Vascular endothelial growth factor (VEGF) Induced Endothelial Cell Migration and Proliferation Depend on a Nitric Oxide–Mediated Decrease in Protein Kinase Cδ Activity

Yukitaka Shizukuda, Shaoqing Tang, Ryoji Yokota, J. Anthony Ware

Abstract—Vascular endothelial growth factor (VEGF) promotes angiogenesis and endothelial cell (EC) migration and proliferation by affecting intracellular mediators, only some of which are known, distal to its receptors. Protein kinase C (PKC) participates in the function of VEGF, but the role of individual PKC isoenzymes is unknown. In this study, we tested the importance of the activity of specific PKC isoenzymes in human EC migration and proliferation in response to VEGF. PKCδ specific activity was depressed by the addition of VEGF (by 41±8% [P<0.05] at 24 hours) in human umbilical vein ECs (HUVECs) and in a HUVEC-derived EC line, ECV, without changing the total amount of either protein or mRNA encoding PKCδ. Neither basic fibroblast growth factor (FGF-2) nor serum altered PKCδ specific activity. The VEGF-induced decrease of PKCδ activity, which began at 8 hours after stimulation, was strongly blocked by pretreatment with the nitric oxide (NO) synthase inhibitor N\textsuperscript{G}-monomethyl-L-arginine in HUVECs; NO release peaked within 2 hours after stimulation. An exogenous NO donor, sodium nitroprusside, also decreased PKCδ activity. The inhibition by N\textsuperscript{G}-monomethyl-L-arginine of VEGF-induced HUVEC migration and proliferation, but not that induced by FGF-2 or serum, suggested that the decrease in PKCδ via NO pathway is required for VEGF-induced EC migration and proliferation. Overexpression of PKCδ in ECV cells specifically prevented EC response to VEGF but not to FGF-2 or serum. Thus, we conclude that suppression of PKCδ activity via a NO synthase mechanism is required for VEGF-induced EC migration and proliferation, but not for that induced by FGF-2 or serum. (Circ Res. 1999;85:247-256.)

Key Words: endothelium n growth factor n nitric oxide synthase n intracellular signaling n angiogenesis

Vascular endothelial growth factor (VEGF) strongly induces both physiological and pathological angiogenesis and has been used clinically to promote angiogenesis in patients with limb ischemia. Physiological responses to VEGF that can contribute to angiogenesis include mitogenic and promigratory effects, chemotaxis, and release of proteolytic enzymes and their inhibitors. VEGF differs from other growth factors, including basic fibroblast growth factor (FGF-2), because it can increase vascular permeability, but its ability to induce capillary tube formation appears to be synergistic with FGF-2.

The PKC family consists of a large group of individual phospholipid-dependent serine-threonine kinase isoenzymes involved in cellular signaling. Inhibition of PKC activity with the chemical inhibitor calphostin prevents VEGF-induced vascular growth, but the role of specific isoenzymes in the processes that constitute angiogenesis is unknown, because the specificity of most PKC inhibitors for individual isoenzymes has not been established. Overexpression or inhibition of some of the individual PKC isoenzymes, including PKCα, PKCδ, and PKCθ, have diverse effects on EC migration and proliferation. In particular, an increase in PKCδ activity reduces serum-induced proliferation of ECs by slowing passage through the S phase of the cell cycle in rat microvascular ECs. In contrast, inhibition of PKCθ prevents serum-induced EC proliferation and migration, whereas enhancing PKCα activity does not affect proliferation but promotes EC migration. Thus, each PKC isoenzyme appears to have specific substrates and activators and to mediate distinct EC events that constitute angiogenesis.

Which, if any, of the specific PKC isoenzymes is critical for VEGF-induced EC function is not known. We chose to...
investigate the role of PKCδ in VEGF-induced EC proliferation and migration, because NO, which is a key mediator generated by VEGF, reduces PKCδ activity in peritoneal macrophages, and because of the inhibitory effect of enhanced PKCδ expression on proliferation and cell cycle progression in rat microvascular cells. To determine the specificity of these effects for VEGF signaling, we compared the importance of PKCδ and NO synthase in EC function in response to another angiogenic growth factor, FGF-2.

Materials and Methods

Cell Cultures, Growth Factors, and Other Materials

Human umbilical vein ECs (HUVECs) were cultured in M199 medium containing 20% newborn calf serum (NCS, GIBCO-BRL), 5% human serum (Gemini Bio-Products, Inc), 50 μg/mL ascorbic acid, 1.6 mmol/L L-glutamine, 5 μg/mL bovine brain extract (Clonetec Corp), 7.5 μg/mL endothelial growth supplement (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 μM heparin. HUVECs of the third to fifth passage were used for all experiments. Subconfluent HUVECs were synchronized with the above-mentioned media with reduced NCS (10%) and lacking endothelial growth supplements. After 24 hours of synchronization, VEGF (a gift from Dr Michael Simons, Beth Israel Deaconess Medical Center, Boston, Mass) in a final concentration of 20 ng/mL, FGF-2 (Sigma, 20 ng/mL), or 20% NCS was added to the cell media depending on the experimental protocol. In the experiments in which a NO synthase inhibitor was used, Nω-nomethyl-L-arginine (L-NMMA, Sigma) was added to the media at a final concentration of 500 μmol/L. In experiments to investigate effects of suppression of total PKC activity on EC migration and proliferation, 100 μmol/L phorbol 12-myristate 13-acetate (PMA; Sigma) was added to media 24 hours before the experimental protocols to downregulate PKC activity. In experiments in which a NO donor was used, sodium nitroprusside (SNP, Sigma) at the final concentration of 500 μmol/L to 1 mmol/L was added at the time that the protocols began.

Immortalized human ECs (ECV cells, obtained from American Type Culture Collection, Manassas, VA) were cultured in M199 (GIBCO-BRL) supplemented with 10% FBS (GIBCO-BRL) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Subconfluent cells were synchronized for 24 hours in serum-free media before the addition of growth factors. Growth factors were added as described above, except that 10% FBS was used instead of NCS for serum stimulation.

ECV cells were used in experiments in which PKCδ was overexpressed. The full-length cDNA encoding PKCδ was cloned into pCDNA3 mammalian expression vector (Invitrogen) using the EcoRI restriction endonuclease enzyme site. The construct was transfected into ECs using the lipofectin method (GIBCO-BRL). Cells that stably overexpressed either the vector alone or PKCδ were selected by resistance to neomycin.

Immunoblot Analysis

Cell lysates were prepared by addition of 2 mL of lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.57 mol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, and 4.2 μmol/L leupeptin) per 1×10^6 cells. The immunoblot was performed using an anti-PKCδ-specific monoclonal antibody (Transduction Laboratories) as previously described.

Northern Transfer Analysis

Subconfluent monolayers of ECs in 100-mm plates were treated with PBS twice and then treated with Trizol reagent (GIBCO-BRL). Twenty micrograms of total RNA extracted was subjected to electrophoresis on a 1.5% formaldehyde-agarose gel and transferred to a nylon-based transfer membrane (GeneScreen Plus) according to the manufacturer’s recommendation. The blot was hybridized with a random-primed cDNA probe at 65°C for 3 hours in a rapid Northern hybridization solution (Quick-Hyb, Stratagene, Inc, La Jolla, Calif). Blots were washed under high-stringency conditions and subjected to autoradiography.

Kinase Activity Assay

Subconfluent monolayers of ECs (in 100-mm plates) were treated with trypsin, counted, and then washed with PBS. HUVECs (4.0x10^5) or ECV cells (2.0x10^5) were resuspended in 1 mL of ice-cold lysis buffer for 10 minutes and were then homogenized by repeated aspiration through a 21-gauge needle. Cell debris was removed by centrifugation at 3500 rpm at 4°C for 15 minutes.

To determine the specific activity of PKCδ, the PKCδ was immunoprecipitated using an anti-PKCδ-specific monoclonal antibody (Transduction Laboratories) from the whole-cell lysate. The kinase assay was carried out according to the methods described previously. The reaction mixture did not contain additional calcium acetate for this assay. The presence of PKCδ was confirmed by immunoblotting with an anti-PKCδ antibody. PKC activity was normalized to the cell number and expressed as the percentage of PKC activity measured simultaneously in unstimulated ECs that were cultured in the absence of serum and growth factors for 2 days. In the study, PKCα specific activity was measured, cell lysates were immunoprecipitated with a polyclonal antibody against PKCα (Santa Cruz Biotechnology Inc), and the specific activity was measured with the method described above, except the reaction buffer contained 3 mmol/L calcium acetate.

RT-PCR of VEGF Receptors

RT-PCR was performed with the SuperScript One-Step RT-PCR System (GIBCO-BRL) according to the manufacturer’s instructions. A PCR cycle (94°C for 1 minute, 50°C for 1.5 minutes, and 72°C for 1 minute) was repeated for 35 cycles. To detect the VEGF receptor 1 (flt-1) and VEGF receptor 2 (KDR/flk-1), the following primers were used, respectively, as previously reported: sense primer 5’-CAGCGGCTTTTGTGGAAGACTCAC-3’ and antisense primer 5’-ACTTCTCCTGTCATCTTGGAC-3’ (corresponding to 735 bp), and sense primer 5’-CAAAATAAGTCGGAGAGG-3’ and antisense primer 5’-ATGACGATGGAAGATGCC-3’ (corresponding to 819 bp). As a positive control, mRNA of GAPDH was amplified by using sense primer 5’-TGAAGTCGAGTTCAAGCGTTG-3’ and antisense primer 5’-CATGTCGCCCATGACCTCCAC-3’ (corresponding to 983 bp).

Measurement of Total NO Release

The ECs were cultured in 6-well plates (Corning Glass Works). After subconfluence was achieved, ECs were synchronized for 24 hours as previously described, and then medium was replaced with medium without phenol red in the presence and absence of VEGF. After incubation in 30 minutes to 24 hours, ECs were washed twice with PBS and replaced with fresh medium. After a 30-minute incubation with fresh medium, this medium was collected and total NO was measured with a nitrate/nitrite colorimetric assay kit (Cayman Chemical) according to the manufacturer’s instruction. The absorbance at 540 nm was measured with a plate reader. The measured amount (nmol) was divided by 30 and normalized to the number of ECs in the well from which the medium was collected.

Cell Proliferation Analysis

EC growth was determined by counting the cells with a hemocytometer under ×50 magnification. Subconfluent cells were seeded at 2.0x10^4 per 16-mm plate in 1 mL of medium and synchronized for 24 hours before the addition of a growth factor. After the indicated periods, the ECs were washed with PBS, treated with trypsin, and suspended in medium for counting.

Endothelial Migration Assay

ECs were cultured on Corning 6-well plates in complete medium until confluent. After a 24-hour synchronization, the monolayer was wounded by scraping ~800 μm with a 200-μL pipette tip (Conti
nental Laboratory Products, Inc). The distance of the gap was measured as previously described.14

Statistical Analysis
All data are presented as mean±SE. Comparisons of the effects among the different growth factors, time points, and cell lines were performed by ANOVA. Multigroup comparison was carried out using Bonferroni-modified t tests. P<0.05 was accepted as statistically significant.

Results
PKCδ Specific Activity After Growth Factor Stimulation in HUVECs
In experiments to test whether the activities of PKCδ were changed by serum or growth factors in HUVECs, VEGF decreased PKCδ specific activity to 58.8±7.5% of the control value (n=9, P<0.05). Neither FGF-2 nor serum, on the other hand, influenced the PKCδ specific activity significantly (Figure 1A). Similar experiments were conducted in the presence of the NO synthase inhibitor L-NMMA in HUVECs. Suppression of PKCδ activity by VEGF was completely blocked by L-NMMA. PKCδ activity with FGF-2 or serum stimulation was not affected by NO inhibition (Figure 1B). Thus, the suppression of PKCδ activity by VEGF was completely blocked by L-NMMA. PKCδ activity with FGF-2 or serum stimulation was not affected by NO inhibition (Figure 1B). Thus, the suppression of PKCδ activity by NO synthase inhibitors was completely blocked by L-NMMA. PKCδ activity with FGF-2 or serum stimulation was not affected by NO inhibition (Figure 1B). Thus, the suppression of PKCδ activity by NO synthase inhibitors was completely blocked by L-NMMA. PKCδ activity with FGF-2 or serum stimulation was not affected by NO inhibition (Figure 1B). Thus, the suppression of PKCδ activity by NO synthase inhibitors was completely blocked by L-NMMA. PKCδ activity with FGF-2 or serum stimulation was not affected by NO inhibition (Figure 1B).

PKCδ Specific Activity After SNP Treatment
To assess whether an exogenous NO donor can suppress PKCδ specific activity, SNP at 500 μmol/L to 1 mmol/L was added to the medium and incubated for 24 hours. PKCδ specific activity of HUVECs decreased significantly with 1 mmol/L of SNP in HUVECs (84.5±2.0% of unstimulated cells, n=6, P<0.05). A significant decrease in PKCδ specific activity was seen also in ECV cells with 500 μmol/L (Figure 3). This result further supports the finding that release of NO decreases PKCδ activity.

PKCδ Protein and mRNA After Growth Factor Stimulation With VEGF Stimulation
Possibilities for the decrease in PKCδ enzymatic activity after VEGF stimulation include a VEGF-mediated reduction in the

![Figure 1. PKCδ specific activity after various growth factors in HUVECs in the absence of L-NMMA (A) and in the presence of L-NMMA (B). The activity is normalized to that of unstimulated ECs. Data are mean±SE of 4 to 9 separate experiments. *P<0.05 vs unstimulated control.](http://circres.ahajournals.org/)

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Time Course of NO Release and PKCδ Suppression With VEGF in HUVECs and ECV Cells
In HUVECs, PKCδ specific activity started to decline 8 hours after VEGF stimulation after a peak NO within 2 hours after VEGF stimulation. The suppression of PKCδ activity reached its maximum at ~16 hours of treatment (Figure 2A). A similar relation between PKCδ activity and NO release was seen in ECV cells (Figure 2B). In ECV cells, PKCδ activity seemed to decrease somewhat faster than that in HUVECs and reached a steady state at ~8 hours of treatment with VEGF. These results demonstrate that NO release peaks before the decrease in PKCδ activity with VEGF in both HUVECs and ECV cells and that PKCδ specific activity decreases significantly in 8 hours of treatment.

PKCδ Specific Activity After Growth Factor Stimulation With VEGF Stimulation
In experiments to test whether the activities of PKCδ were changed by serum or growth factors in HUVECs, VEGF increased PKCδ specific activity significantly after 24-hour stimulation (136.1±10.8% [n=4, P<0.05 versus unstimulated controls in HUVECs] and 123.3±5.5% [n=4, P<0.05 versus unstimulated controls in ECV cells]) as previously reported.17
levels of mRNA or protein corresponding to PKCδ, or posttranslational mechanisms. Immunoblot analysis failed to detect changes in the amount of PKCδ protein in HUVECs (Figure 4A) and ECV cells (Figure 4B). Similarly, Northern transfer analysis did not show a change in mRNA encoding PKCδ in ECs after stimulation with VEGF in either HUVECs (Figure 4A) or ECV cells (Figure 4B). Thus, the decrease in PKCδ activity after VEGF stimulation was not modulated by
EC wound healing assay. In Figure 6A, the NO synthase inhibitor L-NMMA reduced the VEGF-induced HUVEC migration to the level of that seen in unstimulated cells at 12 hours. L-NMMA did not significantly reduce migration mediated by either FGF-2 or serum (Figure 6B and 6C).

VEGF, FGF-2, and serum increased the number of HUVECs significantly (Figure 6D through 6F). The increase in HUVECs by VEGF was blocked by L-NMMA. The mitogenic effect of FGF-2 or serum, on the other hand, was not suppressed by L-NMMA (Figure 6E and 6F). Thus, HUVEC proliferation induced by VEGF, but not that induced by FGF-2 or serum, required the activation of NO synthase.

Response of ECs to PKC Downregulation by PMA
PMA (100 nmol/L) was added to media of HUVECs at 24 hours before migration and proliferation were measured to investigate whether suppression of total PKC effects on growth factor induced migration and proliferation of HUVECs. PMA downregulates total PKC activity to <8% in HUVECs treated for 16 hours.19 Migration induced by VEGF was significantly blunted (Figure 7A); however, HUVEC migration induced by FGF-2 or serum was not altered (Figure 7B and 7C).

The effect of total PKC downregulation on growth factor-induced HUVEC proliferation is shown in Figure 7D through 7F. Proliferation of HUVECs by VEGF, FGF-2, or serum was almost abolished. These findings suggest that the migratory responses of HUVECs to growth factors were less PKC dependent than were proliferation responses to growth factors, except for those that induced by VEGF.

Response of PKCδ-Overexpressing ECs to VEGF, FGF-2, and Serum
To determine whether VEGF-mediated PKCδ suppression was required for VEGF-induced EC migration and proliferation, we prevented such suppression by overexpression of PKCδ in stable clones of ECs. VEGF did not significantly increase either migration (Figure 8A) or proliferation (Figure 8D) of the PKCδ-overexpressing ECs, in contrast to the case of those ECs that overexpressed vector alone. PKCδ did not inhibit unstimulated EC migration or proliferation. Interestingly, PKCδ-overexpressing ECs, rather than showing inhibited migration and proliferation to FGF-2, actually demonstrated somewhat enhanced migration to this growth factor (Figure 8B) and no effect on serum-induced EC migration and proliferation (Figure 8C and 8F), which suggests that the prevention of proliferation and migration to VEGF was not a nonspecific effect of PKCδ overexpression on EC function. Thus, suppression of PKCδ activity appears to be required for VEGF-induced EC migration and proliferation, but not that induced by FGF-2 or serum.

Discussion
The major findings of this study are that the enzymatic activity of a specific PKC isoenzyme, PKCδ, is decreased by VEGF and that this decrease is mediated by the activation of NO synthase in ECs. This process does not require the KDR/flk-1 receptor for VEGF. Overexpression of PKCδ blunted the ability of VEGF to induce EC migration and proliferation, which suggests that the decrease in PKCδ
activity is necessary for VEGF to induce those events. Our study confirms and extends the recent observation\(^9\) that VEGF-induced EC migration and proliferation are blocked effectively by NO synthase inhibition. The present investigation suggests that NO works at a posttranslational level to decrease VEGF-induced EC migration and proliferation, at least in part by reducing PKC\(\delta\) activity.

The dependence of VEGF on this PKC\(\delta\)-NO mechanism is not shown by all EC mitogens; FGF-2–induced EC proliferation and migration were not influenced by either NO inhibition or PKC\(\delta\) overexpression in our study. The lack of effect on FGF-2 and serum provides a useful control as well; the ability of PKC\(\delta\) overexpression to prevent VEGF-induced EC migration and proliferation is unlikely to reflect a non-specific interference with the EC cell cycle or migratory mechanisms, because a similar effect after FGF-2 and serum would be expected under those circumstances. Our results demonstrate that ECs in which PKC\(\delta\) was overexpressed had responses to FGF-2 or serum that equaled or exceeded the responses of control ECs. Therefore, it appears that PKC\(\delta\) suppression was required for VEGF to induce EC migration and proliferation but not for FGF-2 or serum to induce similar

Figure 6. Effects of NO synthase inhibition by L-NMMA on HUVEC migration (A through C) and proliferation (D through F). \(\square\) denotes unstimulated HUVECs; \(\bigcirc\), HUVECs stimulated with growth factors, and \(\bullet\), HUVECs stimulated with growth factors in the presence of L-NMMA. \(*P < 0.05\) vs unstimulated controls; \(\S P < 0.05\) vs HUVECs treated with both growth factor and L-NMMA. Data are mean ± SE from 2 or 3 independent experiments performed in triplicate.
effects. Thus, the present study provides additional evidence that VEGF and FGF-2 use different intracellular mediators to effect EC functions linked to angiogenesis. Previously, 2 distinct pathways to angiogenesis were identified that used different integrin receptors for vitronectin, as follows: FGF-2 and tumor necrosis factor-α trigger a pathway that requires interaction with the integrin αvβ3; VEGF, on the other hand, uses a pathway that depends on integrin αvβ5.12 We speculate that the requirement for NO and PKCδ suppression for VEGF, but not for FGF-2, may reflect differing control mechanisms for these 2 vitronectin receptors. Our finding that enhanced EC function by FGF-2 or serum did not depend on either the activation of NO synthase or a reduction in PKCδ activity suggests that the downstream signals between VEGF and FGF-2 can be clearly separated and supports distinctive intracellular pathways for each growth factor.

PKCδ overexpression did not alter the serum-induced proliferation of ECV cells, which differed from the results obtained previously in rat microvascular ECs. Several variations in the cellular physiology between large vessel–derived ECs, such as HUVECs and microvascular ECs, have been reported. Such differences include surface adhesion molecule expression,21 prostanoid production,22 and cytokines such as interleukin-1 and tumor necrosis factor-α.23 Our finding
suggests that signaling of serum-induced EC proliferation may also differ between HUVECs and rat microvascular ECs. Also, it is possible that the response to PKC\(\delta\)-overexpressing ECs may be attenuated to certain growth factors, such as VEGF, and responses to another growth factor, such as FGF-2, are enhanced as a compensatory mechanism. Stimulation by serum reflects the combined effects of the multiple growth stimulants present in serum and thus may represent conflicting effects of individual growth factors.

Our results suggest that VEGF-induced EC migration and proliferation are totally dependent on NO synthase activation, in agreement with a recent study.\(^9\) The present study demonstrates that the suppression of PKC\(\delta\) activity after VEGF stimulation also depends on NO synthase activation. Exactly how reduction of PKC\(\delta\) activity facilitates VEGF-induced EC proliferation and migration is unknown. It cannot be ruled out that NO limits VEGF-induced function by means other than reducing PKC\(\delta\) activity, although it appears that such reduc-

Figure 8. Effects of PKC\(\delta\) overexpression on EC migration (A through C) and proliferation (D through F) in response to growth factors. Squares denote ECs in which the vector only was overexpressed without (□) or with (■) growth factor stimulation. Circles denote PKC\(\delta\)-transfected ECs without (○) or with growth factor stimulation (●). *\(P<0.05\) vs unstimulated ECs; #\(P<0.05\) vs PKC\(\delta\)-overexpressing ECs. Data are mean±SE of 6 separate experiments.
tion is necessary. NO has been shown to alter cell cycle progression induced by a number of agents. NO inhibits proliferation of vascular smooth muscle cells by inhibiting cyclin-dependent kinase 2, which is required for cell cycle progression, but whether this occurs via a reduction in PKCβ activity is not clear, nor is it known whether a similar or inverse relationship exists between NO and cyclin-dependent kinase 2 in ECs. Another possibility is that PKCβ affects EC function by altering events downstream of Ras. PKCβ activates Ras-dependent signal transduction; however, the inability of concomitant overexpression of Ras to alter the inhibitory effect of PKCβ in NIH3T3 cells suggests that additional effectors for PKCβ outside of the Ras-dependent pathway are important for the suppression of cell growth.

The precise mechanism of how VEGF decreases PKCβ activity via NO is not known. In murine peritoneal macrophages, lipopolysaccharide with interferon-γ results in a significant increase of NO release that is associated with suppression of mRNA encoding PKCβ. In our study, we found that activation of NO synthase was associated with a reduction in PKCβ specific activity after VEGF stimulation in ECs, but we did not observe changes in the level of either protein or mRNA encoding PKCβ, in contrast to the results in macrophages. Only the activity of the specific PKC isoenzyme was altered by VEGF and could be correlated with EC function, rather than transcription or translation of the PKC isoenzyme. Many PKC isoforms can be regulated by serine/threonine phosphorylation; thus, it is possible that cyclic GMP-dependent protein kinase, which is activated by NO and can regulate overall PKC activity, might be involved in reduction of PKCβ activity after VEGF stimulation. In addition, PKCβ has a novel feature, in that it can become phosphorylated on a tyrosine residue in the catalytic domain. One could speculate that the selective effect of VEGF on PKCβ activity might be related to its ability to alter tyrosine phosphorylation of this isoenzyme.

Nonspecific downregulation of all phorbol ester–sensitive isoforms of PKC blocked the induction of migration induced by VEGF and proliferation induced by VEGF, FGF-2, or serum in our study. These findings indicate that downregulation of some isoforms of PKC other than PKCβ inhibit the migratory and mitogenic effects of VEGF, in contrast to the enhancement of VEGF-induced migration and proliferation caused by downregulation of PKCδ. With VEGF stimulation, the activity of those yet-unidentified isoforms might be increased or unchanged. It is also possible that such isoforms might be involved in the proliferation induced by other growth factors such as FGF-2 or serum. Therefore, previous results that have been based on the use of nonspecific inhibitors or activators of PKC must be interpreted cautiously, because changes in the activity of individual PKC isoforms might be obscured.

In this study, the activity of PKC isoforms is assessed by direct enzymatic assay. In a previous report, VEGF upregulates PKC activity as shown by translocation of PKCα in HUVECs. Although translocation of PKCα by immunoblot analysis was shown, translocation of PKCδ did not occur. In our study, the enzymatic assay used to detect the decrease in PKCδ activity also showed the increase in PKCα in HUVECs and ECV cells stimulated with VEGF for 24 hours, which suggests that the enzymatic assay of individual PKC isoforms may reflect at least one aspect of PKC activation more sensitively than does translocation by immunoblotting.

Our results suggest that KDR/flk-1 receptor is not required for the decrease in PKCδ activity. When VEGF receptors were overexpressed in porcine ECs, which have no intrinsic VEGF receptors, the KDR/flk-1 receptor can mediate VEGF-induced proliferation. A recent report, however, suggests that flt-1 receptor mediates VEGF-induced NO release in both human trophoblasts and HUVECs. Taken together with our data, it would seem that the KDR/flk-1 receptor, although perhaps sufficient, is not necessary for the VEGF-induced response in ECs. It is also possible that a VEGF receptor as yet unidentified may mediate the VEGF-induced NO release.

In conclusion, we have demonstrated that VEGF-induced EC migration and proliferation depends on the reduction of PKCδ activity, an effect that requires the activation of NO synthase. The KDR/flk-1 receptor for VEGF is not required for this process. In contrast, the ability of FGF-2 or serum to induce these effects did not depend on either PKCδ suppression or NO synthase. These findings highlight the differences in signal transduction between different angiogenic factors, which suggests that a therapeutic strategy based on activation or inhibition of intracellular mediators could provide an additional control over the angiogenic response.

Acknowledgment

This study was supported by grants (HL 51043 and HL 47032) from the National Heart, Lung, and Blood Institute.

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_Circ Res._ 1999;85:247-256
doi: 10.1161/01.RES.85.3.247

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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