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Requirement for flow in the blockade of endothelium-derived hyperpolarizing factor (EDHF) by ascorbate in the bovine ciliary artery

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1 We previously reported that ascorbate inhibits endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatation in the bovine perfused ciliary circulation and rat perfused mesentery, but not in rings of bovine or porcine coronary artery.

2 In this study, we have compared the ability of ascorbate to inhibit EDHF-mediated vasodilatation in a single vessel, the bovine long posterior ciliary artery, when perfused and when mounted as rings in a myograph.

3 Both in segments perfused at a flow rate of 2.5 ml min^{-1} and in rings mounted in a myograph, bradykinin and acetylcholine each induced vasodilator responses that were mediated jointly by EDHF and nitric oxide, as revealed by their respective blocking agents, apamin/charybdotoxin, and L-NAME.

4 Ascorbate (50 and 150 μM) induced a time (max at 2–3 h)-dependent inhibition of the EDHF-mediated component of vasodilatation to bradykinin or acetylcholine in perfused segments, but not in rings.

5 Ascorbate (50 μM) failed to inhibit bradykinin-induced vasodilatation at a flow rate of 1.25 ml min^{-1} or below, but produced graded blockade at the higher flow rates of 2.5 and 5 ml min^{-1}. Furthermore, using a pressure myograph where pressure and flow were independently controlled, it was confirmed that the inhibitory action of ascorbate (150 μM) was directly related to flow *per se* and not any associated changes in pressure.

6 Thus, we have shown in the bovine ciliary artery that ascorbate inhibits EDHF-mediated vasodilatation under conditions of flow but not in a static myograph. The mechanism by which flow renders EDHF susceptible to inhibition by ascorbate remains to be determined.
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Abbreviations: BK_{Ca}, large-conductance calcium-activated potassium channel; EDHF, endothelium-derived hyperpolarizing factor; i.d., internal diameter; IK_{Ca}, intermediate conductance calcium-activated potassium channel; L-NAME, N^G-nitro-L-arginine methyl ester; o.d., outer diameter; SK_{Ca}, small-conductance calcium-activated potassium channel

Introduction

The vascular endothelium plays a vital role in the regulation of vasomotor tone through the production of three distinct vasodilator signals, namely nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF; for reviews see Moncada *et al*., 1991; Campbell & Harder, 2001). Although the nature of the EDHF is not fully understood, vasodilatation by this agent is inhibited following blockade of calcium-activated potassium channels of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance on the vascular endothelium using apamin and charybdotoxin, respectively (Waldron & Garland, 1994; Zygmunt & Högestätt, 1996; Doughty *et al*., 1999). We have recently shown that EDHF-mediated vasodilatation in the bovine perfused ciliary circulation and rat perfused mesentery induced by bradykinin or acetylcholine is sensitive to inhibition by ascorbate in a time- (max inhibition at ~120 min) and concentration-dependent manner (McNeish *et al*., 2002; 2003a), that is, at concentrations from 10–150 μM, similar to those occurring in human plasma (Keaney & Vita, 1995; Levine *et al*., 1996). The precise mechanism by which ascorbate inhibits EDHF is unknown, but is likely to involve an antioxidant action, since it can be mimicked by the reducing agents, *N*-acetyl-L-cysteine or dithiothreitol, but not by the redox-inactive analogue, dehydroascorbate (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 (McNeish *et al*., 2002; 2003a).

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Although effective in the bovine perfused ciliary circulation and rat perfused mesentery (McNeish *et al*., 2002), ascorbate does not block EDHF-mediated vasodilatation in rings of bovine or porcine coronary artery (McNeish *et al*., 2003a, b). Why ascorbate was effective in only certain vascular preparations was not clear, but we previously speculated that this might relate to differences in the nature of EDHF at different sites. For example, EDHF has been characterized as such diverse entities as potassium ions, an endogenous cannabinoid, an epoxyeicosatrienoic acid, hydrogen peroxide, or gap junctional spread of endothelial hyperpolarization (for review see Campbell & Harder, 2001), and any one of these might show particular sensitivity to ascorbate. Furthermore, differential sensitivity to ascorbate could potentially relate to differences in vessel size (resistance arterioles *versus* conduit arteries), or to the presence (perfused preparations) or absence (arterial rings in a static tissue bath) of flow.

The aim of this investigation was to compare the ability of ascorbate to inhibit EDHF-mediated vasodilatation in a single vessel, the bovine ciliary artery, when perfused and when mounted in a static system in the absence of flow. Our findings reveal that flow is required in order for ascorbate to inhibit EDHF.

Methods

Preparation of bovine long posterior ciliary artery segments for perfusion and pressure measurement

Bovine eyes were obtained from a local abattoir within 90 min of killing. At the laboratory, a long posterior ciliary artery was cannulated on each eye approximately 2 cm from its insertion into the sclera. These extraocular ciliary artery segments of $\sim$1300 μm internal diameter (i.d.), together with any side branches (usually 2–3 of $\sim$570 μm i.d.) were cut from the eye at their insertion into the sclera and perfused using a peristaltic pump at a rate (except where stated) of 2.5 ml min^{-1} with Krebs solution at 37°C 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. Ciliary artery segments were perfused for an equilibration period of at least 30 min before experiments were begun. 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.).

Experimental protocols with the bovine perfused ciliary artery

In order to observe vasodilator responses in the ciliary artery, the perfusion pressure was first raised to $\sim$120 mmHg using the thromboxane A_2-mimetic, U46619 ($\sim$200 nM). In all experiments, responses to bradykinin or acetylcholine were elicited by injecting into the perfusate 10 μl of the appropriate concentration with a Hamilton micro-syringe. It should be noted that although bradykinin dilated all ciliary artery segments to which it was added, results with acetylcholine were less consistent, with only $\sim$40% producing substantial dilatation, while the remainder dilated poorly or constricted (see Results). Consequently, only segments in which acetylcholine produced at least 20% relaxation were used in experiments. When required, the effects of nitric oxide, cyclooxygenase products and EDHF were blocked using N^G-nitro-L-arginine-methyl ester (L-NAME) (100 μM; Rees *et al*., 1990), indomethacin (3 μM) and the combination of apamin and charybdotoxin (both 100 nM; Waldron & Garland, 1994; Zygmunt & Högestätt, 1996), respectively. Some blocking agents, that is, L-NAME and charybdotoxin, enhanced U46619-induced pressure in this preparation; so, when these were employed, the concentration of the vasoconstrictor was reduced to ensure that the pressure induced was similar to that of control experiments.

When the effects of ascorbate (50 or 150 μM) were examined on vasodilator responses in the perfused ciliary artery, this agent was present in the perfusing fluid for at least 120 min, since previous work had shown this timecourse to result in maximal blockade of EDHF (McNeish *et al*., 2002). Furthermore, experiments involving ascorbate were always compared with time-matched controls. Although most experiments were conducted at a flow rate of 2.5 ml min^{-1}, some were also conducted at different rates (0.3, 0.6, 1.25, 2.5 and 5 ml min^{-1}).

Preparation of bovine long posterior ciliary artery rings for tension measurement

The terminal 2 cm segment of a long posterior ciliary artery was dissected from the eye, cut into 2 mm ring segments and mounted in wire myographs (Multi myograph, model 610; Danish Myo Technology) maintained at 37°C in Krebs solution gassed with O_2 containing 5% CO_2. Isometric tension was recorded and displayed using Myodaq (2.01) and analysed using Myodata (2.02). Tension ($\sim$35 mN) was applied to the vessels to give a transmural pressure equivalent to 100.8 ± 1.3 mmHg, $n = 107$, which is similar to the *in vivo* range for vessels of this size (1303 ± 15 μm i.d., $n = 107$). Some experiments (see Results) were also conducted on rings cut from side branches (567 ± 33 μm i.d., $n = 12$) of the main ciliary artery. Tissues were allowed to equilibrate for 30 min before experiments were carried out.

In order to observe vasodilator responses to bradykinin or acetylcholine, assessed as cumulative concentration–responses curves, rings of bovine long posterior ciliary artery were contracted to $\sim$80% of the maximal U46619-induced tone using a concentration of 10–100 nM. As in perfusion experiments, the possible effects of nitric oxide, cyclooxygenase products and EDHF were blocked using L-NAME (100 μM), indomethacin (3 μM), and apamin/charybdotoxin (both 100 nM), respectively. 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. In experiments examining the effects of ascorbate (50 and 150 μM) on vasodilator responses, tissues were pretreated with the antioxidant for at least 120 min. At the end of each experiment, papaverine (300 μM) was added to the myograph chamber to establish baseline tone and all measurements of U46619-induced tone were taken relative to this.

Pressure myography

In order to investigate the effects of flow and pressure on the ability of ascorbate to inhibit bradykinin-induced vasodilatation, some studies were conducted using a pressure myograph (Living Systems Instrumentation, Burlington, VT, U.S.A.), modified for use with large vessels. Terminal segments (1–2 cm) of ciliary artery $\sim$1620 μm outer diameter (o.d.) were cannulated at both ends and any side branches tied off using surgical silk before being pressurized using the servo-controlled peristaltic pump to $\sim$100 mmHg. The bath (volume 10 ml) contained oxygenated Krebs solution at 37°C and this was refreshed every 60 min. The video dimension analyser was used to measure o.d. since the vessels were not transparent. During the equilibration period (1–2 h), vessels developed myogenic tone, thus obviating the need for a vasoconstrictor. When flow through the vessel was required, this was achieved by delivering Krebs solution at a rate of 5 ml min^{-1} *via* a side arm located proximal to the vessel using a separate peristaltic pump. During these experiments, the pressure was maintained at $\sim$100 mmHg by raising the outflow tubing to an appropriate height. Vasodilator responses were elicited at intervals of 1 h to bradykinin (1 nmol) applied intraluminally in 20 μl boluses *via* a pediatric cannula (Doughty *et al*., 1999). These responses were always elicited when pressurized, but in the absence of flow. In the experiments where the effects of flow were examined, flow was interrupted briefly to permit this. When the effects of ascorbate were examined on bradykinin-induced vasodilator responses in both the presence and absence of flow, it was present in both the extraluminal and intraluminal Krebs solutions for at least 3 h. Since some of these experiments involved prolonged incubation without replacement of the intraluminal fluid, ascorbate was used at a higher concentration of 150 μM to compensate for any decay or tissue uptake of the antioxidant that may potentially have occurred. At the end of each experiment, papaverine (300 μM) was added to determine the level of myogenic tone present.

Drugs and chemicals

Acetylcholine chloride, apamin, ascorbic acid, bradykinin acetate, indomethacin, L-NAME, papaverine (free base) and U46619 (9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$) were all obtained from Sigma (Poole, U.K.). Charybdotoxin was obtained from Latoxan (Valence, France). 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 U46619 (1 mM), which was dissolved in 50% ethanol.

Statistical analysis

Results are expressed as the mean$\pm$s.e.m. of n observations, each from a separate eye. Statistical comparisons were made using one-way analysis of variance and the Bonferroni post-test, 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 bovine perfused ciliary arterial segments

The basal perfusion pressure in ciliary arterial segments at a constant flow of 2.5 ml min^{-1} was 20.9$\pm$3.3 mmHg (n=16) and this was increased to 123.7$\pm$15.7 mmHg (n=16) following infusion of U46619 (0.1–0.2 μM).

In the presence of U46619-induced pressure, bolus doses of bradykinin (0.1 pmol–10 nmol) induced dose-dependent vasodilatation (max 69.0$\pm$6.7%, n=9; Figure 1). This vasodilatation was mediated jointly by EDHF and nitric oxide since it

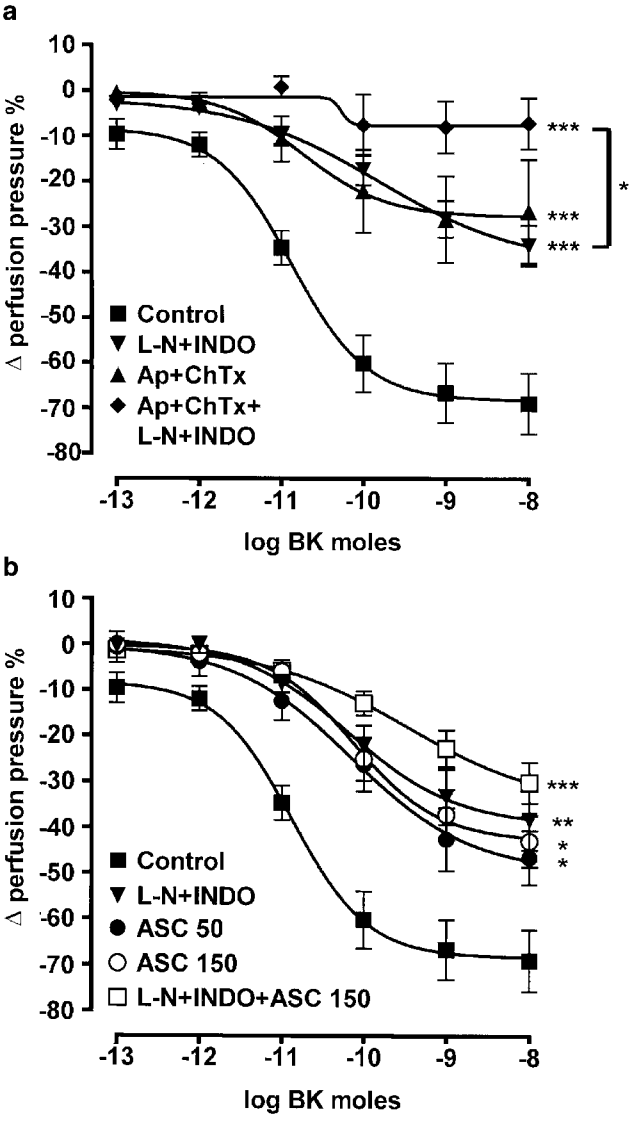

Figure 1 Bradykinin induced dose-dependent vasodilatation of bovine perfused ciliary artery segments. (a) The EDHF-dependent component was inhibited following combined treatment with apamin and charybdotoxin (Ap + ChTx, both 100 nM) and the EDHF-independent component was blocked by L-NAME (L-N, 100 μM) and indomethacin (INDO, 3 μM). (b) Vasodilatation was also inhibited by ascorbate (ASC, 50 or 150 μM, >120 min) alone or in combination with L-NAME and indomethacin. Data are mean$\pm$s.e.m.; $n\geq6$; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ indicate significant differences in maximal vasodilatation from time-matched control or between groups joined by a bracket.

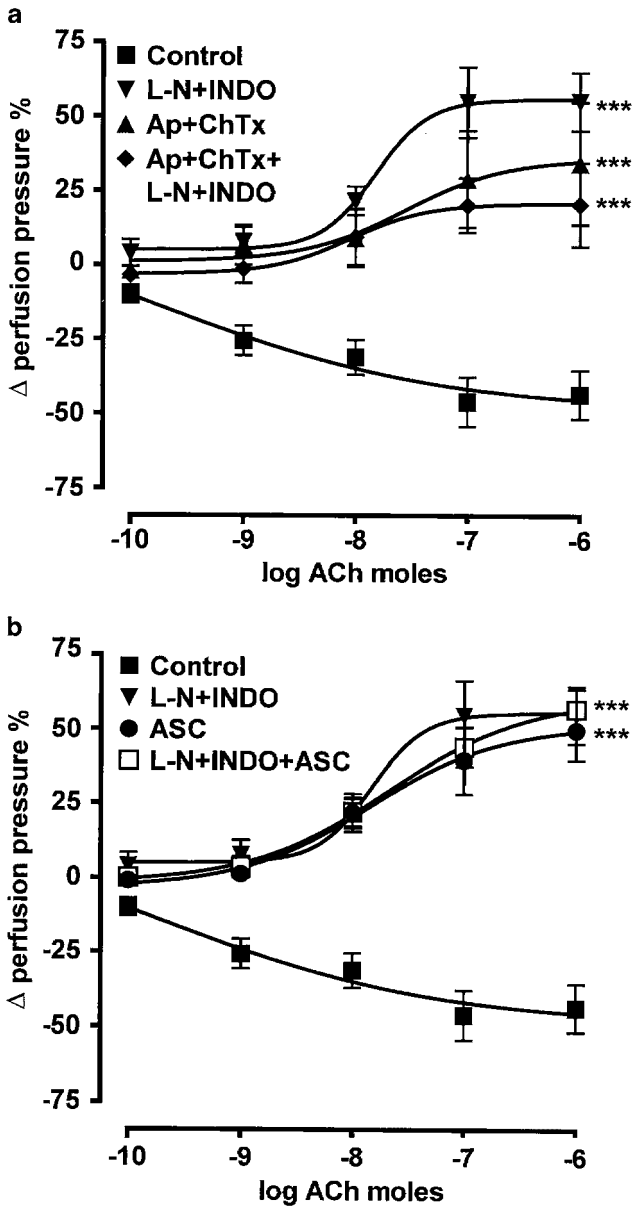

Figure 2 Acetylcholine induced dose-dependent vasodilatation of bovine perfused ciliary artery segments. (a) Vasodilatation was reversed to vasoconstriction following treatment with the EDHF blockers apamin/charybdotoxin (Ap + ChTx, both 100 nM) or with L-NAME (L-N, 100 μM) and indomethacin (INDO, 3 μM). (b) Vasodilatation was also reversed to constriction by ascorbate (ASC, 50 μM, >120 min) alone or in combination with L-NAME and indomethacin. Data are mean ± s.e.m.; $n \geqslant 8$; ***$P<0.001$ indicates differences from time-matched control.

was powerfully inhibited (Figure 1a) following blockade of either EDHF with apamin/charybdotoxin, or of nitric oxide synthase and cyclooxygenase with L-NAME and indomethacin; L-NAME alone was just as effective, but indomethacin alone had no effect (data not shown). Treatment with all the four blocking agents almost abolished vasodilatation. Ascorbate (50 μM, >120 min, Figure 1b), which we have previously shown, abolishes EDHF-mediated vasodilatation in the bovine ciliary vascular bed (McNeish *et al*., 2002; 2003a), also significantly inhibited bradykinin-induced vasodilatation in perfused ciliary artery segments, but it was clearly less effective than the combination of apamin and charybdotoxin. Moreover, increasing the concentration of ascorbate to 150 μM failed to intensify the inhibition. There was a trend towards further inhibition in the additional presence of L-NAME and indomethacin, but substantial vasodilatation remained (max 30.1 ± 4.5%, $n=9$).

Acetylcholine was a less consistent vasodilator in perfused ciliary artery segments than bradykinin: vasodilatation was seen in 32 out of 76 preparations studied, with the remainder exhibiting constriction. Those that dilated to acetylcholine (0.1 nmol–1 μmol) did so in a dose-dependent manner (max 43.6 ± 8.0%, $n=8$; Figure 2). This vasodilatation, like that induced by bradykinin, was mediated jointly by EDHF and nitric oxide since it was reversed to constriction (Figure 2a) by apamin/charybdotoxin or by L-NAME and indomethacin; L-NAME alone was just as effective, but indomethacin alone had no effect (data not shown). A similar reversal of acetylcholine-induced vasodilatation to constriction resulted from treatment with all the four blocking agents together (Figure 2a), or with ascorbate (50 μM), either alone or in the presence of L-NAME and indomethacin (Figure 2b).

The L-NAME -induced increase in U46619-induced perfusion pressure that occurs through blockade of basal nitric oxide release was unaffected following treatment for >120 min with 150 μM ascorbate (111.9 ± 22.8 mmHg, $n=8$, *versus* control of 85.3 ± 11.9 mmHg, $n=13$). Furthermore, the EDHF blockers, apamin/charybdotoxin (both 100 nM), also failed to affect the rise in pressure induced by L-NAME (79.2 ± 11.2 mmHg, $n=8$).

Effects of ascorbate in bovine ciliary arterial rings

Having established that ascorbate inhibits the EDHF-mediated component of vasodilatation induced by bradykinin or acetylcholine in perfused ciliary artery segments, the effects of the antioxidant were then examined in ring preparations of this vessel mounted in a wire myograph. Following induction of tone (88.7 ± 11.4 mN, $n=8$) using U46619, rings of bovine ciliary artery (~1300 μm i.d.) relaxed in a concentration-dependent manner to bradykinin (1 nM–1 μM, max 87.8 ± 4.9%, $n=8$; Figure 3) or acetylcholine (10 nM–10 μM, max 85.6 ± 2.2%, $n=7$; Figure 4). As with perfused segments of ciliary artery, these relaxations were mediated jointly by EDHF and nitric oxide since they were inhibited (Figures 3a and 4a) by apamin/charybdotoxin or by L-NAME and indomethacin; L-NAME alone was just as effective, but indomethacin alone had no effect (data not shown). Treatment with all four blocking agents almost abolished relaxant responses to bradykinin or acetylcholine. In contrast to findings in perfused preparations, treatment with ascorbate (50–150 μM) had no effect on relaxations to bradykinin or acetylcholine and failed to enhance the blockade seen in the presence of L-NAME and indomethacin (Figures 3b and 4b). Ascorbate (150 μM) also failed to affect bradykinin-induced relaxation in rings of small branches (567 ± 33 μm i.d.) of the main ciliary artery (control max 86.3 ± 6.4%; ascorbate max 91.2 ± 2.7%, $n=6$ for both). Also, in contrast to findings in perfused preparations, acetylcholine did not induce contractile responses in rings of ciliary artery in the presence of any or all of the blocking agents. Acetylcholine did, however, induce contraction in endothelium-denuded rings in the absence of all

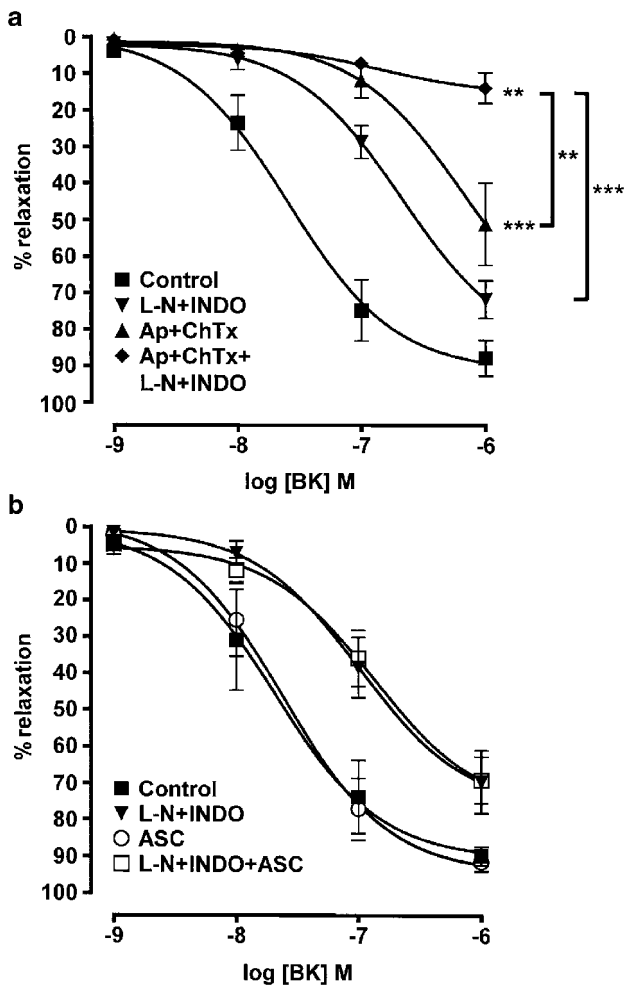

Figure 3 Bradykinin induced concentration-dependent relaxation of bovine ciliary artery rings in a wire myograph. (a) The EDHF-dependent component was inhibited following combined treatment with apamin and charybdotoxin (Ap + ChTx, both 100 nM) and the EDHF-independent component was blocked by L-NAME (L-N, 100 μM) and indomethacin (INDO, 3 μM). (b) Vasodilatation was unaffected by ascorbate (ASC, 150 μM, >120 min) alone or in combination with L-NAME and indomethacin. Data are mean ± s.e.m.; $n \geqslant 6$; $^{**}P<0.01$ and $^{***}P<0.001$ indicate differences in maximal relaxation from time-matched control or between groups joined by a bracket.

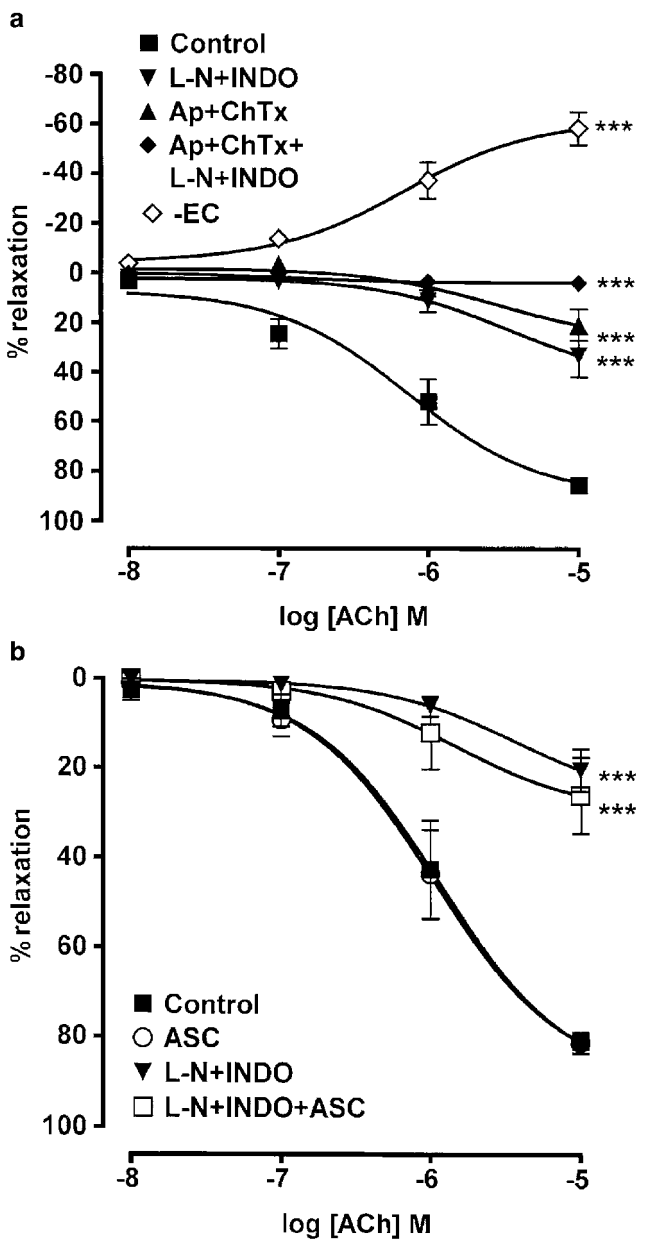

Figure 4 Acetylcholine induced concentration-dependent relaxation of bovine ciliary artery rings in a wire myograph. (a) The EDHF-dependent component was inhibited following combined treatment with apamin and charybdotoxin (Ap + ChTx, both 100 nM) and the EDHF-independent component was blocked by L-NAME (L-N, 100 μM) and indomethacin (INDO, 3 μM). Following endothelial denudation (-EC), acetylcholine-induced relaxation was reversed to constriction. (b) Vasodilatation was unaffected by ascorbate (ASC, 150 μM, >120 min) alone and the antioxidant did not enhance the inhibition induced by L-NAME and indomethacin. Data are mean ± s.e.m.; $n \geqslant 6$; $^{***}P<0.001$ indicates differences from time-matched control.

blocking agents, where it was easier to hold tone at a stable, submaximal level (Figure 4a).

Effect of flow rate on the ability of ascorbate to block EDHF-mediated vasodilatation

Since ascorbate inhibited the EDHF-mediated component of vasodilatation in the ciliary artery when perfused in segments but not when suspended as rings, we considered the possible role of flow. In control segments constricted with U46619, the magnitude of bradykinin (1 nmol)-induced vasodilatation (% reduction in perfusion pressure) was similar across a range of flow rates (0.3–5 ml min^{-1}, Figure 5a) and was well maintained for many hours at each flow rate. The ability of ascorbate (50 μM, 3 h) to inhibit bradykinin-induced vasodilatation was, however, strictly determined by flow rate: no inhibition was seen at 1.25 ml min^{-1} or below, but higher flow rates produced graded inhibition, and at 5 ml min^{-1} vasodilatation was almost abolished.

In a separate series of experiments in which inhibition of bradykinin (1 nmol)-induced vasodilatation had previously been established by ascorbate (50 μM, 3 h) following perfusion at 5 ml min^{-1}, sequentially reducing the flow rate to 2.5, 1.25,

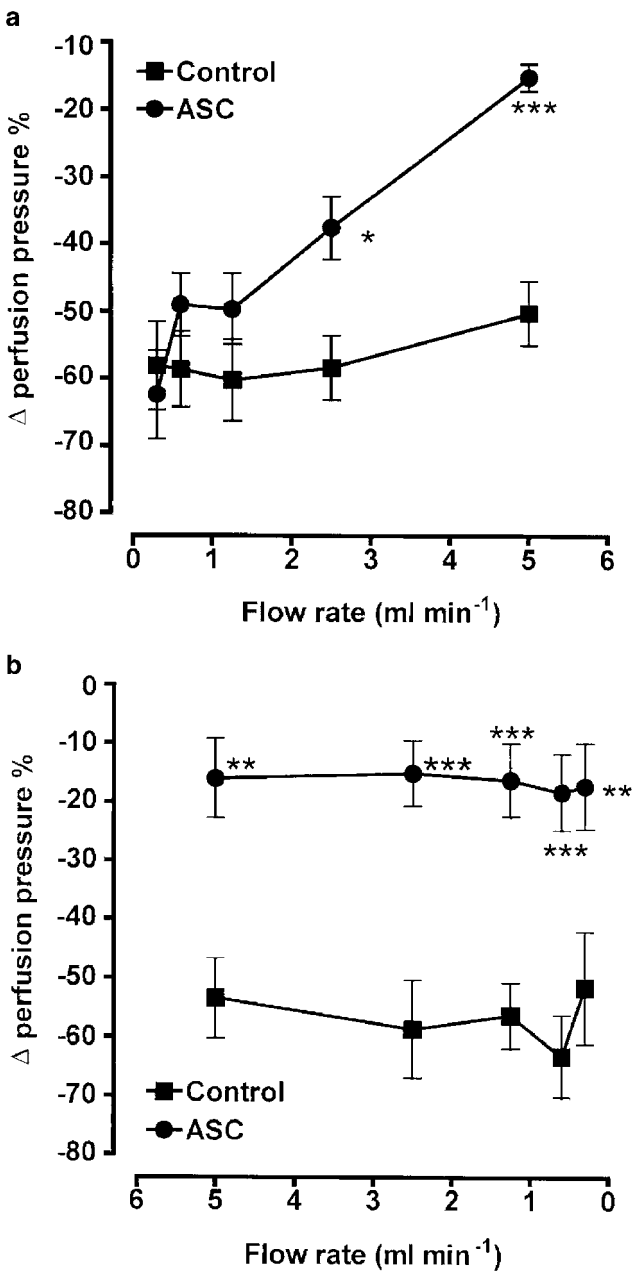

Figure 5 The effect of flow rate on the ability of ascorbate to inhibit bradykinin (1 nmol)-induced vasodilatation in bovine perfused ciliary artery segments. (a) In control preparations, bradykinin-induced vasodilatation was similar at different rates of flow. Ascorbate (ASC, 50 μM, 3 h) inhibited vasodilatation at high but not low rates of flow. (b) In control preparations, sequentially lowering the flow rate from 5 to 0.3 ml min^{-1} had no effect on bradykinin-induced vasodilatation. When inhibition of vasodilatation was established with ascorbate (ASC, 50 μM, 3 h) at 5 ml min^{-1}, sequentially reducing the flow rate to 2.5, 1.25, 0.6 and 0.3 ml min^{-1} had no effect on the level of inhibition observed. Data are mean ± s.e.m.; $n \geq 6$; *$P<0.05$, **$P<0.01$ and ***$P<0.001$ indicate differences from time-matched control.

0.6 and 0.3 ml min^{-1} each for a period of 30 min had no effect on the level of inhibition seen (Figure 5b). The magnitude of bradykinin-induced vasodilatation (% reduction in perfusion pressure) was constant in time-matched control preparations subjected to a similar sequential reduction in flow rate.

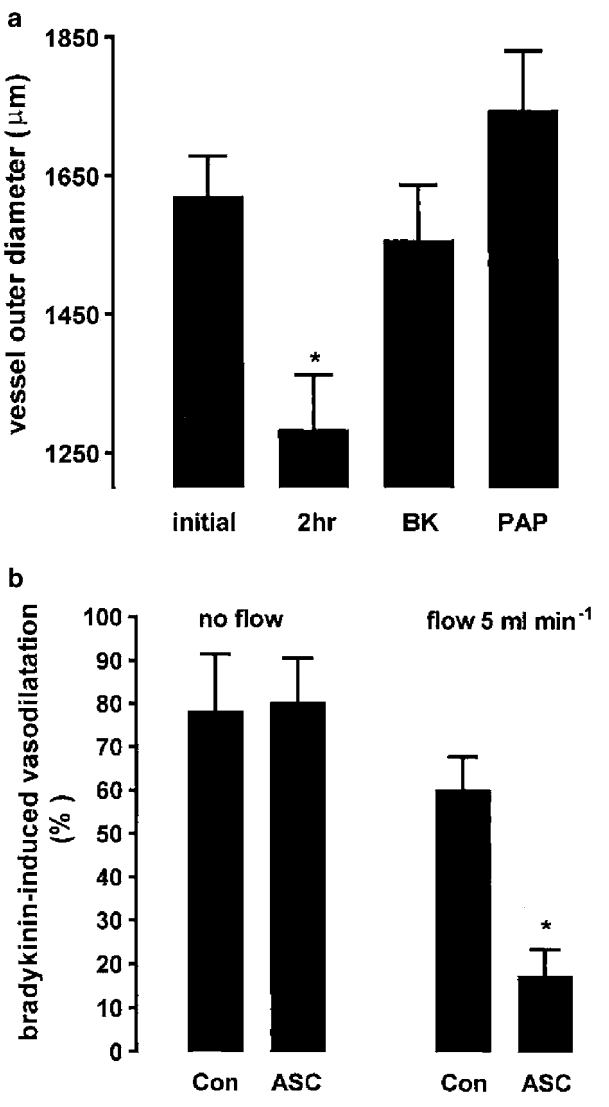

Figure 6 Experiments conducted in a pressure myograph examining the separate effects of pressure and flow on the ability of ascorbate to inhibit bradykinin-induced vasodilatation in bovine ciliary segments. (a) When pressurized to ~100 mmHg, vessels developed myogenic tone, as revealed by a fall in outer diameter between the initial measurement and the end of a 2 h incubation period. This myogenic tone was relaxed powerfully by bradykinin (BK, 1 nmol) and completely by papaverine (PAP, 300 μM). (b) Bradykinin-induced dilatation remained constant for 3 h when pressurized in the absence or presence of flow at 5 ml min^{-1}, or in the presence of ascorbate (ASC, 150 μM) in the absence of flow, but was inhibited significantly by ascorbate in the presence of flow. Data are mean ± s.e.m.; $n \geq 4$; *$P<0.05$ indicates differences from control.

Effects of flow and pressure on the ability of ascorbate to block EDHF-mediated vasodilatation

Although flow appeared critical in determining the ability of ascorbate to block bradykinin-induced vasodilatation, it was not always possible to maintain perfusion pressure at a constant level across all rates of flow by modifying the level of vasoconstriction with U46619. Therefore, in order to determine whether the higher levels of pressure experienced at the faster flow rates rather than flow rate *per se* was responsible for the blocking action of ascorbate, we conducted a series of experiments in a pressure myograph.

When pressurized in the absence of flow to 102 ± 1 mmHg ($n = 16$), segments of ciliary artery (1620 ± 43 μm initial o.d.) developed myogenic tone during the ensuing 2 h incubation period, as revealed by a reduction in o.d. and by the restoration of the original o.d. following treatment with papaverine (300 μM, Figure 6a). Bradykinin (1 nmol) also induced powerful vasodilatation, the magnitude of which remained constant during a further 3 h incubation period when pressurized either at no flow or at a flow rate of 5 ml min^{-1} (Figure 6b). Furthermore, ascorbate (150 μM) had no effect on bradykinin-induced vasodilatation when vessels were pressurized in the absence of flow. In contrast, when pressurized in the presence of flow at 5 ml min^{-1}, ascorbate significantly inhibited bradykinin-induced vasodilatation. The onset of inhibition by ascorbate was time dependent: no block was seen at 1 h, but at 3 h it was maximal.

Discussion

We previously reported that ascorbate induces a highly selective, time- and concentration-dependent inhibition of EDHF-mediated vasodilatation in the bovine perfused ciliary circulation and rat perfused mesentery (McNeish *et al*., 2002), but not in rings of bovine or porcine coronary artery (McNeish *et al*., 2003a, b). The major new finding in this study is that ascorbate inhibits EDHF in a single vessel, the bovine extraocular long posterior ciliary artery, in the presence of flow but not when suspended in a static myograph.

When perfused with Krebs solution at a flow rate of 2.5 ml min^{-1} and constricted with U46619, segments of bovine ciliary artery, together with any side branches, exhibited dose-dependent dilator response to bradykinin. Results with acetylcholine were less consistent: powerful vasodilator responses were seen in $\sim$40% of vessels tested, with the remainder exhibiting weak dilatation or even constriction from the outset. Our previous findings in the bovine perfused ciliary vascular bed, as opposed to the extraocular ciliary artery used in this study, revealed that vasodilator responses to bradykinin or acetylcholine were mediated solely by EDHF, with no contribution by nitric oxide, although basal release of this mediator did exert a tonic vasodepressor action (McNeish *et al*., 2001). By contrast, in the bovine perfused ciliary artery, EDHF and nitric oxide both contributed substantially to agonist-induced vasodilatation, since blockade of either EDHF using apamin/charybdotoxin (Waldron & Garland, 1994; Zygmunt & Högestätt, 1996), or nitric oxide using L-NAME (Rees *et al*., 1990) powerfully inhibited bradykinin-induced dilatation and reversed that induced by acetylcholine to vasoconstriction. We could be certain that apamin/charybdotoxin blocked only EDHF, since, in their presence, the application of L-NAME still produced the expected enhancement of perfusion pressure through inhibition of basal nitric oxide (McNeish *et al*., 2001). As in the ciliary vascular bed (McNeish *et al*., 2001), cyclooxygenase products did not contribute to bradykinin- or acetylcholine-induced dilatation, but indomethacin was routinely employed together with L-NAME to uncover unambiguously the EDHF component. Thus, in the bovine perfused ciliary artery, the combined actions of EDHF and nitric oxide appear vital for induction of vasodilatation, with removal of either component resulting in profound loss of the response. This was particularly true for acetylcholine, probably because of its powerful opposing vasoconstrictor action.

In the perfused ciliary artery, ascorbate produced a slowly developing (maximum at 2–3 h) blockade of EDHF: at the normal plasma concentration of 50 μM (Keaney & Vita, 1995; Levine *et al*., 1996), ascorbate inhibited bradykinin-induced dilatation significantly but not as effectively as apamin/charybdotoxin, whereas that to acetylcholine was reversed to constriction. A higher concentration of 150 μM ascorbate failed to inhibit bradykinin-induced dilatation further; the additional inclusion of L-NAME and indomethacin did produce additional blockade, but substantial residual dilatation could still be elicited ($\sim$30%). It was likely that ascorbate had inhibited the EDHF-mediated component of vasodilatation and not that due to nitric oxide, since, in the presence of the antioxidant, L-NAME still induced the expected elevation of perfusion pressure through blockade of basal nitric oxide activity (McNeish *et al*., 2001). It therefore seemed that EDHF-mediated responses in the perfused artery exhibit the susceptibility to inhibition by ascorbate that we reported previously in the perfused ciliary vascular bed (McNeish *et al*., 2002; 2003a), the only difference being that in the former the blockade of responses to bradykinin is incomplete. Furthermore, the ability of L-NAME to augment pressure through blockade of basal nitric oxide activity demonstrates that ascorbate does not induce nonspecific damage to the endothelium.

When the segments of bovine ciliary artery ($\sim$1300 μm, i.d.) were cut into rings and suspended in a wire myograph, bradykinin and acetylcholine each again evoked concentration-dependent relaxation. As with the perfused artery, relaxation was mediated jointly by EDHF and nitric oxide, but some differences were noted. Specifically, acetylcholine relaxed all ciliary artery rings tested, whereas it dilated only around 40% of perfused arteries. In addition, although acetylcholine-induced relaxation was inhibited significantly by apamin/charybdotoxin, or by L-NAME/indomethacin, even combined blockade failed to uncover the profound constrictor effects seen routinely with acetylcholine in the perfused artery. It is likely that this lack of constrictor responses to acetylcholine was due to the fact that U46619-induced tone was already near maximal because of the difficulty of holding it at a submaximal level in the presence of charybdotoxin. Constrictor responses to acetylcholine were, however, seen in ciliary artery rings following endothelial denudation, where stable, submaximal U46619-induced tone could be obtained. The possibility that differences in the degree of underlying tone ($\sim$120 mmHg U46619-induced pressure for perfused vessels *versus* $\sim$100 mmHg equivalent stretch followed by induction of submaximal U46619 contraction in ring preparations) may also have affected the nature of the constrictor responses to acetylcholine remains to be investigated. Thus, ciliary artery rings in a wire myograph displayed some important differences in behaviour when compared to the perfused preparation, but they nevertheless exhibited a robust EDHF-dependent component of vasodilatation to bradykinin or acetylcholine.

The most striking contrast observed in the ciliary artery was that ascorbate inhibited vasodilator responses in the perfused preparation but not in static rings of either large ($\sim$1300 μm i.d.) or small ($\sim$570 μm, i.d.) diameter. Rings of the smaller diameter side branches were tested separately since they may

have contributed to the resistance of the perfused artery. When we previously observed that ascorbate blocked EDHF in the bovine perfused ciliary vascular bed and rat perfused mesentery but not in rings of bovine or porcine coronary artery (McNeish *et al*., 2002; 2003a, b), we considered the possibility that the differential actions might result from differences in the nature of EDHF at the various vascular sites, or from differences in vessel size (resistance arterioles *versus* conduit arteries). It now seems evident, however, from the present observations on a single blood vessel that the ability of ascorbate to inhibit EDHF is dependent upon a property present during perfusion but absent in a static myograph.

It is well established that shearing forces act as a powerful stimulus to the endothelium, promoting the release of nitric oxide, prostacyclin and EDHF (Boo & Jo, 2003; Busse & Fleming, 2003), as well as affecting morphology (Franke *et al*., 1984; Remuzzi *et al*., 1984) and phenotype (Wasserman *et al*., 2002). We therefore considered the possibility that flow might influence the ability of ascorbate to block EDHF. Indeed, we found a striking relationship between flow rate and the ability of ascorbate to inhibit bradykinin-induced dilatation: at $1.25\,ml\,min^{-1}$ or below, no effect was seen, but at higher flow rates (2.5 and $5\,ml\,min^{-1}$) graded inhibition was produced, with the highest almost abolishing the dilatation. Control experiments showed that bradykinin-induced dilatation (% reduction in perfusion pressure) was constant over this wide range of flow rates ($0.3–5\,ml\,min^{-1}$) and was reproducible for many hours at each flow rate. Furthermore, if inhibition of vasodilatation had already been established with ascorbate at $5\,ml\,min^{-1}$, sequentially lowering the flow rate had no effect on the degree of blockade seen. Thus, induction of inhibition by ascorbate required a period (2–3 h) of high flow, but was not rapidly reversed when the flow rate was dropped to lower levels.

In a perfused preparation, changes in flow result in proportionate changes in perfusion pressure. Nevertheless, it was likely that inhibition of EDHF by ascorbate was a function of flow rate *per se* and not of associated changes in pressure, because in most cases we were able to hold pressure reasonably constant by making adjustments to the level of vasoconstriction with U46619. This conclusion was confirmed using a pressure myograph, where pressure and flow could be independently controlled. When pressurised to $\sim 100\,mmHg$, segments of bovine ciliary artery developed myogenic tone which could be dilated powerfully by bradykinin. In common with findings in a static wire myograph, dilatation was unaffected by treatment with ascorbate in pressurized vessels when flow was absent. In stark contrast, however, dilatation was inhibited by ascorbate in pressurized vessels exposed to flow at $5\,ml\,min^{-1}$. Indeed, inhibition developed in the same time-dependent manner (2–3 h) seen in vessels perfused using our open system. Moreover, since ascorbate was active in pressurized vessels in the presence but not in the absence of flow, that is, when vessel tone was similar in both cases, we can be certain that the conditions required for blockade are unrelated to the level of smooth muscle contraction employed.

At present, we can only speculate about the mechanism by which flow controls the ability of ascorbate to block EDHF. Vascular endothelial cells are well known, however, to discriminate between a variety of haemodynamic forces including, laminar, pulsatile, unidirectional and oscillatory flow, and transduce these signals through distinct calcium-dependent and calcium-independent effector pathways, resulting in modified function or phenotype (for reviews, see Boo & Jo, 2003; Busse & Fleming, 2003). For example, shear stress elevates $[Ca^{2+}]_i$ in endothelial cells (Hoyer *et al*., 1998), but this is transient and therefore unlikely to promote the accompanying sustained increase in nitric oxide release. More recent work has implicated phosphatidylinositol-3-kinase and its downstream targets, serine kinases Akt and protein kinase A, in the process of mechanotransduction (Go *et al*., 1998; Dimmeler *et al*., 1999; Gallis *et al*., 1999). Activation of these pathways leads to phosphorylation of a specific serine residue on endothelial nitric oxide synthase (Ser^{1177} and Ser^{1179} of human and bovine sequences, respectively), with a consequent increase in enzyme activity. The possibility that activation of these calcium-dependent or calcium-independent pathways by flow might modify the EDHF response in some way to permit inhibition by ascorbate warrants further investigation.

The ability of ascorbate at concentrations within the normal plasma range to block EDHF-mediated vasodilatation raises several important issues. For example, if our *in vitro* experiments reflect the behaviour of vascular beds *in vivo*, they would suggest that EDHF activity is normally substantially suppressed in conditions of high flow. Such a situation seems almost inconceivable, since numerous reports describe vasodilator responses attributable to EDHF in living animals (Nishikawa *et al*., 1999; Welsh & Segal, 2000) and humans (Honing *et al*., 2000; Katz & Krum, 2001). Furthermore, the blocking action of ascorbate on EDHF contrasts markedly with its actions on nitric oxide, where enhancement of vasodilator activity is widely reported (Dudgeon *et al*., 1998; Fontana *et al*., 1999; Carr *et al*., 2000; May, 2000). One possible explanation of this paradox is that ascorbate may act *in vivo* as a redox switch to activate EDHF under conditions of oxidant stress. Under such conditions, nitric oxide is destroyed by superoxide anion (Gryglewski *et al*., 1986; Rubanyi & Vanhoutte, 1986) and low molecular weight antioxidants such as ascorbate are rapidly consumed (Frei, 1994). Depletion of ascorbate could then potentially de-repress the EDHF-dependent vasodilator mechanism. Indeed, support for this concept comes from a recent report showing that, in heart failure patients, nitric oxide-dependent vasodilatation is impaired by oxidant stress in the forearm circulation, and EDHF becomes the dominant vasodilator (Katz & Krum, 2001). A similar loss of nitric oxide-mediated, flow-induced dilatation, coupled with upregulation of EDHF, occurs in patients with coronary artery disease (Miura *et al*., 2001). Further evidence of a reciprocal interaction between these two vasodilators is suggested by the findings that nitric oxide impairs EDHF release or production in the porcine coronary circulation both *in vitro* (Bauersachs *et al*., 1996) and *in vivo* (Nishikawa *et al*., 2000). Moreover, there are many reports suggesting that the EDHF-mediated component of vasodilatation becomes fully established only when the nitric oxide-dependent component is inhibited (Kilpatrick & Cocks, 1994; McCulloch *et al*., 1997), a contention supported by the upregulation of EDHF-dependent vasodilatation in the eNOS knockout mouse (Waldron *et al*., 1999; Ding *et al*., 2002). Whatever the precise interaction between these two important vasodilators, our new findings with ascorbate may have important implications in cardiovascular pathologies where nitric oxide-mediated vasodilatation is impaired by oxidant stress.

In conclusion, we have shown in the bovine ciliary artery that ascorbate inhibits EDHF-mediated vasodilatation under conditions of high but not low flow. This is likely to explain our previous finding that ascorbate inhibits EDHF in the bovine perfused ciliary vascular bed and rat perfused mesentery (McNeish *et al*., 2002), but not in rings of bovine or porcine coronary artery (McNeish *et al*., 2003a, b). Further studies are required to elucidate the mechanism by which flow renders EDHF susceptible to inhibition by ascorbate and establish the physiological and pathological consequences of this process.

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