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Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress

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SUMMARY

Endothelium exposed to fluid shear stress (FSS) undergoes cell shape change, alignment and microfilament network remodeling in the direction of flow by an unknown mechanism. In this study we explore the role of tyrosine kinase (TK) activity, intracellular calcium ($[Ca^{2+}]_i$), mechanosensitive channels and cytoskeleton in the mechanism of cell shape change and actin stress fiber induction in bovine aortic endothelium (BAE). We report that FSS induces β-actin mRNA in a time- and magnitude-dependent fashion. Treatment with quin2-AM to chelate intracellular calcium release and herbimycin A to inhibit TK activity abolished BAE shape change and actin stress fiber induction by FSS, while inhibition of protein kinase C with chelerythrine had no effect. Altering intermediate filament structure with acrylamide did not affect alignment or F-actin induction by FSS. Examining the role of the BAE cytoskeleton revealed a critical role for microtubules (MT). MT disruption with nocodazole blocked both FSS-induced morphological change and actin stress fiber induction. In contrast, MT hyperpolymerization with taxol attenuated the cell shape change but did not prevent actin stress fiber induction under flow. Mechanosensitive channels were found not to be involved in the FSS-induced shape change. Blocking the shear-activated current ($I_{K.S}$) with barium and the stretch-activated cation channels (I_{SA}) with gadolinium had no effect on the shear-induced changes in morphology and cytoskeleton. In summary, FSS has a profound effect on endothelial shape and F-actin network by a mechanism which depends on TK activity, intracellular calcium, and an intact microtubule network, but is independent of protein kinase C, intermediate filaments and shear- and stretch-activated mechanosensitive channels.

Key words: Mechanical stress, Mechanotransduction, Cytoskeleton, Beta actin

INTRODUCTION

The manner in which cells sense mechanical forces remains poorly understood. The question is particularly important since hemodynamic forces have been shown to play an important role in regulating cell structure and function in endothelium (Davies and Tripathi, 1993; Malek and Izumo, 1994), smooth muscle (Sumpio et al., 1988) and cardiac myocytes (Sadoshima and Izumo, 1993). The vascular endothelial cell (EC) is subjected at all times to shear forces that act on its surface as a result of the flow of viscous blood (LaBarbera, 1990). Fluid shear stress (FSS) regulates endothelial phenotype by altering its gene expression profile, including growth factors (Hsieh et al., 1991; Malek et al., 1993a; Resnick et al., 1993), vasoactive substances (Sharefkin et al., 1991; Malek et al., 1993b; Nishida et al., 1992), and adhesion molecules (Nagel et al., 1993; Ando et al., 1994). These phenotypic changes are seen in endothelial cells of differing origin including large artery, venous and microvascular (for review see Davies, 1995). One of the most dramatic and obvious responses to shear is the long-term morphological change which the EC experiences under conditions of flow (Fig. 1A). Endothelium undergoes a transition from a polygonal cobblestone-like uniform sheet under static conditions to a uniformly spindle-shaped and aligned monolayer, more akin to its in vivo state (Goode et al., 1977; Nerem et al., 1981). The response is reversible, exhibiting relaxation upon cessation of flow (Remuzzi et al., 1984), is dependent on active protein synthesis (Malek et al., 1993b), and is specific to endothelium since it is seen in neither smooth muscle nor fibroblast cells (Eskin and McIntire, 1988; Malek et al., 1994a). This adaptation to flow is believed to contribute to reducing shear gradients along the EC surface (Barbee et al., 1994). A further response to shear stress is a distinct transformation in the actin cytoskeleton (Herman et al., 1987), resulting in rearrangement of F-actin filaments into bundles of stress fibers aligned in the direction of flow (Wechezak et al., 1985; Kim et al., 1989). This phenomenon is thought to be partly the result of F-actin stress fiber induction (Franke et al., 1984). These cytoskeletal changes are unlikely to be simply cosmetic since the integrity of the actin cytoskeleton has been previously shown to be crucial in resisting fluid-imposed shear stress (Wechezak et al., 1989). The microtubule network (MT)

has also been demonstrated to be affected by shear stress, resulting in transiently preferential positioning of the microtubule-organizing center (MTOC) (Coan et al., 1993). Despite the significant influence of shear stress on the endothelial cytoskeleton and cell shape, little is known about the mechanism of the response.

Vascular endothelium responds functionally to the flow stimulus in the short-term with transient increases in $[Ca^{2+}]_i$ (Ando et al., 1988; Shen et al., 1992) from intracellular stores followed by a longer-term influx from the extracellular compartment (Dull and Davies, 1991; Geiger et al., 1992), and an increase in IP_3 and DAG (Nollert et al., 1990; Prasad et al., 1993). Recently, Prasad et al. (1993) used phospholipase C (PLC) inhibition with neomycin to propose a role for inositol tris-phosphate (IP_3) in EC shape response to flow. It is postulated that the shear-induced increase in IP_3 and DAG may serve to activate protein kinase C (PKC) (Nishizuka, 1986). This presumed shear-activation of PKC may then mediate the cytoskeletal and morphological changes. Our group has recently shown that flow induces an altered pattern of tyrosine phosphorylation in BAE cells with a prominent increase in p-tyr content of a protein of ~65 kDa in response to shear stress of 20 dyn/cm^2. This process was found to be inhibited by $[Ca^{2+}]_i$ chelation with quin-2AM (Di Virgilio et al., 1984) and TK inhibition with herbimycin A (Uehara and Fukazawa, 1991; Malek et al., 1993c). Tyrosine kinase activity can also mediate actin polymerization and cell shape changes. Cell spreading in fibroblasts (Burridge et al., 1992) and morphological changes in platelets (Lipfert et al., 1992) have been shown to be accompanied by changes in p-tyr content of the focal adhesion contact-associated protein pp125^{FAK}. Although shear exerts a significant effect on the MT network, the role of the latter in mediating shear-induced cell shape changes remains undefined. The actin microfilament network has been proposed to generate tension against the underlying substrate while microtubules are believed to counteract shape changes by generating a compressive force against the actin-myosin based contraction (Kolodney and Wysolmerski, 1992; Ingber, 1993). In addition, work by Wang et al. (1993) has suggested the involvement of MTs in resisting torque applied on integrin receptors on the endothelial surface with a partial contribution from intermediate filaments (IF).

Mechanosensitive channels have also been considered as candidates in the transmission of externally-applied flow to internal signaling (Fig. 1B). Two endothelial mechanosensors have been described in aortic endothelial cells: an endothelial cell-specific shear-activated potassium current resulting in cell hyperpolarization ($I_{K.S}$) (Nakache and Gaub, 1988; Olesen et al., 1988; Alevriadou et al., 1993) which is blocked by 1 mM $BaCl_2$ and partially inhibited by tetraethylammonium (TEA), and a gadolinium-sensitive stretch-activated (I_{SA}) non-specific cation channel (Lansman et al., 1987) similar to that found in other cell types (Sachs, 1988; Naruse and Sokabe, 1993). These mechanosensitive channels have been proposed to play a role in sensing and transducing flow-related forces and are thought to be important in modulating nitric oxide release (Cooke et al., 1991), TGF-β induction (Ohno et al., 1995) and ET-1 mRNA downregulation (Malek et al., 1993c) in response to shear stress. Their role in mediating cell shape and F-actin changes in response to flow, however, remains undefined.

In this report, we expose bovine aortic endothelial cells to shear stress in a cone-plate apparatus (Malek et al., 1995) to study the role of shear-induced intracellular calcium release, tyrosine kinase activity, protein kinase C, components of the endothelial cytoskeleton (Fig. 1B) and mechanosensitive channels in the process of morphological change and actin stress fiber induction in response to flow.

MATERIALS AND METHODS

Cell culture

BAE cells (passage 6-15) were harvested from descending thoracic aortas obtained from the local abattoir by collagenase digestion, and verified for Ac-LDL (Biomedical Technologies Inc., Stoughton, MA, USA) uptake (found to be greater than 98%). The cells were cultured in growth medium consisting of Dulbecco's modified Eagle (DME) medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% calf serum (Gibco-BRL), 4 mM L-glutamine, 25 mM Hepes, pH 7.4, 10 units/ml of penicillin, and 6.8 μM of streptomycin, at 37°C, 5% CO_2 in a humidified incubator. Medium was changed to serum-free DME 24 hours prior to applying shear stress.

Chemicals

Quin2-AM (acetoxymethyl ester) was obtained from Molecular Probes (Portland, OR, USA), herbimycin A from Gibco-BRL (Gibco, Gaithersburg, MD, USA), calphostin C and taxol from Calbiochem (San Diego, CA, USA), chelerythrine from LC systems (Woburn, MA, USA), and nocodazole, colchicine, phalloidin, acrylamide, TRITC-phalloidin, $BaCl_2$, $GdCl_3$, and TEA from Sigma Chemicals (St Louis, MO, USA).

Shear stress apparatus

A purpose-built cone-plate viscometer (Malek et al., 1995) was used to subject confluent BAE monolayers to well defined laminar fluid shear stress. Fluid shear stress magnitude and Reynolds number values were computed as previously reported using numerical integration of the expressions derived by Sdougos et al. (1984). Confluent BAE monolayers last fed 24 hours with either serum-free or 10% calf serum in DMEM were exposed to shear stress of 20 dynes/cm^2 for 24 hours. For $BaCl_2$ and $GdCl_3$ experiments, DME medium was modified by replacing sulfate and phosphate salts by their chloride equivalents to avoid precipitation (Cooke et al., 1991).

RNA isolation and hybridization

The acid guanidium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987) was used to isolate total cellular RNA. Northern blot hybridization was performed with a random-primer ^{32}P-labeled β-actin cDNA. Hybridization and densitometric analysis were carried out using an *Eco*RI cDNA fragment of human β-actin (ATCC #77644) and a 1.3 kb *Pst*I fragment of the rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. Following incubation, the blots were serially washed in 2× SSC-1% SDS and 0.2× SSC-1%SDS to a final temperature of 63°C for GAPDH, then exposed to X-ray film (X-Omat-AR film, Kodak, NY, USA) at −80°C. Autoradiograms exposed in the linear range were scanned by a densitometer two-dimensionally, and the signal strength of β-actin mRNA normalized for each sample with respect to the corresponding GAPDH mRNA signal, to compensate for variation in loading and transfer. GAPDH mRNA was chosen because its level is not affected by fluid shear stress (Malek et al., 1992).

Cell morphology analysis

Cells were visualized on an inverted microscope (Olympus IMT-2, Japan) using both phase contrast and Hoffman optics and were photographed using T-Max 400 film (Kodak, NY, USA). Images were then scanned two-dimensionally to serve as input into the image and

shape analysis software. Cell contour was traced manually and connectivity analysis was carried out on the resulting set of blobs using the Image Analyst software package (Automatix, Billerica, MA, USA) on a Macintosh computer system (Apple, Cupertino, CA, USA). The algorithm determined the best-fit ellipse through each cellular contour (between 30 and 50 cells per category) and provided the corresponding cell shape index (CSI) or roundness values for each cellular contour, with 1 representing a perfect circle and 0 a straight line.

Immunostaining

F-actin staining

BAE monolayers were washed three times with warm (37°C) physiological buffered saline (PBS), fixed with 3.7% paraformaldehyde in PBS for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes in PBS, washed in PBS, stained with TRITC-phalloidin for 10 minutes then washed three times in PBS (10 minutes), dehydrated with 100% ethanol and fixed with Möwiol.

Tubulin staining

BAE monolayers were washed three times with warm PBS (37°C) then quickly immersed in cold methanol (–20°C) for 20 minutes. The cells were then rinsed with PBS, incubated with anti-tubulin antibody (Boehringer-Mannheim, Germany) for 10 minutes, washed three times with PBS, incubated with secondary anti-mouse TR-conjugated antibody (Sigma Chemical Co., St Louis, MO) for 10-15 minutes, washed three times 10 minutes with PBS, dehydrated with 100% ethanol and fixed in Möwiol.

Stained cells at a radius of 3 cm, corresponding to a shear stress magnitude of 20 dyn/cm^2 were visualized using an epifluorescence microscope (Model BH-2, Olympus, Japan) and photographed with T-Max (ASA 400) film (Kodak, NY).

Statistics

Data were expressed as mean ± s.e.m. Statistical analysis was performed by analysis of variance and the unpaired Student *t*-test.

RESULTS

Shear stress induces endothelial cell shape change and actin stress fibers

Application of shear stress of physiological magnitude to BAE cells resulted in the formation of actin stress fibers and altered cell shape from the typical cobblestone pattern to fusiform and uniformly aligned in the direction of flow (compare Fig. 2A and B). The shape change response was cell-specific to endothelium since it was not observed in smooth muscle (Malek et al., 1994a; Eskin and McIntire, 1988). The actin staining in control cells revealed numerous fibers arranged in a radial pattern or emerging from the cell edges suggesting generation of tensile force to maintain the cell well-spread and flattened (Fig. 2C). In contrast, the actin staining pattern of cells exposed to shear stress showed prominent actin cables in the direction of the flow with decreased lateral staining (Fig. 2D). BAE monolayers were exposed to 24 hours of moderate shear (20 dyn/cm^2) and stained for F-actin with TRITC-phalloidin, showing the expected cell shape change and induction of numerous actin stress fibers oriented in the direction of flow (Fig. 2D).

Shear stress induces β-actin mRNA

Given the link between fluid shear stress and actin stress fiber induction, we investigated the possible regulation of β-actin expression at the mRNA level. In preliminary experiments conducted at 15 dyn/cm^2 no change was detected even though cell shape change and alignment were seen at this magnitude (Malek et al., 1993a). In other experiments, where a higher shear magnitude of 25 dyn/cm^2 was used in steady laminar mode, we detected an early and significant increase in β-actin mRNA (Fig. 3A), occurring as soon as 1 hour following onset of shear. Densitometric analysis of three sets of experiments conducted at 15, 25 and 45 dyn/cm^2 revealed a time and dose-dependent upregulation of β-actin mRNA by fluid shear stress (Fig. 3B).

Intracellular calcium and tyrosine kinase activity are crucial for endothelial alignment under shear

We explored the role of intracellular calcium and tyrosine kinase activity in the morphological changes and actin stress fiber induction by shear stress. Loading of BAE cells with quin2-AM (10 μM) 1 hour prior to applying shear stress prevented the cell shape change and alignment process (Fig. 4A and B). Similarly, pretreatment of BAE monolayers with herbimycin A (875 nM, 24 hours) inhibited the long-term cell shape change and alignment in BAE monolayers (Fig. 4C and D).

Intracellular calcium and tyrosine kinase activity, but not protein kinase C, are important in the shear-induced cytoskeletal reorganization

In order to evaluate the role of intracellular calcium release and tyrosine kinase activity on the actin stress fiber induction by shear, BAE cell monolayers exposed to 24 hours of steady shear stress were stained for F-actin using TRITC-phalloidin (Fig. 5). We observed that chelation of $[Ca^{2+}]_i$ with quin2-AM interfered completely with the shear-induction of actin stress fibers and cables in the direction of flow. F-actin staining of quin2-AM loaded BAE monolayers showed no detectable difference between cells exposed to shear (Fig. 5B) and cells maintained under static conditions (Fig. 5A). In a similar fashion, pretreatment of BAE monolayers with herbimycin A also prevented the induction of actin stress fiber formation in response to shear (Fig. 5D) compared to static control cells (Fig. 5C). In contrast, inhibition of protein kinase C using either chelerythrine (2 μM) (Herbert et al., 1990) or calphostin C (1 μM) (data not shown) had no effect on actin stress fiber induction and also did not interfere with the expected morphological alignment change (Fig. 5E and F).

Intact microfilaments are crucial for EC response to shear and stabilization with phalloidin interferes with, but does not prevent, alignment

In order to assess the role of the actin microfilaments in the EC alignment in the direction of flow, BAE monolayers were treated with cytochalasin D. However, even low doses (40 nM) induced cell shape rounding with decreased ability to resist shear forces, resulting eventually in sloughing off of the monolayer and cell loss. This result is consistent with a previous report (Wechezak et al., 1989). In contrast, treatment of EC with phalloidin (1 μM) for 24 hours prior to onset of flow (Suttorp et al., 1991) did not prevent EC alignment in response to shear stress (20 dyn/cm^2, 24 hours) (Fig. 6B). The extent of the alignment was, however, visibly decreased compared to untreated cells (Figs 6B and 2B).

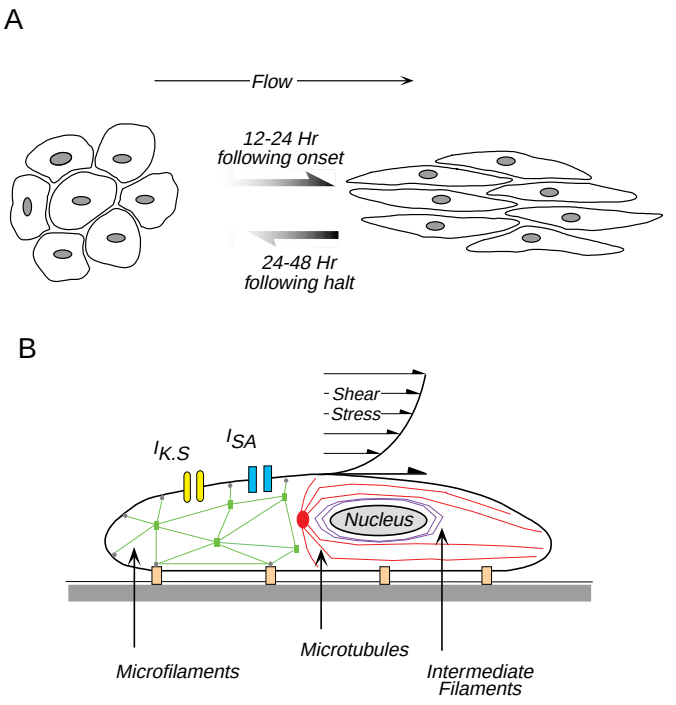


Fig. 1. Schematic of endothelial morphological response to flow and the resultant shear stress. (A) Confluent vascular endothelial cells exposed to flow with a corresponding shear stress magnitude greater than 8-10 dyn/cm^2 undergo morphological alteration from polygonal cobblestone to spindle-shaped with the major axis aligned in the direction of flow. This response requires between 12-24 hours for completion, is dependent on on-going protein synthesis, and is reversible following cessation of flow. The rate of alignment is dependent on shear stress magnitude. (B) Various sensory structures and second messengers play a potential role in the transduction process of shear stress to altered endothelial morphology and F-actin stress fiber induction. These include protein kinase C, cyclic AMP, intracellular calcium ($[Ca^{2+}]_i$), inositol trisphosphate (IP_3), tyrosine kinase (TK) and cytoskeletal elements such as microfilaments (MF), microtubules (MT), and intermediate filaments (IF). Mechanosensitive channels expressed in vascular endothelium include the shear stress-activated potassium current, $I_{K.S}$, and the stretch-activated cation channel, I_{SA}.

An intact microtubule network is essential for the morphological changes and stress fiber induction under shear

We next examined the role of the other components of the endothelial cytoskeleton, namely microtubules and intermediate filaments, on the process of alignment and actin stress fiber induction. BAE monolayers were treated with nocodazole (3.3 μM) to disrupt the tubulin network (Domnina et al., 1985) or with taxol (10 μM) to stabilize microtubules and induce tubulin hyperpolymerization (Howard and Timasheff, 1988) prior to the exposure to shear stress. Long-term (24 hours) exposure of BAE monolayers treated with nocodazole (3.3 μM) (Fig. 7D) or colchicine (data not shown) to fluid shear stress, failed to induce morphological change and alignment in the direction of flow when compared to static matched cells (Fig. 7C). It is worth noting that nocodazole-treated cells (Fig. 7C) appeared to assume a round morphology which was even more uniform than in untreated control cells (Fig. 7A), a finding which was accentuated by exposure to shear (compare Fig. 7D and B).

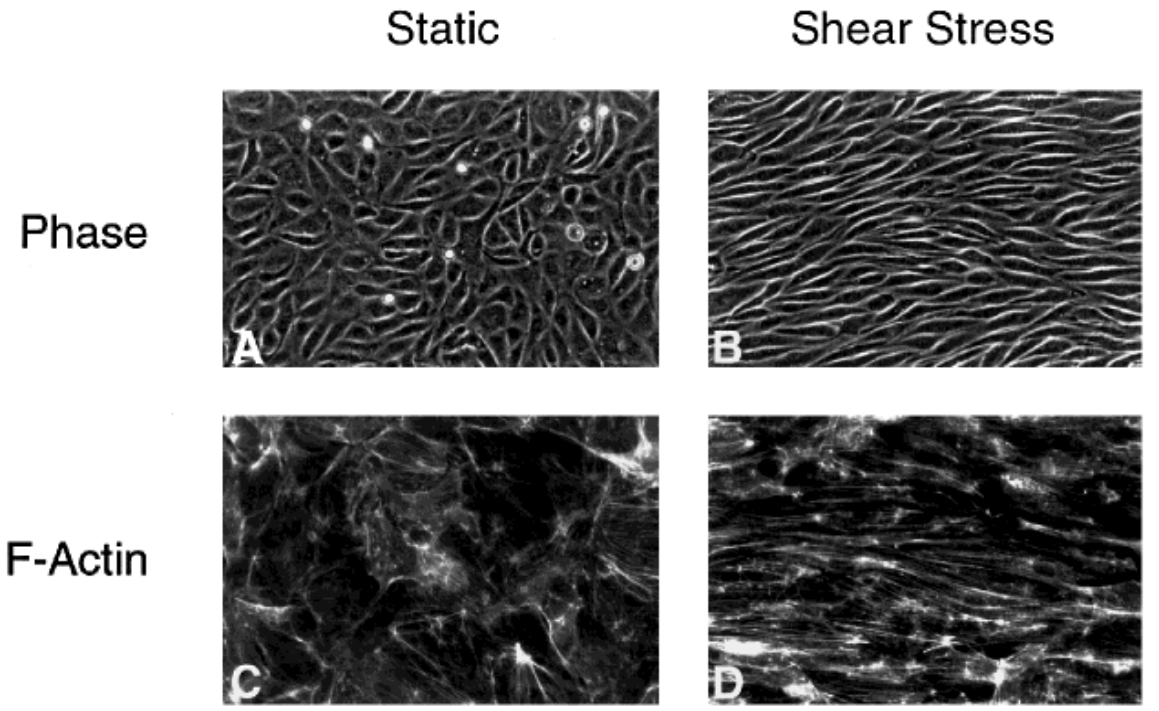


Fig. 2. Fluid shear stress alters endothelial cell shape and microfilament network organization. Phase-contrast micrographs of confluent BAE monolayers reveal a distinct change in cell morphology from the typical cobblestone (A) of static control cells to the fusiform and aligned in the direction of flow (B). The cell shape change is accompanied by alterations in the actin cytoskeleton with induction of actin stress fibers and cables in the direction of flow (from left to right) (D) compared to static controls which show more diffuse and lower intensity staining (C).

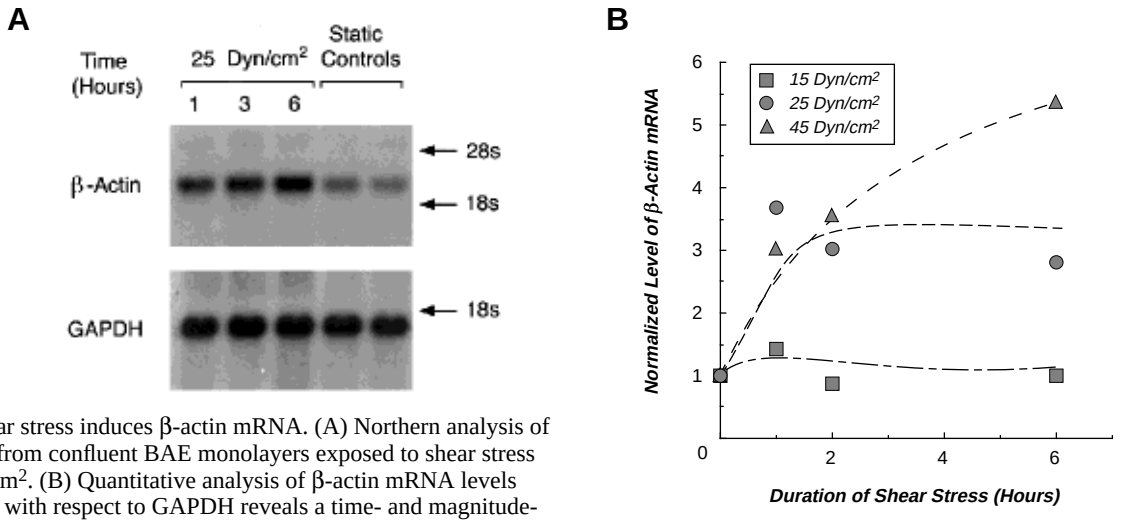


Fig. 3. Shear stress induces β-actin mRNA. (A) Northern analysis of total RNA from confluent BAE monolayers exposed to shear stress of 25 dyn/cm^2. (B) Quantitative analysis of β-actin mRNA levels normalized with respect to GAPDH reveals a time- and magnitude-dependent increase in response to shear stress.

Treatment with taxol was used to increase tubulin polymerization (Howard and Timasheff, 1988). Unlike nocodazole, taxol (Fig. 7F) did not prevent cell shape change and alignment but appeared to attenuate its extent when compared to matched control cells (Fig. 7B). Morphometric analysis enables the detection of the difference in the extent of cell shape change and elongation in response to flow (Fig. 8). Disruption of microtubules with nocodazole had little effect on static cells, but it prevented the decrease in CSI corresponding to the fusiform morphology of aligned cells. On the contrary, shear stress appears to further increase CSI in nocodazole-treated cells, indicating more homogeneously round cells. Taxol treatment attenuated, but did not block, the decrease in CSI corresponding to BAE alignment.

Immunocytochemistry using anti-tubulin antibody was then performed. Fig. 9B illustrates the frank change in microtubule network structure in sheared cells compared to control (Fig. 9A). The MT network assumed a more elongated, compressed, and denser distribution in the direction of flow. Tubulin staining also confirmed the pharmacological action of nocodazole treatment in dissolving the fine fibrillar tubulin pattern into diffuse staining (Fig. 9C and D). In contrast, taxol altered the microtubule staining by increasing its density to a higher steady-state (Fig. 9E), consistent with its ability to cause

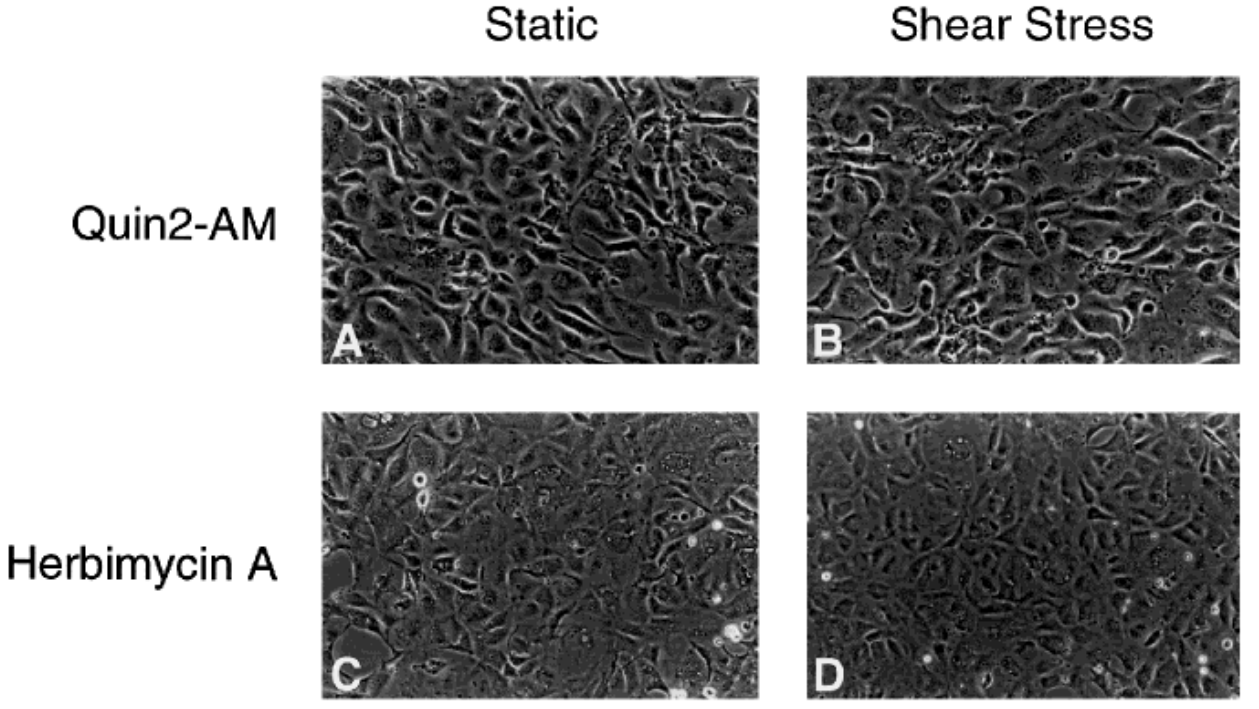


Fig. 4. Dependence of endothelial morphology change on $[Ca^{2+}]_i$ and tyrosine kinase activity. Phase contrast micrographs of BAE cells treated with the permeable calcium ion chelator quin2-AM (10 μM) (A,B), and the tyrosine kinase inhibitor herbimycin A (875 nM) (C,D) fail to undergo change in cell shape under shear stress.

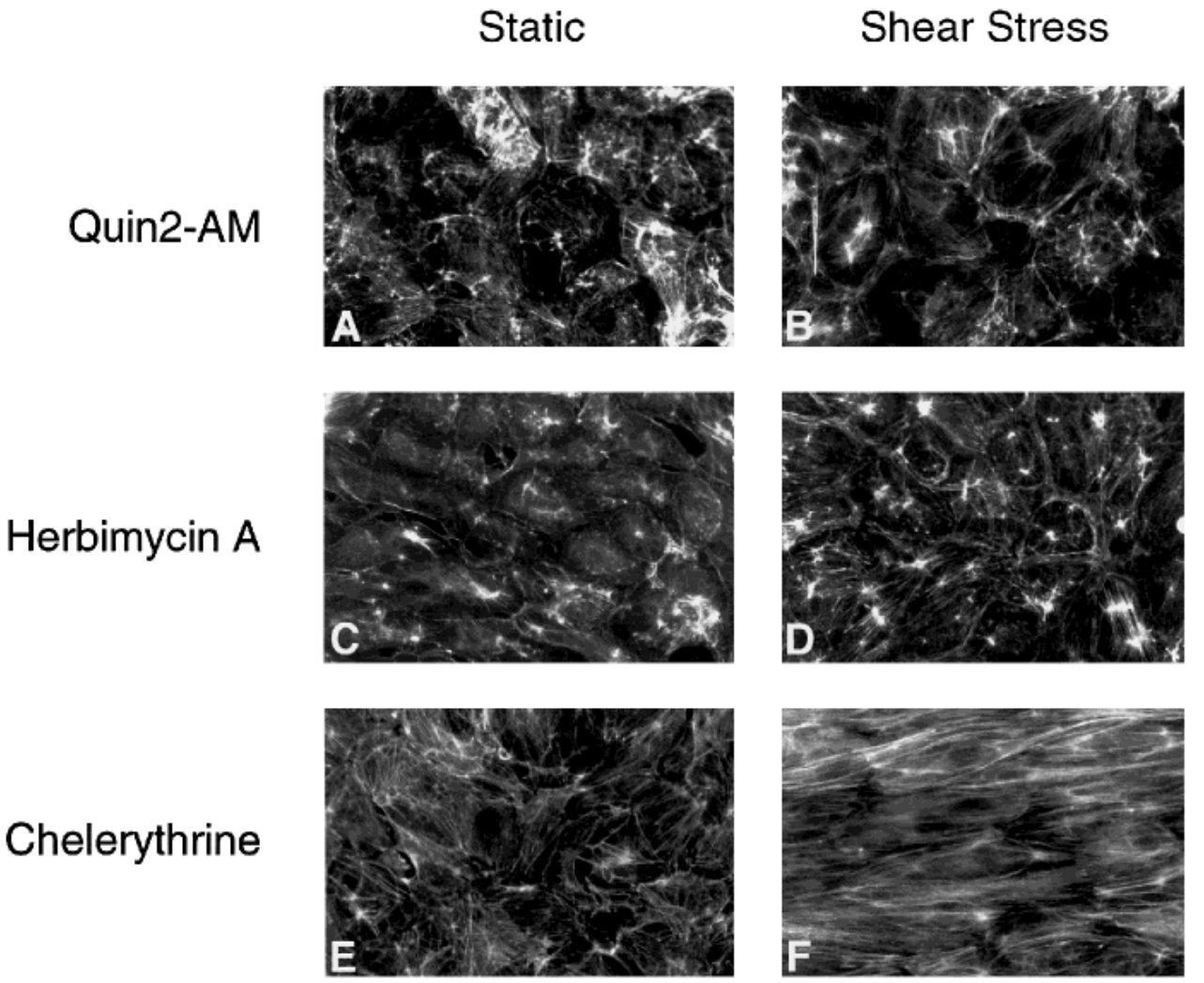


Fig. 5. Dependence of actin stress fiber induction on $[Ca^{2+}]_i$ and tyrosine kinase but not protein kinase C activity. Actin staining with TRITC-phalloidin reveals lack of actin stress fiber induction in the direction of flow for both quin2-AM (10 μM) (A,B) and herbimycin A (875 nM) treatment (C,D) but shows normal response and alignment in monolayers treated with the protein kinase C inhibitor chelerythrine (2 μM) (E,F).

tubulin hyperpolymerization. In response to shear stress, the taxol-treated cells still demonstrated the change in the microtubule network shape (Fig. 9F) but to a lesser degree than control cells. The importance of the tubulin network was then evaluated in the actin stress fiber induction process. Fig. 10C and D show that nocodazole completely interfered with the

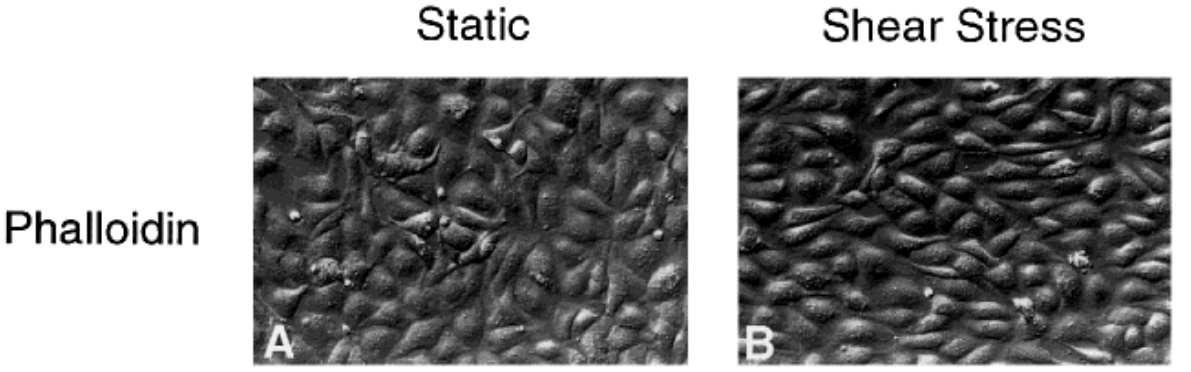


Fig. 6. Phalloidin pretreatment attenuates but does not prevent BAE cell shape change. Hoffman phase contrast micrographs of BAE cells pretreated with 1 μM phalloidin for 24 hours prior to onset of shear stress. The right micrograph reveals alignment of the cells in direction of flow (B), though to a slightly lesser extent than untreated cells (Fig. 2B).

shear stress induction of F-actin (Fig. 10D) compared to control untreated cells (Fig. 10B). In contrast, taxol-treated cells (Fig. 10E and F) still underwent F-actin stress fiber induction in the direction of shear stress in a similar fashion to untreated cells. This result suggests that microtubules are crucial for both alignment and actin stress fiber induction processes.

Acrylamide was used to alter IF network structure (Hay and De Boni, 1991) prior to exposing BAE monolayers to shear stress. Fig. 11 shows that acrylamide (2 mM) did not interfere with the actin stress fiber induction nor with the EC alignment process (compare Fig. 11B to A).

Mechanosensitive channels are not important in either EC alignment nor in stress fiber induction by shear

We next explored the role of mechanosensitive channels, the shear stress activated potassium current, $I_{K.S}$, which is blocked by barium, and the stretch-activated I_{SA} channel, which is inhibited by gadolinium. We employed a low-sulfate DME medium to avoid the precipitation of barium or gadolinium ions. Fig. 12A and B show that cells grown in low-sulfate DME still aligned in response to flow. Neither 1 mM $BaCl_2$ (Fig. 12D compared to C) nor 10 μM $GdCl_3$ (Fig. 12F compared to E) interfered with EC alignment in the direction of flow. F-actin staining also revealed that neither barium (Fig. 13D compared to C) nor gadolinium (Fig. 13F compared to E) appeared to inhibit the actin stress fiber induction in response to fluid shear forces when compared to untreated cells (Fig. 13A and B). Similar findings were also observed with 3 mM TEA (tetraethylammonium) (data not shown).

DISCUSSION

Despite the recognized effects of flow and the resulting shear stress on endothelial cell morphology (Dewey et al., 1981; Levesque and Nerem, 1985; Herman et al., 1987) and cytoskeleton (Franke et al., 1984; Kim et al., 1989), little is known about the mechanism and the transduction structures involved. In this study we have shown that intracellular

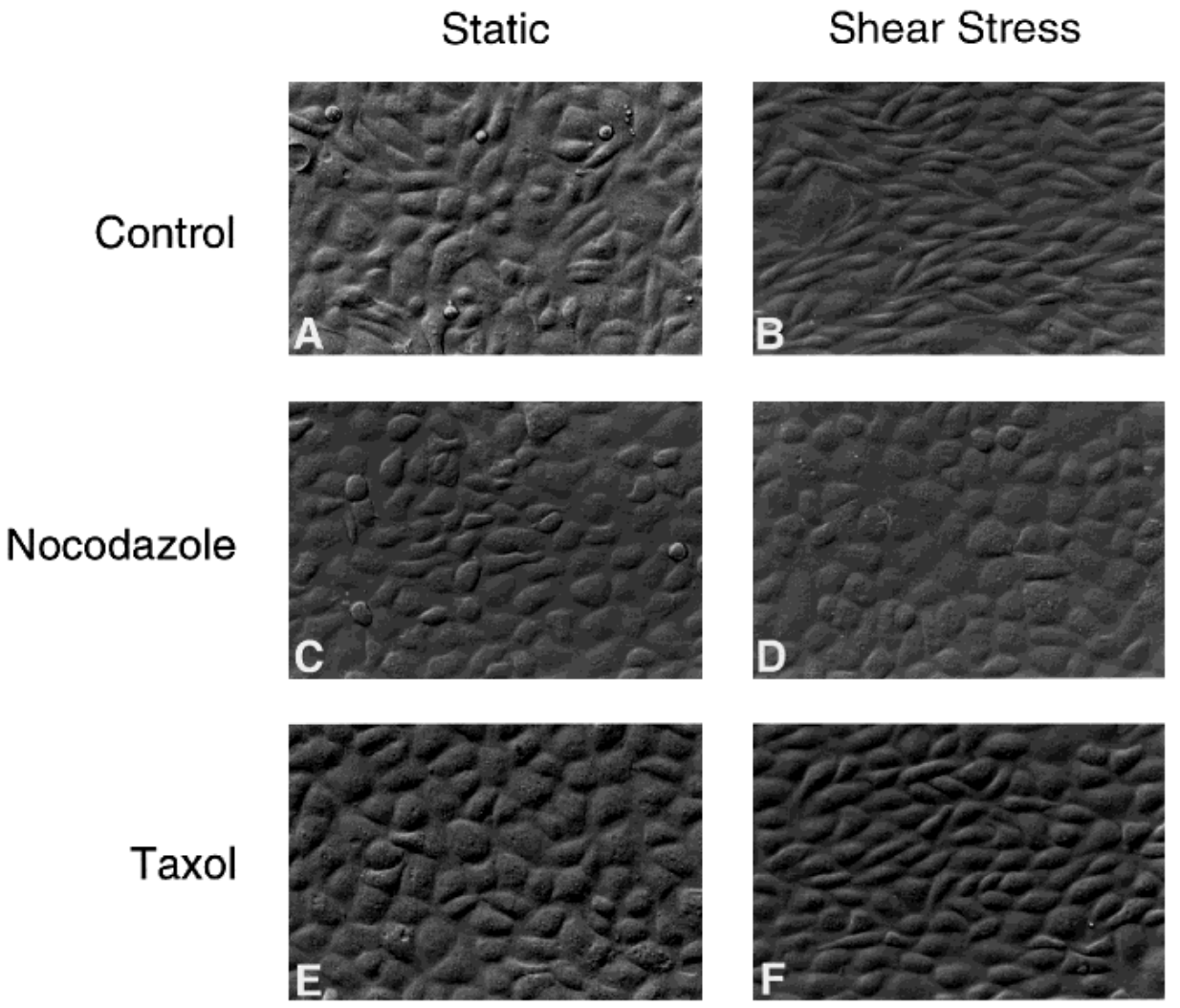


Fig. 7. Microtubules are crucial in the endothelial cell shape response to shear stress. Hoffman phase-modulation micrographs showing static (A,C,E) and shear stress-subjected (B,D,F) cells. Note the lack of BAE alignment in cells subjected to nocodazole (3.3 μM) (D) compared to control (B). In contrast treatment with taxol (1 μM) attenuates, but does not prevent, the cell shape change and alignment in the direction of flow (F) when compared to untreated cells (B).

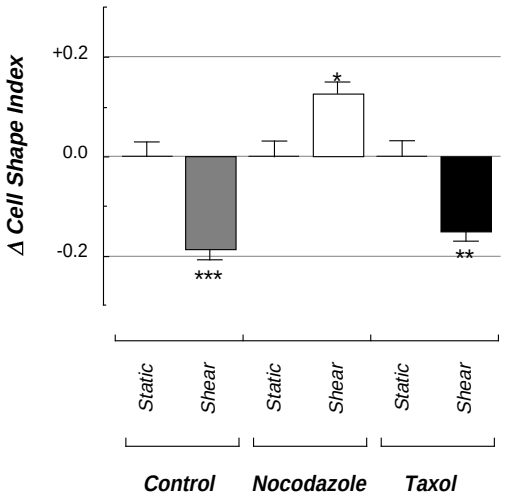


Fig. 8. Morphometric analysis of endothelial cells exposed to flow and MT-altering agents. Quantitative analysis of endothelial cell shape exposed to shear stress. A CSI value of 1 indicates a perfect circle while 0 corresponds to a flat line. Each category represents between 30 and 50 cells (*$P<10^{-3}$, **$P<10^{-4}$, ***$P<10^{-9}$).

calcium, which has been described to increase in response to flow and shear by both ATP-dependent (Dull and Davies, 1991; Mo et al., 1991) and -independent mechanisms (Geiger et al., 1992; Shen et al., 1992) is crucial for the observed phenomenon. Use of alternate intracellular calcium chelators such as BAPTA-AM induced cell shape changes under static conditions and cell death over a 24 hour period (data not shown). Lanthanum chloride (Shen et al., 1992) in low-sulfate medium was also used to block calcium entry into BAE cells but was found to be toxic over the time period necessary to observe alignment and actin stress fiber formation (data not shown). We attempted to explore the role of extracellular calcium by

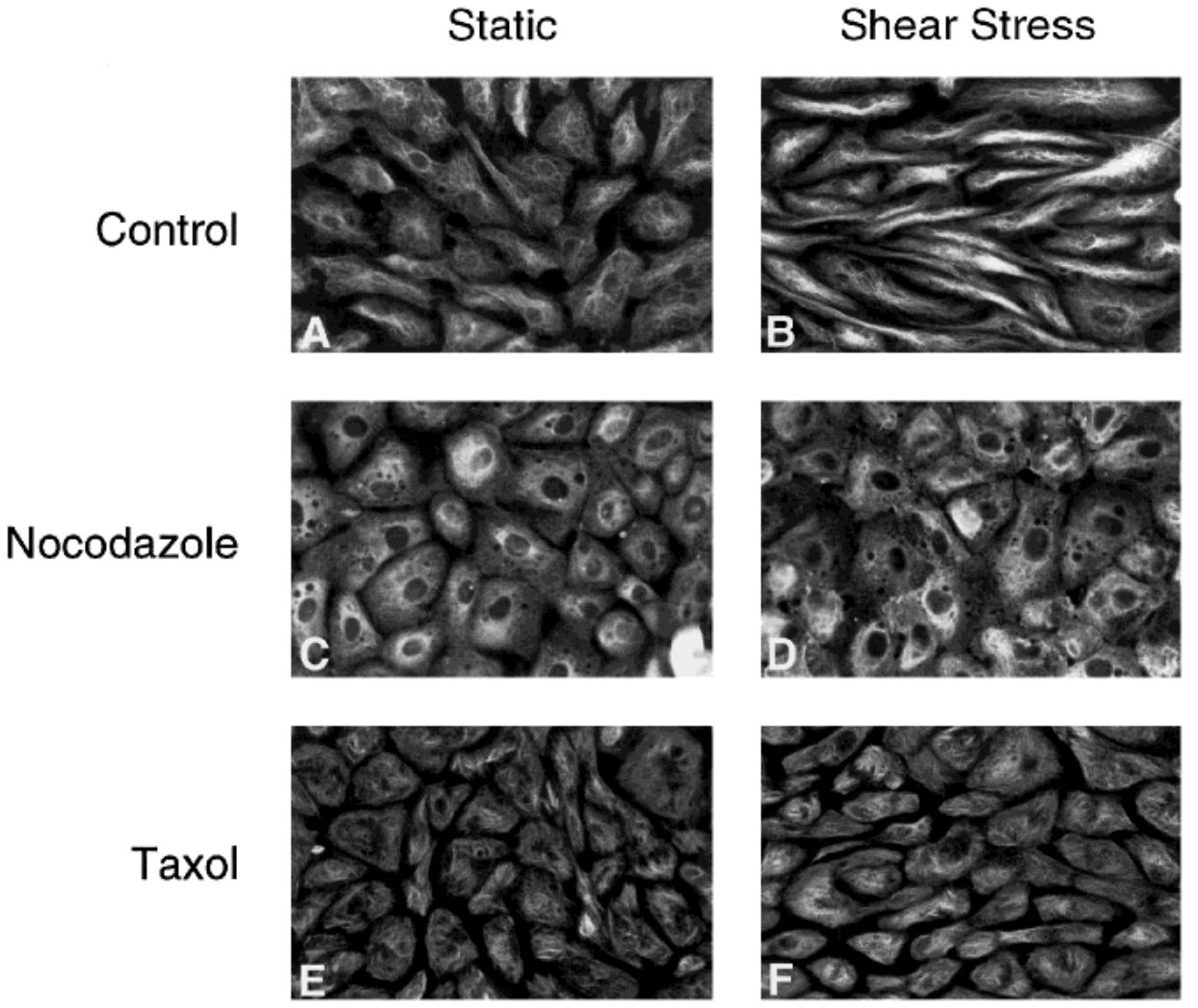


Fig. 9. Immunostaining of confluent BAE monolayers with anti-tubulin antibody. Shear stress (B) induces alignment of the microtubule network along the direction of flow and thinning in the minor axis compared to static controls (A). Nocodazole treatment (C,D) results in diffuse staining consistent with microtubule disruption; cells do not align in the direction of flow (compare D to C). Taxol treatment (E,F) to stabilize microtubules results in a denser and coarser pattern of staining. BAE cells still undergo cell shape change and align with flow (F) but in a less dramatic fashion than untreated cells (B).

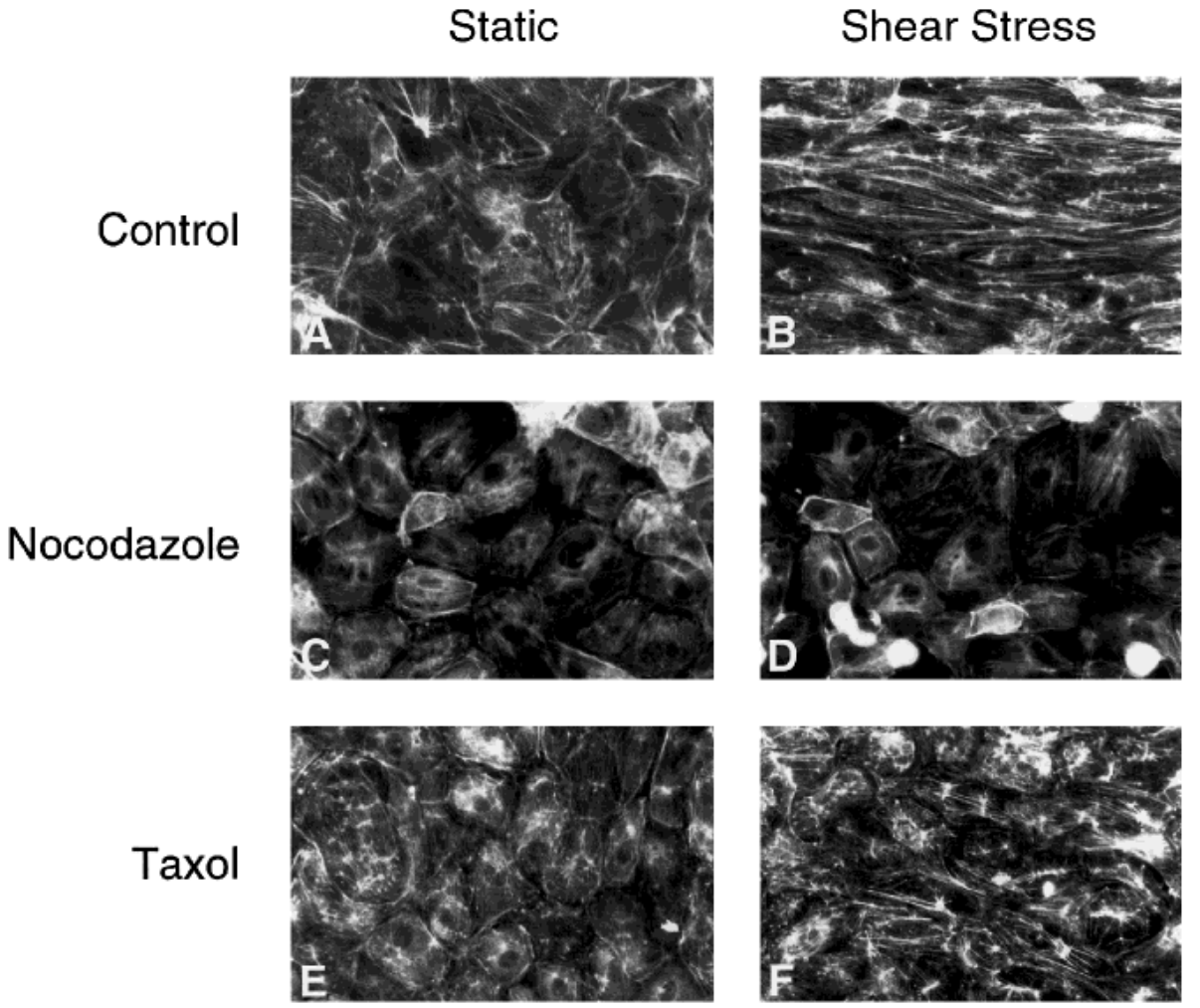


Fig. 10. Microtubules are essential for the shear stress-induced actin stress fiber induction in direction of flow. Compared to untreated controls (A,B), nocodazole-treated cells (C,D) do not show any measurable increase in actin stress fiber induction nor any detectable reorientation of the existing actin fibers in sheared cells (D) compared to static cells (C). In contrast, taxol treatment (E,F) attenuates the extent of actin stress fiber induction but not their orientation.

chelation with EGTA. However, even low concentrations of EGTA added to the medium resulted in cell retraction over 24 hours, sloughing and loss under shear (data not shown), a finding recently corroborated by others (Morita et al., 1994). This may be crucial since Geiger et al. (1992) have shown that the early initial $[Ca^{2+}]_i$ release is followed by a sustained calcium entry from the extracellular space.

We have further shown that tyrosine kinase activity is

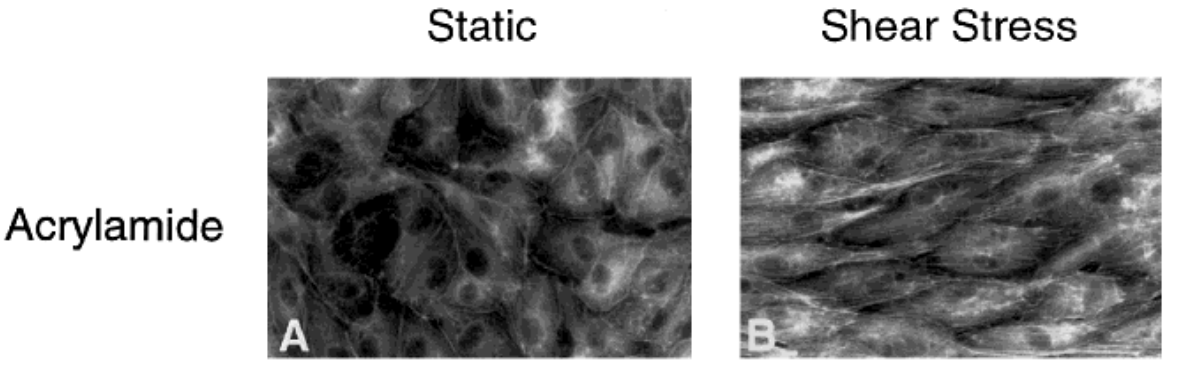


Fig. 11. Intermediate filament reorganization does not interfere with BAE response. Acrylamide (2 mM) treatment did not affect the cellular response of alignment in the direction of shear and F-actin stress fiber induction (compare B to A), arguing against crucial involvement of IF in the response to flow.

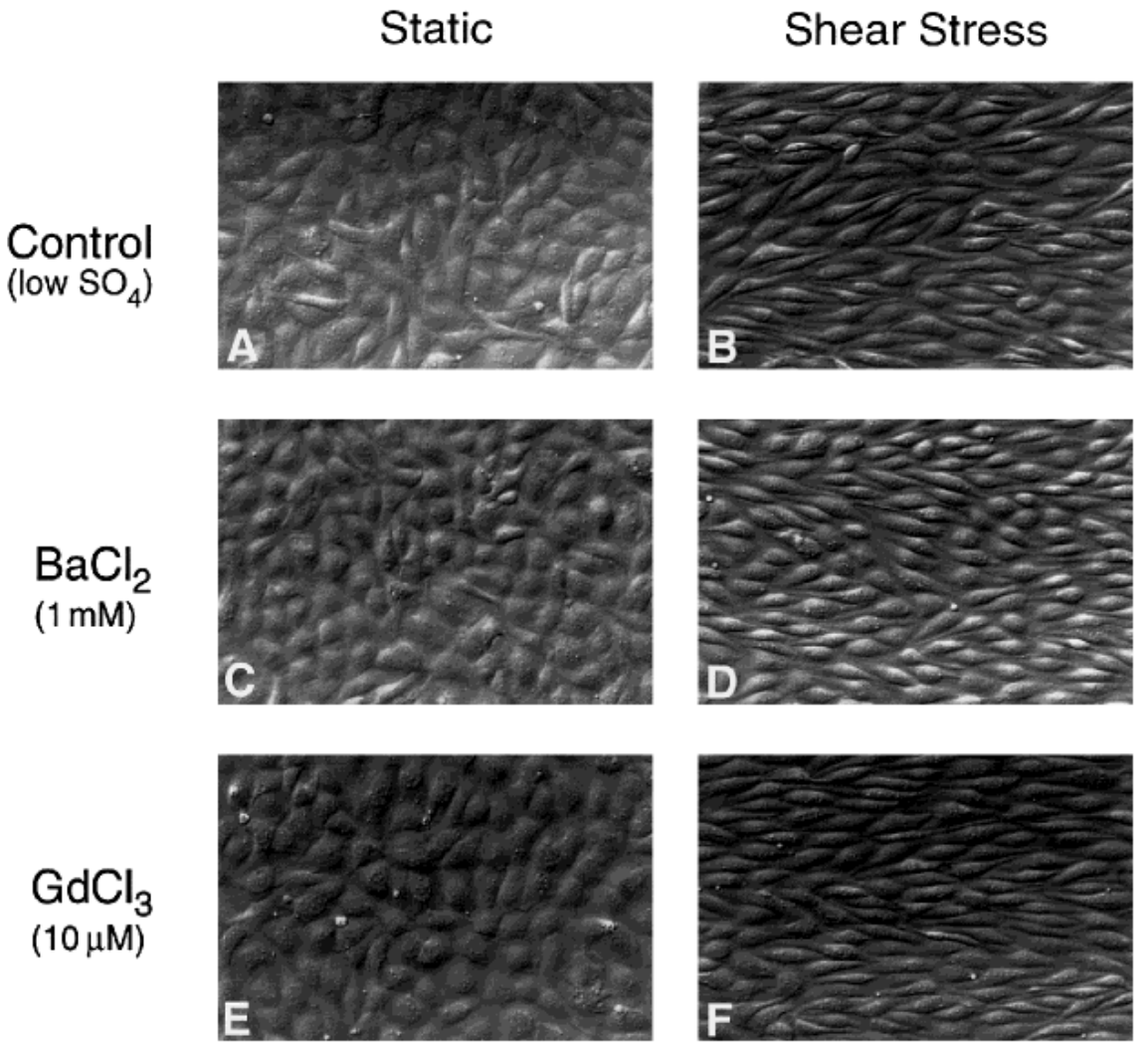


Fig. 12. Mechanosensitive channels do not play a critical role in the BAE cell shape response to flow. A low-sulfate DME medium was used to test the effect of barium (1 mM) and gadolinium (10 μM) ions to block the shear stress-sensitive potassium channel, $I_{K.S}$, and the stretch-activated cation channel, I_{SA}, respectively. The special formulation did not affect the alignment and actin stress fiber induction when compared to regular DMEM (compare B to A and to Fig. 2). Blocking $I_{K.S}$, (D) or I_{SA} (F) failed to interfere with the BAEC alignment and morphological change in response to flow (compare to respective control C and E).

essential since its inhibition with herbimycin A similarly prevented the morphological change and the induction of actin stress fibers. These results concur with other reports showing the importance of tyrosine kinase activity in cell spreading (Burridge et al., 1992) and platelet shape change upon activation (Lipfert et al., 1992). Another TK inhibitor was also tried in our experiments: genistein was found to induce BAE cell rounding and death after a period of 12 hours of actin stress fiber induction (data not shown). The important role of TK may be in mediating focal adhesion site remodeling (Davies et al., 1994) or the recently-documented integrin receptor redistribution to the upstream end of the EC in response to shear stress (Girard and Nerem, 1995). It is not at present clear how TK activity and $[Ca^{2+}]_i$ are linked but we have seen that the shear-induced increase in p-tyr content of a number of proteins is abolished by chelation of intracellular calcium (Malek et al., 1993c), and recent work has demonstrated the importance of TK in the sustained calcium entry phase of agonist response in EC (Fleming et al., 1995). Despite multiple reports suggesting the involvement of protein kinase C in the endothelial response to shear stress (Hsieh et al., 1992), PKC inhibition with chelerythrine did not interfere with either cell shape change nor with stress fiber induction, a finding also seen with calphostin C, another PKC inhibitor acting through a different mechanism. The magnitude of shear stress (20 dyn/cm^2) and cell type (BAE) used in this study is similar to a previous report from our group in which we did not detect significant activation of protein kinase C under similar circumstances (Malek et al., 1993b). The use of another PKC inhibitor, staurosporine (10 nM), resulted in significant cell rounding and was accompanied by cell loss under flow (data not shown). Treatment with the phorbol ester PMA, in an effort to downregulate PKC activity, induced, on its own, cell shape changes which were dramatic and occurred prior to those observed in response to flow (Antonov et al., 1986, and data not shown).

The induction of β-actin mRNA by shear stress of high but

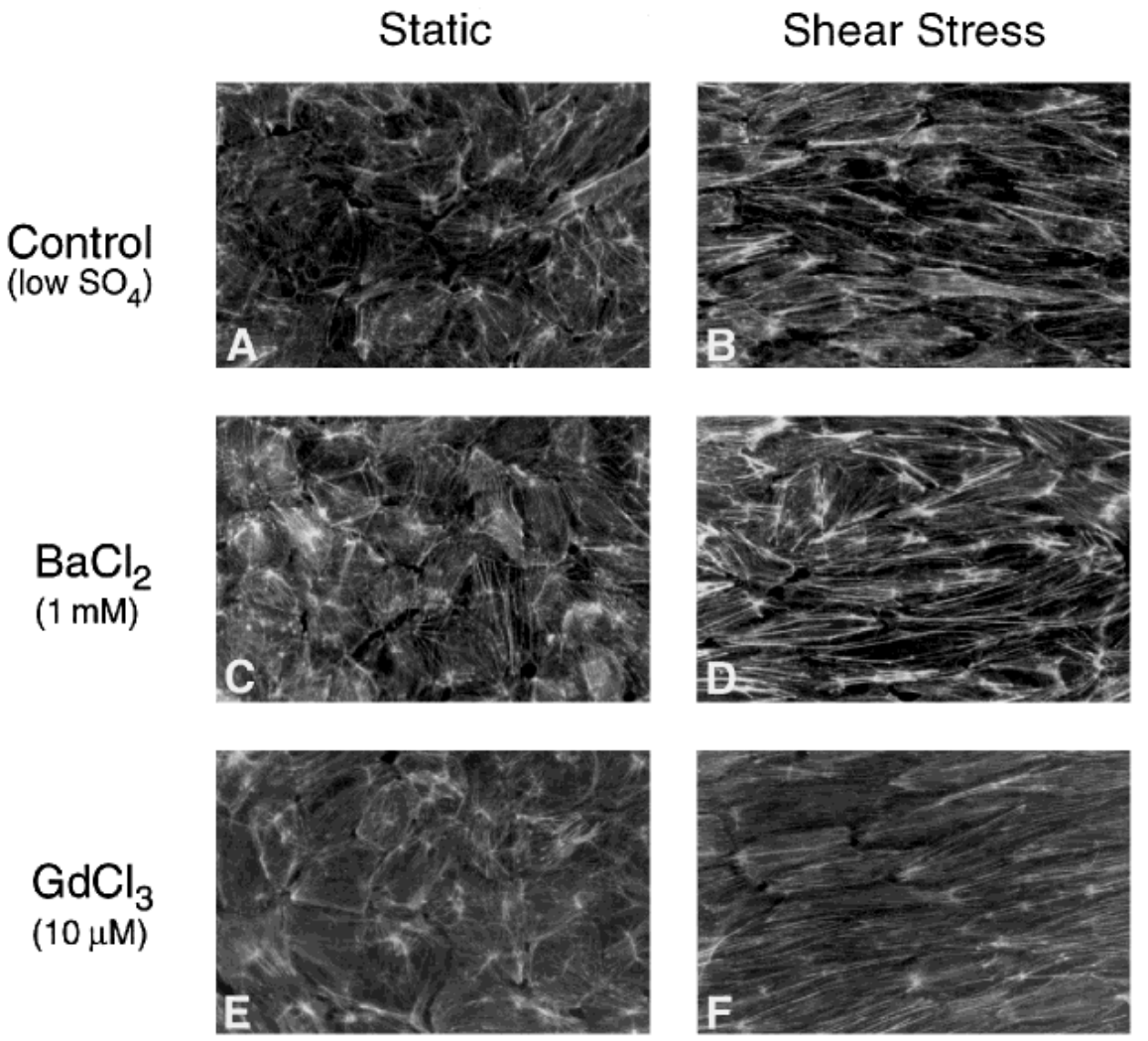


Fig. 13. Mechanosensitive channels are not essential for the F-actin induction by flow. F-actin staining shows that the low-sulfate DME medium (B) shows similar response when compared to regular DME (Fig. 2D). Blocking the shear stress-activated potassium channel, $I_{K.S}$ (C,D) or the stretch-activated cation channel, I_{SA} (E,F) did not interfere with the F-actin stress fiber induction by shear stress.

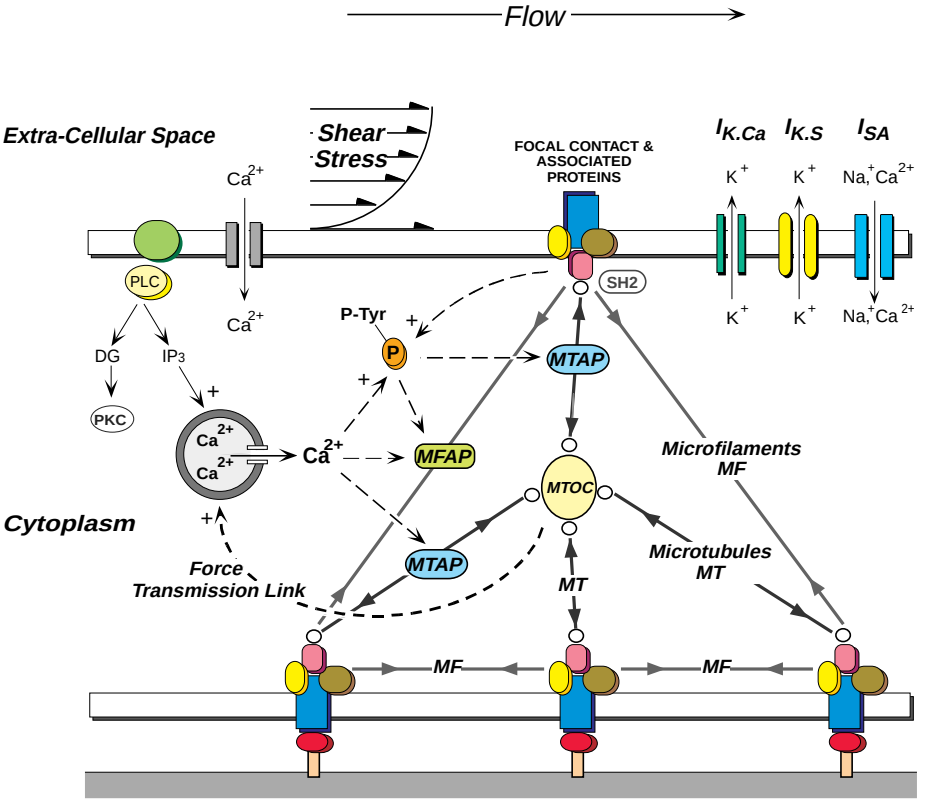
not low magnitude offers a parallel with other gene products which only show induction in BAE cells at elevated magnitudes of shear, namely bFGF (Malek et al., 1993a) and t-PA (Diamond et al., 1990; Malek et al., 1994a). The response is similar to the increased β-actin mRNA seen in hepatocytes subjected to mechanical swelling resulting from hypotonic medium (Schulz et al., 1991). Since shear initially induces actin depolymerization (Morita et al., 1992), the mechanism of increased β-actin mRNA may be similar to that reported in response to cytochalasin D in erythroleukemia cells (Sympson et al., 1993). Shear may also induce a greater turnover of actin including de novo protein synthesis, a phenomenon which would explain the inhibition of the alignment response by cycloheximide (Malek et al., 1992). Such synthesis could be sufficiently important at the higher magnitudes of shear stress as to require novel transcription of β-actin mRNA. Alternatively, increased actin may be required in order to interact with the higher levels of integrin $\alpha_5\beta_1$ subtype (fibronectin receptors) number seen in response to shear (Girard and Nerem, 1995; Sharpless et al., 1993). The mechanism of the increase, which may be the result of a change in β-actin mRNA stability such as that seen in response to tumor necrosis factor (Kohno et al., 1993) or a change in transcription as in the case of cytochalasin D exposure (Sympson et al., 1993), is currently under study.

We have shown that microtubules constitute an essential component of the endothelial morphological response to flow. MTs have also been documented to be important in mediating other shear stress responses, namely both the transient increase (Morita et al., 1992) and sustained downregulation of ET-1 mRNA (Malek et al., 1994b) seen at low and higher levels of shear respectively. It is interesting that disruption of microtubules in itself induced a more uniform cell shape of the EC and that this rounding was further increased by application of shear stress. The relevance of this phenomenon or its mechanism is unclear, however it suggests that the microtubules contribute greatly to basal EC shape, possibly via their action as stiff structures (Ingber, 1993; Kolodney and Wysolmerski, 1992; Heidemann and Buxbaum, 1990). Taxol only slowed and attenuated the alignment in response to shear but did not prevent it, indicating that while MTs are required

for cell shape change, they may also resist it. Our findings underline the central role of MTs and are consistent with the observation that MTs are the first cytoskeletal components to assume orientation during cell shape change (Oakley and Brunette, 1993). Although we do not present time-lapse analysis of MT alignment in response to flow, the alignment of endothelium establishes similar intracellular shear-free planes to those seen in fibroblast spreading on grooved substrata (ibid). In line with Oakley's findings, our results showing that phalloidin did not prevent cell alignment, while nocodazole did, suggest that microtubules may be more important than microfilaments in the alignment in the direction of flow. Despite the important role of intermediate filaments in cell nucleus anchoring and stabilization (Hay and De Boni, 1991), they appear not to be crucial in the EC alignment and actin stress fiber response to shear even though IF have recently been shown to contribute partially to cytoskeletal mechanical characteristics (Wang et al., 1993).

One of the most important findings in this report is the absence of a significant role for the two types of mechanosensitive channels reported in the endothelial cell. Neither 1 mM $BaCl_2$ which blocks the shear-induced K-current (Olesen et al., 1988), nor 3 mM TEA, which blocks calcium-activated K-channels (Cooke et al., 1991) had a significant effect on morphologic or cytoskeletal change. In addition, inhibiting the stretch-activated channel (Lansman et al., 1987; Sachs, 1988) with 10 μM $GdCl_3$ had no detectable effect on the endothelial response to flow. Our findings argue against the involvement of $I_{K.S}$, I_{SA}, or $I_{K.Ca}$ in cell shape and cytoskeletal response to flow though they do not rule out their importance in other functional responses to flow.

We propose a hypothetical model to explain the mechanism



LEGEND

Ca^{2+}	Calcium	MF	Microfilaments
DG	Diacylglycerol	MFAP	Microfilament-associated proteins
IP3	Inositol tris-phosphate	MT	Microtubules
$I_{K.Ca}$	Calcium-activated K-Channel	MTAP	Microtubule-associated proteins
$I_{K.S}$	Shear stress-activated K-Channel	PKC	Protein kinase C
I_{SA}	Stretch-activated cation channel	PLC	Phospholipase C

Fig. 14. Hypothetical model of endothelial morphological and cytoskeletal response to flow. The endothelial cell is subjected to fluid shear stress resulting from blood flow on the cell surface. This stress is transmitted from the luminal surface to focal adhesion contacts through the endothelial cytoskeleton including microtubule and microfilament networks. The thus-transmitted shear increases intracellular calcium and protein tyrosine phosphorylation, both of which play a crucial role in dissolution and reorganization of the cytoskeleton and focal adhesion sites to a new steady state configuration with lower surface shear gradients and an improved hydrodynamic profile with lower drag.

of endothelial alignment in response to flow (Fig. 14). The shear stress acting on the endothelial surface is transmitted throughout the cytoskeleton to the focal adhesion complexes on the abluminal surface. Microtubules are crucial in communicating the intracellular stress since they act in compression and partly contribute, via an undefined force transmission link, to calcium release from intracellular stores in parallel with the calcium released from shear-induced IP_3 increase. Concomittantly, the transmitted shear alters the mechanical configuration of focal adhesion-associated proteins and their tyrosine kinase activity, yielding increased tyrosine phosphorylation of an intermediate protein. Together the latter and the increased intracellular calcium act to shift assembly/disassembly dynamics of both microfilament and microtubule networks and induce migration of focal adhesion sites. The end result is a morphological steady state configuration with lower surface shear gradients and an improved hydrodynamic profile and lower drag. This microstructural remodeling is independent of PKC, and $I_{K.S}$, I_{SA} and $I_{K.Ca}$.

In summary, we have shown that the primordial endothelial response of alignment and actin stress fiber induction in the direction of flow is dependent on intracellular calcium, tyrosine kinase activity and the presence of microtubules but is independent of protein kinase C, intermediate filaments, and the known endothelial mechanosensitive channels. Further study is required to determine the dynamic interrelationship of these components in response to flow.

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