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Mechanotransduction through the endothelial cytoskeleton: mediation of flow- but not agonist-induced EDRF release

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1 We have used a cascade bioassay system and isolated arterial ring preparations to investigate the contribution of the endothelial microfilament and microtubule cytoskeleton to EDRF release evoked by time-averaged shear stress and by acetylcholine in rabbit abdominal aorta.

2 Cytochalasin B (1 μM) and phalloidin (100 nM) were used to depolymerize and stabilize, respectively, F-actin microfilaments. Colchicine (500 nM) was used to inhibit tubulin dimerization and thus disrupt the microtubule network. Experiments were performed before or 1 h after administration of agents to the donor perfusate or organ bath.

3 In cascade bioassay studies, time-averaged shear stress was manipulated with dextran (1–4% w/v, 80,000 MW), to increase perfusate viscosity. EDRF release induced by increased perfusate viscosity was significantly ($P<0.01$) attenuated by cytochalasin B, phalloidin and colchicine.

4 Endothelium-dependent relaxations to acetylcholine (0.01–30 μM) in cascade bioassay and in isolated aortic ring preparations were unaffected by pretreatment with any of these agents both in terms of their EC_{50} and maximal responses. Endothelium-independent relaxations to sodium nitroprusside (0.001–10 μM) were similarly unaffected.

5 We conclude that the endothelial F-actin microfilament and microtubule networks are involved in the mechanotransduction pathway for flow-evoked EDRF release in rabbit abdominal aorta. However, these cytoskeletal elements appear to play no role in acetylcholine-induced EDRF release in this tissue.

Keywords: Rabbit aorta; time-averaged shear stress; cytoskeleton; endothelium-derived relaxing factor (EDRF)

Introduction

Vascular endothelial cells release the potent endogenous nitrovasodilator endothelium-derived relaxing factor (EDRF), now identified as the nitric oxide (NO) radical formed from L-arginine by the action of NO synthase (Moncada *et al.*, 1991), in response to the mechanical force of hydrodynamic shear stress arising from blood flow (Griffith, 1994). Synthesis of NO in response to both flow and agonists is dependent on an elevation in endothelial intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) levels as the constitutive NO synthase is Ca^{2+}-dependent (Moncada *et al.*, 1991; Griffith, 1994). It is still unclear how endothelial cells convert a mechanical stimulus into a biochemical response but a number of potential mechanotransduction pathways have been proposed. Flow can increase the open-state probability of lanthanun-sensitive stretch-activated Ca^{2+} channels within the endothelial membrane (Lansman *et al.*, 1987) and also induce membrane hyperpolarization through activation of an inwardly rectifying K^+ current (Olesen *et al.*, 1988). This hyperpolarization provides a driving force for Ca^{2+} entry into the non-excitable endothelial cell down its electrochemical gradient. In rabbit aorta it has been shown that ATP-sensitive and multiple Ca^{2+}-activated K^+ channels are involved in flow-related NO release (Cooke *et al.*, 1991; Hutcheson & Griffith, 1994). Flow can also induce phosphoinositide hydrolysis to generate inositol 1, 4, 5-trisphosphate (IP_3) and stimulate release of Ca^{2+} from intracellular stores (Nollert *et al.*, 1990; Bhagyalakshi *et al.*, 1992) which may in turn activate capacitative Ca^{2+} entry (Schilling *et al.*, 1992). Modulation of endothelial Cl-HCO_3^- and Na^+-H^+ exchange has been demonstrated in response to flow (Zeigelstein *et al.*, 1992). The resulting changes in cytosolic pH can influence endothelial $[Ca^{2+}]_i$ and cNOS activity (Ziegelstein *et al.*, 1992; Fleming *et al.*, 1993). However, it remains to be elucidated how the flow signal is transmitted into the cell and transduced at the sites described above.

In the present study we have focused on the possibility that structural changes in the endothelial cytoskeleton may provide a transduction pathway between shear stress and NO synthesis. The endothelial cytoskeleton is a complex network of interconnected tension-bearing microfilaments, microtubules and intermediate filaments that span the intracellular space and are anchored at the plasma membrane by integrins and focal adhesion sites. These filaments play a key role in determining cell shape, adhesion to the substratum and neighbouring cells and cell proliferation, spreading and migration (Gottlieb *et al.*, 1991). Shear stress is capable of altering endothelial cell shape and aligning them in the direction of flow (Dewey *et al.*, 1981; Langille & Adamson, 1981; Davies & Tripathi, 1993) and furthermore, can induce formation of endothelial stress fibres demonstrating an effect on actin microfilament organisation (Franke *et al.*, 1984; Wechezak *et al.*, 1989; Davies & Tripathi, 1993). This is reinforced by the finding that porcine aortic cells exposed to shear stress show an increase in actin turnover with a rapid depolymerization of F-actin to G-actin detectable after only 5 min exposure (Morita *et al.*, 1993; 1994). Furthermore, a role for actin microfilaments in calcium mobilization in isolated endothelial cells in response to mechanical stimuli has also been demonstrated (Diamond *et al.*, 1993; Oike *et al.*, 1994). However, there is still little evidence linking the endothelial cytoskeleton to release of vasoactive mediators in response to changes in shear stress. In the present study we have used a cascade bioassay system and endothelium-intact arterial ring preparations to investigate the potential role of actin- and tubulin-related components of the endothelial cytoskeleton in

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flow- and agonist-evoked release of the potent vasodilator, NO. Cytochalasin B, which caps the end of a growing actin filament preventing polymerization, phalloidin, an agent that associates with and stabilizes F-actin by reducing the rate of filament depolymerization, and colchicine, a microtubule disrupter that binds to the α and β tubulin dimers and inhibits polymerization were used to interfere with cytoskeletal function (Gottlieb *et al.*, 1991; Morita *et al.*, 1993).

Methods

Experiments were performed on abdominal aortae from male New Zealand white rabbits (2.5 kg) which had been killed by injection of sodium pentobarbitone (120 mg kg^{-1}, i.v.). The tissues were transferred to cold (4°C) gassed (95% O_2, 5% CO_2, pH 7.4) Holman's solution of the following composition (mM): NaCl 120, KCl 5, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 11, sucrose 10, containing indomethacin (10 μM). N^G-nitro-L-arginine methyl ester (L-NAME) was used as a specific inhibitor of NO formation from L-arginine in both cascade bioassay and organ bath experiments.

Cascade bioassay studies

The bioassay system used to quantify NO release in response to changes in time-averaged shear stress has been described previously (Hutcheson & Griffith, 1994). Briefly, a 3 cm section of rabbit abdominal aorta was cannulated and placed horizontally in an organ chamber filled with oxygenated buffer at 37°C. For control experiments the endothelium of the donor vessel was removed by gentle abrasion of the luminal surface with a metal rod, or the donor vessel replaced by rubber tubing. In many preparations the functional presence of endothelium was confirmed by bioassay of NO release in response to a submaximal concentration of acetylcholine (1 μM), which generally resulted in ca. 30% relaxation of the detector tissue. The presence or absence of endothelium was confirmed histologically by *en face* silver staining at the end of each experiment.

The preparations were perfused at a mean flow rate of 9 ml min^{-1} by a Watson-Marlow peristaltic pump (Type 503U). Perfusion pressure was monitored continuously by a pressure transducer (SensoNor 840) positioned immediately proximal to the infusion cannula. Maximum damping of the circuit was introduced by an air filled compliance chamber also connected at this point and this maintained the amplitude of the pressure pulse at $\leqslant$2 mmHg. To monitor NO release from the donor rabbit aorta, a 2 mm-wide ring segment of endothelium-denuded rabbit aorta, connected to an isometric force transducer (ADInstruments FT-102), was positioned directly below the organ bath outlet, the transit time between the donor and the recipient being ca. 2 s. The recipient tissue was allowed to equilibrate for 1 h at a resting tension of 2 g, before being preconstricted by direct superfusion with phenylephrine, to a final calculated concentration of 300 nM.

To study changes in viscosity and thus time-averaged shear stress at a constant mean flow rate of 9 ml min^{-1}, dextran (80,000 mol.wt.; 1–4% w/v) was added to the perfusate. Perfusate viscosity was measured with a clinical viscometer (Luckham). Responses to agonist stimulation were also assessed by construction of cumulative concentration-response curves to acetylcholine added to the perfusate. For these experiments, atropine (3 μM) was superfused over the recipient ring to eliminate any direct vasoconstrictor effect.

To investigate the role played by the endothelial actin and microtubule cytoskeleton the experiments were repeated after a 1 h perfusion of donor aorta and recipient ring with cytochalasin B (1 μM), phalloidin (100 nM) and colchicine (500 nM). Relaxations of the recipient ring to sodium nitroprusside were assessed prior to and following incubation with these agents to determine their effects on smooth muscle function.

Isolated ring preparations

Isolated endothelium-intact rabbit aortic ring preparations were used to examine the endothelium-dependent responses to acetylcholine, 2–3 mm long segments being suspended in 7 ml organ baths containing gassed Holman's solution at 37°C, and connected to a force transducer (ADInstruments FT-102) to monitor continuously changes in isometric force. The rings were left to equilibrate at a resting tension of 1 g for an hour and then contracted with a submaximal concentration of phenylephrine (300 nM). Once the response had stabilized, cumulative concentration-response curves were constructed. After frequent washings to return tension to the previous baseline value, the experiments were repeated in the presence of cytochalasin B (1 μM), phalloidin (100 nM) and colchicine (500 nM). These agents were added 1 h before administration of acetylcholine. Relaxations to sodium nitroprusside were again assessed in an identical fashion to determine any effects of these agents on smooth muscle function.

Immunofluorescence studies

Rabbit aortic endothelial cells were harvested from inverted vessels by exposure to collagenase (2 mg ml^{-1}) in medium E199. The cells were centrifuged at 1400 r.p.m. for 5 min at room temperature, resuspended and cultured on fibronectin-coated coverslips in complete culture medium (medium E199 containing 10% foetal bovine serum, 10% new born bovine serum, glutamine (6 mM), penicillin (200 u ml^{-1}) and streptomycin (200 μg ml^{-1})). The cells used in this study were primary nonconfluent cultures and were characterized by their ability to incorporate fluorescent labelled acetylated low density lipoprotein (Voyta *et al.*, 1984). Cells were incubated for 1 h at 37°C in the presence and absence of cytochalasin B (1 μM) and colchicine (500 nM). In studies to stain for actin microfilaments the cells were then fixed for 15 min at room temperature in 3% paraformaldehyde in phosphate buffered saline (PBS). In some cases the cells were then washed and incubated at room temperature with 0.1% (v/v) Triton X-100 in PBS for 5 min to permeabilize the cells while in other experiments the permeabilization stage was omitted. To localize the F-actin the cells were then incubated for 1 h with phalloidin conjugated to fluorescein isothiocyanate (FITC) (100 nM). Microtubules were labelled by indirect immunofluorescence with monoclonal mouse antibodies directed against α-tubulin. Cells were fixed and permeabilized as described above before incubation with the first antibody for 45 min at 37°C followed by an identical incubation with FITC-labelled goat anti-mouse immunoglobulin G as secondary antibody. The stained cells were mounted in 50% glycerol in PBS and examined under a Nikon inverted Photomicroscope equipped with epifluorescence optics.

Drugs

Acetylcholine, phenylephrine, dextran 80, indomethacin, atropine cytochalasin B, phalloidin, FITC-labelled phalloidin, colchicine, paraformaldehyde, triton X-100 and N^G-nitro-L-arginine methyl ester were obtained from Sigma Limited, Poole, U.K. All drugs were dissolved in Holman's buffer with the exception of cytochalasin B (dimethylsulphoxide), phalloidin and FITC-phalloidin (ethanol), and indomethacin (5% w/v $NaHCO_3$ in distilled water). Monoclonal mouse anti-α-tubulin and FITC-labelled goat anti-mouse IgG were also obtained from Sigma Limited, Poole, U.K.

Statistics

All data are given as mean $\pm$ s.e.mean, where n denotes the number of animals studied for each data point. Statistical analysis for viscosity studies was assessed by repeated measures of ANOVA followed by Bonferroni multiple compar-

isons test and for acetylcholine and SNP studies the Student's t test for paired data was used. $P<0.05$ was considered as significant.

Results

Effects of cyctoskeletal modulators

To confirm the effectiveness of cytochalasin B and colchicine we performed fluorescent staining of the rabbit aortic endothelial cytoskeleton applying the same conditions used in the functional studies. A 1 h incubation of cultured aortic endothelial cells with cytochalasin B (1 μM) resulted in virtually complete disintegration of the actin microfilaments (Figure 1a and b). A similar degree of disruption of endothelial microtubules was observed, using indirect immunofluorescence, following a 1 h incubation with colchicine (500 nM) (Figure 1d and e). In vessels that were not permeabilized with Triton X-100, staining of endothelial F-actin could be observed following a 1 h incubation of the vessel with FITC-labelled phalloidin (Figure 1c).

Cascade bioassay studies

Effects on altered perfusate viscosity To study the relationship between NO release and longitudinal shear stress, dextran 1–4% (w/v) was added to the donor perfusate, increasing its viscosity successively from 0.73 mPas to 0.98, 1.24, 1.57, and 1.97 mPa respectively, this highest viscosity being only slightly

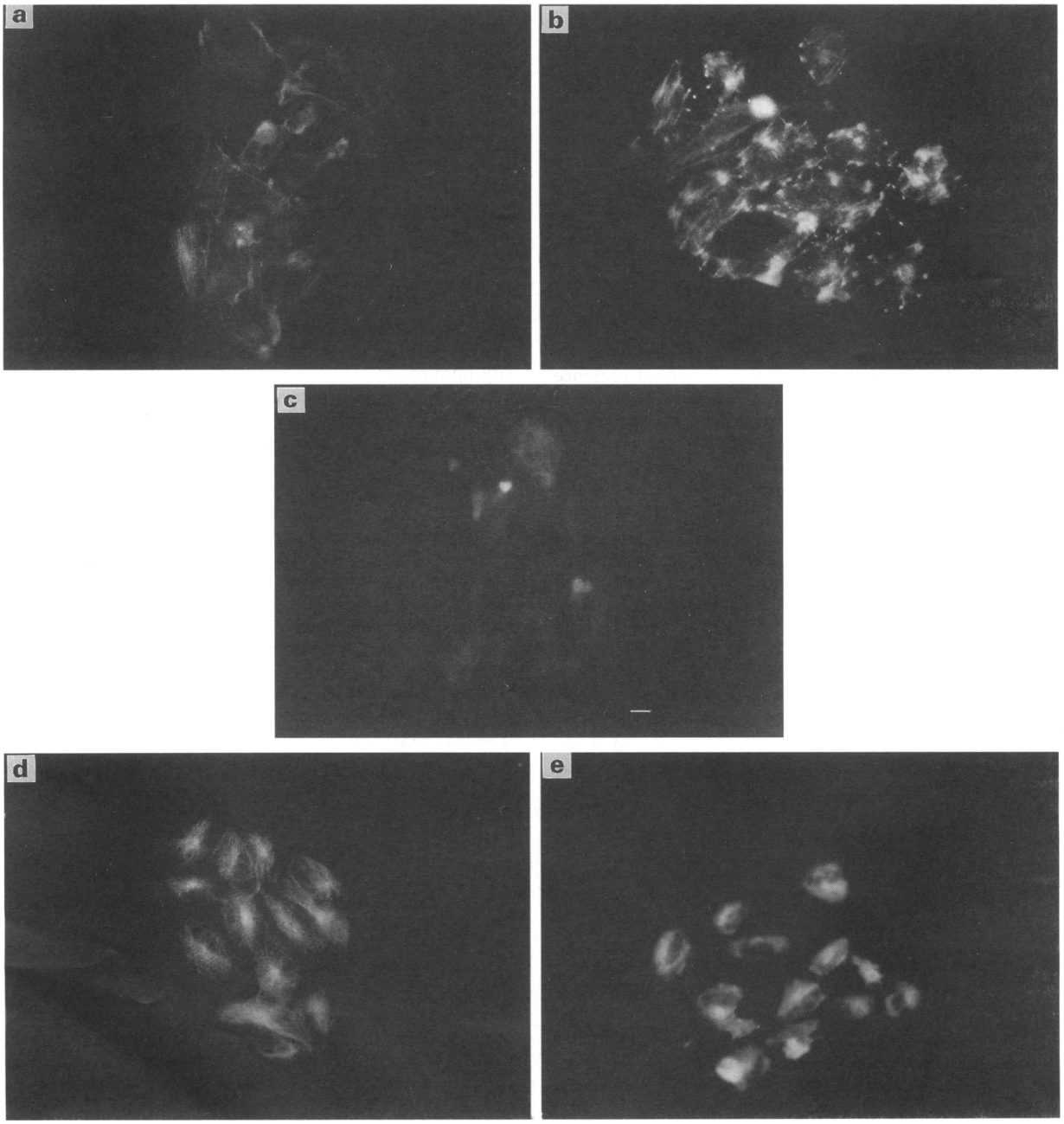

Figure 1 Effect of actin microfilament and microtubule disruptors on the cytoskeleton of rabbit aortic endothelial cells. Actin filaments were directly stained using fluoroscein isothiocyanate (FITC) conjugated phalloidin (a-c), whereas an indirect immunofluoresce technique using a monoclonal mouse anti-α-tubulin antibody and FITC conjugated monoclonal goat anti-mouse IgG antibody was employed to stain microtubules (d, e). The cells were incubated for 1 h in the absence (a) and presence of 1 μM cytochalasin B (b) and in the absence (d) and presence of 500 nM colchicine (e) before staining. Staining of actin microfilaments could be observed in non-permeabilized cells following a 1 h incubation with FITC-conjugated phalloidin (C) indicating that phalloidin is capable of exerting an effect within the endothelial cell at this time point. Calibration bar is 10 μm.

below that of blood *in vivo* (2.5–6 mPa.s, depending on shear rate). On the basis of diameter 1.5–2 mm, flow rate 9 ml min^{-1}, and viscosity 1.97 mPa, the calculated intimal shear stress would therefore be in the order of ~10 dyne cm^2 which is within the physiological range for small conduit arteries (Hutcheson & Griffith, 1994). Each increment in the concentration of dextran caused reversible relaxation of the recipient tissue, which was maintained for the duration of stimulation ($\geqslant$15 min). Pre-incubation of the donor aorta with L-NAME for 45 min significantly attenuated dextran-induced relaxations ($P<0.001$; $n=4$) (Figure 2) but a small relaxation was still observed. It has previously been shown that this L-NAME-insensitive relaxation is a non-specific effect of dextran on the recipient ring (Hutcheson & Griffith, 1994). Mean perfusion pressure (6.2±0.3 mmHg) was unaffected by changes in viscosity or addition of L-NAME. Incubation of the bioassay system with cytochalasin B (1 μM), phalloidin (100 nM) or colchicine (500 nM) significantly ($P<0.01$; $n=5$ for each agent) attenuated the viscosity-related relaxations observed following addition of dextran to the perfusate (Figures 3 and 4). There was no effect of these agents on the non-specific relaxations of the recipient ring induced by dextran when the donor vessel was replaced with rubber tubing (Figures 3 and 4).

Effects on acetylcholine-induced responses

To investigate agonist-evoked NO release cumulative concentration-response curves were constructed to acetylcholine (10 nM–30 μM). Endothelium-independent relaxations were also determined with sodium nitroprusside (1 nM–30 μM). Addition of acetylcholine to the donor perfusate induced relaxations of the recipient ring that could be completely abolished by 45 min pre-incubation of the donor with L-NAME (100 μM). There was no significant difference in acetylcholine-induced relaxations for vessels before and after incubation with cytochalasin B (1 μM), colchicine (500 nM) or phalloidin (100 nM) both in terms of the EC_{50} and the maximal responses ($n=5$ for each agent; Table 1). Relaxations to sodium nitroprusside were unaffected by cytochalasin B; however, EC_{50} values were significantly smaller following incubation with colchicine or phalloidin suggesting a minor degree of sensitization to this agent by these cytoskeletal modulators ($n=5$ for each agent; Table 2). Cytochalasin B significantly attenuated precontraction of the recipient ring to phenylephrine (pooled acetylcholine and sodium nitroprusside data; control = 3.12±0.19 g; cytochalasin B = 2.73±0.2 g; $P<0.01$, $n=10$).

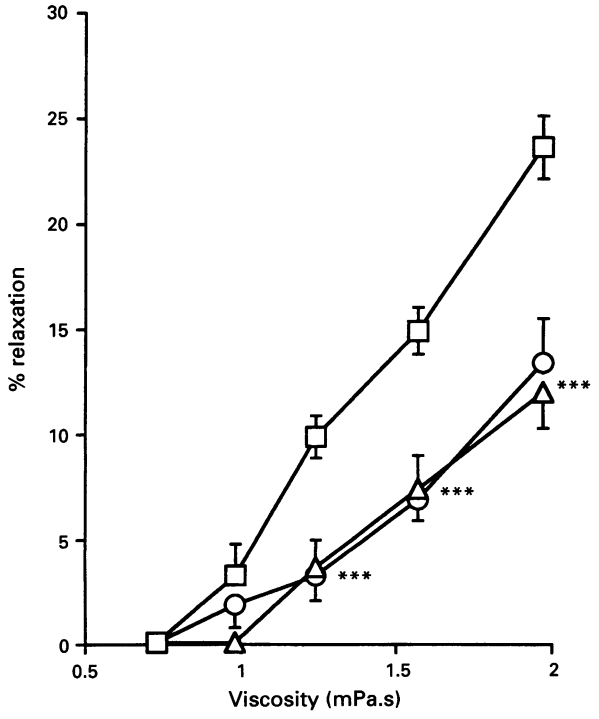

Figure 2 Relaxations of the preconstricted endothelium-denuded recipient aortic ring in response to increases in perfusate viscosity passing through the endothelium-intact donor aorta (□). The response was significantly attenuated ($P<0.001$; $n=4$) by L-NAME (100 μM; △) implying that it is mediated by release of NO. When the donor vessel was replaced by rubber tubing a similar relaxation to that seen following incubation with L-NAME was observed (○, $n=3$). There was therefore a small direct relaxation to dextran.

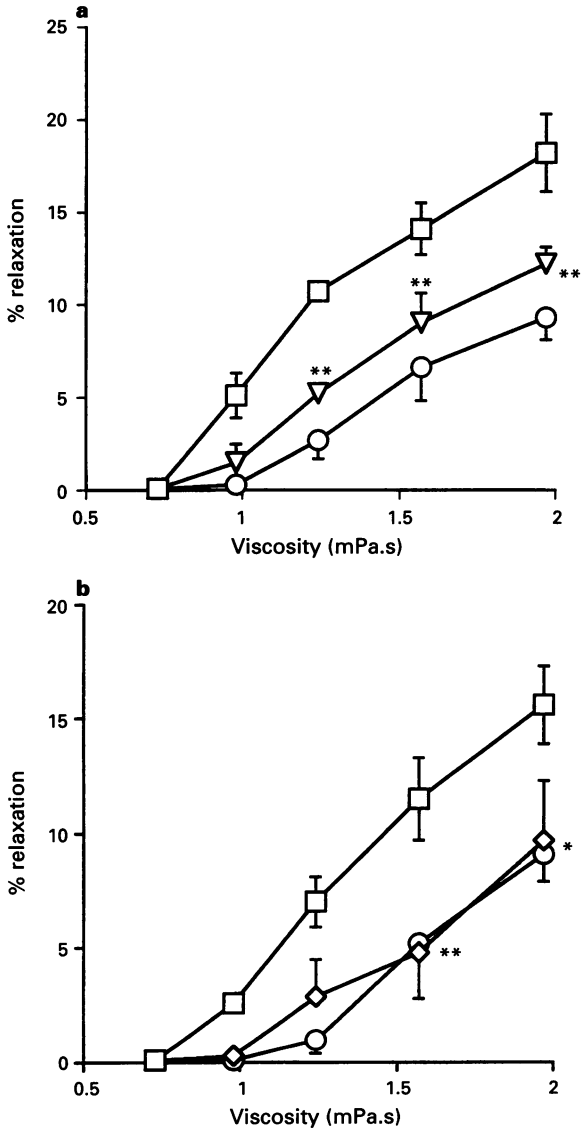

Figure 3 Effects of actin microfilament modulators on viscosity-related NO release. (a) Cytochalasin B (▽) an actin microfilament filament disruptor significantly ($P<0.01$; $n=5$) attenuated viscosity-related NO release. (b) Stabilizing the endothelial actin microfilament network with phalloidin (◇) also significantly ($P<0.01$; $n=5$) depressed release of NO in response to increased viscosity. The action of these agents can be entirely attributed to a reduction in NO release as they have no effect on either the residual relaxation observed when the donor vessel is replaced with rubber tubing (○) or relaxations of the recipient aortic ring to sodium nitroprusside (see Tables 1 and 2). (□) Control.

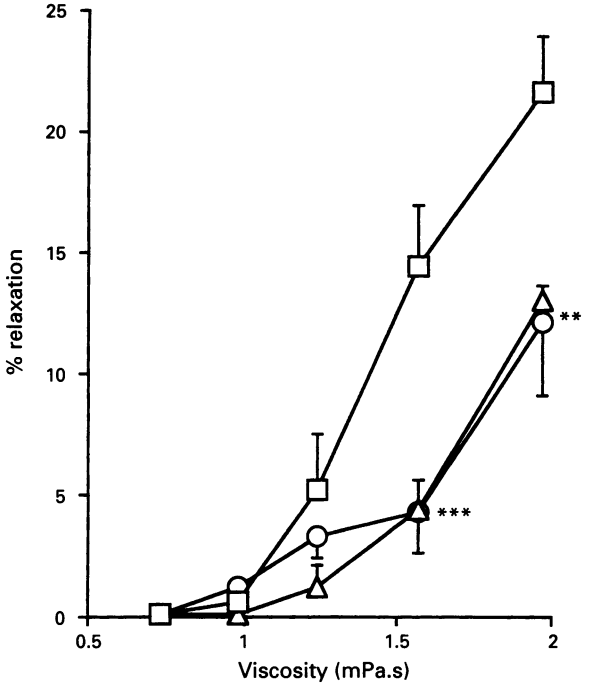

Figure 4 Incubation with colchicine, a microtubule disruptor significantly ($P<0.01$, $n=5$; △) attenuated the viscosity-related relaxations of the recipient rabbit aortic ring which again could be attributed to inhibition of NO release as this agent had no effect on either the direct relaxant action of dextran on the recipient (rubber donor, ○) or on relaxations to sodium nitroprusside (see Tables 1 and 2). (□) Control.

Isolated arterial ring studies Concentration-response curves to acetylcholine (10 nM−30 μM) and sodium nitroprusside (1 nM−30 μM) were determined in isolated ring segments of endothelium-intact rabbit abdominal aorta. Preincubation of the rings with L-NAME (100 μM) abolished the endothelium-dependent relaxant responses, confirming their dependence on NO synthesis (not shown). Cytochalasin B, phalloidin and colchicine exerted no significant effect on the EC_{50} or maximal responses to either acetylcholine ($n=5$ for each agent; Table 1) or sodium nitroprusside ($n=3$ for each agent; Table 2). Cytochalasin B again significantly attenuated phenylephrine-induced precontraction (pooled acetylcholine and sodium nitroprusside data: control = 2.95 ± 0.23 g; cytochalasin B = 2.33 ± 0.18 g; $P<0.001$, $n=10$).

Discussion

We have used preparations of rabbit abdominal aortae in cascade bioassay and as isolated rings to investigate the role of the endothelial microfilament and microtubule cytoskeleton in the mechanotransduction pathway associated with flow-related NO release and to evaluate any role they may play in response to agonist stimulation. Cytochalasin B, an F-actin depolymerizing agent, phalloidin, an F-actin stabilizing agent and colchicine, a tubulin dimerization inhibitor were employed to assess the contribution of the actin microfilament and microtubule lattice. The possible contribution of prostanoids was excluded by the presence of indomethacin in all experiments.

Before interpretation of the results it is important to consider two points; (1) whether these agents act predominantly at the level of the endothelial cell, and (2) whether the endothelial cells are effectively loaded with these agents to achieve the

Table 1 Effects of cytoskeletal modulators on recipient aortic ring contractility and responses to sodium nitroprusside and acetylcholine expressed as the EC_{50} and maximal relaxation

	Sodium nitroprusside			*Acetylcholine*		
	EC_{50} (−log M)	*Maximal response* (%)	*Contraction* (g)	*EC_{50}* (−log M)	*Maximal response* (%)	*Contraction* (g)
Control	6.68 ± 0.19	68.5 ± 10.7	3.26 ± 0.16 ($n=5$)	6.2 ± 0.16	30.3 ± 2.1	3.03 ± 0.35 (n = 5)
Cytochalasin B	6.99 ± 0.14	90.9 ± 11.7	2.9 ± 0.28 (n = 5)	6.22 ± 0.06	27.4 ± 3.2	2.57 ± 0.35** (n = 5)
Control	6.48 ± 0.07	87.4 ± 12.8	2.91 ± 0.18 (n = 5)	6.34 ± 0.04	30.5 ± 2.9	2.45 ± 0.27 (n = 5)
Phalloidin	6.71 ± 0.12*	93 ± 10.2	2.98 ± 0.13 (n = 5)	6.27 ± 0.05	26.7 ± 5.6	2.66 ± 0.28 (n = 5)
Control	6.51 ± 0.09	89.4 ± 3.4	3.28 ± 0.19 (n = 5)	6.34 ± 0.09	46.9 ± 4.4	2.25 ± 0.14 (n = 5)
Colchicine	6.95 ± 0.17*	98.5 ± 7.8	3.39 ± 0.13 (n = 5)	6.25 ± 0.03	40.7 ± 5.5	2.12 ± 0.08 (n = 5)

There was no effect of cytochalasin B, phalloidin and colchicine on relaxations of the recipient ring in response to acetylcholine. However, incubation with phalloidin and colchicine but not cytochalasin B significantly ($P<0.05$; $n=5$) reduced the median effective dose of sodium nitroprusside although there was no effect of any of these agents on maximal relaxations. It should also be noted that cytochalasin B significantly attenuated ($P<0.01$, $n=5$; acetylcholine study) the contractile response of the recipient ring to phenylephrine

Table 2 Effects of cytoskeletal modulators on contratility of isolated aortic rings and responses to sodium nitroprusside and acetylcholine expressed as the EC_{50} and maximal relaxation

	Sodium nitroprusside			*Acetycholine*		
	EC_{50} (−log M)	*Maximal response* (%)	*Contraction* (g)	*EC_{50}* (−logM)	*Maximal response* (%)	*Contraction* (g)
Control	6.54 ± 0.16	91.7 ± 2.8	3.06 ± 0.14 ($n=5$)	6.15 ± 0.04	65.8 ± 2.5	2.81 ± 0.52 ($n=5$)
Cytochalasin B	6.86 ± 0.18	99.7 ± 6.5	2.68 ± 0.25 ($n=5$)	6.21 ± 0.04	75.7 ± 2.9	2.25 ± 0.29 ($n=5$)
Control	6.61 ± 0.15	65.3 ± 8.5	2.68 ± 0.24 ($n=5$)	6.33 ± 0.15	47.5 ± 4.4	2.4 ± 0.08 ($n=5$)
Phalliodin	7.11 ± 0.14	64 ± 5.9	2.75 ± 0.35 ($n=5$)	6.25 ± 0.06	42 ± 3.1	2.7 ± 0.29 ($n=5$)
Control	6.5 ± 0.18	84.1 ± 6.5	2.86 ± 0.18 ($n=5$)	6.32 ± 0.07	78.4 ± 7.4	3.31 ± 0.52 ($n=5$)
Colchicine	6.97 ± 0.13	88.8 ± 7.2	2.75 ± 0.17 ($n=5$)	6.39 ± 0.12	76 ± 5.5	3.34 ± 0.55 ($n=5$)

Modulation of endothelial cytoskeleton in isolated rings of rabbit abdominal aorta had no effect on responses to sodium nitroprusside nor acetylcholine both in terms of their EC_{50} and maximal relaxations. As in Table 1 it again should be noted that cytochalasin B attenuated the contractile response of the isolated rings to phenylephrine.

required effect. Phalloidin and colchicine had no significant effect on phenylephrine-induced recipient artery tone in cascade bioassay nor on contractions of isolated rings. Furthermore relaxations to the nitrovasodilator, sodium nitroprusside, were also unaffected in cascade bioassay and in isolated rings by these agents, indeed, the isolated rings appeared to be more sensitive to SNP following incubation with these agents. However, cytochalasin B significantly attenuated recipient tone and reduced, though not significantly, contractions of isolated rings suggesting an effect of this agent on smooth muscle actin microfilaments. Nevertheless, relaxations to sodium nitroprusside were unaffected by this agent implying that cytochalasin B does not compromise the ability of the arterial rings to detect NO release. Thus, any effects of these agents can be interpreted as an effect on endothelial function e.g. NO release rather than on smooth muscle contractility. To determine that these agents were entering the endothelial cells in effective concentrations we used fluorescent stains for F-actin and tubulin. The fluorescence experiments demonstrated that 1 h perfusions of aortic endothelial cells with cytochalasin B and colchicine induced extensive disruption of the actin microfilament and microtubule networks, respectively. Furthermore, although some workers have considered phalloidin to be membrane impermeable (Oike *et al.*, 1994), we were able to stain non-permeabilized vessels with a 1 h incubation with FITC conjugated phalloidin implying that this agent can enter endothelial cells within the time scale used in the functional studies as reported by Morita *et al.* (1993). We are therefore confident that these agents are effectively loaded into the donor endothelium.

Endothelial cell shape is determined by the organisation of actin microfilaments, microtubules and intermediate filaments which make up the cytoskeleton. The contractile protein actin in its polymeric or filamentous (F-actin) form is organised into microfilament bundles that confer tension to the cell. Microtubules, formed from the polymerization of tubulin, in conjunction with intermediate filaments, primarily vimentin in endothelial cells, provide cell rigidity. Numerous studies have demonstraed that haemodynamic shear stress arising from blood flow is capable of influencing endothelial cell morphology (Dewey *et al.*, 1981; Langille & Adamson, 1981; Davies & Tripathi, 1993; Girard & Nerem, 1995) suggesting that the cytoskeleton is sensitive to this mechanical force. Indeed, it has been shown that shear stress induces actin depolymerization in porcine isolated aortic endothelial cells (Morita *et al.*, 1993; 1994) and reorganisation of actin microfilaments into stress fibres *in vivo* and *in vitro* suggesting a role for the endothelial cytoskeleton in stress transmission (Franke *et al.*, 1984; Wechezak *et al.*, 1989; Kim *et al.*, 1989; Davies & Tripathi, 1993; Girard & Nerem, 1995). In the present study we have shown that graded increases in the viscosity of the donor vessel perfusate, and thus time-averaged intimal shear stress, enhance NO release. Cytochalasin B, phalloidin and colchicine all significantly attenuated viscosity-related NO release consistent with the involvement of endothelial actin microfilaments and microtubules in mechanotransduction of this stimulus.

It should be noted that, as we have previously reported, the relaxation of the recipient ring induced by dextran in cascade bioassay is only partly meditaed by NO (Hutcheson & Griffith, 1994). There is also a small non-specific effect of dextran on the detector tissue possibly as a consequence of a direct action on vascular smooth muscle secondary to an associated increase in osmolality, although an interaction with the constrictor, phenylephrine, cannot be discounted. There was no effect on any of the cytoskeletal modulators used in this study on this small direct component implying a specific action of these agents on viscosity-related NO release. This is further emphasized by the findings that acetylcholine-induced NO release is also unaffected by these agents both in cascade bioassay and in isolated ring preparations. Interestingly, Hecker *et al.* (1993) have reported that flow can amplify release of NO induced by acetylcholine suggesting that, in cascade bioassay, responses to acetylcholine involve both a flow- and an agonist-evoked component. However, in the present study, disruption of the flow responses with cytoskeletal modulators has no effect on acetylcholine-evoked relaxations in cascade bioassay. Furthermore, we have been unable to demonstrate any amplification of the acetylcholine response with increasing perfusate viscosity in our bioassay (data not shown). There was similarly no effect of increasing concentrations of dextran on acetylcholine- and sodium nitroprusside-induced relaxations in the organ bath studies (data not shown).

Integrins and focal adhesion sites provide anchorage for the cytoskeletal elements at the luminal and abluminal surface of the cell, respectively, and both are responsive to shear stress (Wang *et al.*, 1993; Davies *et al.*, 1994). Wang *et al.* (1993) have recently demonstrated that the endothelial cytoskeleton confers an internal tension or prestress to the cell, 75% generated by actin microfilaments and 25% by a combination of microtubules and intermediate filaments, that is focused at these sites. They have proposed that the endothelial cytoskeleton utilises a tension integrity (tensegrity) system whereby the cytoskeleton functions as a single integrated unit that can respond dynamically to alterations in internal tension sensed by integrins. Interestingly, integrins also bind to the endothelial extracellular matrix and thus may provide a link to the intracellular microfilaments (Burridge *et al.*, 1988) and potentially a transmission pathway from possible extracellular flow sensors into the cell. Indeed, it has been proposed that endothelial cell surface glycoproteins but not proteoglycans may play a role in perceiving shear stress (Suarez & Rubio, 1991) and both proteoheparan sulphate and sialic acid residues within the endothelial glycocalyx have been proposed as potential flow sensors (Bevan & Siegel, 1991; Hecker *et al.*, 1993). Shear stresses upon the cytoskeleton may also be transmitted to neighbouring cells via focal adhesion sites thus providing intercellular communication of shear stress levels (Ingber, 1991; Davies & Tripathi, 1993).

The possible mechanotransducer sites whereby this transmitted force can be converted to changes in $[Ca^{2+}]_i$ in order to stimulate NO synthesis have yet to be elucidated. In a variety of cell types, F-actin has been shown to be associated with stretch-activated channels; however, it is unclear whether this linkage is direct or as a result of an indirect effect upon the tension exerted on the plasma membrane. Interestingly, it has recently been reported that the Cl^- current activated by swelling of human umbilical vein endothelial cells is unaffected by cytoskeletal disruption (Oike *et al.*, 1994). A mechanical linkage between cytoskeletal microfilaments (F-actin and tubulin) and G-proteins, phospholipase C and tyrosine kinase has also been demonstrated and thus shear-related microfilament reorganisation may potentially influence Ca^{2+} mobilization through these second messenger systems (Horvath *et al.*, 1990; Goldschmidt-Clermont *et al.*, 1990; Rasenick *et al.*, 1990). A role for phospholipase A_2 is unclear as release of Ca^{2+} from internal stores in response to mechanical stimulation can be mimicked by arachidonic acid in human umbilical vein but not bovine aortic endothelial cells (Oike *et al.*, 1994; Diamond *et al.*, 1994). Recently, Bourgignon *et al.* (1993) have reported that IP_3-sensitive intracellular stores are directly linked to the cytoskeleton in mouse T-lymphoma cells possibly providing a more direct transduction pathway. Indeed, swelling-induced increases in human umbilical vein endothelial $[Ca^{2+}]_i$; which is mediated by release of Ca^{2+} from internal stores, can be blocked with phalloidin, an F-actin stabilizer, implicating a role for actin depolymerization in this mechanotransduction pathway (Oike *et al.*, 1994). In contrast, capacitative Ca^{2+} influx in response to store depletion does not involve the F-actin cytoskeleton in this cell type (Oike *et al.*, 1994).

In conclusion, our findings are consistent with the hypothesis that the endothelial actin microfilament and microtubule cytoskeleton participate in the mechanotransduction pathway for viscosity- and therefore time-averaged shear stress-related

but no acetylcholine-evoked NO release in rabbit aorta. It would appear that the signal is passed by dynamic turnover of cytoskeletal elements rather than a simple transmission down a rigid connection since both disruption and stabilization of actin microfilaments attenuate the flow response.

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