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RESEARCH PAPER

Flow-induced enhancement of vasoconstriction and blockade of endothelium-derived hyperpolarizing factor (EDHF) by ascorbate in the rat mesentery

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Background and purpose: We previously reported that ascorbate inhibits flow- and agonist-induced, EDHF-mediated vasodilatation in the bovine ciliary circulation. This study examined whether ascorbate had similar actions in the rat mesenteric vasculature.
Experimental approach: The effects of ascorbate were examined both in rat second order mesenteric arterial rings suspended in a static wire myograph and the rat mesentery perfused at different rates of flow.
Key results: Ascorbate (50 μM) had no effect on U46619-induced tone or acetylcholine-induced, EDHF-mediated vasodilatation in either rings of mesenteric artery or the perfused mesentery at rates of flow below 10 ml min^{-1}. At higher rates of flow, ascorbate produced two distinct effects in the rat mesentery: a rapid and maintained enhancement of vasoconstrictor tone and a slow (max at 3 h) inhibition of acetylcholine-induced, EDHF-mediated vasodilatation. The enhancement of vasoconstrictor tone appeared to be due to inhibition of flow-induced EDHF-like activity, since it was endothelium-dependent, but could be elicited during blockade of nitric oxide synthase and cyclooxygenase. Despite this, the classical inhibitors of EDHF, apamin and charybdotoxin, failed to affect the ascorbate-induced enhancement of tone, although they inhibited acetylcholine-induced vasodilatation.
Conclusions and implications: Ascorbate inhibits both flow- and agonist-induced EDHF in the rat mesentery. The strikingly different timecourses of these two effects, together with their differential sensitivity to apamin and charybdotoxin, suggest that the flow- and agonist-induced EDHFs in the rat mesenteric vasculature may either be different entities or operate by different mechanisms.
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Keywords: artery; ascorbate; EDHF; endothelium; endothelium-derived hyperpolarizing factor; flow; nitric oxide; vasodilatation

Abbreviations: CHAPS, 3-[(cholamidopropyl)dimethyl-ammonio]1-propanesulphonate; EDHF, endothelium-derived hyperpolarizing factor; L-NAME, N^G-nitro-L-arginine methyl ester

Introduction

Three distinct vasodilator signals produced by the vascular endothelium, namely nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF), play a critical role in the regulation of vasomotor tone (for reviews see Moncada *et al*., 1991; Campbell and Harder, 2001). The identity of EDHF remains the subject of debate, but potential candidates include a cytochrome P450 metabolite of arachidonic acid, C-type natriuretic peptide, hydrogen peroxide, a cannabinoid, K^+ ions or the spread of endothelial hyperpolarization via myoendothelial gap junctions (for reviews see Campbell and Harder, 2001; Busse *et al*., 2002; Griffith, 2004). Although the nature of EDHF is not fully understood, vasodilatation by this agent is inhibited following blockade of calcium-activated potassium channels of small and intermediate conductance on the vascular endothelium using apamin and charybdotoxin, respectively (Waldron and Garland, 1994; Zygmunt and Högestätt, 1996; Doughty *et al*., 1999).

We have shown that EDHF-mediated vasodilatation induced by bradykinin or acetylcholine in the bovine perfused ciliary circulation is inhibited by physiological levels (10–150 μM) of ascorbate (McNeish *et al*., 2002). Inhibition by ascorbate is not immediate, but develops steadily over ~2 h. Furthermore, the inhibition of EDHF induced by ascorbate appears highly selective, since the vasodilator actions of

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endothelium-derived nitric oxide, the nitrovasodilator, glyceryl trinitrate, and the K_{ATP} channel opener, levcromakalim, remain entirely unaffected (McNeish *et al*., 2002; McNeish *et al*., 2003b).

Although effective in the bovine perfused ciliary circulation (McNeish *et al*., 2002), ascorbate does not block EDHF-mediated vasodilatation in rings of bovine or porcine coronary artery (McNeish *et al*., 2003a, b). Indeed, work on a single vessel, the bovine extraocular long posterior ciliary artery, demonstrated that flow was necessary to uncover the ability of ascorbate to inhibit EDHF-mediated vasodilatation; inhibition was seen when the vessel was perfused, but not when rings were studied in a static myograph (Nelli *et al*., 2004).

Our recent work on the bovine extraocular long posterior ciliary artery shows that in addition to inhibiting agonist-induced, EDHF-mediated vasodilatation, ascorbate enhances vasoconstrictor tone (Stirrat *et al*., 2006). This enhancement of tone develops over the same slow time course (~2 h max) as the blockade of agonist-induced EDHF, and arises not from a constrictor action *per se*, but from inhibition of a tonic vasodilator influence. Indeed, it appeared to result from inhibition of flow-dependent EDHF-like activity, since it could be elicited in the presence of flow but not in static rings, and was endothelium-dependent, but did not involve nitric oxide or prostacyclin.

We have demonstrated previously that ascorbate also inhibits agonist-induced, EDHF-mediated vasodilatation in the rat perfused mesentery (McNeish *et al*., 2002). The aim of this investigation was to determine if this inhibition is also dependent upon the presence of flow and associated with a flow-dependent enhancement of vasoconstrictor tone. A preliminary report on these findings has already been published (Stirrat *et al*., 2005).

Materials and methods

Preparation of rat mesenteric artery rings for tension measurement
Male Wistar rats (150–180 g) were killed by concussion followed by exsanguination. Segments of second-order mesenteric artery (internal diameter following stretch 293 ± 6 μm, $n = 24$) were trimmed free of fat and adhering connective tissue, and mounted in wire myographs (Multi myograph, model 610; Danish Myo Technology) maintained at 37 °C in 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. Isometric tension was recorded and displayed using Myodaq (2.01) and analysed using Myodata (2.02). Tissues were allowed to equilibrate for 30 min before experiments were carried out, and the effects of nitric oxide and cyclooxygenase products were blocked in all experiments using N^G-nitro-L-arginine methyl ester (L-NAME) (100 μM) and indomethacin (5 μM), respectively.

Experiments were conducted to examine the effects of ascorbate (50 and 150 μM) on 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619)-induced tone and on acetylcholine-induced, EDHF-mediated dilator responses during a 3-h period. To examine the effects of ascorbate on U46619-induced tone, rings were placed under tension (~2.5 mN) and contracted to ~20% of the maximum (max 11.3 ± 0.5 mN, $n = 8$, in a separate control set of tissues) using a concentration of 3–10 nM to give a transmural pressure equivalent to 54.3 ± 5.8 mm Hg, $n = 12$, which is close to the *in vivo* range for vessels of this size. These conditions left ample scope for any further potential contraction by ascorbate. In contrast, to observe EDHF-mediated relaxant responses to acetylcholine, assessed as cumulative concentration–responses curves, mesenteric artery rings were contracted to ~80% of maximal U46619-induced tone using a concentration of 1 μM. Relaxation was expressed as a percentage of this U46619-induced tone.

Preparation of the rat perfused mesentery for pressure recording
The superior mesenteric artery was cannulated and the mesenteric arterial vasculature dissected from the intestines and suspended in a heated organ bath as described previously (McNeish *et al*., 2002). The mesentery was then perfused using a peristaltic pump (Minipuls 3, Gilson, Anachem Ltd., Luton, UK) at a flow rate of 15 ml min^{-1} (unless otherwise stated) with Krebs solution at 37 °C gassed with 95% O_2/5% CO_2 and 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, UK).

Experimental protocols with the rat perfused mesentery
To observe EDHF-mediated dilator responses to acetylcholine, the perfusion pressure was raised by ~100 mm Hg using the thromboxane-mimetic, U46619 (10–100 nM). The effects of nitric oxide and cyclooxygenase products were blocked using L-NAME (100 μM) and indomethacin (5 μM), respectively. Vasodilator responses to acetylcholine were elicited by injecting into the perfusion fluid 10 μl volumes of the appropriate concentration using a Hamilton microsyringe and expressed as a percentage of the U46619-induced perfusion pressure. Injections of acetylcholine were given at 15 min intervals, either to produce a dose–response curve (1 pmol–1 μmol) or to observe reproducibility to a single, submaximal concentration (10 nmol) during a 3-h period.

As will be seen in the Results, ascorbate (50 μM) produced two effects in the perfused mesentery at the standard flow rate of 15 ml min^{-1}: a rapidly developing and sustained enhancement of U46619-induced pressure and a slowly developing (max at 3 h) inhibition of acetylcholine (10 nmol)-induced, EDHF-mediated dilatation. Furthermore, the flow dependence of these actions of ascorbate (50 μM) was studied by conducting a series of experiments at different rates of flow (5, 10, 15 and 20 ml min^{-1}). As in previous experiments, U46619 (10–100 nM) was used to raise the perfusion pressure by ~100 mm Hg and L-NAME (100 μM) and indomethacin (5 μM) were present throughout the 3-h study period. Time-matched control experiments were conducted in the absence of ascorbate to determine the reproducibility of the dilator action of acetylcholine (10 nmol) at the different flow rates. The selectivity of the

blockade of acetylcholine-induced, EDHF-mediated vasodilatation by ascorbate was assessed by examining the effects of the antioxidant on dilatation to another agent, the K_{ATP} channel opener, levcromakalim (5 nmol).

The role of the endothelium in the ability of ascorbate to enhance U46619-induced pressure was investigated by damaging the endothelial layer using the detergent, 3-[(cholamidopropyl)dimethyl-ammonio]1-propanesulphonate (CHAPS) (Randall and Hiley, 1988; McNeish *et al*., 2001). In these experiments, conducted at a flow rate of 15 ml min^{-1}, U46619 (10 nM) was used to raise the perfusion pressure to 96.1 ±7.7 mm Hg ($n=8$), and vasodilator responses to acetylcholine (10 nmol) and levcromakalim (5 nmol) were obtained in the presence of L-NAME (100 μM) and indomethacin (5 μM). CHAPS (0.3%) was then infused into the perfusion fluid for either 2 or 8 min using a syringe pump (model SP120CE-300, WPI, Stevenage, UK). An injection artefact was present during the infusion of CHAPS, but following washout, the perfusion pressure was not significantly different following the 2-min treatment (79.8 ±5.1 mm Hg, $n=8$). The perfusion pressure did not, however, recover spontaneously with washout after 8-min treatment with CHAPS, but was restored (to 100.9 ±2.5 mm Hg, $n=10$) by increasing the concentration of U46619 to 30 nM. Dilatations to acetylcholine (10 nmol) and levcromakalim (5 nmol) were taken as an index of endothelial and smooth muscle integrity, respectively. The ability of ascorbate (50 μM) to elicit a rise in perfusion pressure following treatment with CHAPS (2 and 8 min) was then assessed.

The role of EDHF in the ability of ascorbate to enhance U46619-induced pressure was investigated using the EDHF blockers, apamin and charybdotoxin (Waldron and Garland, 1994; Zygmunt and Högestätt, 1996). In these experiments, conducted at a flow rate of 15 ml min^{-1}, U46619 (100 nM) was used to raise the perfusion pressure by ~100 mm Hg and vasodilator responses to acetylcholine (10 nmol) were obtained in the presence of L-NAME (100 μM) and indomethacin (5 μM). Apamin and charybdotoxin (both 100 nM) were added to the perfusate and remained present thereafter. Inhibition of acetylcholine (10 nmol)-induced dilatation was taken as evidence of successful inhibition of EDHF. The subsequent ability of ascorbate (50 μM) to elicit a rise in perfusion pressure was then assessed.

Statistical analysis

Results are expressed as the mean ± s.e. mean of n separate observations, each from a separate preparation. Graphs were drawn and statistical comparisons made using one-way analysis of variance with Bonferroni's post-test with the aid of a computer program, Prism (GraphPad, San Diego, USA). A probability (P) $\leqslant 0.05$ was considered significant.

Drugs and chemicals

Acetylcholine chloride, apamin, ascorbic acid, CHAPS, indomethacin, L-NAME, U46619 were all obtained from Sigma (Poole, UK). Catalase (bovine liver) was obtained from Calbiochem (Beeston, UK) and charybdotoxin was obtained from Latoxan (Valence, France). Levcromakalim was a gift from GlaxoSmithKline (Harlow, UK). 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^{-1}), levcromakalim (0.1 M stock), which was dissolved in 70% ethanol and U46619 (1 mM), which was dissolved in 50% ethanol.

Results

Effects of ascorbate on rat mesenteric arterial rings

The thromboxane-mimetic, U46619 (3–10 nM), induced tone of 1.8 ±0.8 mN (16.1 ±7.1% of max, $n=6$) in rat second-order mesenteric arteries treated with the nitric oxide synthase inhibitor, L-NAME (100 μM), and the cyclooxygenase inhibitor, indomethacin (5 μM), present. Ascorbate (50 μM) had no effect on this tone at any point during a 3-h period (data not shown).

Following induction of tone (9.2 ±1.3 mN, 81.6 ±11.8% max, $n=6$) using U46619 (1 μM), subsequent addition of acetylcholine (10 nM–10 μM) induced concentration-dependent, EDHF-mediated relaxation (max 89.1 ±4.4%, Figure 1). Treatment with ascorbate (50 μM) for 3 h had no effect on this acetylcholine-induced, EDHF-mediated relaxation. A higher concentration of ascorbate (150 μM) also failed to affect tone or acetylcholine-induced relaxation (data not shown).

Effects of ascorbate on pressure in the rat perfused mesentery

The basal perfusion pressure in rat mesenteric vascular beds in the presence of L-NAME (100 μM) and indomethacin (5 μM) at a standard constant flow rate of 15 ml min^{-1} was 54.2 ±3.3 mm Hg and this was raised to 121.6 ±9.2 mm Hg ($n=17$) using U46619 (70–100 nM). Treatment with

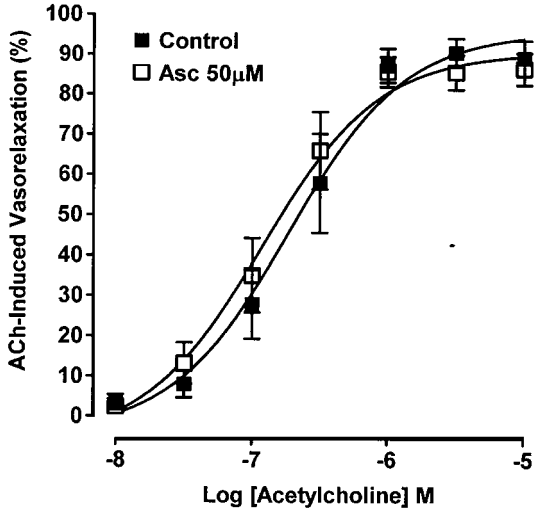

Figure 1 Cumulative log concentration–response curves showing endothelium-derived hyperpolarizing factor-mediated vasorelaxation to acetylcholine (10 nM–10 μM) in rat second-order mesenteric arteries under control conditions and following treatment with ascorbate (Asc, 50 μM). Data are mean ± s.e. mean of six observations.

ascorbate (50 μM) induced a rapid and maintained rise in pressure (Figures 2 and 3) in the presence of U46619-induced tone.

The ability of ascorbate (50 μM) to enhance U46619-induced pressure was examined at a range of flow rates: no significant rise was seen at 5 ml min^{-1}, but rises occurred at flow rates between 10 and 20 ml min^{-1}, with an optimum at 15 ml min^{-1} (Figure 3).

Endothelial dependence of the ascorbate-induced enhancement of pressure in the perfused mesentery

Experiments were conducted with the detergent, CHAPS (0.3%, 2 or 8 min), at the standard flow rate of 15 ml min^{-1} to determine if the enhancement of U46619-induced pressure by ascorbate was endothelium-dependent. Perfusion with CHAPS (0.3%) for 2 min significantly inhibited both acetylcholine (10 nmol)-induced, EDHF-mediated dilatation and the rise in perfusion pressure induced ascorbate (50 μM), but had no effect on the endothelium-independent dilatation to levcromakalim (5 nmol; Figure 4). Perfusion with CHAPS for 8 min produced more powerful inhibition of the actions of acetylcholine or ascorbate, but also significantly inhibited dilatation to levcromakalim.

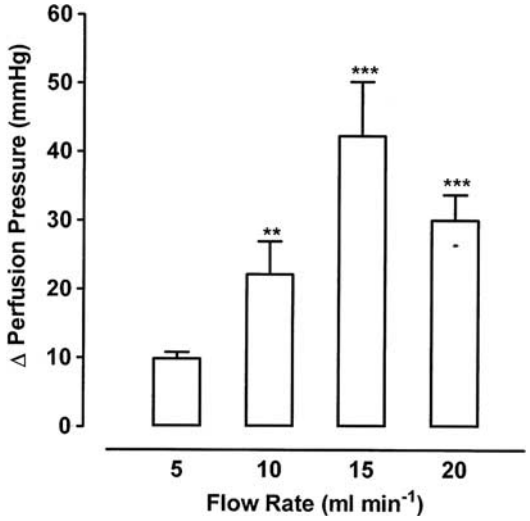

Figure 3 Enhancement of U46619-induced pressure produced by ascorbate (50 μM) in the rat mesentery perfused at different rates of flow (5–20 ml min^{-1}). Data are the mean ± s.e. mean of 8–10 observations. $^{**}P<0.01$ and $^{***}P<0.001$ indicate a significant rise in pressure when compared to time-matched controls.

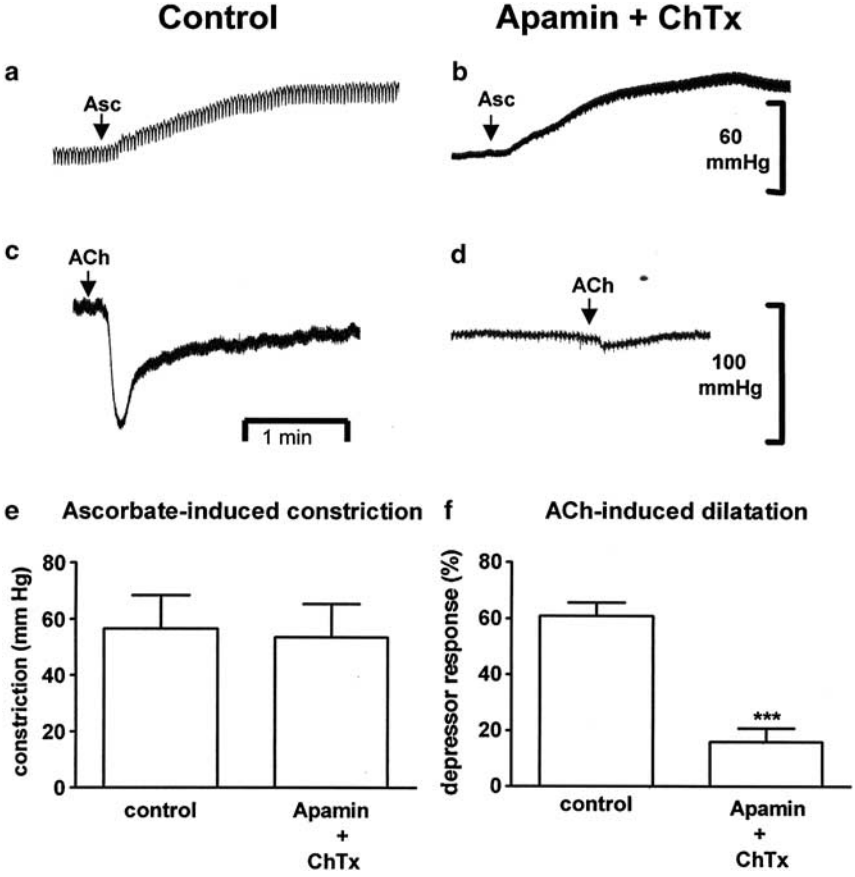

Figure 2 Experimental traces showing in rat control mesenteric vascular beds perfused at a constant flow rate of 15 ml min^{-1} that (**a**) ascorbate (Asc; 50 μM) produces a rapid and sustained rise in U46619-induced pressure and (**c**) acetylcholine (ACh, 10 nmol) produces a vasodepressor response. Apamin and charybdotoxin (ChTx; both 100 nM) had no effect on the constriction to ascorbate (**b**), but blocked the vasodepressor response to acetylcholine (**d**). Histograms showing ascorbate-induced constriction (**e**) and acetylcholine-induced dilatation (**f**) in control tissues and in those treated with apamin and charybdotoxin. Data are mean of 4–6 observations. $^{***}P<0.001$ indicates a significant difference from control.

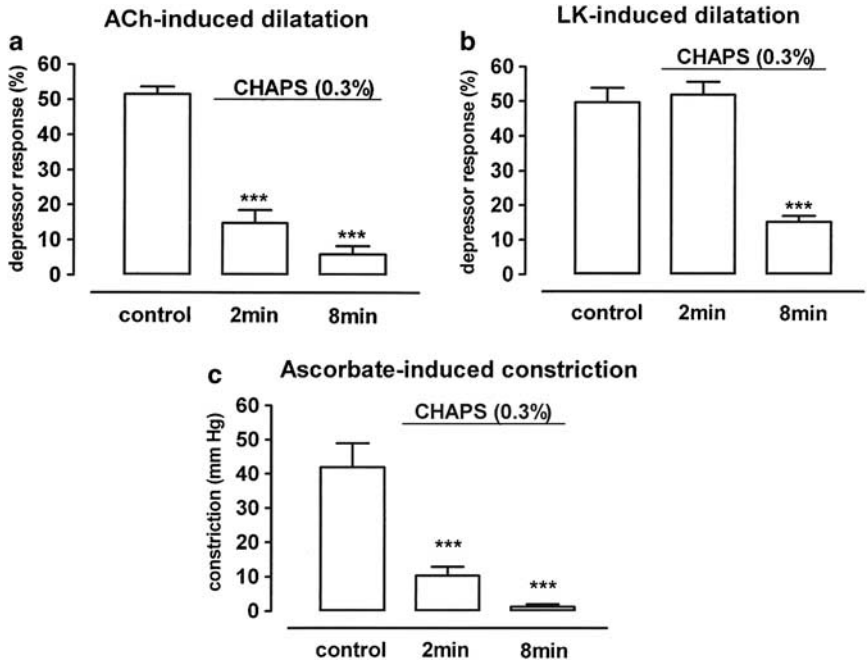

Figure 4 Effects of CHAPS (0.3%), perfused for 2 or 8 min, on the vasodepressor response to (**a**) acetylcholine (Ach; 10 nmol) and (**b**) levcromakalim (LK; 5 nmol), and on the constrictor response to (**c**) ascorbate (50 μM). Data are the mean ± s.e. mean of 6–10 observations. ***$P<0.001$ indicates a significant difference from control.

Treatment with the EDHF inhibitors, apamin and charybdotoxin (both 100 nM), powerfully inhibited acetylcholine (10 nmol)-induced dilatation (Figure 2). Apamin and charybdotoxin had no effect, however, on the ability of ascorbate (50 μM) to enhance perfusion pressure (Figure 2).

Effects of ascorbate on acetylcholine-induced, EDHF-mediated dilatation in the perfused mesentery

Following the induction of tone with U46619 at the standard flow rate of 15 ml min^{-1}, the addition of acetylcholine (1 pmol–1 μmol) produced dose-dependent, EDHF-mediated dilatation (max 61.0 ± 5.9%, $n=10$, Figure 5a). Moreover, the dilatation to a single dose of acetylcholine (10 nmol) repeated every 15 min remained fairly constant during a 3-h period (Figure 5b). The addition of ascorbate (50 μM) led, however, to a slowly developing blockade of acetylcholine (10 nmol)-induced, EDHF-mediated dilatation (Figure 5b).

The ability of ascorbate (50 μM) to inhibit acetylcholine (10 nmol)-induced, EDHF-mediated dilatation was also examined at a range of flow rates: no significant inhibition was seen at 5 ml min^{-1}, but blockade occurred at flow rates between 10 and 20 ml min^{-1}, with an optimum at 15 ml min^{-1} (Figure 5b). In each case, the inhibition developed steadily during the 3-h incubation period.

The ability of ascorbate (50 μM, 3 h) to block acetylcholine-induced dilatation at the optimum flow rate of 15 ml min^{-1} appeared selective, since dilator responses to the K_{ATP} opener, levcromakalim (5 nmol), were unaffected (control 39.0 ± 5.3%; ascorbate-treated 34.3 ± 7.1%, $n=8$).

A recent report (Liu *et al*., 2006) suggests that catalase inhibits flow-mediated dilatation in the rat mesenteric artery. We found, however, that catalase (1200 and 3600 U ml^{-1}) had no effect on U46619-induced perfusion pressure at the standard flow rate of 15 ml min^{-1} (Δ perfusion pressure of 2.1 ± 4.3 and –3.9 ± 1.3 mm Hg, respectively, both $n=8$). Catalase (3600 U ml^{-1}) also had no effect on acetylcholine (10 nmol)-induced, EDHF-mediated vasodilatation (control 49.8 ± 5.4%; catalase-treated 43.9 ± 3.4%, $n=10$).

Discussion

Our findings show that ascorbate enhances vasoconstrictor tone and blocks agonist-induced, EDHF-mediated vasodilatation in the rat perfused mesentery. The time course of these two events differs markedly, with the former occurring immediately and the latter developing slowly, over 2–3 h. Ascorbate also enhances tone and blocks agonist-induced EDHF in the bovine ciliary vasculature, but here, both actions follow the same slow (2–3 h) time course (McNeish *et al*., 2002, 2003b; Nelli *et al*., 2004; Stirrat *et al*., 2006).

In the bovine perfused long posterior ciliary artery, the enhancement of tone arises, not from a constrictor action *per se*, but through inhibition of a tonic vasodilator activity (Stirrat *et al*., 2006). In fact, it appeared to be due to inhibition of flow-dependent EDHF activity, since it was seen in perfused vessels but not in static rings, was endothelium-dependent but did not involve nitric oxide or prostanoids and was mimicked by the EDHF blockers, clotrimazole or TRAM-34. The similar, slow time course of this action and the associated inhibition of acetylcholine- or bradykinin-induced vasodilatation (McNeish *et al*., 2002, 2003b) would suggest that ascorbate exerts a common inhibitory action on the EDHF(s) evoked both by flow and by agonists. This might

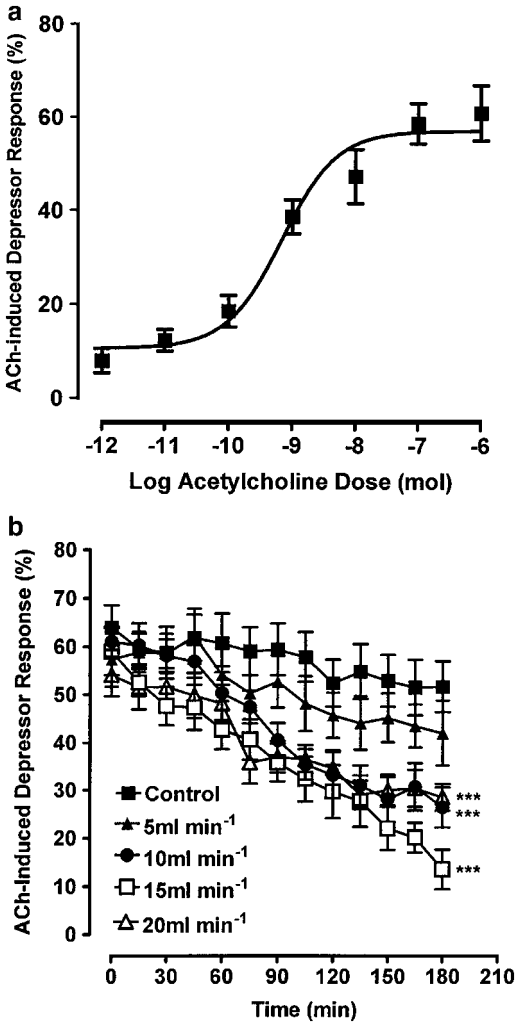

Figure 5 (**a**) Dose–response curve showing the endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodepressor response elicited by individual doses of acetylcholine (1 pmol–1 μmol) in the rat mesentery perfused at 15 ml min^{-1}. (**b**) Inhibition of the EDHF-mediated depressor response to acetylcholine (10 nmol) following treatment with ascorbate (50 μM) in rat mesentery perfused at a range of flow rates (5–20 ml min^{-1}). The control shows the reproducibility of the acetylcholine-induced depressor response in the absence of ascorbate when perfusion was conducted at 15 ml min^{-1}. Data are the mean ± s.e. mean of 8–10 observations. ***$P<0.001$ indicates a significant difference from control.

further suggest that the flow-induced and agonist-induced EDHFs are similar entities or operate by a common mechanism in the bovine ciliary vasculature.

In contrast, in the perfused rat mesentery, the striking difference in the time courses of the ascorbate-induced enhancement of vasoconstrictor tone and the inhibition of acetylcholine-induced, EDHF-mediated vasodilatation, argues against a common mechanism of action. We found that the rapid enhancement of vasoconstrictor tone by ascorbate appeared flow-dependent, since it was absent both in second-order mesenteric arteries mounted in a static wire myograph and in the perfused rat mesentery at low rates of flow (<10 ml min^{-1}), but present in the latter at higher rates of flow. Experiments were then conducted with the detergent, CHAPS (Randall and Hiley, 1988; McNeish *et al.*, 2001), to test the endothelial dependence of the ascorbate-induced enhancement of vasoconstriction. While treatment with CHAPS for 8 min caused clear disruption to smooth muscle function, as indicated by a profound fall in tone and impaired endothelium-independent dilatation to levcromakalim, treatment for 2 min had no such detrimental action. Indeed, 2-min treatment with CHAPS appeared to damage selectively the endothelium without compromising smooth muscle function, since after washout it had no effect on U46619-induced pressure or the endothelium-independent vasodilatation induced by levcromakalim, but blocked acetylcholine-induced, EDHF-mediated vasodilatation. The ascorbate-induced enhancement of vasoconstriction was also blocked, suggesting that it too was endothelium-dependent. It occurred, however, in the presence of an inhibitor of nitric oxide synthase and cyclooxygenase, suggesting that it might arise as a result of inhibition of flow-induced EDHF. Despite this, the 'classical' EDHF blockers, apamin and charybdotoxin (Waldron and Garland, 1994; Zygmunt and Högestätt, 1996), had no effect on the ascorbate-induced enhancement of constriction, although they did, as expected, inhibit acetylcholine-induced, EDHF-mediated vasodilatation in the rat mesentery.

These findings raise the possibility that in the rat mesentery, the flow-induced EDHF may be different from the 'classical' apamin- and charybdotoxin-sensitive, agonist-induced EDHF. Indeed, this conclusion is consistent with a recent report showing that the flow-induced EDHF in the rat mesenteric artery is inhibited by catalase but unaffected by apamin and charybdotoxin, whereas the reverse is the case for the agonist-induced EDHF (Liu *et al.*, 2006). On the basis of these observations, the authors concluded that the flow- and agonist-induced EDHFs were different entities in the rat mesenteric artery, and that the former was, on the basis of the selectivity of catalase, most likely to be hydrogen peroxide. Ascorbate can participate in a number of reactions in biological systems that lead to the depletion of hydrogen peroxide (Halliwell and Gutteridge, 1989), and this could potentially explain the rapid enhancement of vasoconstrictor tone we observed occurring through inhibition of flow-induced EDHF in the rat mesentery. We found, however, that catalase at concentrations similar to those used by Liu *et al.* (2006) had no effect on U46619-induced tone. On the basis of these results, we feel it is unlikely that the ascorbate-induced enhancement of tone arises through removal of hydrogen peroxide. Others have proposed that hydrogen peroxide is the agonist-induced EDHF in mouse and human mesenteric arteries and in porcine coronary microvessels (Matoba *et al.*, 2000; Shimokawa and Morikawa, 2005), but on the basis of the lack of effect of catalase, this appears not to be the case in the bovine ciliary vasculature or the guinea pig carotid artery (McNeish *et al.*, 2002; Gluais *et al.*, 2005). More specifically, for the rat mesenteric vasculature, our present findings and those published previously (Liu *et al.*, 2006) show a lack of effect of catalase, thus arguing against a role for hydrogen peroxide as the agonist-induced EDHF.

The second major aim of this study was to determine if our previously reported ability of ascorbate to inhibit agonist-induced, EDHF-mediated vasodilatation (McNeish *et al.*,

2002) was dependent upon the presence of flow in a manner similar to that seen in the bovine ciliary vasculature (Nelli *et al*., 2004). In fact, flow was vital; ascorbate had no effect on acetylcholine-induced, EDHF-mediated vasodilatation either in rat second-order mesenteric arteries in a static wire myograph or in the perfused mesentery at low rates of flow, but produced graded inhibition in the latter at rates of flow of 10 ml min^{-1} and above, with an optimum at 15 ml min^{-1}. Thus, these new findings strengthen the view that flow is required to uncover the ability of ascorbate to inhibit agonist-induced EDHF (McNeish *et al*., 2003b; Nelli *et al*., 2004). The slow time course of the inhibition (max at 3 h) of agonist-induced EDHF by ascorbate in the rat mesentery is roughly similar to that seen in the bovine ciliary vasculature (McNeish *et al*., 2002), but strikingly different to the rapid enhancement of vasoconstrictor tone in the former. The precise mechanism by which ascorbate inhibits agonist-induced EDHF is unknown, but experiments in the bovine ciliary vasculature suggest that it 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).

In conclusion, the results of the present study show that plasma levels of ascorbate produce two distinct effects on the rat mesentery: a rapid enhancement of vasoconstrictor tone, through inhibition of a flow-induced EDHF, and a slowly developing inhibition of agonist-induced EDHF. The different time courses of these two actions are consistent with recent findings that the flow- and agonist-induced EDHFs in the rat mesenteric vasculature may be different entities or operate by different mechanisms.

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Conflict of interest

The authors state no conflict of interest.

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