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Differential effects of ascorbate on endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation in the bovine ciliary vascular bed and coronary artery

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1 The ability of ascorbate to inhibit endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation was compared in the bovine perfused ciliary vascular bed and isolated rings of coronary artery.

2 Acetylcholine-induced, EDHF-mediated vasodilatation of the ciliary circulation was blocked following inclusion of ascorbate (50 μM, 120 min) in the perfusion fluid. The blockade was highly selective since ascorbate had no effect on the vasodilator actions of the K_{ATP} channel opener, levcromakalim, nor on the tonic vasodepressor action of basally released nitric oxide.

3 The possibility that concentration of ascorbate by the ciliary body was a prerequisite for blockade to occur was ruled out, since EDHF was still blocked when the anterior and posterior chambers were continuously flushed with Krebs solution or when both the aqueous and vitreous humour were drained.

4 Ascorbate at 50 μM failed to affect bradykinin- or acetylcholine-induced, EDHF-mediated vasodilatation in rings of bovine coronary artery. Raising the concentration to 3 mM did produce blockade of EDHF, but this was nonselective, since vasodilator responses to endothelium-derived nitric oxide were also inhibited.

5 Thus, ascorbate (50 μM) is not a universal blocker of EDHF. Whether its ability to block in the bovine ciliary circulation, but not in the coronary artery, is due to differences in the nature of EDHF at the two sites, differences in vessel size (resistance arterioles *versus* conduit artery), the presence or absence of flow, or to some other factor remains to be determined.
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Abbreviations: BK_{Ca}, large conductance calcium-sensitive potassium channel; EDHF, endothelium-derived hyperpolarizing factor; IK_{Ca}, intermediate conductance calcium-sensitive potassium channel; K_{ATP}, ATP-sensitive potassium channel; L-NAME, N^G-nitro-L-arginine methyl ester; SK_{Ca}, small conductance calcium-sensitive potassium channel

Introduction

It is now well established that the antioxidant actions of ascorbate protect endothelium-derived nitric oxide from destruction by superoxide anion under conditions of oxidant stress (Dudgeon *et al*., 1998; Fontana *et al*., 1999). Indeed, acute administration of ascorbate to patients restores the impaired nitric oxide-mediated vasodilatation seen in a range of cardiovascular pathologies, including essential hypertension (Taddei *et al*., 1998; Natali *et al*., 2000), atherosclerosis (Levine *et al*., 1996), hypercholesterolaemia (Ting *et al*., 1997), chronic heart failure (Hornig *et al*., 1998; Ellis *et al*., 2001) and in insulin-dependent (Timmi *et al*., 1998) and non-insulin-dependent (Ting *et al*., 1996) diabetes mellitus. In spite of these promising reports, however, long-term treatment with vitamin C does not appear to improve 5-year survival rates in any form of cardiovascular disease (Collins *et al*., 2002), although a slowed progression of cardiac transplant-associated coronary atherosclerosis has been reported (Fang *et al*., 2002).

In contrast to its ability to enhance the activity of nitric oxide, we recently reported that ascorbate blocks the endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation induced by acetylcholine or bradykinin in the perfused ciliary vascular bed of the bovine eye (McNeish *et al*., 2002b). The lowest effective concentration of ascorbate was 10 μM and maximal blockade occurred at 150 μM. Thus, blockade of EDHF is seen well within the normal range of plasma concentrations of ascorbate found in humans (mean 46 μM, range 30–150 μM) (Keaney & Vita, 1995; Levine *et al*., 1996). The mechanism by which ascorbate blocks EDHF-mediated vasodilatation remains unresolved, but is likely to result from an antioxidant action, since the redox-inactive analogue, dehydroascorbate, is ineffective. In addition, blockade by ascorbate appears to be selective, since vasodilator responses to the nitrovasodilator, glyceryl trinitrate, are

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unaffected. Although infusion of only 10–150 μM ascorbate into the ciliary vascular bed was required, it is possible that the blockade of EDHF arose as a result of the ability of the eye to concentrate this antioxidant. Specifically, the epithelial cells of the ciliary body actively transport ascorbate supplied by the ciliary vasculature (Millar & Kaufman, 1995; Mead *et al*., 1996), such that concentrations of $\sim$1 mM are found in the aqueous and vitreous humour of humans and mammals (Davson, 1980; Halliwell & Gutteridge, 1989). We did not test this possibility directly, but it seemed unlikely because 50 μM ascorbate was also able to block acetylcholine-induced, EDHF-mediated vasodilatation in the rat perfused mesenteric arterial bed (McNeish *et al*., 2002b), where concentration of the antioxidant would not be expected.

The aim of the present study was to explore further the ability of ascorbate to block EDHF-mediated vasodilatation. Specifically, our new findings show that: (i) the blockade of EDHF by ascorbate (50 μM) in the bovine ciliary circulation is highly selective, since vasodilatation induced by endothelium-derived nitric oxide or the K_{ATP} channel opener, levcromakalim, remains unaffected; (ii) the accumulation of ascorbate by the ciliary body is not a prerequisite for blockade of EDHF to occur; (iii) ascorbate at 50 μM is not a universal inhibitor of EDHF, since vasodilatation induced by bradykinin or acetylcholine in the bovine coronary artery was completely unaffected. Much higher concentrations of ascorbate (1–3 mM) did inhibit EDHF responses, but this arose from a nonselective action, since vasodilatation mediated by endothelium-derived nitric oxide was also powerfully blocked.

Methods

Preparation of the ciliary vascular bed of the bovine eye for perfusion

The ciliary vascular bed of the bovine eye was perfused using the constant flow method as previously described (McNeish *et al*., 2001). In brief, bovine eyes obtained from a local abattoir were cannulated within 90 min of killing through a long posterior ciliary artery and perfused at 37°C 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 O_2 containing 5% CO_2. Flow was commenced at $\sim$0.2–0.5 ml min^{-1} and raised in 5–10 increments to a final constant rate of 2.5 ml min^{-1} over a 50-min period (increasing the flow rate too rapidly led to damage of the microvasculature in the eye). When this final flow rate was achieved, eyes were perfused for an equilibration period of at least 30 min. 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.). Only eyes that had a basal perfusion pressure of $<$60 mmHg after the equilibration period were used for further study.

Experimental protocols with the bovine ciliary vascular bed

In order to observe vasodilator responses in the bovine eye, the perfusion pressure was first raised to $\sim$130 mmHg using the thromboxane A_2-mimetic, U46619 ($\sim$300 nM). In all experiments, responses to acetylcholine or levcromakalim were elicited by injecting into the perfusate 10 μl of the appropriate concentration with a Hamilton microsyringe. We have previously demonstrated that vasodilator responses elicited by acetylcholine are mediated solely by EDHF and do not involve a contribution by nitric oxide or a cyclooxygenase product (McNeish *et al*., 2001). Consequently, inhibitors of nitric oxide synthase or cyclooxygenase were not required to study EDHF-mediated responses in this preparation.

When the effects of ascorbate (50 μM) were examined on vasodilator responses in the bovine eye, this agent was present in the perfusing fluid for at least 120 min, since previous work had shown this to result in maximal blockade of EDHF (McNeish *et al*., 2002b). Since the epithelium of the ciliary body actively transports ascorbate (Millar & Kaufman, 1995), it was possible that concentration of the antioxidant above the 50 μM infused was a prerequisite for it to block vasodilator responses. We therefore attempted to test this hypothesis by examining the ability of ascorbate (50 μM) in the perfusion fluid to block acetylcholine-induced, EDHF-mediated vasodilatation after adopting two different experimental approaches to prevent the accumulation of this antioxidant. Firstly, since the ciliary body is located in the posterior chamber of the eye, we ensured that its ability to accumulate ascorbate was prevented by continuously flushing this chamber with Krebs solution (without ascorbate) at a rate of 0.25 ml min^{-1}, after insertion of an inflow cannula through the cornea and behind the iris, and an outflow cannula in the anterior chamber. The second approach involved making an incision in the cornea and removing the lens, thus permitting the aqueous humour to drain freely. Following this, an incision was made around the optic nerve at its insertion into the sclera, forming an opening through which the vitreous humour was removed. This permitted perfusion of the ciliary vasculature in a preparation devoid of both aqueous and vitreous humour.

Assay of ascorbate

Samples ($\sim$1.5 ml) of aqueous and vitreous humour were removed from bovine eyes using a syringe and their ascorbate content measured using a modification of an established spectrophotometric assay (Reguera *et al*., 1997; Barrales *et al*., 1998). Briefly, 0.5 ml volumes each of Fe(III)Cl_3 (1 mM, in 10 mM HCl), sodium ferrozine (10 mM, aqueous) and hexamethylenetetramine buffer (HTMA, 0.23 M, pH 5) were added to 0.5 ml of samples or authentic ascorbate standards (1–500 μM). The final volume was adjusted to 5 ml with distilled water, following which they were incubated at 25°C for 10 min, then spun at 100 $\times$ *g* for 15 min at 4°C. Absorbance was then measured at 562 nm using a spectrophotometer (Pye Unicam, model SP6-550). The limit of detection was $\sim$10 μM ascorbate and the standard curve linear to 500 μM. When necessary, samples were diluted with distilled water to bring them into the linear range. Moreover, loss of absorbance following incubation of samples of aqueous and vitreous humour with ascorbate oxidase (1.5 u ml^{-1}, 20 min, 25°C) was employed to confirm the authenticity of the measured ascorbate.

Preparation of bovine left anterior descending coronary artery rings

Sections of myocardium containing the left anterior descending coronary artery were cut from bovine hearts at a local abattoir and transported to the laboratory in Krebs solution. The coronary artery was then dissected out, cut into 2.5 mm ring segments, suspended between two stainless-steel hooks within 10 ml organ baths and maintained at 37°C in Krebs solution gassed with O_2 containing 5% CO_2. Tension was recorded isometrically with Grass FTO3C transducers. Resting tension was adjusted to 2*g* and tissues were allowed to equilibrate for 60 min before experiments were carried out, during which time the tension was readjusted to 2*g*, if required.

In order to observe vasodilator responses, rings of bovine coronary artery were contracted to about 60% ($15 \pm 2g$) of the maximal U46619-induced tone using a concentration of 10–100 nM. Some blocking agents, that is, N^G-nitro-L-arginine methyl ester (L-NAME) and charybdotoxin, enhanced U46619-induced tone in this preparation, so when these were employed, the concentration of the vasoconstrictor was reduced to ensure that the level of tone achieved was similar to that of control experiments. It should be noted that although bradykinin powerfully relaxed all coronary artery rings to which it was added, acetylcholine was a less consistent relaxant. Consequently, only rings in which acetylcholine produced at least 50% relaxation (21 out of 63 tested) were used in experiments.

When EDHF-mediated vasodilatation to bradykinin or acetylcholine was to be studied, the nitric oxide synthase inhibitor, L-NAME (100 μM), and the cyclooxygenase inhibitor, indomethacin (3 μM), were present throughout. In contrast, when nitric oxide-mediated vasodilatation was to be studied, indomethacin (3 μM) and the EDHF blockers, apamin and charybdotoxin (both 100 nM), were present throughout; apamin and charybdotoxin, respectively, block the SK_{Ca} and IK_{Ca} channels that underlie the EDHF response (Waldron & Garland, 1994; Zygmunt & Högestätt, 1996).

Experiments were conducted to determine the effects of ascorbate (50 μM–3 mM) on: (i) the EDHF-mediated component of vasodilatation to bradykinin or acetylcholine (performed in the presence of indomethacin and L-NAME); (ii) the nitric oxide-mediated vasodilatation to bradykinin (indomethacin, apamin and charybdotoxin present); (iii) vasodilatation to the nitric oxide donor, glyceryl trinitrate; and (iv) vasodilatation to the K_{ATP} channel opener, levcromakalim (Quast & Cook, 1989; Edwards & Weston, 1993). In these experiments, ascorbate was present in the bathing fluid for at least 180 min before vasodilator responses were elicited.

Drugs and chemicals

Acetylcholine chloride, apamin, ascorbate oxidase, ascorbic acid, bradykinin acetate, HTMA, indomethacin, L-NAME, sodium ferrozine and U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) were all obtained from Sigma (Poole, U.K.). Charybdotoxin was obtained from Latoxan (Valence, France). Glyceryl trinitrate (10% w w^{-1} in lactose) was a gift from Napp Laboratories (Cambridge, U.K.). Levcromakalim was a gift from GlaxoSmithKline (Harlow, U.K.). All drugs were dissolved and diluted in 0.9% saline except indomethacin (0.01 M stock), which was dissolved in Na_2CO_3 (1 M), and levcromakalim (0.1 M stock), which was dissolved in 70% ethanol, and U46619 (1 mM), which was dissolved in 50% ethanol. When ascorbate was used at concentrations of 1 and 3 mM, the stock solution (1 M) was first neutralized using 1 M NaOH, so as to avoid lowering the pH of the Krebs in organ chambers.

Statistical analysis

Results are expressed as the mean $\pm$ s.e.m. of *n* separate observations, each from a separate eye or coronary arterial ring; for the latter, the number of animals from which they were obtained is also given. Vasodilator responses are expressed as percentage reduction of U46619-induced perfusion pressure (bovine eye) or tone (bovine coronary artery). Graphs were drawn and statistical comparisons made using Student's *t*-test, or one-way analysis of variance with Bonferroni's post-test, as appropriate, with the aid of a computer program, Prism (GraphPad, San Diego, U.S.A.). A probability (*P*) less than or equal to 0.05 was considered significant.

Results

Effects of ascorbate in the bovine ciliary vascular bed

As previously reported (McNeish *et al*., 2002a), treatment with ascorbate (50 μM, >120 min) blocked the acetylcholine (10 nmol)-induced, EDHF-mediated fall in U46619-induced perfusion pressure and uncovered a normally suppressed vasoconstrictor response in the bovine perfused ciliary vascular bed (Figure 1).

Experiments were conducted to test whether concentration of ascorbate by the ciliary body (Millar & Kaufman, 1995) was a prerequisite for blockade of EDHF-mediated vasodilatation to occur. The endogenous concentration of ascorbate in the aqueous and vitreous humour of bovine eyes measured within 1 h of death was 1.1 ± 0.1 and 0.9 ± 0.1 mM (each $n = 8$), respectively. These concentrations did not change following perfusion of the ciliary vascular bed for 120 min with either normal Krebs solution or Krebs containing ascorbate (50 μM; Figure 2). When the anterior and posterior chambers of the eye were continuously flushed with Krebs solution at a rate of 0.25 ml min^{-1} to prevent accumulation of ascorbate, the concentration in the aqueous humour fell to below detectable levels ($\sim$10 μM), whereas that of the vitreous humour did not change. Nevertheless, flushing the anterior and posterior chambers failed to prevent the blockade of acetylcholine-induced, EDHF-mediated vasodilatation produced by the presence of ascorbate (50 μM, >120 min) in the perfusion fluid (Figures 1 and 2). Control experiments showed that flushing the chambers themselves had no effect on acetylcholine-induced vasodilatation when the ciliary vascular bed was perfused with Krebs lacking ascorbate. Moreover, when the ciliary vascular bed was perfused following removal of the aqueous and vitreous humour, the presence of ascorbate (50 μM, >120 min) in the perfusion fluid still blocked the EDHF-mediated vasodilator response to acetylcholine (Figure 1).

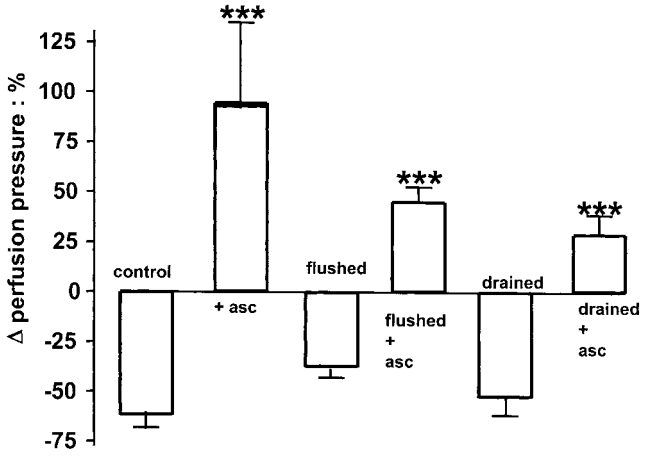

Figure 1 Histogram showing that intra-arterial injection of acetylcholine (10 nmol) induces EDHF-mediated vasodilatation (control) in the ciliary vascular bed of the bovine perfused eye. This response is blocked, and a normally suppressed vasoconstrictor response uncovered, following prolonged treatment with ascorbate (+asc, 50 μM, >120 min). Moreover, when the anterior and posterior chambers of the eye were continuously flushed with Krebs solution to prevent any accumulation of ascorbate in the aqueous humour (flushed), or when both the aqueous and vitreous humour were removed (drained), normal vasodilator responses to acetylcholine were seen, provided the arterial perfusate lacked ascorbate. Nevertheless, when ascorbate (50 μM, >120 min) was added to the perfusion fluid under these conditions, it still resulted in blockade of the EDHF-mediated vasodilatation (flushed + asc, drained + asc, respectively). Data represent the mean ± s.e.m. of five to eight observations, each from a different eye. ***$P<0.001$ indicates a significant blockade of the EDHF-mediated vasodilatation by ascorbate.

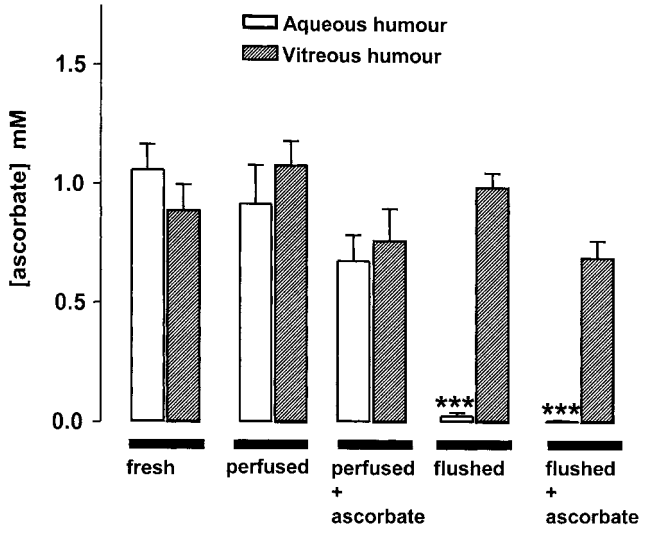

Figure 2 Histogram showing the concentration of ascorbate found in samples of aqueous and vitreous humour taken from freshly obtained bovine eyes (fresh), and from eyes perfused via a ciliary artery for 120 min with normal Krebs solution (perfused) or Krebs solution containing ascorbate (perfused + ascorbate, 50 μM). Also shown are the levels of ascorbate found following continuous flushing of the anterior and posterior chambers with Krebs solution in eyes whose ciliary vascular bed has been perfused with either normal Krebs solution (flushed) or Krebs containing ascorbate (flushed + ascorbate, 50 μM). Data represent the mean ± s.e.m. of six to nine observations, each from a different eye. ***$P<0.001$ indicates a significant difference from the level of ascorbate in fresh eyes.

Selectivity of the blockade by ascorbate in the ciliary vascular bed

Although nitric oxide does not contribute to acetylcholine-induced vasodilatation in the bovine ciliary vascular bed, basal release of this mediator exerts a tonic depression of vascular tone in this preparation (McNeish *et al*., 2001). Abolition of this tonic depressor action following treatment with L-NAME (100 μM) resulted in an increase in U46619-induced perfusion pressure (30.0 ± 6.0 mmHg, $n=7$). Following treatment with ascorbate (50 μM, >120 min), which blocked acetylcholine-induced, EDHF-mediated vasodilatation, L-NAME (100 μM) still produced this increase in perfusion pressure (30.5 ± 5.9 mmHg, $n=6$).

Furthermore, treatment with ascorbate (50 μM, >120 min) had no effect on the vasodilatation induced by the K_{ATP} channel opener, levcromakalim (0.1–30 nmol, Figure 3).

Effects of ascorbate on rings of bovine coronary artery

Following treatment with the cyclooxygenase inhibitor, indomethacin (3 μM), and induction of U46619-induced tone, bradykinin (0.1–300 nM) induced concentration-dependent vasodilatation (Figure 4). This vasodilatation was mediated jointly by nitric oxide and EDHF, since in the additional presence of L-NAME (100 μM), the residual vasodilatation induced by bradykinin (max 83.7 ± 1.8%) was mediated solely by EDHF, because it was almost abolished following treatment with apamin (100 nM), either alone or in combination with charybdotoxin (100 nM, Figure 4a). In contrast, in the additional presence of apamin and charybdotoxin (both 100 nM), the residual vasodilatation induced by bradykinin (max 87.0 ± 5.6%) was mediated solely by nitric oxide, since it was abolished by L-NAME (100 μM, Figure 4b).

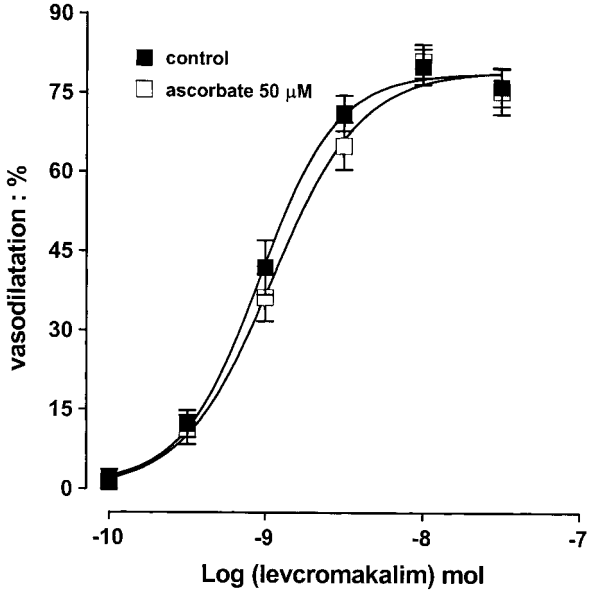

Figure 3 Dose–response curves showing that the ability of levcromakalim to induce vasodilatation in the bovine ciliary vascular bed is unaffected by treatment with ascorbate (50 μM, >120 min). Data represent the mean ± s.e.m. of six to seven observations, each from a different eye.

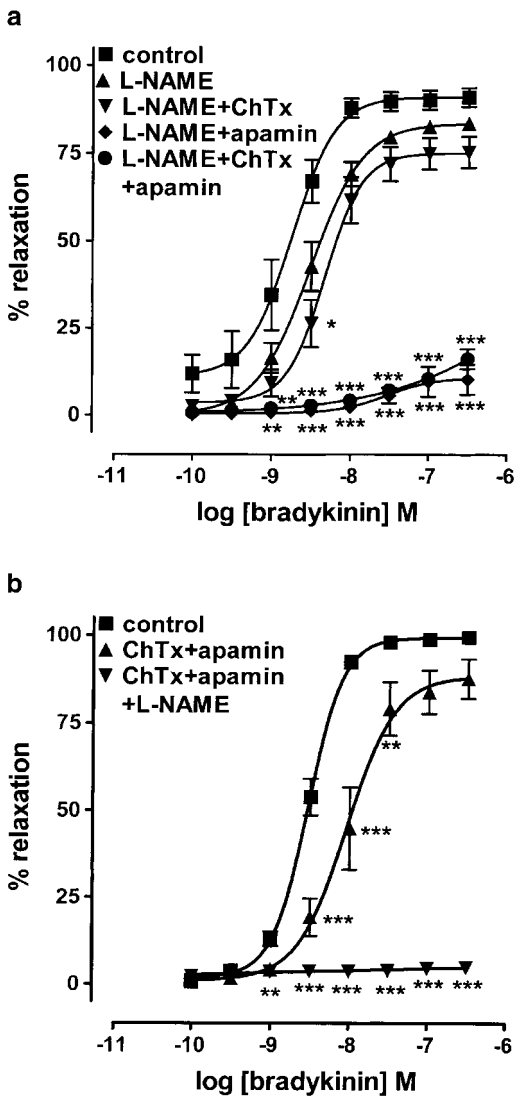

Figure 4 Following treatment of rings of bovine coronary artery with indomethacin (3 μM), bradykinin induces concentration-dependent relaxation through release of both EDHF and nitric oxide. (a) In the additional presence of L-NAME (100 μM), the residual vasodilatation was mediated solely by EDHF, since it was almost abolished by apamin (100 nM) alone or in combination with charybdotoxin (ChTx, 100 nM). (b) In the additional presence of the EDHF blockers, apamin and charybdotoxin (both 100 nM), the residual vasodilatation was mediated solely by nitric oxide, since it was abolished by L-NAME. Data represent the mean $\pm$ s.e.m. of eight to fifteen observations, each from a different vessel from five to seven animals. $^{*}P<0.05$, $^{**}P<0.001$ and $^{***}P<0.001$ indicate a significant difference from control.

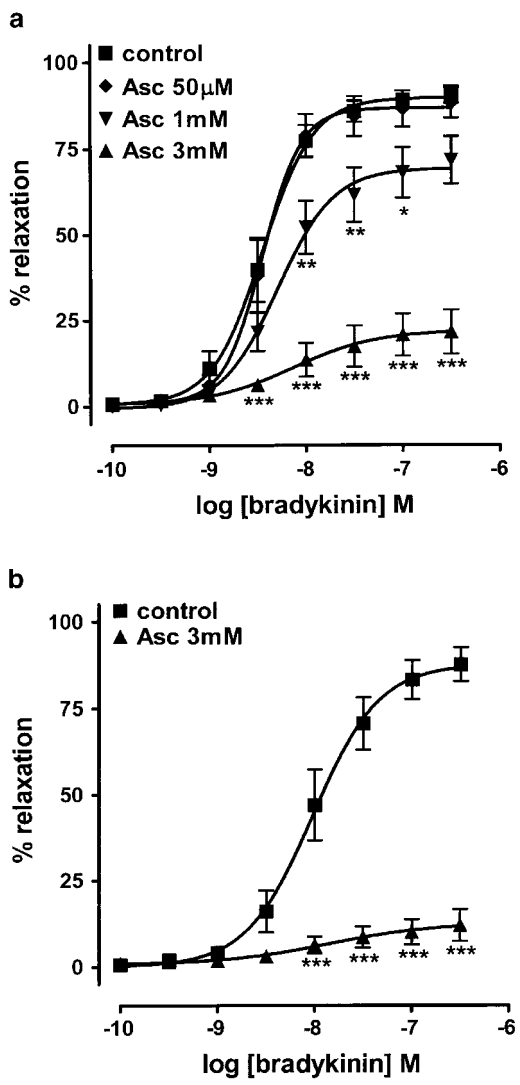

Figure 5 (a) Bradykinin-induced, EDHF-mediated vasodilatation of bovine coronary artery rings (obtained in the presence of L-NAME and indomethacin) was unaffected following treatment for 180 min with ascorbate at 50 μM, but was blocked at concentrations of 1 or 3 mM. (b) Bradykinin-induced, nitric oxide-mediated vasodilatation of bovine coronary artery rings (obtained in the presence of indomethacin and the EDHF blockers, apamin and charybdotoxin) was blocked following treatment with 3 mM ascorbate. Data represent the mean $\pm$ s.e.m. of seven to fifteen observations, each from a different vessel from four to seven animals. $^{*}P<0.05$, $^{**}P<0.001$ and $^{***}P<0.001$ indicate a significant difference from control.

In contrast to our findings in the ciliary vascular bed, bradykinin-induced, EDHF-mediated vasodilatation (conducted in the presence of L-NAME 100 μM) in the bovine coronary artery was unaffected following treatment for 180 min with ascorbate at 50 (Figure 5a) or even 150 μM (data not shown). When the concentration of ascorbate was increased to 1 or 3 mM, however, concentration-dependent blockade of EDHF-mediated vasodilatation was seen (Figure 5a). Similarly, ascorbate at 50 μM failed to affect acetylcholine-induced, EDHF-mediated vasodilatation, but at 3 mM almost complete blockade was seen (Figure 6). Moreover, at a concentration of 3 mM, ascorbate also blocked bradykinin-induced, nitric oxide-mediated vasodilatation (conducted in the presence of apamin and charybdotoxin, both 100 nM) in the coronary artery (Figure 5b), but vasodilatation induced by glyceryl trinitrate (1–300 nM) or levcromakalim (10–300 nM) was unaffected (Figure 7).

Discussion

This study confirms our previous report that the presence of 50 μM ascorbate in the perfusing fluid leads to blockade

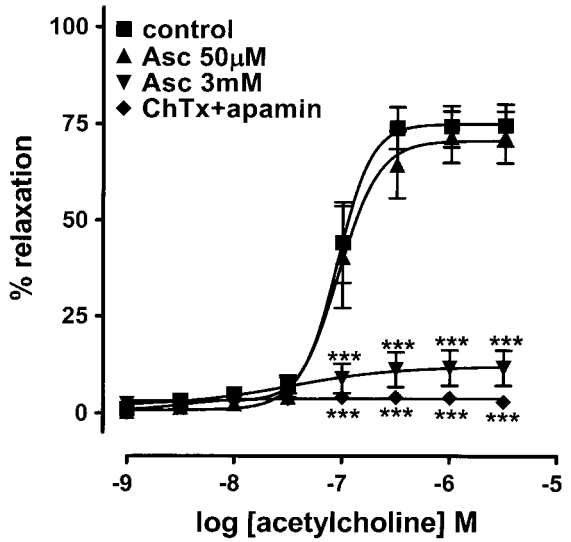

Figure 6 Following treatment of rings of bovine coronary artery with indomethacin (3 μM) and L-NAME (100 μM), acetylcholine induces concentration-dependent vasodilatation. This vasodilatation was mediated by EDHF, since it was abolished in the combined presence of apamin and charybdotoxin (ChTx, both 100 nM). Acetylcholine-induced, EDHF-mediated vasodilatation was unaffected following treatment for 180 min with ascorbate at 50 μM, but was blocked at 3 mM. Data represent the mean$\pm$s.e.m of five to seven observations, each from a different vessel from four to six animals. ***$P<0.001$ indicates a significant difference from control.

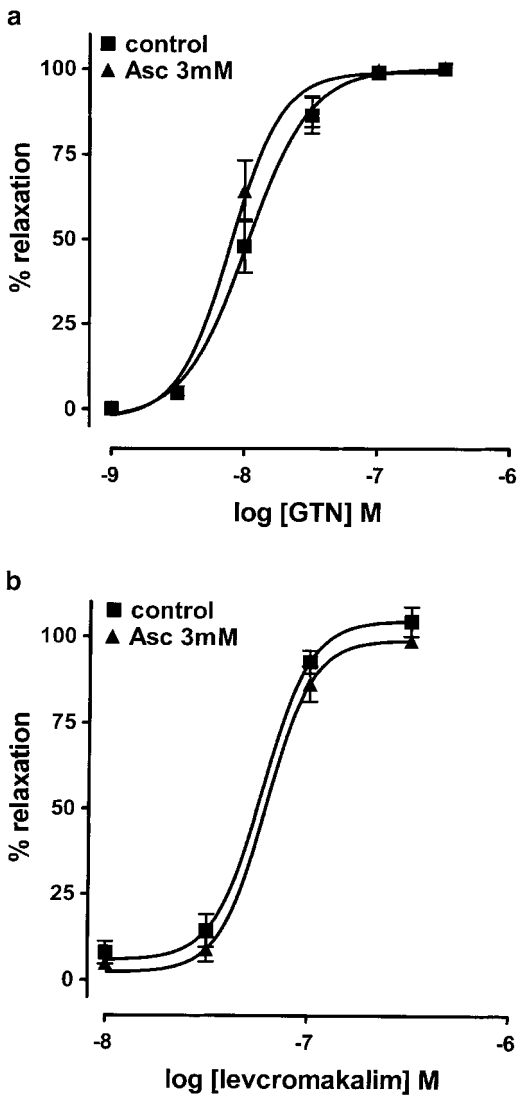

Figure 7 Treating bovine coronary arteries for 180 min with ascorbate (3 mM) had no effect on vasodilatation to (a) glyceryl trinitrate (GTN) or (b) levcromakalim. Data represent the mean$\pm$s.e.m. of four to eight observations, each from a different vessel from four to five animals.

of EDHF-mediated vasodilatation in the ciliary vascular bed of the bovine eye (McNeish *et al*., 2002b). A concentration of 50 μM ascorbate is roughly similar to the plasma level in humans (mean 46 μM, range 30–150 μM) (Keaney & Vita, 1995; Levine *et al*., 1996), perhaps suggesting that in the eye, EDHF-mediated vasodilatation is normally greatly suppressed. Nevertheless, it is possible that the actual concentration that blocks EDHF is considerably higher, because ascorbate supplied *via* the ciliary circulation is actively transported by the epithelial cells of the ciliary body (Millar & Kaufman, 1995; Mead *et al*., 1996), such that levels of around 1 mM are found in both aqueous and vitreous humour of eyes from a wide range of mammalian species and humans (Davson, 1980; Halliwell & Gutteridge, 1989).

Our own measurements of 1.1 ± 0.1 and 0.9 ± 0.1 mM ascorbate in the aqueous and vitreous humour, respectively, of freshly obtained eyes are in keeping with published values for this species (1.1 and 0.5 mM, respectively) (Davson, 1980). Moreover, these levels were maintained following perfusion of the ciliary circulation for 120 min with Krebs solution, although surprisingly they appeared to fall slightly when the perfusate contained ascorbate (50 μM). Some consumption of the antioxidant in redox reactions might have been expected during this period, but the epithelia lining the chamber have such avid transport and metabolic activity that any dehydroascorbate formed is rapidly reduced back to ascorbate (Bode *et al*., 1991, 1993). Thus, the levels of ascorbate in both aqueous and vitreous humour appear to be tightly regulated.

As expected, when we continuously flushed the anterior and posterior chambers of the eye with normal Krebs solution to prevent any concentration of ascorbate by the ciliary body, the level of the antioxidant in the aqueous humour fell below our limit of detection (~10 μM). The level in the vitreous humour did not change, however, indicating that the integrity of the barrier separating the ocular chambers remained intact. Moreover, the act of flushing the anterior and posterior chambers had no effect by itself on the ability of acetylcholine to induce EDHF-mediated vasodilatation in the ciliary vascular bed. Nevertheless, flushing to prevent any accumulation of ascorbate failed to influence the ability of the antioxidant (50 μM) to block EDHF. It was therefore likely that a concentration of 50 μM ascorbate was sufficient to block EDHF in the ciliary vascular bed, and that no further accumulation in the aqueous humour was required. This conclusion was supported by experiments in which the aqueous and vitreous humour were both removed and the ciliary circulation perfused without either its endogenous

reservoirs of ascorbate or the ability to concentrate it. Under these conditions, acetylcholine-induced, EDHF-mediated vasodilator responses were identical to those obtained when the eye remained intact. Nevertheless, when 50 μM ascorbate was included in the perfusion fluid, the antioxidant retained its ability to block EDHF. Thus, it is almost certain that accumulation of ascorbate in the aqueous or vitreous humour is not a prerequisite for blockade of EDHF to occur and that a level of 50 μM itself is sufficient.

We previously reported that the ability of ascorbate to block EDHF in the bovine ciliary circulation appeared selective, since vasodilator responses to the nitric oxide donor, glyceryl trinitrate, remained unaffected (McNeish *et al*., 2002b). The findings of the present study support and extend these observations. Specifically, although nitric oxide plays no role in the endothelium-dependent vasodilator actions of acetylcholine or bradykinin in the ciliary vascular bed, basal release of this mediator exerts a tonic vasodilator action, since infusion of the nitric oxide synthase inhibitor, L-NAME, enhances U46619-induced vasoconstriction (McNeish *et al*., 2001). We now find that under conditions where EDHF is blocked by 50 μM ascorbate, this tonic vasodilator action of nitric oxide remains active, since L-NAME was still able to enhance U46619-induced vasoconstriction. From this we can conclude that when ascorbate blocks EDHF, this does not result from a nonselective action on the vascular endothelium. In addition to this, ascorbate (50μM) had no effect on the vasodilator actions of levcromakalim, which relaxes vascular smooth muscle through the opening of K_{ATP} channels (Quast & Cook, 1989; Edwards & Weston, 1993). Thus, the ability of ascorbate (50 μM) to block EDHF in the ciliary vascular bed appears entirely selective.

To date, the effects of ascorbate on EDHF have only been investigated in the bovine ciliary circulation and rat mesentery; in both cases blockade was observed (McNeish *et al*., 2002b). In these two beds, vascular tone is controlled by resistance vessels, so we wished to determine if similar blockade was seen in a large conduit artery. The bovine coronary artery was chosen for this purpose, since in this tissue vasodilatation is mediated jointly by endothelium-derived nitric oxide and EDHF (Drummond *et al*., 2000; Pratt *et al*., 2001). When the nitric oxide component is blocked with L-NAME, $\sim$90% relaxation can still be attained through EDHF working alone. Similarly, if the EDHF component is blocked with apamin and charybdotoxin (Waldron & Garland, 1994; Zygmunt & Högestätt, 1996), $\sim$90% relaxation can still be attained through NO working alone. In fact, as previously reported (Drummond *et al*., 2000), apamin alone almost abolished the EDHF response, but we always included charybdotoxin to remove the small component contributed by IK_{Ca} channels.

In contrast to our findings in the bovine ciliary circulation, ascorbate at a concentration of 50 μM, or even 150 μM, failed to inhibit bradykinin- or acetylcholine-induced, EDHF-mediated vasodilator responses in the coronary artery. Raising the concentration to 1 and 3 mM, did, however, lead to concentration-dependent blockade. Similarly, 50 μM ascorbate had no effect on acetylcholine-induced, EDHF-dependent vasodilatation, but 3 mM produced powerful blockade. Nevertheless, the nature of the blockade with ascorbate at 3 mM was different from that seen at 50 μM in the ciliary circulation. Although even 3 mM ascorbate had no effect on the endothelium-independent vasodilator actions of glyceryl trinitrate or levcromakalim, suggesting a degree of selectivity in the blockade produced, bradykinin-induced, nitric oxide-mediated vasodilatation (observed in the presence of the EDHF blockers, apamin and charybdotoxin) was profoundly inhibited. Indeed, millimolar concentrations of ascorbate have previously been found to block the actions of nitric oxide (de Saram *et al*., 2002). Thus, the blockade of EDHF produced by millimolar concentrations of ascorbate in the bovine coronary artery may arise through nonselective disruption of endothelial function, while that produced in the ciliary circulation by a concentration of 50 μM seems entirely selective.

An explanation for the ability of ascorbate (50 μM) to block EDHF in the bovine ciliary circulation but not in the coronary artery was not immediately apparent. Since the EDHF response in the ciliary circulation is powerfully blocked by charybdotoxin, with little, if any, additional block with apamin (McNeish *et al*., 2001), whereas that in the coronary artery is almost abolished by apamin alone (Drummond *et al*., 2000; this study), we considered the possibility that ascorbate might selectively block the IK_{Ca} channel. This explanation seems unlikely, however, since in a preliminary investigation we have found that ascorbate also fails to block in the porcine coronary artery (McNeish *et al*., 2002a), where the charybdotoxin-sensitive IK_{Ca} channel significantly contributes to the EDHF response (Burnham *et al*., 2002; Sollini *et al*., 2002). Another possibility could simply be that the nature of the EDHF is different at the two vascular sites. Indeed, there has been speculation that the EDHF in porcine and bovine coronary arteries is different from that in other tissues and may be an epoxyeicosatrienoic acid generated from arachidonic acid by cytochrome P_{450} (Bauersachs *et al*., 1994; Fisslthaler *et al*., 1999). Alternatively, the ability of ascorbate to block might be related to vessel size, such that it occurs in small resistance vessels (ciliary and mesenteric vascular beds) but not in a large conduit artery (coronary artery), perhaps reflecting the higher density of myoendothelial gap junctions in vessels of small diameter through which the EDHF response could be transmitted (Sandow & Hill, 2000; Berman *et al*., 2002). Yet another possibility is that blockade by ascorbate might occur in the presence (perfused vascular beds) but not in the absence (coronary artery rings) of flow. Indeed, the shearing forces generated by flow can themselves promote the activation of EDHF (Dube & Canty, 2001; Miura *et al*., 2001). Moreover, an intact vessel may behave differently from one cut into rings. Further study will, however, be required to investigate these alternative possibilities.

In conclusion, our findings suggest that concentration of ascorbate in the aqueous humour is not a prerequisite for blockade of EDHF to occur in the ciliary vascular bed of the bovine eye and that the normal plasma concentration of $\sim$50 μM ascorbate is highly effective. Moreover, the blockade is highly selective, since basal nitric oxide activity and the vasodilator effects of the K_{ATP} channel opener, levcromakalim, were completely unaffected. Ascorbate at this concentration failed, however, to block EDHF in the bovine coronary artery, demonstrating that the antioxidant is not a universal inhibitor of EDHF. Millimolar concentrations of ascorbate did inhibit EDHF in the coronary artery, but this action appeared to result from dysfunction of the endothelium, since

endothelium-dependent, nitric-oxide-mediated vasodilatation was also impaired. The mechanism accounting for the ability of ascorbate to block EDHF in the bovine ciliary circulation, but not the coronary artery, remains to be determined.

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