Sequential Activation of Protein Kinase C (PKC)-α and PKC-ε Contributes to Sustained Raf/ERK1/2 Activation in Endothelial Cells under Mechanical Strain*

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Endothelial cells (ECs) are constantly subjected to hemodynamic forces including cyclic pressure-induced strain. The role of protein kinase C (PKC) in cyclic strain-treated ECs was studied. PKC activities were induced as cyclic strain was initiated. Cyclic strain to ECs caused activation of PKC-α and -ε. The translocation of PKC-α and -ε but not PKC-β from the cytosolic to membrane fraction was observed. An early transient activation of PKC-α versus a late but sustained activation of PKC-ε was shown after the onset of cyclic strain. Consistently, a sequential association of PKC-α and -ε with the signaling molecule Raf-1 was shown. ECs treated with a PKC inhibitor (calphostin C) abolished the cyclic strain-induced Raf-1 activation. ECs under cyclic strain induced a sustained activation of extracellular signal-regulated protein kinases (ERK1/2), which was inhibited by treating ECs with calphostin C. ECs treated with a specific Ca2+-dependent PKC inhibitor (Go 6976) showed an inhibition in the early phase of ERK1/2 activation but not in the late and sustained phase. ECs transfected with the antisense to PKC-α, the antisense to PKC-ε, or the inhibition peptide to PKC-ε reduced strain-induced ERK1/2 phosphorylation in a temporal manner. PKC-α mediated mainly the early ERK1/2 activation, whereas PKC-ε was involved in the sustained ERK1/2 activation. Strained ECs increased transcriptional activity of Elk1 (an ERK1/2 substrate). ECs transfected with the antisense to each PKC isoform reduced Elk1 and monocyte chemotactic protein-1 promoter activity. Our findings conclude that a sequential activation of PKC isoform (α and ε) contribute to Raf/ERK1/2 activation, and PKC-ε appears to play a key role in endothelial adaptation to hemodynamic environment.

Vascular endothelial cells (ECs) are constantly under the influence of hemodynamic forces including flow-induced shear stress and pressure-generated cyclic strain. These hemodynamic forces play an essential role in maintaining vascular integrity by inducing the release of vasoactive substances and modulating gene expression (1, 2). Studies have examined how intracellular signals are involved in transmitting mechanical forces into second messengers and subsequently gene expression (3, 4). Shear flow stimulates the signals involved in the ERK1/2 and JNK pathways. These signals may result in the induction of various genes’ expression including platelet-derived growth factor (5), Egr-1 (6), c-fos (7), monocyte chemotactic protein-1 (MCP-1) (8), and intercellular adhesion molecule-1 (ICAM-1) (9). Because rhythmic distension of the vessel wall is a component of pulsatile flow, cyclic strain on vessel walls plays an important role in modulating gene expression. Earlier studies from our laboratory showed that ECs under cyclic strain increase their expression of MCP-1 (10–12), ICAM-1 (13, 14), and early growth response-1 (Egr-1) (15). Signaling pathways involving ERK1/2 and c-Jun N-terminal kinase participate in mechanical force-induced gene expression (3, 12, 16). However, the initial events and the following networks of signaling pathways are still poorly understood.

Cyclic strain to ECs activates intracellular second messengers. Activation of protein kinase C (PKC) is associated with an increase of phosphatidyl inositol turnover and intracellular calcium (17). PKC is activated by diacylglycerol (DAG), which is derived either from phosphatidylinositol (PI) or phosphatidylinositol (PC). PKC isoforms in human ECs have been identified that cover PKC-α, PKC-δ, PKC-ε, and PKC-ζ (18). PKC-α belongs to a Ca2+-dependent group, and the isoforms PKC-ε and PKC-ζ belong to a Ca2+-independent group. Studies have indicated that PKC is involved in shear stress- and cyclic strain-induced gene expression of platelet-derived growth factor and Et-1 in ECs (19, 20). Indeed, PKC-ε is required for fluid shear stress-mediated activation of ERK1/2 in ECs (21). In smooth muscle cells, stretching promotes DNA synthesis via activation of PKC (22). Our previous studies demonstrated that cyclic strain to ECs increases gene expression of MCP-1, which is regulated by PKC (10). Further studies indicated that cyclic strain induces the Ras/Raf-1/ERK1/2 signaling pathway and results in an increase of gene expression of MCP-1 and Egr-1 (11, 15). The upstream signaling pathway and/or signaling network that lead to activation of Ras/Raf-1/ERK1/2 by cyclic strain remain unclear. Among the likely signaling networks, different PKC isoforms have been shown to modulate the ERK1/2 signaling pathway under different stimuli (23, 24). However, direct evidence of any of the PKC isoforms being involved in the signaling pathway during endothelial response to cyclic strain has not been clearly defined. In the present study, we demonstrate that ECs subjected to cyclic strain...
crease PKC activities and that PKC-α and PKC-ε are sequentially activated for Raf/ERK1/2 activation. PKC-α and PKC-ε contribute to the early and late phase of ERK1/2 activation, respectively, in cyclic strain-treated ECs. The consequence of these PKCs being activated by cyclic strain leads to cellular adaptation including gene induction in ECs. Our results provide direct evidence of PKC isoforms’ participation in signaling transduction in ECs under a hemodynamic environment.

MATERIALS AND METHODS

In Vitro Cyclical Strain on ECs—The strain unit Flexcell FX-2000 (Flexcell, McKeensport, PA) consisted of a vacuum unit linked to a valve controlled by a computer program (25). Bovine aortic ECs cultured on a flexible membrane base were deformed by a sinusoidal negative pressure that produced an average strain of 12% at a frequency of 1 Hz.

PKC Activity Assay—ECs were scraped in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM KCl, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture) and sonicated. Total cell lysate was collected, and PKC activity was detected based on an enzyme-linked immunosorbent assay that utilizes a synthetic peptide and a monoclonal antibody that recognizes the phosphorylated form of the peptide (Upstate Biotechnology, Inc., Lake Placid, NY).

Separation of PKC and Immunoblot Analysis—ECs were scraped into buffer containing 2-mercaptoethanol and protease inhibitors. After sonication and centrifugation, the supernatants and pellets were collected as cytosolic and membrane fractions. For experiments to detect the phosphorylation in PKC, total cell lysate was used. Proteins were extracted in buffer containing SDS and subjected to SDS-PAGE. The PKC isoforms were analyzed with PKC monoclonal antibodies (Transduction Laboratories). To detect serine phosphorylation in PKC, antibody to phospho-PKC-α (Ser657) or phospho-PKC-ε (Ser719) (Transduction Laboratories) was used. Antibody-antibody complexes were detected using horseradish peroxidase-labeled rabbit anti-mouse IgG and an ECL detection system (Amersham Pharmacia Biotech). For detection of phosphorylated ERK1/2 (pERK1/2), PERK1/2 antibody (Transduction Laboratories) was used. For the Raf activation detection, antibody specific to the phosphorylated activation site (Ser402; Transduction Laboratories) was used. Antigen-antibody complexes were detected using an enzyme-linked immunosorbent assay that utilizes a synthetic peptide and a monoclonal antibody that recognizes the phosphorylated form of the peptide (Upstate Biotechnology, Inc., Lake Placid, NY).

DNA Plasmids, Transfection, and Luciferase Assays—An Elk1 transduction pathway-reporting system was obtained from Stratagene (La Jolla, CA) that contains plasmids 

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\text{Gal} / \text{Elk1-(307-428)} \quad \text{and} \quad \text{Gal4-Luc.}
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An MCP-1 promoter construct (−540 base pairs) containing the luciferase reporter gene (26) was also used. Transfection was performed using the LipofectAMINE method (Life Technologies, Inc., Gaithersburg, MD) that contains plasmids GAL/Elk1-(307–428) and GAL4-Luc. As shown in Fig. 1B, ECs under cyclic strain rapidly induced phosphorylation of Ser657 on PKC-α and maintained that at an activated form for up to 3 h. In contrast, the phosphorylation of Ser719 on PKC-ε showed apparent activation after 1 h of strain treatment and maintained that up to 6 h.

Immediately following lysis of ECs after strain treatment, the cytosolic and membrane fractions were separated. Different PKC isoforms from each fraction were analyzed. Three major PKC isoforms (i.e. PKC-α, -β, and -ε) were identified with respective antibodies. ECs, after cyclic strain for 5 min, rapidly induced their PKC-α activity as shown by the strong PKC-α migration from the cytosolic to the membrane fraction (Fig. 2). These PKC-α then gradually retreated to the cytosolic fraction. The majority of PKC-α returned to the cytosolic fraction at 6 h after continuous cyclic strain. Interestingly, the PKC-ε transmigration was not detected at the early phase but instead became apparent at 1 h, reached maximal activation at 3 h, and remained in an activated form even at 6 h after cyclic strain treatment (Fig. 2). In contrast to the transmigration of PKC-α and PKC-ε from the cytosolic to the membrane fraction, the PKC-β isoform was not activated and remained in the cytosolic fraction during the entire 6-h cyclic strain treatment. These observations are consistent with the sequential pattern of serine phosphorylation on active sites of PKCs shown in Fig. 1B. Our results clearly demonstrate a sequential transmigration of PKC-α and PKC-ε to the membrane fraction in ECs after the onset of cyclic strain.

Sequential Association of PKC-α and PKC-ε with Raf-1 in Cyclic Strain-treated ECs—The direct phosphorylation of Raf-1 by PKC isoforms has been suggested as an activation mechanism of PKC on the Raf-1/ERK1/2 signaling pathway (31, 32). We previously demonstrated that cyclic strain activates the Ras/Raf-1/ERK1/2 pathway (14). To demonstrate that PKC is involved in Raf-1 activation, ECs were pretreated with a PKC inhibitor, calphostin C, followed with cyclic strain treatment. As shown in Fig. 3A, the cyclic strain, while inducing an increased phosphorylation of Raf-1 in its activation site, was significantly blunted by treating ECs with calphostin C. To further elucidate the role of PKC isoforms in the Raf-1/ERK1/2 signaling cascade, the association of each PKC isoform with Raf-1 in strained ECs was assessed by immunoprecipitating Raf-1 with monoclonal antibodies from total cellular extracts of ECs and by immunoblotting with antibodies to PKC-α or PKC-ε. Although an equal amount of Raf-1 was showed in the immune complex, PKC isoform association with Raf-1 occurred in a temporal manner (Fig. 3B). In ECs under static conditions, some PKC-ε was associated with Raf-1, but PKC-α was not. However, ECs subjected to cyclic strain for 5 min resulted in a rapidly increased association of PKC-α with Raf-1. This association of PKC-α with Raf-1 reeded after 3 h of cyclic strain.
contrast, PKC-ε was strongly associated with Raf-1 at this time point. These findings of Raf-1 phosphorylation via PKC and sequential association of PKC isoforms with Raf-1 are consistent with the finding of temporal transmigration of PKC-α and PKC-ε in ECs after cyclic strain treatment.

Sequential Activation of PKC-α and -ε Contributes to Strain-induced ERK1/2 Activation—We previously demonstrated that cyclic strain to ECs induces Egr-1 gene expression, which is predominantly mediated via the Ras/Raf-1/ERK1/2 signaling pathway (15). To further confirm that the ERK1/2 signaling pathway is involved, ECs after cyclic strain for various intervals were lysed, and phosphorylated ERK1/2 was analyzed with Western blotting using anti-phosphorylated ERK1/2 antibody. Cyclic strain to ECs, similar to those ECs after phorbol ester treatment, rapidly induced ERK1/2 activity (Fig. 4A).

**Fig. 1.** Cyclic strain induces protein kinase C activity. A, ECs were exposed to 12% strain for various time intervals. Total PKC activities of cell homogenates were measured by a nonradioactive assay as described under “Materials and Methods.” Results are expressed as mean ± S.E. from five experiments. *, p < 0.05 versus static control ECs. B, ECs subjected to cyclic strain for various time intervals were collected. Total cell lysate was subjected to SDS-PAGE and immunoblotted with phospho-PKC-α (Ser657), phospho-PKC-ε (Ser113), or PKC-α antibody. Antibody to PKC-α was used to indicate that an equal amount of protein was applied on each gel lane. Results are representative of three separate experiments with similar results.

**Fig. 2.** Sequential transmigration of PKC-α and PKC-ε to membrane in cyclic strain-treated ECs. ECs subjected to cyclic strain for various time intervals were collected and separated into cytosolic (c) and membrane fractions (m). Equal amounts of total proteins from each cell sample were subjected to SDS-PAGE. PKC-α, PKC-β, and PKC-ε isoforms were identified with respective monoclonal antibodies by Western blot. Results (mean ± S.E.) shown are from three separate experiments of respective PKC-α and PKC-ε. *, p < 0.05 versus cytosolic fraction in controls. #, p < 0.05 versus membrane fraction in controls.
strain, were inhibited after treating ECs with calphostin C (Fig. 4B). Consistently, cyclic strain-induced ERK1/2 kinase activity, as analyzed by \( ^{32}P \) phosphorylation of myelin basic protein, was inhibited in ECs pretreated with calphostin C (Fig. 4C). In contrast to the activation of PKC-\( \epsilon \), PKC-\( \alpha \) activation is \( Ca^{2+} \)-dependent. To differentiate which PKC isoforms contribute to the early phase versus the late but sustained phase of ERK1/2 activity, ECs were pretreated with a specific \( Ca^{2+} \)-dependent PKC inhibitor, Go 6976, and then subjected to cyclic strain. As shown in Fig. 4D, the early phase of cyclic strain-induced ERK1/2 activity was significantly inhibited after Go 6976 treatment of ECs. In contrast, strain-induced ERK1/2 activity in the late but sustained phase was not affected by this inhibitor treatment. These results support that a sequential activation of PKC-\( \alpha \) and PKC-\( \epsilon \) is involved in cyclic strain-induced ERK1/2 activation in ECs.

Antisense Oligonucleotides to PKC-\( \alpha \) or -\( \epsilon \) Inhibit Cyclic Strain-induced ERK1/2 Activity—To further confirm the role of each PKC isoform in strain-induced ERK1/2 activity, ECs were pretreated with antisense to PKC-\( \alpha \) or PKC-\( \epsilon \). ECs transfected with an antisense (2 \( \mu \)mol/liter) to a particular PKC isoform significantly reduced the protein expression of that PKC isoform in ECs (Fig. 5, A–D). Consistently, antisense to PKC-\( \alpha \) and PKC-\( \epsilon \) significantly inhibited PKC activity in ECs after cyclic strain for 5 min and 3 h, respectively (Fig. 5E). When ECs were subjected to cyclic strain for 5 min, only those ECs transfected with antisense to PKC-\( \alpha \) showed an inhibition of ERK1/2 phosphorylation (Fig. 6A). In contrast, ECs transfected with the scramble oligonucleotides did not affect ERK1/2 activity. This indicates that PKC-\( \alpha \) is required for early ERK1/2 activity. However, PKC-\( \epsilon \) did not play a significant role at the late phase of ERK1/2 activation, since antisense PKC-\( \alpha \)-transfected ECs did not inhibit ERK1/2 phosphorylation at 3 h after cyclic strain treatment (Fig. 6B). In contrast, ECs transfected with an antisense to PKC-\( \epsilon \) abolished the strain-induced ERK1/2 activity at this later phase. PKC-\( \epsilon \) involved in late phase of ERK1/2 was further confirmed by the inhibition of ERK1/2 activity in strained ECs transfected with the inhibitory peptide to PKC-\( \epsilon \) (Fig. 6B). All of these data demonstrate that PKC-\( \alpha \) is required for the early phase, while PKC-\( \epsilon \) contributes mainly to the late and sustained phase of cyclic strain-induced ERK1/2 activation in ECs.

Cyclic Strain-induced PKC Activation Increases Transcriptional Activity of Elk1 and MCP-1—When the Ras/Raf-1/ERK1/2 signaling pathway is triggered, it leads to the activation of downstream transcriptional factors including activator protein-1 and ternary complex factors Elk1/TCF (ternary complex factors). Since PKC isoforms contribute to strain-induced ERK1/2 activity and ERK1/2 activation increases the transcriptional activity of Elk1 by phosphorylation, we thus investigated whether PKC isoforms elicit the transcriptional activity of Elk1. To demonstrate Elk1 activity, plasmid GAL4/Elk1-307–428, which encodes the fusion protein of the GAL4/DNA-binding domain fused to the activation domain of Elk1, was
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PKC-α was involved in an early activation, whereas PKC-ε was activated at a late but sustained phase. Second, ECs treated with a PKC inhibitor abolished the Raf-1 phosphorylation by cyclic strain. Third, a sequential association of PKC-α and PKC-ε with the signaling molecule Raf-1 was shown. Fourth, a PKC inhibitor inhibited strain-induced ERK1/2 activation, indicating a crucial role of PKC in the Raf/ERK1/2 signaling pathway. Fifth, ECs treated with a Ca²⁺-dependent PKC inhibitor (Go 6976 (Go)) showed an inhibition in the early phase but not the late sustained phase of ERK1/2 activation. Sixth, ECs transfected with the antisense to PKC-α inhibited only early and not late ERK1/2 activation, whereas the antisense or inhibitory peptide to PKC-ε suppressed late, sustained ERK1/2 activation. Consistently, an antisense to PKC-α did not inhibit late ERK1/2 activation. All of these data confirm that PKC-α and PKC-ε are sequentially activated and are required for Raf/ERK1/2 activation. Furthermore, ECs treated with an antisense to each PKC isoform significantly reduced the transcriptional activity of Elk1, a downstream substrate of ERK1/2. As such, these results indicate that the sequential activation of PKC-α and PKC-ε is essential for Raf/ERK1/2 activation in cyclic strain-treated ECs. The activation of ERK1/2 and its downstream Elk-1 activation may result in gene induction.

Early studies indicated that PKC activities are increased in ECs under shear or cyclic strain treatment (33). Our previous studies showed that PKC is involved in cyclic strain-induced Et-1 and MCP-1 gene expression (10, 30). Cyclic strain to ECs results in a biphasic increase in DAG (17) that corresponds to early transient PKC activity followed by sustained elevated PKC activity (33). Although PKC involvement in mechanical force-induced endothelial responses has been acknowledged (33), studies have indicated only that specific isoforms PKC-ε and PKC-β are involved in shear-induced endothelial response (21, 34). The mechanisms as to what and how PKC isoforms are involved in cellular responses to mechanical forces remain unclear. The present study shows that PKC-α and PKC-ε are sequential activated and are involved in cyclic strain-induced Raf/ERK1/2 activation. Several lines of evidence support this notion. First, the transmigration of PKC-α and PKC-ε from the cytosolic to the membrane fraction was a sequential event (i.e. PKC-α was involved in an early activation, whereas PKC-ε was activated at a late but sustained phase). Second, ECs treated with a PKC inhibitor abolished the Raf-1 phosphorylation by cyclic strain. Third, a sequential association of PKC-α and PKC-ε with the signaling molecule Raf-1 was shown. Fourth, a PKC inhibitor inhibited strain-induced ERK1/2 activation, indicating a crucial role of PKC in the Raf/ERK1/2 signaling pathway. Fifth, ECs treated with a Ca²⁺-dependent PKC inhibitor (Go 6976 (Go)) showed an inhibition in the early phase but not the late sustained phase of ERK1/2 activation. Sixth, ECs transfected with the antisense to PKC-α inhibited only early and not late ERK1/2 activation, whereas the antisense or inhibitory peptide to PKC-ε suppressed late, sustained ERK1/2 activation. Consistently, an antisense to PKC-α did not inhibit late ERK1/2 activation. All of these data confirm that PKC-α and PKC-ε are sequentially activated and are required for Raf/ERK1/2 activation. Furthermore, ECs treated with an antisense to each PKC isoform significantly reduced the transcriptional activity of Elk1, a downstream substrate of ERK1/2. As such, these results indicate that the sequential activation of PKC-α and PKC-ε is essential for Raf/ERK1/2 activation in cyclic strain-treated ECs. The activation of ERK1/2 and its downstream Elk-1 activation may result in gene induction.

PKC-α belongs to the family of conventional protein kinases that are Ca²⁺-dependent, whereas PKC-ε is a novel protein kinase. It is well recognized that when ECs are subjected to hemodynamic forces, Ca²⁺ mobilization plays an essential role in endothelial responses (20, 35). When ECs are under hemodynamic treatment, a rapid increase of [Ca²⁺]i (35, 36), inositol trisphosphate and DAG (17) occurs. DAG is produced from hydrolysis of PI and PC (17). DAG derived from PI after phospholipase C activation is responsible for the translocation of PKC-α (37). This transient DAG release coupled with Ca²⁺ mobilization may activate PKC-α during the early response of ECs to cyclic strain. PC hydrolysis, however, provides a sustained source of DAG in growth factor-stimulated cells that is compatible with the signaling activity required for long-term response (38). Although PKC-α, -δ, -ε, -ζ, and -γ have been iden-
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identified in ECs (18, 21), only PKC-ε has been implicated to be involved in shear-induced ERK1/2 activity (21). In the present cyclic strain study, PKC-α participated in early ERK1/2 activity. Although signaling events by which each PKC isoform activates ERK1/2 remain to be further characterized, PKC involvement in Raf-1 activation and sequential association of PKC isoforms with Raf-1 indicate that PKC-α and PKC-ε contribute to Raf-1/ERK1/2 activation. In addition, the fact that the antisense to each PKC isoform temporally inhibits ERK1/2 activity also suggests that these two PKC isoforms contribute

![Figure 5](image5.png)

**FIG. 5.** Antisense oligonucleotides to PKC-α or PKC-ε decrease the PKC isoform expression in ECs. ECs were either transfected with a scramble oligonucleotide (Sc) or an increasing concentration of antisense to PKC-α (A) or PKC-ε (C) for 6 h. Two days after transfection, ECs were lysed, and the same amount of protein was subjected to Western analysis using respective anti-PKC-α, -PKC-β, or -PKC-ε antibody. The specificity of each antisense to PKC isoform was shown in B and D. Results (mean ± S.E.) are representative of three separate experiments with similar results. *, p < 0.05 versus PKC-α or PKC-ε in controls. PKC-β has no change in its protein expression and is shown as an internal control. E, ECs were transfected with antisense to PKC-α and PKC-ε and subjected to mechanical strain. The total PKC activities were accessed as described under “Materials and Methods.” Data are shown as relative activity (mean ± S.E.). *, p < 0.05 versus strained ECs which have been transfected with corresponding scramble oligonucleotides.

![Figure 6](image6.png)

**FIG. 6.** PKC-α and PKC-ε sequentially regulate cyclic strain-induced ERK1/2 phosphorylation. A, ECs were transfected with either sense, antisense, or scrambled (Sc) oligonucleotides (2 μmol/liter) to PKC-α or PKC-ε for 6 h. Two days after transfection, ECs were subjected to cyclic strain for 5 min (S5'). B, ECs were similarly transfected with sense or antisense oligonucleotides (2 μmol/liter) to PKC-α or PKC-ε or inhibition peptide to PKC-ε (5 μmol/liter, PKC-εi) for 6 h. Two days after transfection, ECs were subjected to cyclic strain for 3 h (S3h). Total cell lysate was collected for Western analysis using an antibody to pERK1/2. Equal amounts of protein applied to each lane are shown by the ERK for each lane. Results are representative of three separate experiments.
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versus and activate Raf-1 (31, 32, 39). PKC−/H9251 and PKC−/H9280 both PKC−/H9251 activations and our current findings, it is strongly suggested that induced Egr-1 expression (15). Based on these previous obser-

Previous study demonstrated that the serum response element in the promoter region of Egr-1 is involved in cyclic strain-

PKC−/H9280 overexpression was shown to induce a sustained induction. In addition to sequential activation of PKC−/H9280 signaling pathway that leads to activation of ERK1/2 and gene

The present study clearly demonstrates that PKC isoforms are essential signaling molecules for transduction of cyclic strain. PKC−/H9251 and PKC−/H9280 act as Raf-1 activators that lead to a prolonged effect on the MAPK signaling pathway and gene induction. Recent studies indicate that lack of fluid shear flow triggers apoptosis in ECs (50). The activation of PKC isoforms by cyclic strain serves not only as a signaling response but is also important for cellular growth and survival. Elucidating the signaling mechanism mediated via PKC isoforms in ECs during hemodynamic changes is key for further understanding of endothelial dysfunction during atherosclerosis, hypertension, and reperfusion-induced vascular injuries.

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