Role of Ca\(^{2+}\) and Protein Kinase C in Shear Stress–Induced Actin Depolymerization and Endothelin 1 Gene Expression

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Abstract  Vascular endothelial cells adapt to changes in blood flow by altering the cell architecture and by producing various substances. We have previously reported that low shear stress induces endothelin 1 (ET-1) expression in endothelial cells and that this induction is mediated by depolymerization of actin fiber. In the present study, we examined the role of Ca\(^{2+}\) and protein kinase C (PKC) in shear stress–induced actin depolymerization and subsequent ET-1 gene expression. Exposure of cultured porcine aortic endothelial cells to low shear stress (5 dyne/cm\(^2\)) for 3 hours increased the ratio of G-actin to total actin from 54±0.8% to 80±1.0%. This shear stress–induced actin depolymerization was completely blocked by chelation of extracellular Ca\(^{2+}\) with EGTA and partially inhibited by intracellular Ca\(^{2+}\) chelation with the tetraacetoxymethyl ester of BAPTA (BAPTA/AM). Pretreatment with staurosporine, a PKC inhibitor, or desensitization of PKC by treatment with 12-O-tetradecanoylphorbol 13-acetate (TPA) for 24 hours also resulted in partial inhibition of shear stress–induced actin depolymerization. Although PKC activation by TPA mildly increased G-actin content, the effect of TPA and shear stress on actin depolymerization was not additive. Moreover, shear stress–induced ET-1 gene expression was inhibited by EGTA, BAPTA/AM, and staurosporine to a degree similar to the inhibition of actin depolymerization. In contrast, ET-1 gene expression induced by cytochalasin B, an actin-disrupting agent, was not affected by staurosporine. These results suggest that shear stress can induce actin fiber depolymerization through, at least in part, Ca\(^{2+}\)- and PKC–dependent pathways and that the resultant actin depolymerization leads to induction of ET-1 gene expression in a PKC-independent manner. (Circ Res. 1994;75:630-636.)

Key Words  • shear stress  • Ca\(^{2+}\)  • protein kinase C  • actin  • endothelin 1

In the cardiovascular system, blood flow is optimally maintained by various factors. Hemodynamic shear stress produced by blood flow itself is also among the regulatory factors, and endothelial cells are indispensable in the response of the vascular bed to shear stress. Under shear stress, endothelial cells are elongated and aligned in the direction of flow.\(^1\)\(^-\)\(^3\) These morphological changes seem to be caused by reorganization of actin microfilaments into stress fibers, thick actin filament bundles containing myosin, tropomyosin, and \(\alpha\)-actinin under shear stress,\(^4\)\(^-\)\(^8\) and to play a role in endothelial adhesion and integrity against damage imposed by shear stress.\(^8\)\(^-\)\(^9\) In addition to changes in cellular architecture, shear stress affects the metabolism of endothelial cells. An increase in flow induces several rapid responses, including K\(^+\) channel activation,\(^1\)\(^0\) intracellular Ca\(^{2+}\) increase,\(^1\)\(^1\)\(^-\)\(^1\)\(^3\) cytosolic acidification,\(^1\)\(^4\) and the activation of phosphatidylinositol turnover\(^1\)\(^5\)\(^-\)\(^1\)\(^\text{6}\) in endothelial cells. These rapid responses are followed by increases in the production of various substances, such as endothelium-derived relaxing factor or nitric oxide,\(^1\)\(^7\)\(^-\)\(^1\)\(^\text{8}\) prostacyclin,\(^1\)\(^\text{9}\)\(^-\)\(^2\)\(^0\) tissue plasminogen activator,\(^2\)\(^1\) and platelet-derived growth factor (PDGF).\(^2\)\(^2\) These substances may contribute to the shear stress–induced changes in vascular tone and organization of vascular wall components.

Previously, we have shown that the expression of endothelin 1 (ET-1), a potent vasoconstrictor peptide, in endothelial cells is enhanced by shear stress.\(^2\)\(^3\) Furthermore, we demonstrated that the shear stress–induced ET-1 gene expression is mediated by actin fiber depolymerization\(^2\)\(^4\) and hypothesized that the actin cytoskeleton can serve not only as a transmitter of mechanical force but also as a signal transducer to the nucleus, increasing gene expression in response to shear stress. In the present study, we further investigated the intracellular mechanisms regulating the actin-mediated response to shear stress in endothelial cells. We show that Ca\(^{2+}\) and protein kinase C (PKC) are involved in shear stress–induced actin-mediated ET-1 gene expression.

Materials and Methods

Cell Culture

Endothelial cells were prepared from adult porcine thoracic aortas and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and standard amounts of penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air. The preparation was characterized by the typical “cobblestone” appearance and staining for factor VIII antigen. For all experiments, confluent monolayers between 5 and 10 passages were grown in 28.3-cm\(^2\) culture dishes. Twenty-four hours before experiments, cells were washed twice with phosphate-buffered saline and maintained in 3.0 mL of serum-free DMEM.
Shear Stress Apparatus

We used the shear stress–producing cone–plate apparatus first reported by Dewey et al., with some modification as previously described. Briefly, we inserted a stainless-steel cone (54 mm in diameter) directly into a 28.3-cm² culture dish (60 mm in diameter). The whole apparatus was placed in an ordinary CO₂ incubator at 37°C in 5% CO₂/95% air. The cone was rotated at 80 rpm to produce circumferential laminar flow. The calculated shear stress was 5 dyne/cm². To confirm the existence of laminar flow on the culture plate, we observed the phenomenon of cell alignment after 24 hours.

Measurement of G-Actin Content

Monomeric G-actin content of endothelial cells was determined by the DNase inhibition assay developed by Blikstad et al. with some modifications by Hinshaw et al. Calf thymus DNA (80 µg/mL) was dissolved in 0.1 mol/L Tris-Cl (pH 7.5), 4 mmol/L MgSO₄, and 1.5 mmol/L CaCl₂ and filtered. DNase I (10 µg/mL) was stocked in 0.125 mol/L Tris-Cl (pH 7.5), 5 mmol/L MgCl₂, 2 mmol/L CaCl₂, 1 mmol/L Na₃, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). Working solutions were prepared by 100-fold dilution in 20 mmol/L imidazole (pH 7.5), 30 mmol/L NaCl, and 15% glycerol. Cells were lysed in 500 µL HBBS containing 1% Triton X-100, 2 mmol/L MgCl₂, 2 mmol/L EGTA, 0.2 mmol/L ATP, 0.5 mmol/L dithiothreitol, and 0.1 mmol/L PMSF. G-Actin contents were determined by mixing 5 µL cell lysate and 5 µL DNase solution with 1.5 mL DNA solution, followed by timed sedimentation absorbance at 260 nm. For measurements of total actin, 10 µL of guanidine solution (a solution containing 1.5 mol/L guanidine HCl, 1 mol/L NaCH₃CO₂, 1 mmol/L CaCl₂, 1 mmol/L ATP, and 20 mmol/L Tris-Cl, pH 7.5) was added to each reaction mixture to dissociate filamentous (F)-actin. A standard curve was obtained by plotting absorbance at 260 nm at 120 seconds after addition of 0.1 to 4 µg bovine muscle actin. G-Actin and total actin levels were standardized by total cellular protein determined by the BCA protein assay (Fierce Chemical Co). G-Actin data were expressed as a percentage of total actin.

Northern Blot Analysis

Total cellular RNA was extracted by the LiCl-urea technique from endothelial cells and quantified by measuring absorbance at 260 nm. RNA samples (5 µg) were heat-denatured in formaldehyde, electrophoresed through 1.2% agarose/formaldehyde gels, and transferred to nylon membranes (Hybond N, Amersham) by standard procedures. As a probe, we used the 0.8-kb HindIII-HindIII DNA fragment from porcine ET-1 cDNA clone pET4. The fragment was labeled with [α-³²P]dCTP by using a standard random-primed reaction. The specific activity was 1 x 10⁶ cpm/µg. The membranes were hybridized for 24 to 48 hours at 42°C in 50% formamide, 1% sodium dodecyl sulfate (SDS), 0.98 mol/L NaCl, 0.25 mg/mL salmon sperm DNA, and 1 x 10⁶ cpm/µL of the probe. Membranes were washed twice in 2× standard saline citrate (SSC) containing 1% SDS at 65°C, twice in 2× SSC/1% SDS at 37°C, and once in 0.1× SSC/1% SDS at 37°C for 15 minutes each and were then exposed to Kodak X-Omat AR film (Eastman Kodak) with intensifying screens at −80°C. The membranes were subsequently stripped and rehybridized with a ³⁵P-labeled β-actin probe to determine an internal standard of total RNA content. The photographic density of 28S and 18S ribosomal RNA also served as an internal control. To quantify ET-1 mRNA levels, we scanned autoradiographs with a 2222-020 UltraScan XL laser densitometer running the LKB 2400 GELSCAN software package (LKB Instruments). To correct differences in loading, we divided the signal density of each RNA sample hybridized to the ET-1 probe by that hybridized to the β-actin probe. The corrected density for each sample was then divided by that of the control and presented in a relative unit.

Results

Effect of Ca²⁺-Chelating Agents on Shear Stress-Induced Changes in G-Actin Content

In a previous report, we showed that low shear stress (5 dyne/cm²) causes a significant increase in G-actin content and in stress fiber alignment in endothelial cells thereafter. Total actin contents were not changed at any point by shear stress, suggesting that the increase in G-actin content is due to actin depolymerization rather than de novo actin synthesis. In the present study, we examined the effects of several agents on G-actin content under shear stress to investigate the involvement of second messenger systems in shear stress–induced actin depolymerization.

First, we incubated endothelial cells in Ca²⁺-free DMEM plus the Ca²⁺-chelating agent EGTA (2 mmol/L) and assessed the role of extracellular Ca²⁺ in shear stress–induced actin depolymerization. As shown in Fig 1, the G-actin content was 54±0.8% of total actin in quiescent endothelial cells and 80±1.0% in endothelial
cytosolic cells under content doses of higher than 3 hours caused inhibited the shear in G-actin.

These results suggest that shear stress-induced G-actin increase was a nonspecific toxic effect of staurosporine, the involvement of PKC in shear stress-induced actin depolymerization was further investigated by using PKC-activating phorbol ester TPA. Fig 3 shows that short exposure of endothelial cells to TPA (1 μmol/L) caused a weak but significant increase in G-actin content. This result suggests that activation of PKC can shift the actin equilibrium to a monomeric state. Fig 3 also shows that the effects of TPA were not additive to those of shear stress on actin depolymerization.

The effect of PKC desensitization by 24-hour exposure to TPA was also examined. In quiescent cells, G-actin content was not affected by long exposure to TPA (data not shown). However, shear stress–induced increase in G-actin content was significantly suppressed by ~70% by PKC desensitization (Fig 4). Thus, we confirmed that shear stress–induced actin depolymerization is dependent, at least in part, on PKC activity.

**Effect of Ca²⁺-Chelating Agents and Staurosporine on Shear Stress–Induced ET-1 mRNA Increases**

To study the coupling of shear stress–induced Ca²⁺ and PKC-dependent actin depolymerization with ET-1 gene induction, we examined the effects of EGTA (2 mmol/L), BAPTA/AM (2.5 μmol/L), and staurosporine (0.1 μmol/L) on shear stress–induced ET-1 gene expression. After pretreatment with these agents for 1 hour, endothelial cells were exposed to shear stress, and ET-1 mRNA levels were quantified by Northern analysis. Shear stress–induced increases in ET-1 mRNA were almost abolished by EGTA (Fig 5). BAPTA/AM and staurosporine also decreased shear stress–induced ET-1
mRNA increases by ≈40% and 70%, respectively (Figs 5 and 6). Although the inhibitory effect of BAPTA/AM is not so clear, the effects of these agents on ET-1 gene expression seem to be in parallel with their effects on shear stress–induced increases in G-actin contents. These results, together with our previous data using actin-disrupting agents, indicate that shear stress–induced Ca²⁺- and PKC-dependent actin depolymerization is coupled with the induction of ET-1 gene expression.

**Effect of Stauroporine on Cytochalasin B–Induced ET-1 mRNA Increases**

We have shown that PKC is involved in shear stress–induced actin depolymerization. To test whether ET-1 gene expression induced by disruption of actin fiber is PKC dependent, we examined the effect of staurosporine on ET-1 gene expression induced by cytochalasin B, which is a PKC-independent inhibitor of actin polymerization. In agreement with our previous study, 1 µmol/L cytochalasin B significantly increased ET-1 mRNA levels in endothelial cells. The cytochalasin B–induced ET-1 gene expression was not affected by 0.1 µmol/L staurosporine (Fig 7). These results suggest that the pathway from actin depolymerization to ET-1 gene expression is independent of PKC activity; ie, PKC is involved in shear stress–induced ET-1 gene expression at a level upstream from actin depolymerization.

**Discussion**

In a previous study, we showed that hemodynamic shear stress induces ET-1 gene expression in vascular endothelial cells via actin fiber depolymerization. This implicates the shear stress–induced actin fiber depolymerization not only in the cytoskeletal reorganization as an adaptation to flow but also in gene induction, which may contribute to the regulation of vascular tone in response to flow. In the present study, we demonstrate that (1) shear stress–induced increases in cytosolic G-actin content were completely blocked by EGTA and partially inhibited by BAPTA/AM, staurosporine, and long-term treatment with TPA; (2) PKC activation by short exposure to TPA caused a mild increase in G-actin content, and the TPA-induced increases in G-actin content were not additive to that induced by shear stress; (3) EGTA, BAPTA/AM, and staurosporine inhibited shear stress–induced ET-1 gene expression to a degree similar to shear stress–induced actin depolymerization; and (4) cytochalasin B–induced ET-1 gene expression was not affected by staurosporine. These observations indicate that shear stress–induced increases in cytosolic G-actin content and subsequent ET-1 gene expression are dependent on the presence of extracellular and intracellular Ca²⁺ and PKC activity and that PKC is not involved in the pathway from actin depolymerization to ET-1 gene induction (Fig 8).

Actin microfilaments, one of the major cytoskeletal components, are diffusely distributed as peripheral and
central bundles in endothelial cells. The equilbrial state between G- and F-actin and the subsequent redistribution of microfilaments are associated with several cell functions, such as migration and reendothelialization after intimal injury. In the case of endothelial cell migration, the equilibrium between the two forms of actin is changed in favor of F-actin, and actin fibers are reorganized after reendothelialization. Hemodynamic shear stress also causes redistribution of actin microfilaments, resulting in the formation of stress fibers and central actin bundles containing myosin, tropomyosin, and α-actinin in endothelial cells. In our present report, we have shown that shear stress induces actin depolymerization in endothelial cells through a Ca²⁺ and PKC-dependent pathway.

Shear stress is known to elevate intracellular Ca²⁺ concentration and to stimulate phosphoinositide turnover in endothelial cells. The stimulation of phosphoinositide turnover results in the production of inositol trisphosphate (IP₃) and diacylglycerol (DG). IP₃ triggers Ca²⁺ release from sarcoplasmic reticulum and causes the elevation of intracellular Ca²⁺ concentration. DG activates PKC, which leads to a diverse set of responses, including gene expression. Thus, our present study suggests that the shear stress-induced intracellular Ca²⁺ elevation and PKC activation may be, at least in part, responsible for the actin depolymerization. The present study also demonstrates that shear stress-induced actin depolymerization is completely dependent on extracellular Ca²⁺, suggesting that Ca²⁺ entry across plasma membrane may be essential for actin depolymerization. In contrast to the effect of EGF, BAPTA/AM could only incompletely inhibit shear stress–induced increases in G-actin content and ET-1 mRNA levels. It may be possible that the used dose of BAPTA/AM was insufficient for the complete suppression of shear stress–induced intracellular Ca²⁺ elevation. However, the parallelism between its effects on G-actin content and ET-1 mRNA levels supports the possible implication of intracellular Ca²⁺ in shear stress–induced actin-mediated ET-1 gene expression.

The state of equilibrium of actin microfilaments is regulated by a variety of actin-associated proteins in diverse types of cells. The activity of several actin-associated proteins is affected by intracellular Ca²⁺ and PKC. For example, gelsolin, which can seve actin filaments and nucleate actin filament assembly, is activated by submicromolar Ca²⁺. Another actin-associated protein, MARCKS, is bound to Ca²⁺-calmodulin and phosphorylated by PKC, resulting in the inhibition of its actin filament cross-linking activity. In fact, agonist-mediated intracellular Ca²⁺ elevation can cause the activation of gelsolin and promote actin disassembly in various kinds of cells, such as platelets and neutrophils. These inducible changes in actin filaments are thought to be associated with important cellular functions, including lamellar extension and chemotaxis. Thus, it is possible that actin-associated protein(s) may link the stimulation of shear stress to actin depolymerization and that this phenomenon may be important for subsequent cellular rearrangement in direction to flow.

In our previous study, we have demonstrated that shear stress–induced actin-mediated ET-1 gene expression is dependent on the integrity of microtubules. In addition, the arrangement of microtubular structure as well as actin cytoskeleton is affected by shear stress in
endothelial cells. Thus, it is possible that Ca\(^{2+}\) and/or PKC may be involved in shear stress–induced increases in G-actin content and ET-1 mRNA through the pathway regulated by the microtubular structure. Further investigations are needed to elucidate the interaction between shear stress–induced changes in actin microfilaments and microtubules and the site of Ca\(^{2+}\) and PKC involvement.

Our present study also confirms the coupling of shear stress–induced actin depolymerization with ET-1 gene expression by showing that EGTA, BAPTA/AM, and staurosporine inhibit both shear stress–induced actin depolymerization and ET-1 gene expression in a similar manner. In addition, cytochalasin-induced ET-1 gene expression was not affected by staurosporine. This result, taken together with our previous results, indicates that PKC is involved in shear stress–induced ET-1 gene expression at a level upstream from actin depolymerization.

In contrast, several reports have demonstrated that shear stress downregulates ET-1 gene expression in bovine aortic endothelial cells and human umbilical vein endothelial cells. In bovine endothelial cells, this downregulation of ET-1 gene expression is independent of PKC and cAMP. This discrepancy between these reports and our present study using low shear stress of 5 dynes/cm\(^2\) may be explained by the existence of dual effects of shear stress on ET-1 gene expression: PKC-dependent stimulation at low shear stress and cGMP-dependent inhibition at high shear stress. The balance of both these effects seems to be different according to the origin of endothelial cells as well as the magnitude of shear stress imposed on endothelial cells. Our present results are consistent with the former effect and have identified the involvement of PKC in the pathway from shear stress recognition to actin depolymerization, which leads to ET-1 gene induction.

Several other genes are reported to be induced by shear stress. Recently, Resnick et al have demonstrated that shear stress–induced expression of PDGF-B chain gene is mediated by a cis-acting shear stress–responsive element in the promoter region and that the core sequence GAGCC also exists in the promoter region of tissue-type plasminogen activator, transforming growth factor-\(\beta_1\), and intercellular adhesion molecule-1. The core sequence, however, is not found within the known published sequence of the ET-1 promoter region. Further, the character of shear stress–induced expression of PDGF-B chain gene is different from that of ET-1 gene. Whereas shear stress–induced ET-1 gene expression is partially inhibited by staurosporine and completely inhibited by EGTA, shear stress–induced PDGF-B chain gene expression is almost completely blocked by staurosporine but is not affected by EGTA in human umbilical vein endothelial cells.

Although this discrepancy may be due to a species difference, we can hypothesize that shear stress–induced expression of ET-1 gene and that of other genes including PDGF-B chain may be mediated by different signal transduction pathways.

In summary, shear stress can induce actin fiber depolymerization through a Ca\(^{2+}\)– and PKC-dependent pathway, followed by an increase in ET-1 gene expression in endothelial cells. Thus, Ca\(^{2+}\) and PKC-dependent changes in actin fiber equilibrium may be important not only for rearrangement of cell shape in response to shear stress but also for signal transduction to gene expression.

Acknowledgments

This study was supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan; Japanese Heart Foundation; Uehara Memorial Foundation; and SANDOZ Geron-ontological Research Foundation. We thank Chie Fujinami for her excellent technical assistance. We also thank Drs Mu-En Lee and Stella Kourembanas for critical reading of the manuscript and helpful discussions.

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Role of Ca2+ and protein kinase C in shear stress-induced actin depolymerization and endothelin 1 gene expression.
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Circ Res. 1994;75:630-636
doi: 10.1161/01.RES.75.4.630

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