Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2008 May ; 28(5): 919–924. doi:10.1161/ATVBAHA.108.162842.

Differential regulation of VEGF signaling by PKCα and PKCε in endothelial cells

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Abstract

Objective—Vascular endothelial growth factor (VEGF) stimulates pro-angiogenic signal transduction and cell function in part through activation of protein kinase C (PKC). Our aim was to examine how individual isoforms of PKC affect VEGF action.

Methods and Results—Transfection of bovine aortic endothelial cells with small interfering RNA (siRNA) targeting either PKCα, δ, or ε caused a reduction in the cognate PKC protein by 76–89% without changing expression of non-targeted isoforms. Downregulation of PKCε abrogated VEGF-stimulated phosphorylation of Akt at Ser473 and eNOS at Ser1179 and decreased VEGF-stimulated NO synthase activity in intact cells. In contrast, PKCα knockdown increased Akt and eNOS phosphorylation, while PKCδ knockdown had no significant effect. PKCε knockdown also decreased VEGF-stimulated Erk1/2 phosphorylation and abolished VEGF-stimulated DNA synthesis. Consistent with an effect on several pathways of VEGF signaling, VEGF receptor-2 (VEGFR2) tyrosine phosphorylation and expression of VEGFR2 protein and mRNA was decreased by 81, 90, and 84%, respectively, during knockdown of PKCε, but increased during PKCα knockdown.

Conclusions—By regulating VEGFR2 expression and activation, PKCε expression is critical for activation of Akt and eNOS by VEGF and contributes to VEGF-stimulated Erk activation, whereas PKCα has opposite effects.

Introduction

In patients with ischemia in the myocardium and lower limbs, vascular endothelial growth factor (VEGF) is a central regulator of collateral artery formation through arteriogenesis or angiogenesis (1). Expression of VEGF and its receptors are decreased in the myocardium of animal models of type 2 diabetes and in diabetic patients (2,3), and VEGF, VEGF receptors, and capillary density are decreased in animal models of obesity-associated insulin resistance (2,4). This may contribute to a more sinister course of chronic ischemia in patients with diabetes compared with nondiabetic patients or worsen prognosis during recovery from acute myocardial infarction. Therefore, there is a compelling need to identify therapeutic targets that can enhance VEGF action in patients with myocardial or peripheral ischemia, particularly in patients with diabetes.

VEGF-stimulated angiogenesis is mediated by several pathways of intracellular signaling. VEGF phosphorylates Akt through activation of phosphatidylinositol 3-kinase and, in turn, Akt phosphorylates several substrates, several of which have been shown to be important for

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Disclosures
None.

VEGF-stimulated angiogenesis. Among them are forkhead transcription factors, including FoxO1 and FoxO3a (5–7) and glycogen synthase kinase-3β (GSK3β) (8). Furthermore, Akt activates eNOS through phosphorylation on S1179 (9,10). VEGF-stimulated cell proliferation and angiogenesis involve several other signaling pathways than Akt, among them activation of Erk1/2 (11,12).

It has long been known that phorbol esters, which are synthetic analogs of diacylglycerol and activators of protein kinase C (PKC), can promote angiogenesis (13). VEGF activates PKC, and PKC activity contributes to VEGF-stimulated endothelial cell proliferation (11,14). However, limited information is available about the isoforms of PKC that are important for VEGF action. In the current study, our aim was to characterize how individual PKC isoforms affect VEGF signaling and VEGF-stimulated cell function. Using RNA interference, we show here that PKCε is critical for Akt and eNOS activation induced by VEGF, and that VEGF-stimulated Erk phosphorylation and DNA synthesis are significantly dependent on PKCε expression. The influence of PKCε expression on both Akt and Erk signal transduction pathways, often presumed to have limited cross-talk, is explained by the finding that downregulation PKCε dramatically decreases tyrosine phosphorylation and expression of VEGF receptor-2/kinase insert domain-containing receptor (VEGFR2/KDR) protein and mRNA. In contrast, PKCα negatively regulates VEGF signaling and VEGFR2 expression and activation.

Methods

A detailed methods description is included in the Online Data Supplement (please see http://atvb.ahajournals.org). Experiments were performed in primary bovine aortic endothelial cells (BAEC). Cytosol and membrane fractions of cell lysate were prepared by ultracentrifugation. BAEC were transfected with 21-mer small interfering RNA (siRNA) complexed to Lipofectamine 2000 (Invitrogen, Carlsbad, CA). NO synthase activity was assayed in intact BAEC culture as previously described (15) with modifications. DNA synthesis was measured by labeling with 5-bromo-2′-deoxyuridine (BrdU) using a commercially available kit (Roche Applied Science, Penzberg, Germany). mRNA was measured by TaqMan real-time PCR (Applied Biosystems, Foster City, CA).

Results

Endothelial cells express several isoforms of PKC, including PKCα, β1, β2, δ, θ, ε, and ζ, depending on species and vascular bed (16,17). The known pro-angiogenic effects of phorbol ester is likely caused by activation of conventional and novel PKC isoforms (18). We therefore characterized the presence of conventional and novel PKCα, β1, β2, δ, θ, and ε in BAEC used for the present study. PKCα, δ, and ε were detected at the protein level in whole cell lysate (fig. 1). When bovine brain was used as a positive control, reverse transcription real-time PCR with primers targeting the sequence common to PKCβ1 and β2 amplified isolated RNA, and Western blotting detected PKCβ1 and β2 protein in tissue lysate (data not shown). However, PKCβ mRNA was not detected in RNA isolated from BAEC, and PKCβ1, β2 or θ protein was not detected in whole cell lysate (results not shown).

In order to specifically inhibit individual PKC isoforms, we designed siRNA targeting bovine PKCα, δ, and ε. Transfection of BAEC with these siRNAs downregulated PKCα, δ, and ε protein levels by 76±3, 89±3, and 76±2%, respectively ($p<0.005$, fig. 1). Importantly, each siRNA only changed the expression of the targeted isoform and not any of the two other isoforms (fig. 1). Unstimulated Akt phosphorylation was not changed by PKCα or δ knockdown, but decreased by 47±8% during PKCε knockdown ($p<0.001$, fig. 2a and b).

Unexpectedly, PKCε knockdown completely inhibited VEGF-stimulated Akt phosphorylation (1.7±0.1 -fold increase in the control siRNA condition, $p<0.001$; a non-significant 1.1±0.2 -fold increase during PKCε knockdown; fig. 2). In contrast, PKCα knockdown increased VEGF-stimulated Akt phosphorylation (1.7±0.1 and 2.3±0.4 -fold during control and PKCα knockdown conditions, respectively, $p<0.05$, fig. 2). PKCδ knockdown did not significantly change VEGF-stimulated Akt phosphorylation (fig. 2). Downregulation of PKC isoforms selectively affected VEGF signaling, because PKCα, δ, or ε knockdown did not change Akt phosphorylation stimulated by insulin (supplemental fig. I) or insulin-like growth factor (data not shown).

We next evaluated the effect of knockdown of individual PKC isoforms on VEGF-stimulated eNOS phosphorylation. Basal levels of eNOS Ser1179 phosphorylation was not significantly changed by knockdown of PKCα, δ, or ε (fig. 2a and b). However, VEGF-stimulated eNOS phosphorylation was increased during PKCα knockdown, unchanged during PKCδ knockdown, and completely inhibited by PKCε knockdown (5.2±0.5, 6.1±0.1, 4.2±0.3, and 1.7±0.3 -fold increase in the control, PKCα, PKCδ, and PKCε siRNA conditions, respectively; maximal levels as well as fold-increase of eNOS phosphorylation statistically different from the control condition during knockdown of PKCα and ε ($p<0.05$), but not δ; fig. 2). In turn, changes in VEGF-stimulated NO synthase activity, measured in intact cell cultures by conversion of ^{3}H-L-arginine to ^{3}H-L-citrulline, largely paralleled Akt and eNOS phosphorylation, although with less pronounced effects. Thus, PKCε knockdown decreased VEGF-stimulated NO synthase activity by 54±12% ($p<0.05$, fig. 3a). PKCα knockdown increased (by 14±12%) and PKCδ knockdown decreased (by 15±18%) VEGF-stimulated NOS activity, although these changes were not statistically significant (fig. 3a).

The involvement of PKCε in VEGF signaling was not confined to Akt/eNOS signaling. VEGF increased phosphorylation of Erk1/2 by 10±1.8 -fold after transfection of BAEC with control siRNA (at Thr203/Tyr205 in bovine Erk1/p44 MAPK, homologous to residues Thr202/Tyr204 in human Erk1) (fig. 2a and b). However, PKCε knockdown decreased both maximal levels and the increase in Erk1/2 phosphorylation stimulated by VEGF (2.7±0.9 -fold increase during PKCε knockdown, $p<0.05$ compared to the control siRNA condition, fig. 2). During PKCα knockdown, unstimulated Erk1/2 phosphorylation decreased to 55±17% of the level during the control siRNA condition ($p<0.05$, fig. 2). The maximal level of Erk phosphorylation stimulated by VEGF was not changed by PKCα knockdown, but the relative increase was greater due to the reduction in the unstimulated condition (19.8±2.1 -fold increase during PKCα knockdown). Unstimulated Erk1/2 phosphorylation was not significantly changed during PKCδ and ε knockdown, and VEGF-stimulated Erk1/2 phosphorylation was not changed by PKCδ knockdown (fig. 2).

To evaluate cellular function known to be regulated by Erk signaling, we measured DNA synthesis by BrdU incorporation. PKCα knockdown increased BrdU incorporation without VEGF stimulation to 239% of the control value ($p<0.001$, fig. 3b), but the unstimulated BrdU incorporation did not change during PKCδ or ε knockdown. VEGF increased BrdU incorporation by 34% in the control siRNA condition ($p<0.01$, fig. 3b) and by 14% and 39% during PKCα and δ knockdown ($p<0.05$, fig. 3b). In contrast, PKCε knockdown completely inhibited VEGF-stimulated BrdU incorporation (fig. 3b).

The findings that Akt and Erk signal transduction, which are usually considered to have limited cross-talk, were both affected by PKCε knockdown, pointed to a role for PKCε early in VEGF signal transduction. We therefore studied VEGFR2 activation, which is responsible for most known actions of VEGF, including activation of eNOS (1). PKCε knockdown decreased VEGF-stimulated VEGFR2 tyrosine phosphorylation by 81±6% compared to transfection with control siRNA ($p<0.001$, fig. 4), whereas PKCα and δ

knockdown increased tyrosine phosphorylation by 38±18 and 33±12%, respectively ($p<0.05$, fig. 4). These changes reflected changes in VEGFR2 protein expression. PKCε knockdown caused a dramatic reduction in VEGFR2 protein to only 10±2% of the control value ($p<0.001$, fig. 4). In contrast, PKCα and δ increased VEGFR2 protein by 31±6 and 46±2%, respectively ($p<0.05$, fig. 4). VEGF stimulation caused a 12±3% decrease in VEGFR2 protein in cells transfected with control siRNA, likely representing ligand-stimulated downregulation of the receptor ($p<0.05$, fig. 4). A VEGF-stimulated decreased in VEGFR2 protein (by 17±3%) was preserved during PKCα knockdown, but not detectable during PKCδ or ε knockdown.

In VEGFR2 immunoblots (fig. 4), the less intense, slower migrating (200 kDa) band may represent an intermediary, cytosolic form, whereas the more intense, upper (230 kDa) band may represent the fully N-glycosylated receptor expressed at the cell surface (19). Of note, PKCε knockdown reduced both bands, suggesting that it affects transcription, translation, or early post-translational modification rather than promoting internalization and degradation of the surface-bound receptor (20). Therefore, we measured VEGFR2 mRNA expression with real-time PCR during knockdown of PKCα, δ, and ε. A second siRNA targeting a different region of PKCε mRNA was used to minimize the possibility that the effect on VEGFR2 expression was due to an off-target effect. Compared with the control siRNA condition, VEGFR2 mRNA expression decreased by 84±7 and 83±6% during PKCε knockdown with the original and additional siRNA, respectively ($p<0.001$, fig. 5a). Thus, PKCε appears to regulate VEGFR2 at the mRNA level. PKCα knockdown had no significant effect, whereas PKCδ increased VEGFR2 mRNA by 16±1% ($p<0.001$, fig. 5a).

VEGF signal transduction during PKC knockdown could change because VEGFR2 expression results in a secondary change in VEGFR1/Fms-like tyrosine kinase-1 (Flt-1) expression. Indeed, PKCα and PKCδ knockdown increased VEGFR1 mRNA epxression to 240±20 and 178±1% of the control siRNA condition, respectively ($p<0.01$, fig. 5b). However, VEGFR1 mRNA did not change significantly during PKCε knockdown with either of the two siRNAs (fig. 5b). We could not detect VEGFR1 protein or VEGFR1-mediated signal transduction by Western blotting in these cells. VEGFR1 protein was not detectable in whole cell lysate (using two different commercially available antibodies) and VEGFR1 or phosphotyrosine was not detectable in immunoprecipitates with VEGFR1 antibody with or without stimulation with VEGF; furthermore, placental growth factor, which activates VEGFR1 but not VEGFR2, did not increase phosphorylation of eNOS, Akt, Erk, or p38 (data not shown). Thus, VEGFR1 protein is likely expressed at a very low level compared to VEGFR2 in BAEC. Changes seen in VEGF signal transduction during PKCα and δ knockdown could be influenced by upregulation of VEGFR1, but there is no indication that changes in VEGF signaling during PKCε knockdown can be attributed to changes in VEGFR1 mRNA expression.

Discussion

It is well-recognized that PKC activation is pro-angiogenic and that PKC mediates several actions of VEGF, including activation of eNOS. However, the involvement of specific isoforms of PKC in VEGF action has been insufficiently described. In the current study, using siRNA-mediated specific downregulation of PKC isoforms, we have demonstrated for the first time that expression of PKCε is critical for VEGF-stimulated phosphorylation of Akt and eNOS and for catalytic activity of NO synthase in endothelial cells. Furthermore, PKCε was shown to contribute to VEGF-stimulated Erk phosphorylation and DNA synthesis. In contrast, experiments with RNA interference indicated that PKCα inhibited VEGF-stimulated Akt and eNOS phosphorylation and negatively regulated DNA synthesis. These changes in signal transduction and cell function can be explained by the finding that

VEGFR2 expression and activation appears to be critically dependent on PKCε, but inhibited by PKCα. Importantly, PKCα and ε downregulation selectively affected VEGF signaling, as insulin-stimulated Akt phosphorylation was unaffected by PKC knockdown.

Although PKCε was previously shown to translocate from the cytosol to the membrane fraction in human umbilical vein endothelial cells during VEGF stimulation (11), a role for PKCε in regulating VEGFR2 expression or downstream VEGF signaling has not been reported before. VEGFR2 downregulation during PKCε knockdown could be regulated at many different levels, including regulation of transcription (21,22) or mRNA stability, or through internalization and degradation of VEGFR2 protein after ubiquitinylation (23–25). For example, activating protein-1 (AP-1), nuclear factor-κB (NF-κB), and Sp1 have been implicated in regulation of VEGFR2 expression, and PKCε has been shown to regulate all of these transcription factors during hypoxia (26,27). Regulation of VEGFR2 at the level of transcription or mRNA stability seem likely because PKCε knockdown caused a large reduction in VEGFR2 mRNA, and because a decreased intensity was seen in the 230 kDa-band on VEGFR2 immunoblots and in a 200 kDa-isoform which may represent a cytosolic, immature form of VEGFR2 (fig. 4) (19). Studies are ongoing in our laboratory to test these possibilities.

It is likely that PKCε has significant effects *in vivo* on vascular function. In PKCε knockout mice, chronic hypoxia was reported to result in excessive pulmonary hypertension and right ventricular hypertrophy compared to wild-type mice (30). Furthermore, PKCε knockout mice lacked the induction of eNOS gene expression observed in wild-type mice, suggesting a role for PKCε in regulating eNOS expression (30). In the current study, although PKCε knockdown caused a dramatic reduction in VEGFR2 mRNA and protein expression and near-complete inhibition of VEGF-stimulated Akt and eNOS phosphorylation, NO synthase activity and Erk phosphorylation were only moderately affected. However, certain signaling pathways and biological actions of VEGF may be partially preserved despite substantial reduction of VEGFR2 expression. This is suggested by the fact that heterozygous knockout of VEGFR2 are phenotypically normal, whereas homozygous deletion of the VEGFR2 gene in mice is embryonically lethal (31).

Our current data demonstrating that PKCα knockdown increased VEGF-stimulated Erk phosphorylation and DNA synthesis are consistent with a previous publication from our group showing that downregulation of PKCα protein levels with antisense oligonucleotides increased the VEGF-stimulated growth of BAEC (14). This effect may be specific for VEGF action. Thus, PKCα had the opposite effect on signal transduction stimulated by fibroblast growth factor-2 (FGF2) in a previous study (33), where FGF2-stimulated eNOS Ser1179 phosphorylation increased during PKCα overexpression and decreased after PKCα downregulation (33). We previously reported that adenovirus-mediated expression of PKCβ1 and β2 inhibited VEGF-stimulated Akt activation in endothelial cells (32). It is likely that PKCβ isoforms can inhibit VEGF-stimulated Akt activation if they are present at certain expression levels, but in the current study the BAEC used did not contain detectable levels of the β isoforms.

PKCδ knockdown increased expression and VEGF-stimulated tyrosine phosphorylation of VEGFR2, but did not significantly affect downstream signaling in the form of phosphorylation of Akt, eNOS, or Erk, or increase in NO synthase activity or DNA synthesis. However, PKCδ knockdown inhibited VEGF-stimulated reduction of VEGFR2 protein expression. A previous publication has shown that PMA treatment of endothelial cells causes downregulation of VEGFR2, and that the receptor is internalized and degraded after phosphorylation of serine 1188 and 1191 in its carboxyterminus (28). It is possible that PKCδ is necessary for this process. As internalization of the receptor has been shown to be

necessary for full effects of VEGF stimulation (29), PKCδ knockdown may have complex effects on VEGFR2 expression and downstream signaling, which should be characterized in future studies.

In conclusion, this study has documented that PKCε expression is critical for VEGF-stimulated phosphorylation and activation of Akt and eNOS, and that VEGF-stimulated Erk phosphorylation and DNA synthesis are significantly dependent on PKCε expression. In contrast, PKCα has inhibitory effects on VEGF-stimulated phosporylation of Akt and eNOS and on DNA synthesis. These effects can be explained by a critical role for PKCε in maintaining VEGFR2 gene expression, and a negative modulation of VEGFR2 expression by PKCα. These findings have potential implications for prevention and treatment of disease, because enhancing PKCε activity may be of use in promoting VEGF-stimulated angiogenesis in patients with ischemia in the myocardium or lower limbs, while inhibiting PKCε may find use in preventing diabetic retinopathy or tumor angiogenesis.

Acknowledgments

Sources of Funding

The Danish Medical Research Council (grant 22-01-0498), the Danish Heart Foundation (grant 01-2-2-79-22946) and a Mary K. Iacocca Research Fellowship supported C.R-M. This study was also supported by NIH grant NIDDK R01 DK53105 to G.L.K.

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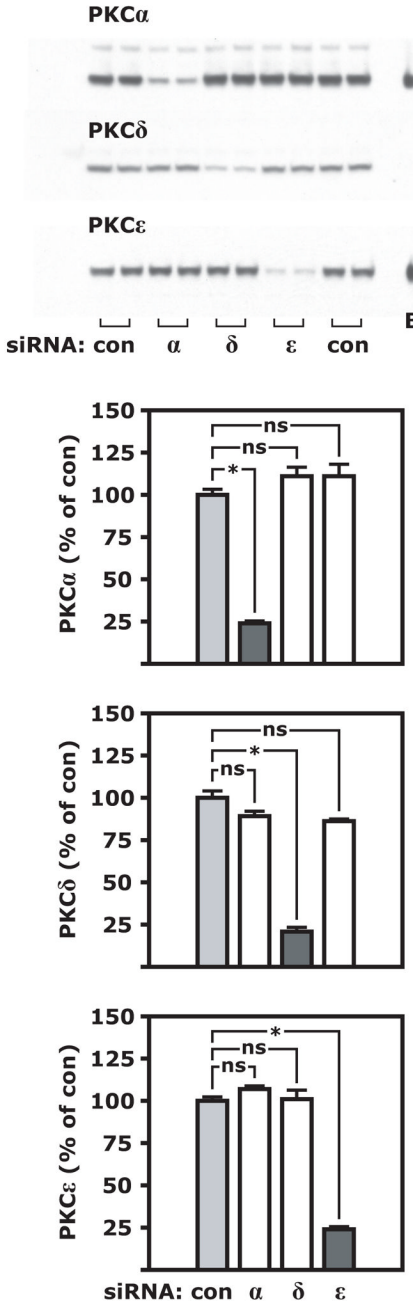
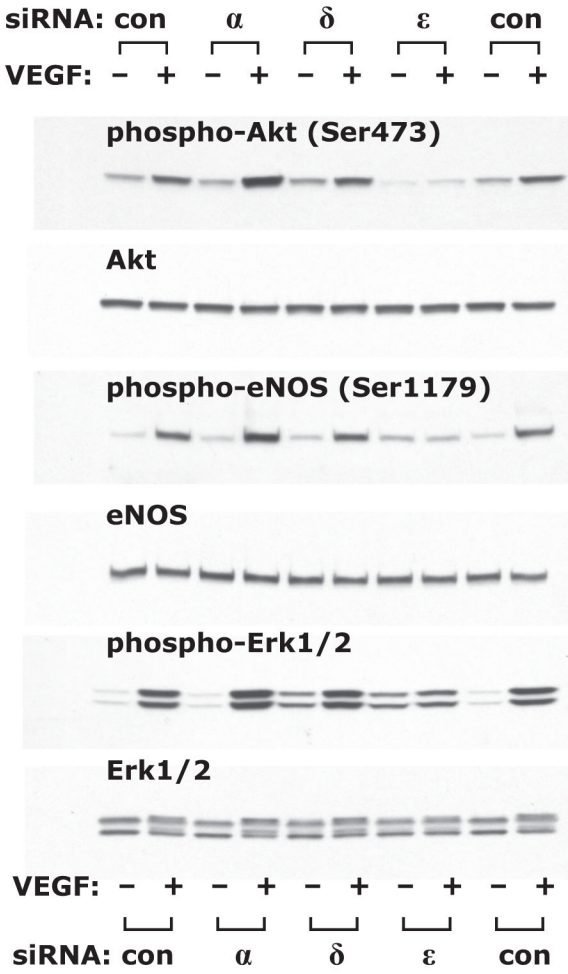


Figure 1. siRNA-mediated knockdown of PKC isoforms

BAEC were transfected with siRNA targeting a gene not present in mammalian cells (“con”) or targeting PKCα (“α”), PKCδ (“δ”) or PKCε (“ε”). Cells were used 3 days after transfection, including 16 hours of serum starvation. In the representative immunoblots, shown above the mean data from three independent experiments, control conditions are loaded on both the left and right side of the gel. Abbrevations: “B”, bovine brain lysate, used as a positive control; “M”, molecular weight marker.



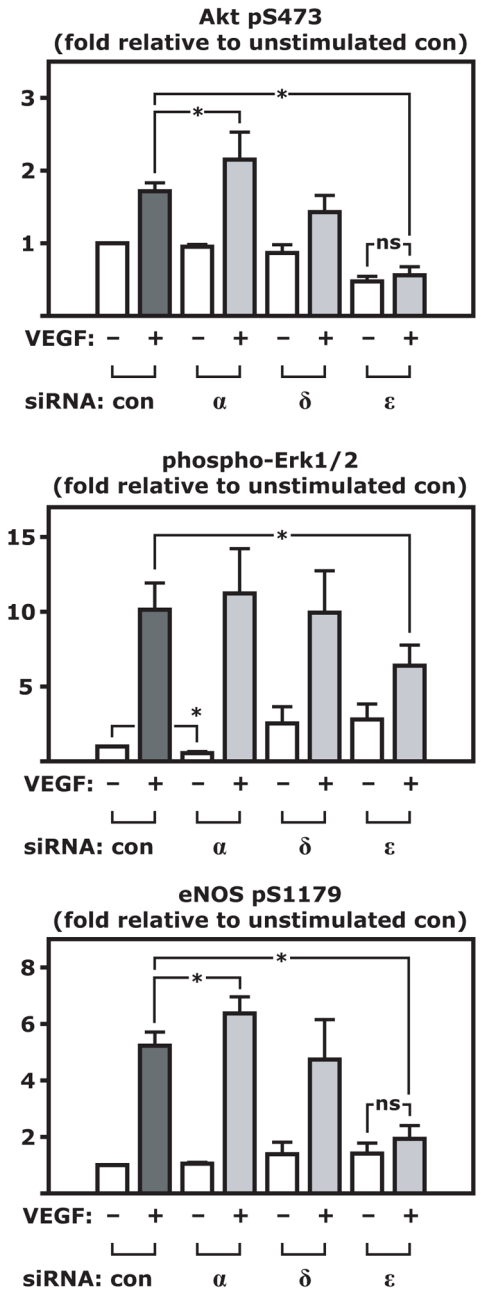


Figure 2. VEGF-stimulated phosphorylation of Akt, eNOS and Erk1/2 during siRNA-mediated knockdown of PKC isoforms

BAEC were transfected with siRNA, grown, and serum-starved as described in figure 1. Cultures were then stimulated with VEGF (1 nM, 5 minutes). *a.* In the representative immunoblots, control conditions are loaded on both the left and right side of the gel. *b.* In the graph, mean data from 3–5 independent experiments are based on densitometry of the phospho-protein normalized to the control condition ("*", $p<0.05$). Abbrevations: "Akt pS473", Akt phosphorylated on Ser473; "eNOS pS1179", eNOS phosphorylated on Ser1179; "p-Erk1/2", Erk1 phosphorylated on Thr203 and Tyr205 and Erk2 phosphorylated on Thr185 and Tyr187.

Fig. 3a

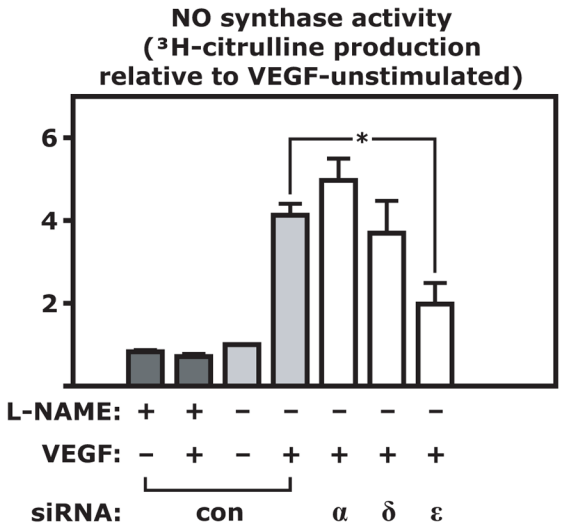


Fig. 3b

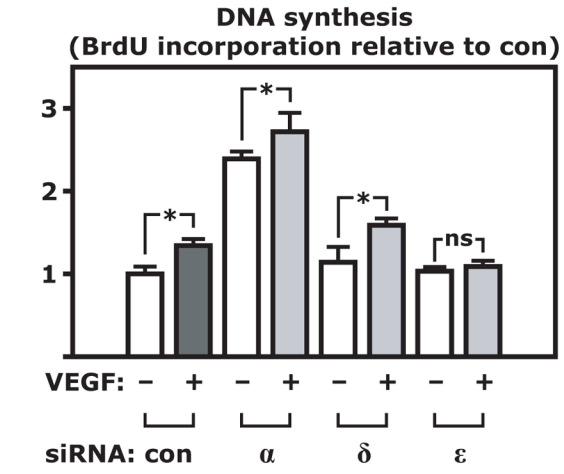


Figure 3. VEGF-stimulated NO synthase activity in intact cells and VEGF-stimulated DNA synthesis during siRNA-mediated knockdown of PKC isoforms

a. Cultures were prepared as described in figure 1, then starved of L-arginine for 3 hours. L-NAME was added for the last 30 minutes, as indicated, before incubation with ^{3}H-L-arginine and VEGF (1 nM) for 10 minutes. NO synthase activity was measured as the radioactivity of ^{3}H-L-citrulline in the cell lysate (n=3; "*", $p<0.05$). *b.* Two days after transfection with siRNA, equal numbers of BAEC were plated in each well of a 96-well plate in serum-free media with or without VEGF (1 nM). Cultures were labeled with BrdU for the last 8 hours of a total of 24 hours of VEGF stimulation and BrdU incorporation was measured by ELISA (n=5; "*", $p<0.05$).

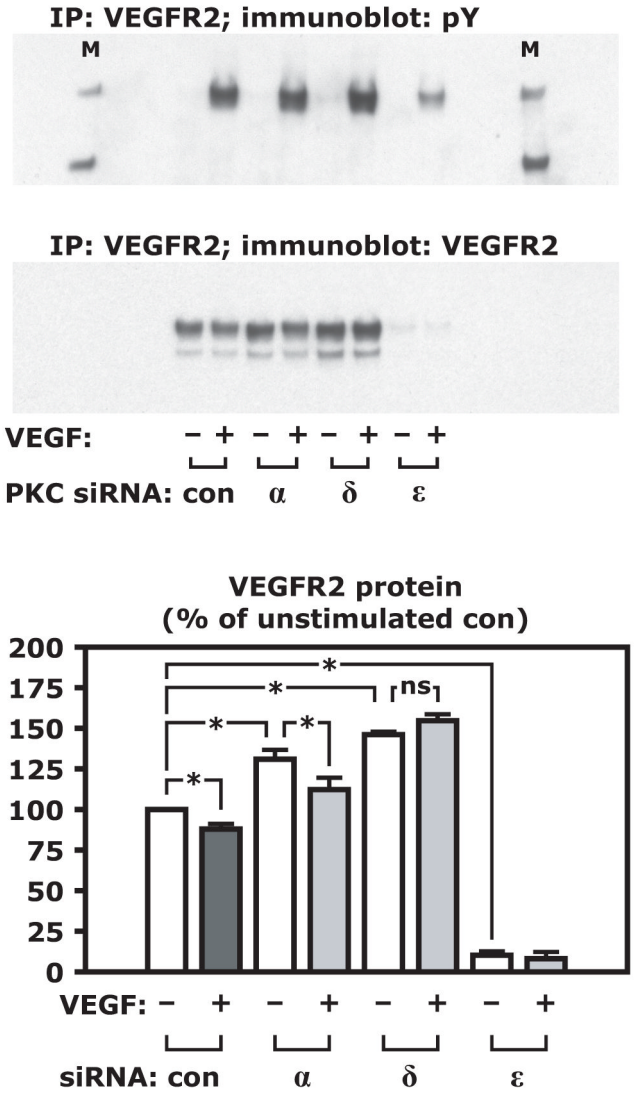


Figure 4. Tyrosine phosphorylation and protein expression of VEGFR2 in BAEC during siRNA-mediated knockdown of PKC isoforms

BAEC were transfected with siRNA, grown, and serum-starved as described in figure 1, then stimulated with VEGF (1 nM, 5 minutes). Immunoprecipitation was performed with a VEGFR2 antibody followed by immunoblotting with a phospho-tyrosine antibody or VEGFR2 antibody. Representative immunoblots are shown above a graph of mean values from 4–7 independent experiments based on densitometry of VEGFR2 immunoblots normalized to the control condition ("*", $p<0.05$).

Fig. 5a

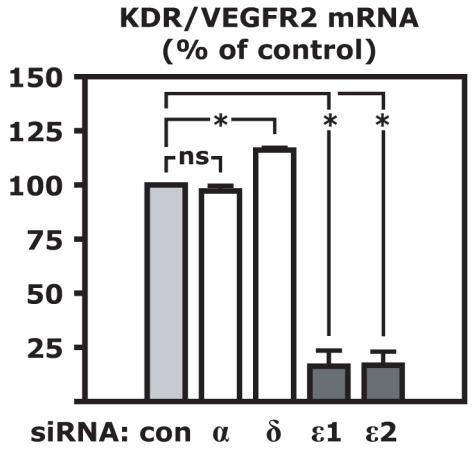


Fig. 5b

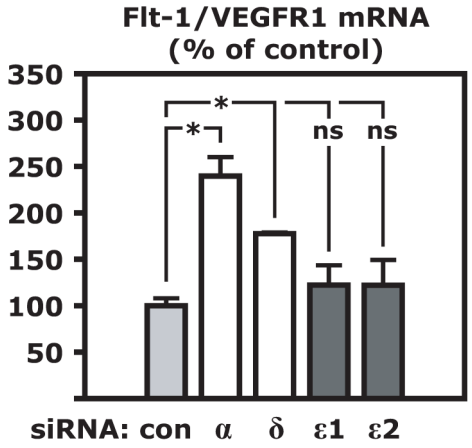


Figure 5. mRNA expression VEGFR2 and VEGFR1 in BAEC during siRNA-mediated knockdown of PKC isoforms

BAEC were transfected with siRNA, including the PKCε siRNA used in the previous experiments (abbreviated "ε1" here) and a second PKCε ("ε2"), grown, and serum-starved as described in figure 1. VEGFR2 (*a*) or VEGFR1 (*b*) mRNA was measured in cell lysate by real-time PCR (n=3; "*", $p<0.05$).