PKC-ε Is Required for Mechano-sensitive Activation of ERK1/2 in Endothelial Cells*

(Received for publication, May 22, 1997, and in revised form, August 27, 1997)

Oren Traub‡, Brett P. Monia§, Nicholas M. Dean§, and Bradford C. Berk‡¶

From the ¶Department of Pathology and Department of Medicine, Division of Cardiology, University of Washington, Seattle, Washington 98195 and §Department of Molecular Pharmacology, ISIS Pharmaceuticals, Carlsbad, California 92008

Mechano-sensitive regulation of endothelial cells (EC) function by shear stress is critical for flow-induced vasodilation and gene expression. Previous studies by our laboratory demonstrated that shear stress activates the 44- and 42-kDa extracellular signal-regulated kinases (ERK1/2) in EC in a time- and force-dependent manner. ERK1/2 activation was inhibited by protein kinase C (PKC) down-regulation with phorbol 12,13-dibutyrate (1 μM for 24 h) but not by calcium chelation with BAPTA-AM (acetoxymethyl ester of BAPTA) (75 μM for 30 min), suggesting that a novel PKC isoform (δ, ε, η, θ) mediates shear stress-induced ERK1/2 activation. Western blotting with PKC isoform-specific antibodies demonstrated expression of PKC-α, -ε, and -ζ isoforms in EC. PKC-ε was specifically inhibited by transfection with antisense PKC-ε phosphorothioate oligonucleotides (1,000 nM for 6 h). Antisense treatment decreased PKC-ε protein levels by 80 ± 13% after 72 h and completely inhibited shear stress-stimulated ERK1/2 activation. Scrambled PKC-ε oligonucleotides and antisense PKC-α and PKC-ζ oligonucleotides had no effect on ERK1/2 activity. PKC-ε appeared specific for mechano-sensitive ERK1/2 activation, as antisense PKC-ε oligonucleotides did not inhibit ERK1/2 activation by EGF or bradykinin but did inhibit ERK1/2 activation upon EC adhesion to fibronectin. These results define a pathway for shear stress-mediated ERK1/2 activation and establish a new function for PKC-ε as part of a mechano-sensitive signal transduction pathway in EC.

Mechanical stimuli are important modulators of cellular function in tissues, particularly in the cardiovascular system (1). A key physical force experienced by endothelial cells (EC) by virtue of their unique location in the vessel wall is fluid shear stress created by blood flow. Changes in fluid shear stress have been shown to release vasoactive mediators such as nitric oxide (2) as well as modulate gene expression such as e-cfos (3), platelet-derived growth factor (4), and endothelial nitric oxide synthase (5). These hemodynamically regulated events may contribute to the pathogenesis of vascular disease, as atherosclerotic plaques are preferentially localized to areas of the vascular system that experience low shear and turbulence (6).

Two members of the mitogen-activated protein kinase family, ERK1 and ERK2, which are known to modulate cell physiology and gene expression in many different ways (7), have been reported to be activated by shear stress in endothelial cells (8). However, whereas growth factor-mediated stimulation of ERK1/2 has been well defined (9), the upstream signaling pathway that leads to activation of ERK1/2 by shear stress remains unexplored.

Shear stress has also been shown to activate phospholipase C (10), resulting in the cleavage of phosphatidylinositol bisphosphate into inositol 1,4,5-trisphosphate, a calcium-mobilizing second messenger, and diaicylglycerol, an activator of protein kinase C (PKC). Indeed, recent studies have implicated PKC in cellular responses to shear stress, such as endothelin-1 production (11), platelet-derived growth factor expression (12), and cytoskeletal reorganization (13). Previous studies by our laboratory suggested that PKC is also required for the fluid shear stress-mediated activation of ERK1/2 (8). In the current paper, we investigated the role of PKC in the shear stress-mediated signaling and show that PKC-ε but not PKC-α or PKC-ζ is required for ERK1/2 activation by shear stress.

MATERIALS AND METHODS

Cell Culture—Bovine aortic EC were isolated from fetal calf aortas and maintained in M199 (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Cells used in experiments were passage <6, as ERK1/2 kinase activation by shear decreased in later passages. For experiments employing antisense oligonucleotides against human PKC isoforms, EC were obtained from human umbilical veins (HUVEC) as described previously (14). Cells at passages between 1 and 3 were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 20% fetal bovine serum (HyClone Laboratories, Inc.), heparin (Sigma), and endothelial cell growth factor (kindly provided by Dr. R. Ross).

Shear Stress Experiments—Cells were grown in 2 cm × 4 cm slides of tissue culture plastic cut from the bottom of tissue culture dishes. Upon reaching 95% confluence, cells were rinsed free in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† An Established Investigator of the American Heart Association. To whom correspondence should be addressed: Box 357710, The University of Washington, Seattle, WA 98195. Tel.: 206-616-6600; Fax: 206-616-1580; E-mail: bbkerk@u.washington.edu.

§ This work was supported by Medical Scientist Training Program Grant NIGM-GM07266 (to O. T.), American Heart Association National-in-aid 94014290 (to B. C. B.), and National Institutes of Health Grant IH PO1 Hl18645. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†¶ From the Department of Pathology and Department of Medicine, Division of Cardiology, University of Washington, Seattle, Washington 98195 and Department of Molecular Pharmacology, ISIS Pharmaceuticals, Carlsbad, California 92008.

The abbreviations used are: EC, endothelial cells; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; FMA, phorbol 12-myristate 13-acetate; HUVEC, human umbilical vein endothelial cells; PLL, poly-I-lysine; PDBu, phorbol 12,13-dibutyrate; PI 3-kinase, phosphotyrosine 3-kinase; BAPTA-AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.
Shear Stress Activation of ERK1/2 Requires PKC-ε

7.4) was added. Cell lysates were prepared by scraping, sonication, and centrifugation (5 min, 4 °C, 14,000 rpm in microcentrifuge). Sample protein concentrations were determined by DC protein (Bio-Rad) analysis. For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose filters (Hybond, Amersham). To ensure quantitative transfer of proteins, the filters were stained with Ponceau S. The membrane was blocked for 2 h at room temperature with a commercial blocking buffer (Life Technologies, Inc.). The blots were incubated overnight at 4 °C with the primary antibody (phospho-specific ERK1/2 antibody was obtained from New England Biolabs; non-specific ERK1 and ERK2 antibodies and PKC isoform-specific antibodies were obtained from Santa-Cruz Biological) followed by incubation for 1–2 h with a secondary antibody (horseradish peroxidase-conjugated). Immunoreactive bands were visualized by chemiluminescence (Amersham Corp. or Pierce).

Separation of Membrane and Cytosolic Fractions for PKC Localization—Cells from one 150-mm culture dish were scraped into 0.75 ml of fractionation lysis buffer (20 mM Tris-HCl, 10 mM EDTA, 5 mM EGTA, 5 mM 2-mercaptoethanol, 10 mM benzamidine, 1 mg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, 0.1 mg/ml ovalbumin, and 0.1 mM α-motaprin, pH 7.4) on ice. After incubation for 5 min, cells were disrupted with a Dounce homogenizer (50 strokes), and centrifugation was performed (100,000 × g for 1 h). The supernatant was saved as the cytosolic fraction. The pellet (membrane fraction) was washed once with lysis buffer then resuspended in 150 μl of lysis buffer that contained 1% Triton X-100 and was solubilized for 1 h at 4 °C before sonication. Proteins then underwent Western blot analysis as described above.

PKC Activity Assay by Histone Phosphorylation—Cells from one 150-mm culture dish were scraped into 1.0 ml of assay buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, 1 mM EGTA, 10 mM phosphate, 10 μM α-motaprin, and 10 μM leupeptin with 1% Triton X-100) on ice. Immunoprecipitation and immune complex recovery was performed for PKC-α (using 25 μg/reaction sample), PKC-ε (using 50 μg/reaction sample), and PKC-ζ (using 75 μg/reaction sample) as described above. Protein samples were placed in a reaction mixture containing 20 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 200 μM CaCl₂, cofactor mixed micelle preparation containing dioxane (16 μg/ml), and phosphatidylserine (240 μg/ml) in 0.3% Triton X-100 as PKC cofactors, 0.4 mg/ml histone-H1, and [γ-32P]ATP. For Ca²⁺-free experiments, 1.0 mM EGTA was substituted for CaCl₂. Also, inhibitors (10 mM staurosporine) or activators (2 mM PMA; 2 mM inactive phorbol) were added directly to the assay buffer. The mixture was incubated for 10 min at 30 °C, at which point the reaction was halted by adding 6× Laemmli buffer, and proteins were subjected to SDS-PAGE under reducing conditions using 15% polyacrylamide, and the gel was allowed to dry overnight. Histone-H1 phosphorylation was detected by autoradiography and quantified by densitometry.

Transfection Protocol for PKC-isoform Antisense Oligonucleotides—HUVEC were grown to 95% confluence in 60-mm tissue culture dishes for transfection. Lipofectin (Life Technologies, Inc.) was prepared in 0.2 ml of Opti-MEM containing 5 μg of Lipofectin/μl oligonucleotide and equilibrated for 45 min. A series of antisense oligonucleotides directed against the PKC isoforms were screened, and the most active sequences were identified. PKC-α: antisense oligonucleotide (5TCTCGCTGTGT-GAGTTTCA), scrambled oligonucleotide (GTTTACATCGGCTTCT-GO); PKC-ε: antisense oligonucleotide (CTAGAGGGCCGAGTGGTCACC-T), scrambled oligonucleotide (TACCGFAAAGCGCGCTGTGGG); PKC-ζ: antisense oligonucleotide (CAGCGCGCGCGCGCTCAACC), scrambled oligonucleotide (AAGCGCGACACGGCGCTTC). Antisense or scrambled phosphorothioate oligonucleotides were prepared at concentrations of 1, 3, and 10 μM in 0.2 ml of Opti-MEM and equilibrated for 1 min. The Lipofectin and oligonucleotide solutions were then mixed gently and incubated at room temperature for 15 min before being diluted to a final volume of 2 ml with Opti-MEM to give oligonucleotide concentrations of 100, 300, and 1,000 nM. The cells were washed with sterile PBS and treated with Opti-MEM/Lipofectin/oligonucleotide mixture for 6 h before being returned to RPMI with 20% fetal calf serum. Cells were harvested 3 days later because preliminary experiments (not shown) determined that antisense PKC oligonucleotides were unable to reduce protein levels when EC were harvested earlier. The 3-day period followed transfection was necessary to degrade existing PKC, consistent with protein half-lives of PKC proteins reported by other investigators (15).

Adhesion Experiments—HUVEC were incubated at 37 °C in PBS containing 2 mM EDTA for 5 min and detached from dishes by gentle pipeting as described previously (16). The cells were washed three times with RPMI 1640, collected by low speed centrifugation, and resus-

RESULTS

Fluid Shear Stress Leads to Activation of ERK1/2—Stimulation of ERK1/2 by fluid shear stress was measured by Western blotting with a phosphospecific ERK antibody. Compared with static conditions, fluid shear stress at 12 dynes/cm² activated ERK1/2 with a peak at 10 min and return to baseline by 60 min (Fig. 1, A and C). These data show activation kinetics similar to those previously reported by our laboratory using other techniques (8, 17). Western blotting with an antibody for ERK1/2 that detects both the phosphorylated and unphosphorylated form of the kinases showed that cellular ERK1/2 levels remained constant throughout the shear stress time course (Fig. 1, B). These results demonstrate that ERK1/2 is phosphorylated in response to fluid shear stress with time course similar to receptor agonists, such as thrombin and EGF (8).

ERK1/2 Activation by Shear Stress is PKC-dependent and Calcium-independent—Several investigators have reported that PKC is activated in response to various mechanical stimuli such as stretch, pressure, and shear (1). To determine the role of PKC in ERK1/2 activation by shear stress, cells were exposed to 1 μM PDBu for 24 h before shear stress to down-regulate PKC. ERK1/2 activation by shear stress was significantly inhibited by PDBu pretreatment (28 ± 3% control), as shown by immunoblotting with the ERK1/2 phosphospecific antibody (Fig. 2). Pretreatment with the protein kinase inhibi-
Shear Stress Activation of ERK1/2 Requires PKC-ε

Effect of PDBu Treatment on PKC Isoform Expression—Because shear stress-mediated activation of PKC was significantly attenuated by 24 h pretreatment with PDBu, we determined the time-dependent change in PKC isoform levels (Fig. 4). Whereas brief stimulation with PMA (200 nM, 10 min) had no effect on PKC levels, prolonged exposure of cells to PDBu caused down-regulation of PKC-α (100% by 24 h) and PKC-ε (100% by 12 h). PKC-ζ levels were unaffected. PDBu treatment had no effect on either cellular ERK1/2 levels (Fig. 4, bottom) or on EGF-mediated ERK1/2 activation (data not shown). These results are consistent with the characteristics of the different PKC isoforms described above and suggest that PKC-ζ is not involved in ERK1/2 activation by shear stress as it was unaffected by PDBu treatment.

Measurement of PKC Activity by Translocation Assay—To determine whether the PKC isoforms expressed in EC translocate upon cell stimulation, the intracellular localization of the PKC isoforms was determined by centrifugal fractionation, SDS-PAGE separation, and Western blotting (Fig. 5). Western analysis showed that in the unstimulated state, both PKC-α and PKC-ζ were evenly distributed in the cytosolic and membrane fractions, whereas PKC-ε was localized solely to the membrane fraction. After stimulation with PMA (200 nM, 10 min), PKC-α translocated to the membrane fraction, but little difference was observed in the distribution of PKC-ε and PKC-ζ. Since PKC-ε was already localized to the membrane fraction (although whether nuclear or membrane is unknown) and because there was little difference in cellular localization of PKC-ζ in response to PMA, this method of measuring PKC activity would not be useful in determining whether PKC-ε and PKC-ζ are activated by shear stress.

Measurement of PKC Activity by Histone Phosphorylation—Another method to measure PKC activity is by phosphorylation of a PKC substrate. Since specific substrates for each isoform are not available, we measured the activity of the PKC isoforms by immunoprecipitating each isoform and then performing an immune complex kinase assay with a universal PKC substrate, histone-H1. In this assay, PKC-α activity was inhibited by the addition of staurosporine, exclusion of Ca²⁺ (below basal levels discovered and little-studied PKC-μ isoform. Because ERK1/2 activation by shear stress is phorbol ester-responsive but calcium independent, our results suggest that some member(s) of the novel class (δ, ε, θ, η) is involved in the signaling pathway that leads to activation of ERK1/2. To determine which PKC isoforms were expressed in EC, we performed Western blotting with isoform-specific antibodies on EC lysates. EC express primarily three PKC isoforms: PKC-α, PKC-ε, and PKC-ζ (Fig. 3), whereas no significant immunoreactivity was detected for PKC-β, -γ, -δ, -θ, -η, and -λ/κ. Thus, the only member of the novel class present in EC is PKC-ε.

Effect of PDBu Treatment on PKC Isoform Expression—Because shear stress-mediated activation of PKC was significantly attenuated by 24 h pretreatment with PDBu, we determined the time-dependent change in PKC isoform levels (Fig. 4). Whereas brief stimulation with PMA (200 nM, 10 min) had no effect on PKC levels, prolonged exposure of cells to PDBu caused down-regulation of PKC-α (100% by 24 h) and PKC-ε (100% by 12 h). PKC-ζ levels were unaffected. PDBu treatment had no effect on either cellular ERK1/2 levels (Fig. 4, bottom) or on EGF-mediated ERK1/2 activation (data not shown). These results are consistent with the characteristics of the different PKC isoforms described above and suggest that PKC-ζ is not involved in ERK1/2 activation by shear stress as it was unaffected by PDBu treatment.

Measurement of PKC Activity by Translocation Assay—To determine whether the PKC isoforms expressed in EC translocate upon cell stimulation, the intracellular localization of the PKC isoforms was determined by centrifugal fractionation, SDS-PAGE separation, and Western blotting (Fig. 5). Western analysis showed that in the unstimulated state, both PKC-α and PKC-ζ were evenly distributed in the cytosolic and membrane fractions, whereas PKC-ε was localized solely to the membrane fraction. After stimulation with PMA (200 nM, 10 min), PKC-α translocated to the membrane fraction, but little difference was observed in the distribution of PKC-ε and PKC-ζ. Since PKC-ε was already localized to the membrane fraction (although whether nuclear or membrane is unknown) and because there was little difference in cellular localization of PKC-ζ in response to PMA, this method of measuring PKC activity would not be useful in determining whether PKC-ε and PKC-ζ are activated by shear stress.

Measurement of PKC Activity by Histone Phosphorylation—Another method to measure PKC activity is by phosphorylation of a PKC substrate. Since specific substrates for each isoform are not available, we measured the activity of the PKC isoforms by immunoprecipitating each isoform and then performing an immune complex kinase assay with a universal PKC substrate, histone-H1. In this assay, PKC-α activity was inhibited by the addition of staurosporine, exclusion of Ca²⁺ (below basal levels
or to basal levels with PMA stimulation; data not shown), or exclusion of both Ca\(^{2+}\) and cofactors (diolein and phosphatidylserine, Fig. 6A). The addition of PMA to the reaction mixture potentiated PKC-\(\alpha\) activity. Activity of the PKC-\(\varepsilon\) isoform was also inhibited by staurosporine and removal of Ca\(^{2+}\) and cofactors but was not affected by removal of Ca\(^{2+}\) alone (Fig. 6B). The addition of PMA stimulated PKC-\(\varepsilon\) activity.

The activity of PKC-\(\zeta\) was not inhibited by staurosporine, removal of Ca\(^{2+}\), or removal of both Ca\(^{2+}\) and cofactors (Fig. 6C). Further, PMA added directly to the assay was unable to stimulate activity. These results confirm that EC PKC-\(\zeta\) is a calcium-independent and phorbol ester-unresponsive PKC isoform.

Adding PMA to the reaction mixture stimulated activity of PKC-\(\alpha\) and PKC-\(\varepsilon\), but pretreating cells with PMA before immunoprecipitation failed to stimulate PKC activity as measured by histone phosphorylation (see far right, Fig. 6A). These results suggest that immunoprecipitation separates PKC from cellular inhibitors and activators that regulate agonist-stimulated activity. In fact, no change in immunoprecipitated PKC activity was noted for any PKC isoform when cells were pretreated with PMA, shear stress, or thrombin (data not shown). Therefore, this assay is useful to characterize the effects of calcium, phorbol, and staurosporine in vitro on the separate isoforms but is not useful to measure the effects of physiological stimuli on intact cells.

**PKC Antisense Oligonucleotides Are Specific and Effective**—It appears that among the PKC isoforms present in EC, PKC-\(\varepsilon\) is the most likely isoform to mediate shear stress ERK1/2 signaling. This conclusion is based on the findings in
cleotides were specific for PKC-e responding PKC isoforms was observed (data not shown). However, in cells treated with antisense PKC oligonucleotides were maintained in static culture or exposed to shear stress. Antisense or scrambled PKC oligonucleotides did not alter the ERK1/2 activation by shear stress. In contrast, cells treated with antisense PKC oligonucleotides demonstrated minimal nonspecific effects of the transfection protocol. Similar specificity and efficacy of 1,000 nM antisense oligonucleotides for 6 h before returning the cells to media containing serum. Three days after transfection, cells were washed with HBSS, and lysates were prepared for Western blot analysis using 25 μg of protein and PKC isoform-specific antibodies.

Figs. 2, 4, and 6 that the PKC isoform is 1) phorbol ester-responsive, 2) calcium-independent, and 3) inhibited by staurosorine. Further, these data suggest that neither PKC-α nor PKC-ζ is involved in this signaling process, as PKC-α is calcium-dependent and PKC-ζ is phorbol ester-unresponsive and resistant to inhibition by staurosorine.

To establish the role of PKC-e in shear stress-mediated activation of ERK1/2, we decided to inhibit each expressed PKC isoform individually and measure changes in ERK1/2 activation. Since specific pharmacologic inhibitors of the separate PKC isoforms are currently unavailable, antisense phosphorothioate oligonucleotides and their corresponding scrambled controls for the different PKC isoforms were employed. Antisense oligonucleotides have previously been employed to inhibit expression of PKC-α in mouse and human cell lines in an isoform-specific manner (21, 22). HUVEC were transfected with antisense PKC-e oligonucleotides for 6 h, and the cells were harvested 3 days later for analysis. Protein levels for PKC-e were reduced in a concentration-dependent manner with reductions of 22 ± 10%, 25 ± 6%, and 80 ± 13% at 100, 300, and 1,000 nM antisense PKC-e oligonucleotides, respectively (Fig. 7). Expression of PKC-α and PKC-ζ isoforms was not significantly affected at any concentration of antisense PKC-e oligonucleotide, indicating that the antisense oligonucleotides were specific for PKC-e. PKC-e levels were not affected by treatment with 1,000 nM scrambled PKC-e oligonucleotides, demonstrating minimal nonspecific effects of the transfection protocol. Similar specificity and efficacy of 1,000 nM antisense PKC-α and PKC-ζ oligonucleotides for their corresponding PKC isoforms was observed (data not shown).

Antisense PKC-e Oligonucleotides Block Shear Stress-mediated ERK1/2 Activation—Several studies have demonstrated that many PKC isoforms are able to activate ERK1/2 in a stimulus-specific manner, including PKC-α, PKC-e, and PKC-ζ (23–25). To determine the effect of inhibiting different PKC isoforms on ERK1/2 activation by shear stress, cells treated with antisense PKC oligonucleotides were maintained in static culture or exposed to shear stress. Antisense or scrambled PKC-α, -e, -ζ oligonucleotide treatment did not affect base-line phosphorylation of ERK1/2 (Fig. 8A, left three lanes). Antisense or scrambled PKC-α or -ζ oligonucleotides did not alter the ERK1/2 activation by shear stress. However, in cells treated with antisense PKC-e oligonucleotides, shear stress-mediated activation of ERK1/2 was completely inhibited (Fig. 8A, far right). Scrambled PKC-e oligonucleotide treatment had no effect on ERK1/2. Further, antisense PKC-e oligonucleotides had no effect on bradykinin or EGF-induced ERK1/2 activation, demonstrating that ERK1/2 was still capable of being activated through mechanisms independent of PKC-e (Fig. 8B). Treatment with antisense PKC-e oligonucleotides inhibited PMA-induced ERK1/2 activation by only 35%. The inability of antisense PKC-e oligonucleotides to completely inhibit PMA-induced ERK1/2 activation is likely due to PKC isoforms other than PKC-e that can also activate ERK1/2 in response to PMA.

Antisense PKC-e Oligonucleotides Block ERK1/2 Activation by Adhesion to Fibronectin—We previously showed that ERK1/2 was activated when EC adhered to fibronectin but not when cells adhered to PLL, suggesting a role for integrins in ERK1/2 activation in EC (16). To determine the role of PKC-e in these integrin-mediated pathways, we compared activation of ERK1/2 during adherence to PLL and fibronectin in the presence and absence of PKC-e oligonucleotides (Fig. 9). Adherence to fibronectin for 10 min increased ERK1/2 activity by 4.3-fold compared with cells maintained in suspension. Adherence to PLL for 10 min, in contrast, caused no significant increase in ERK1/2 activity. Antisense PKC-e oligonucleotides completely inhibited ERK1/2 activation by fibronectin, whereas scrambled PKC-e oligonucleotides had minimal inhibitory effect. There was no significant difference in cell morphology or the extent of cell spreading in the presence of antisense PKC-e oligonucleotides. Thus, PKC-e appears to be required for integrin-mediated activation in EC.
FIG. 9. Effect of antisense PKC-ε oligonucleotide treatment on ERK1/2 activation by adhesion. HUVEC were treated with either 1,000 nm antisense PKC-ε oligonucleotides or 1,000 nm scrambled PKC-ε oligonucleotides for 6 h before returning the cells to media with serum. Three days after transfection, EC were washed free of culture medium, and approximately 10⁶ cells were placed onto 60-mm bacteriologic plastic dishes coated with fibronectin or PLL and incubated at 37 °C for 10 min or kept in suspension. Lysates were analyzed by Western blot using a phosphospecific ERK antibody.

DISCUSSION

The major finding of this study is that PKC-ε is a component of a mechano-sensitive signal transduction pathway that leads to the activation of ERK1/2 in endothelial cells. Further, this pathway is specific for PKC-ε, as PKC-α and PKC-ζ are not required for the activation of ERK1/2. These results, combined with observations from other investigators that shear stress stimulates changes in cellular physiology and gene expression (26), provide evidence that mechanical stimuli can activate signal transduction pathways in a manner similar to conventional agonist-receptor-initiated signaling events. These results define a pathway for shear stress-mediated ERK1/2 activation and establish a new function for PKC-ε in EC.

Two upstream mechanisms for the activation of PKC-ε in response to shear stress may be proposed based on previous studies. First, phospholipase C is activated by shear stress (10), resulting in the cleavage of phosphatidylinositol bisphosphate and generation of inositol 1,4,5-trisphosphate and diacylglycerol. PKC-ε is similar to the classical PKC isoforms in that it is activated by diacylglycerol (20); thus, one mechanism for activation of PKC-ε is through shear stress-mediated generation of diacylglycerol. Second, other activators of PKC-ε such as phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, may be increased in EC in response to fluid shear stress. Reports show that both of these phosphoinositides generated by PI 3-kinase activity are potent and selective activators of the novel class of PKC isoforms and have little effect on the classical or atypical PKC isoforms (27). To date, no studies have been published regarding changes in PI 3-kinase activity in response to fluid shear stress. Moriya et al. (28) reported that both the PI 3-kinase and the phospholipase C pathway can activate PKC-ε in a cell-specific and stimulus-specific manner. The specificity of the PI 3-kinase pathway for the novel PKC isoforms suggests that analysis of PI 3-kinase activity in response to flow will be an interesting area for future studies.

The plasma membrane sensor or receptor responsible for transducing mechanical stimuli into biochemical signaling events remains undefined. Several molecules have been proposed as candidate mechanotransducers, including integrins and mechano-sensitive ion channels such as the inward rectifying K⁺ channel and the stretch-activated Ca²⁺ channel (26). Whereas it is unlikely that mechano-sensitive ion channels could result in the selective activation of PKC-ε, integrins have been shown to activate many cellular kinases, including PKC (29). Because antisense PKC-ε oligonucleotides completely inhibited adhesion (i.e. integrin)-induced ERK1/2 activation, the present findings suggest an important role for PKC-ε in integrin-mediated signaling in EC. In two previous studies, we found that integrins played an essential role in activation of ERK1/2 by shear stress in EC (16, 17). Thus, the present data support the concept that integrins participate as mechanoreceptors, although additional experiments should be performed to determine which integrins activate PKC-ε.

Although signal transduction events by which PKC-ε activates ERK1/2 remain to be determined, it seems likely that PKC-ε interacts directly with Raf-1 to activate ERK1/2. Activation of ERK1/2 by growth factors has been shown to occur via activation of Ras and subsequent recruitment of Raf-1 to the plasma membrane. Raf-1, in turn, activates MEK-1, the direct upstream activator of ERK1/2 (7). This pathway also appears to be activated in EC by shear stress, since two groups have demonstrated Ras activation by shear (30, 31). We have shown that inhibiting PKC-ε with antisense oligonucleotides completely blocked shear stress-mediated ERK1/2 activation, indicating that PKC-ε directly participates in the signaling cascade leading to ERK1/2 activation (e.g. by its kinase activity) or that the constitutive presence of PKC-ε is required for normal function of some component of the signaling cascade (e.g. by regulating gene expression). Whereas the current data cannot distinguish between these two possibilities, several recent studies suggest that PKC-ε acts directly on Raf-1. Cai et al. (32) showed that overexpression of active PKC-ε resulted in increased Raf-1 activity even in the context of dominant negative Ras. These investigators also demonstrated that PKC-ε stimulated Raf-1 in baculovirus-infected Sf9 cells and was able to directly activate Raf in vitro. Schaap et al. (33) also found that overexpression of PKC-ε isofrom activated ERK1/2, but that ERK1/2 activation was not present if PKC-ε was overexpressed in a dominant-negative Raf-1-expressing cell line. A study by Cacape et al. (34) demonstrated that PKC-ε acts downstream of Ras but upstream of Raf-1 to induce oncogenic transformation in PC12 cells. Finally, several studies demonstrate that activation of Raf-1 and ERK1/2 in vascular smooth muscle cells by platelet-derived growth factor (35, 36), angiotensin II (35, 36, 38), serotonin (39), thrombin (40), or ATP (41) was inhibited by phorbol ester pretreatment or antisense PKC oligonucleotide treatment (23). Whereas these studies suggest that PKC activates ERK1/2 through Raf-1 in vascular smooth muscle as well, studies to determine whether PKC directly phosphorylates and activates Raf-1 in vascular smooth muscle cells are still required to confirm these observations.

PKC-ε-mediated activation of ERK1/2 is likely to be an important pathway for the EC response to shear stress. ERK1/2 has been shown to phosphorylate and activate pp90ε, allowing phosphorylation of S6 protein and increased protein synthesis. ERK1/2 is an upstream regulator of Elk-1 and may stimulate ternary complex formation, which increases fos expression, which has been shown to be induced by shear stress. ERK1/2 may also be involved in regulation of endothelial nitric oxide synthase based on observations that NO production is regulated by shear stress (43) and that endothelial nitric oxide synthase has multiple consensus sites for phosphorylation by ERK1/2 (44). Kuchan and Frangos (45) reported that whereas initial release of NO was Ca²⁺-independent, sustained NO production in response to shear was Ca²⁺-independent. Since the shear stress signaling pathway involving PKC-ε described in this paper is Ca²⁺-independent, it may be involved in sustained NO production stimulated by shear stress. Finally, other PKC-ε substrates such as the cytoskeletal proteins, caldesmon (37), calponin (38), and filamentous actin (42) may contribute to the rearrangement in EC cytoskeleton in chronically induced...
by flow.

The present study demonstrates the utility of antisense PKC oligonucleotides to explore the cellular role of specific PKC isoforms. Whereas the traditional pharmacological PKC inhibitors employed to investigate the role of PKC are likely to possess differential affinities for the separate PKC isoforms, they are unlikely to be specific for individual PKC isoforms. Indeed, in the present study, staurosporine was an effective inhibitor of PKC-α and PKC-ε but did not affect PKC-ζ activity. The use of antisense oligonucleotides has been shown to be effective in several studies (21, 22) and, as demonstrated here, can be used to study isoform specific events. In summary, these data are the first to identify a specific PKC isoform required for transduction of mechanical stimuli. Specifically, we showed that PKC-ε, but not PKC-α or PKC-ζ, serves as a mechanosensitive mediator for activation of ERK1/2 by shear stress in endothelial cells. Further characterization of this signal transduction pathway will yield greater insight into the mechanisms by which mechanical stimuli regulate biological processes of the vascular wall.

Acknowledgments — We thank Kathy McGraw, Loren Miraglia, and Robert McKay for expert technical assistance.

REFERENCES

1. Watson, P. A. (1991) FASEB J. 5, 2013–2019
2. Furchgott, R. F., and Vanhoutte, P. M. (1989) FASEB J. 3, 2007–2018
3. Hsieh, H. J., Li, N. Q., and Frangos, J. A. (1993) J. Cell. Physiol. 154, 143–151
4. Matsuoka, M., Fischl, R. S., Neren, B. N., Alexander, R. W., and Berk, B. C. (1993) Am. J. Physiol. 265, H3–H8
5. Uematsu, M., Navas, J. P., Nishida, K., Ohara, Y., Murphy, T. J., Alexander, R. W., Neren, R. M., and Harrison, D. G. (1993) Circulation 88, I-184
6. Ku, D. N., Giddens, D. P., Zarins, C. K., and Glagov, S. (1985) Arteriosclerosis 5, 293–302
7. Pellegrini, S. L., and Sanghera, J. S. (1992) Science 257, 1355–1356
8. Tseng, H., Peterson, T., and Berk, B. C. (1995) Circ. Res. 77, 869–878
9. Lange-Carter, C. A., Pleiman, C. M., Gardiner, A. M., Blumer, K. J., and Johnson, G. L. (1993) Science 260, 315–319
10. Nollert, M. U., Eskin, S. G., and McIntire, L. V. (1990) Biochem. Biophys. Res. Commun. 170, 281–287
11. Kuchan, M. J., and Frangos, J. A. (1993) Am. J. Physiol. 264, H150–H156
12. Biswas, P., Abdou, H. E., Kiyomoto, H., Wenzel, U. O., Grandaliano, G., and Choudhury, G. G. (1995) FEBS Lett. 373, 146–150
13. Girard, P. R., and Neren, B. M. (1993) Front. Med. Biol. Eng. 5, 31–36
14. Gimbrone, Jr., M. A. (1976) Prog. Hemost. Thromb. 3, 1–29
15. Woodgett, J. R., and Hunter, T. (1987) Mol. Cell. Biol. 7, 85–96
16. Takahashi, M., and Berk, B. C. (1996) J. Clin. Invest. 98, 2623–2631
17. Ishida, T., Peterson, T. E., Kovach, N., and Berk, B. C. (1996) Circ. Res. 79, 310–316
18. Shen, J., Lusczynskas, F. W., Connolly, A., Dewey, C. F. J., and Gimbrone, M. A. (1992) Am. J. Physiol. 262, C384–C390
19. Geiger, R. V., Berk, B. C., Alexander, R. W., and Neren, R. M. (1992) Am. J. Physiol. 262, C1411–C1417
20. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498
21. Dean, N. M., McKay, R., Condon, T. P., and Bennett, C. F. (1994) J. Biol. Chem. 269, 16416–16424
22. Dean, N. M., and McKay, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11762–11766
23. Liao, D.-P., Monia, B., Dean, N., and Berk, B. C. (1997) J. Biol. Chem. 272, 6146–6150
24. Clark, K. J., and Murray, A. W. (1995) J. Biol. Chem. 270, 7097–7103
25. Young, S. W., Dickens, M., and Tavaré, J. M. (1996) FEBS Lett. 384, 181–184
26. Davies, P. F. (1995) Physiol. Rev. 75, 519–566
27. Liscovitch, M., and Cantley, L. C. (1994) Cell 77, 329–334
28. Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S., Mizuno, K., Takenawa, T., Fukui, Y., Watanebe, Y., Ozaki, S., and Ohno, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 151–155
29. Vuori, K., and Ruoslahti, E. (1993) J. Biol. Chem. 268, 24859–24862
30. Li, Y.-S., Shyy, J. Y., Li, S., Lee, J., Su, B., Kafri, M., and Chien, S. (1996) Mol. Cell. Biol. 16, 5947–5954
31. Jo, H., Sipos, K., Go, Y.-M., Law, R., Rong, J., and McDonald, J. M. (1997) J. Biol. Chem. 272, 1395–1401
32. Cai, H., Smola, U., Wiedler, V., Eisenmann, T.-J., Dietz, M.-M., Moscat, J., Rapp, U., and Cooper, G. M. (1997) Mol. Cell. Biol. 17, 732–741
33. Schaap, D., et al. (1997) Circulation 95, 20232–20236
34. Cacace, A. M., Guadagno, S. N., Krauss, R. S., Fabbro, D., and Weinstein, I. B. (1993) Oncogene 8, 2095–2104
35. Liao, D. F., Duff, J. L., Daum, G., Pelech, S. L., and Berk, B. C. (1996) Circ. Res. 78, 1007–1014
36. Plevin, R., Scott, P. H., Robinson, C. J., and Gould, G. W. (1996) Biochem J. 318, 657–663
37. Horowitz, A., Clement-Chomienne, O., Walsh, M. P., and Morgan, K. G. (1996) Am. J. Physiol. 271, C598–C604
38. Arai, H., and Escobedo, J. A. (1996) Mol. Pharmacol. 50, 522–528
39. Hershenson, M. B., Cha, T. S., Abe, M. K., Gumes, I., Kelleher, M. D., Solway, J., and Rosner, M. R. (1995) J. Biol. Chem. 270, 19968–19973
40. Trejo, J., Connolly, A. J., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 21536–21541
41. Yu, S. M., Chen, S. F., Lau, Y. T., Yang, C. M., and Chen, J. C. (1996) J. Biol. Chem. 271, 20232–20236
42. Araki, H., and Escobedo, J. A. (1996) Mol. Pharmacol. 50, 1050–1059
43. Prekeris, R., Mayhew, M. W., Cooper, J. B., and Terrian, D. M. (1996) J. Cell Biol. 132, 77–80
44. Corson, M. A., Berk, B. C., Navas, J. P., and Harrison, D. G. (1993) Circulation 88, I-183
45. Berk, B. C., Corson, M. A., Peterson, T. E., and Tseng, H. (1995) J. Biochem. 186, 1439–1450
46. Kuchan, M. J., and Frangos, J. A. (1994) Am. J. Physiol. 266, C628–C636