid 10 μl volumes of stock solution using a Hamilton micro-syringe, and expressed as a percentage of the U46619-induced perfusion pressure.

As will be seen in the Results, the ability of ascorbate (50 μM) to induce a rapid constriction and a slowly developing (max at 3 h) blockade of acetylcholine-induced, EDHF-mediated dilatation (Stirrat et al., 2008) was abolished by the Cu^{2+} ion chelator, cuprizone (30 μM), and enhanced by $CuSO_4$ (0.5 μM). As a consequence, except when otherwise stated, all further experiments with ascorbate (50 μM) were conducted on tissues perfused with Krebs containing $CuSO_4$ (0.5 μM). A number of agents were investigated for their ability to inhibit the constrictor and EDHF-blocking actions of ascorbate in the presence of $CuSO_4$. These were: the superoxide scavengers, superoxide dismutase (SOD, 100 u/ml), tiron (300 μM) and Tempol (1 mM); the NADPH oxidase inhibitor, diphenyleneiodonium (3 μM); the xanthine oxidase inhibitor, allopurinol (100 μM); the mitochondrial inhibitors, rotenone (1 μM) and myxothiazole (0.3 μM); the hydrogen peroxide scavenger, catalase (1200 u/ml); and the hydroxyl radical scavenger, mannitol (10 mM).

Time-matched control experiments were conducted in the absence of ascorbate and $CuSO_4$ to determine the reproducibility of the dilator action of acetylcholine. In addition, the selectivity of the blockade of acetylcholine-induced, EDHF-mediated vasodilatation by ascorbate in the presence of $CuSO_4$ was assessed by examining the effects on dilatation to the endothelium-independent agent, levcromakalim (5 nmol).

Experiments were conducted to determine if $FeCl_3$ (0.5 and 100 μM) could mimic the ability of $CuSO_4$ to facilitate ascorbate (50 μM)-induced constriction and blockade of acetylcholine-induced, EDHF-mediated dilatation. Before these experiments were conducted, Cu^{2+}-free conditions were produced by purging the perfusion system with Krebs containing cuprizone (30 μM). Cuprizone was not, however, present during these experiments to guard against any possibility of chelation of Fe^{3+} ions.

Other experiments were conducted to determine if hydrogen peroxide (30 and 50 μM) could mimic the ability of ascorbate to induce constriction and blockade of acetylcholine-induced, EDHF-mediated dilatation. These experiments were conducted in the presence of cuprizone (30 μM) to ensure any effects seen were due to hydrogen peroxide per se and not to other reactive oxygen species formed following its interaction with trace Cu^{2+} ions.

2.3. Assay of hydrogen peroxide formation

The ability of Cu^{2+} and Fe^{3+} ions to catalyse the formation of hydrogen peroxide from ascorbate was assessed using the FOX2 (ferrous oxidation in xylenol orange, version 2) method (Nourooz-Zadeh et al., 1994). The FOX2 reagent was prepared by adding one volume of solution 1 (1 mM xylenol orange and 2.5 mM ammonium ferrous sulphate in 250 mM sulphuric acid) to 9 volumes of solution 2 (4.4 mM butylated hydroxytoluene in HPLC grade methanol). Experiments were carried out at 37 °C in organ baths filled with the same Krebs solution used for the biological experiments and gassed with 95% O_2/5% CO_2·$CuSO_4$ (0.5 μM) or $FeCl_3$ (100 μM) was added to the Krebs solution and 100 μl samples (time zero) taken for assay. Ascorbate (50 μM) was then added to the Krebs solution and 100 μl samples taken for assay at 2, 30, 60, 120 and 180 min. Once taken, samples were immediately added to 1 ml of FOX2 reagent and incubated at room temperature for 30 min. Absorbance at 560 nm was read using a Pye Unicam SP6-550 spectrophotometer, with hydrogen peroxide levels assessed using standards of known concentration (0–100 μM). Fresh standards and blanks were prepared for each time point because their absorbance did not remain stable during the 3 h duration of experiments.

2.4. Drugs and chemicals

Acetylcholine chloride, ammonium ferrous sulphate, allopurinol, ascorbic acid, butylated hydroxytoluene, cuprizone, deferoxamine, diphenyleneiodonium, indomethacin, myxothiazole, L-NAME (N^G-nitro-L-arginine methyl ester), mannitol, rotenone, Tempol, tiron and U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$) were all obtained from Sigma. Catalase (bovine liver) was obtained from Calbiochem. Hydrogen peroxide was obtained from BDH. Levcromakalim was a gift from GlaxoSmithKline (Harlow, UK). Xylenol orange disodium salt was obtained from Fluka. All drugs were dissolved and diluted in 0.9% saline except indomethacin (1 mM stock), which was dissolved in Na_2CO_3 (0.4 mg/ml), rotenone (10 mM stock in ethanol), levcromakalim (0.1 M stock in 70% ethanol), cuprizone and U46619 (10 mM and 1 mM stocks, respectively, in 50% ethanol), allopurinol, diphenyleneiodonium and myxothiazole (0.1 M, 30 mM and 3 mM stocks, respectively, in DMSO).

2.5. Statistical analysis

Results are expressed as the mean ± S.E.M. of n separate observations, each from a separate tissue. Graphs were drawn and statistical comparisons made using one-way analysis of variance and Bonferroni's post-test with the aid of a computer program, Prism (GraphPad, San Diego, USA). A probability (P) less than or equal to 0.05 was considered significant.

3. Results

3.1. Effects of ascorbate on the rat perfused mesentery in the presence of Cu^{2+} or Fe^{3+} ions

In the rat mesenteric vascular bed, perfused at 15 ml/min in the presence of L-NAME (100 μM) and indomethacin (3 μM), perfusion pressure was raised to ~120 mm Hg using U46619 (10–100 nM). Under these conditions, acetylcholine (10 nmol) induced powerful EDHF-mediated dilatation (49.8 ± 5.4%, $n = 8$; Fig. 2). As previously reported (Stirrat et al., 2008), treatment with ascorbate (50 μM) induced a rapid rise in perfusion pressure (53.8 ± 11.8 mm Hg, $n = 9$; Figs. 1 and 2) and a slowly developing blockade of acetylcholine-