A Protein Kinase Ce-Anti-apoptotic Kinase Signaling Complex Protects Human Vascular Endothelial Cells against Apoptosis through Induction of Bcl-2

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Endothelial cell apoptosis is associated with vascular injury and predisposes to atherogenesis. Endothelial cells express anti-apoptotic genes including Bcl-2, Bcl-XL and survivin, which also contribute to angiogenesis and vascular remodeling. We report a central role for protein kinase Ce (PKCe) in the regulation of Bcl-2 expression and cytoprotection of human vascular endothelium against apoptosis. Using myristoylated inhibitory peptides, a predominant role for PKCe in vascular endothelial growth factor-mediated endothelial resistance to apoptosis was revealed. Immunoblotting of endothelial cells infected with an adenovirus expressing a constitutively active form of PKCe (Adv-PKCe-CA) or control Adv-β-galactosidase demonstrated a 3-fold, PKCe-dependent increase in Bcl-2 expression, with no significant change in Bcl-XL, Bad, Bak, or Bax. The induction of Bcl-2 inhibited apoptosis induced by serum starvation or etoposide, and PKCe activation attenuated etoposide-induced caspase-3 cleavage. The functional role of Bcl-2 was confirmed through overexpression of constitutively active PKCe, suggesting PKCe acts downstream of phosphoinositide 3-kinase. Co-immunoprecipitation studies demonstrated a physical interaction between PKCe and Akt, which resulted in formation of a signaling complex, leading to optimal induction of Bcl-2. This study reveals a pivotal role for PKCe in endothelial cell cytoprotection against apoptosis. We demonstrate that PKCe forms a signaling complex and acts co-operatively with Akt to protect human vascular endothelial cells against apoptosis through induction of the anti-apoptotic protein Bcl-2 and inhibition of caspase-3 cleavage.

Experimental models suggest that vascular endothelial injury is the earliest detectable event in atherogenesis and that chronic endothelial dysfunction precedes structural lesions (1). Endothelial dysfunction is a feature of chronic renal disease, post-transplant vasculopathy (2), systemic lupus erythematosus, and inflammatory rheumatic diseases (3) and represents an independent risk factor for accelerated atherosclerosis. Endothelial cell (EC) apoptosis occurs preferentially at sites predisposed to atherosclerosis, where denudation of vascular endothelium enhances the risk of plaque development and local thrombosis (4). Moreover, the observation that aging and exposure to oxidized low density lipoprotein or reactive oxygen species increases EC apoptosis implies a role in the initiation of atherosclerosis (5). Apoptotic EC are themselves pro-thrombotic and release microparticles that may induce EC dysfunction (6) and precipitate thrombosis (7). Although considerable progress has been made in identifying the pro-inflammatory pathways involved and in the management of established atherosclerosis, development of disease prevention strategies requires detailed understanding of endogenous mechanisms of endothelial cytoprotection.

Vascular endothelial growth factor (VEGF) represents a family of multifunctional glycoproteins, which in addition to their fundamental role in vasculogenesis and angiogenesis, control endothelial homeostasis through the regulation of survival signals (8). The cytoprotective actions of VEGF-A include induction of the anti-apoptotic genes Bcl-2 and A1 (9). In addition, VEGF increases endothelial nitric oxide (NO) biosynthesis (10, 11), induces expression of the cytoprotective enzyme heme oxygenase-1 (12), contributes to the maintenance of an anti-thrombotic endothelial surface through release of prostacyclin (10), and enhances protection against complement-mediated injury (13). These mechanisms may contribute to the cytoprotective effects of VEGF.

The abbreviations used are: EC, endothelial cell(s); VEGF, vascular endothelial growth factor; PKC, protein kinase C; PI-3K, phosphoinositide 3-kinase; PDBu, phorbol 12,13-dibutyrate; m.o.i., multiplicity of infection; ifu, infectious units; FBS, fetal bovine serum; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; ICAM, intercellular adhesion molecule 1; vWF, von Willebrand factor; hTERT, human telomerase reverse transcriptase; HVEC, human umbilical vein endothelial cell(s); ANG, angiotensin; eNOS, endothelial nitric oxide synthase; INT333, 1-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate.
VEGF in vivo (14), and their importance may be reflected in the side effects associated with the anti-VEGF-A monoclonal Ab bevacizumab therapy, including hypertension and thrombosis (15).

Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases, divided on the basis of their structure and response to phosphatidylserine, calcium, and diacylglycerol, into classical (cPKCα, -βI, -βII, -γ), novel (nPKCδ, -ε, -η, -θ), and atypical (aPKCζ and λ/ι) isozymes, with PKCζ/ι forming a distinct fourth group (16). The presence of multiple, highly conserved PKC isozymes suggests they have distinct roles, a hypothesis supported by emerging data from the study of PKC isozyme-deficient mice, which while revealing essential functions for individual isozymes, also suggests the presence of functional redundancy (for review, see Ref. 17).

PKC isozymes demonstrate a cell-type and stimulus-specific influence on apoptosis, with classical isozymes PKCα and βII/ΙΙI reported to be both pro- and anti-apoptotic and the atypical isozymes PKCζ and λ/ι predominantly anti-apoptotic (for review, see Ref. 18). The novel isozymes PKCδ and PKCe typically exert opposite effects (19, 20). PKCδ has been implicated in the initiation of apoptosis (19) at the level of the mitochondria (21) and in its amplification through interactions with caspase-3 (18, 22). In contrast, PKCe is an important cell survival factor and may act as an oncogene (23). PKCe is anti-apoptotic, promoting survival of interleukin-3-dependent human myeloid (24), Jurkat (25), and glioma cell lines (26). Moreover, expression of PKCe correlates with resistance to chemotherapy and metastasis in prostate and breast carcinomas (27, 28). In vivo studies have demonstrated that during ischemic preconditioning, activation of PKCe in cardiomyocytes protects against apoptosis (29, 30), whereas targeted disruption of PKCe inhibits the beneficial effect of preconditioning (31, 32).

Despite its potential, the role of PKCe in EC survival and resistance to vascular injury remains relatively unexplored. VEGF activates PKCε in vascular EC (33, 34), and using the complement regulatory protein decay-accelerating factor as a VEGF target gene, we have previously identified PKCe as a regulator of EC resistance to complement-mediated injury (35). Herein we have investigated the hypothesis that PKCe plays a pivotal role in the regulation of VEGF-activated effector mechanisms against vascular endothelial injury. We demonstrate that PKCe acts downstream of phosphoinositide 3-kinase (PI-3K) and forms a signaling complex with Akt, acting co-dependently to protect primary human vascular EC against apoptosis through induction of the anti-apoptotic protein Bcl-2 and inhibition of caspase-3 cleavage.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Antibodies used were: anti-PKCe from Santa Cruz Biotechnology (Santa Cruz, CA), anti-ICAM-2 from BD Biosciences, and anti-phospho-PKCe (Ser729) from Upstate Ltd. (Dundee, UK). Anti-Akt, anti- phospho-Akt (Ser473), and antibodies against Bcl-2, Bcl-X1, Bad, Bak, and Bax were all from Cell Signaling, (Beverly, MA). Phorbol 12,13-dibutyrate (PDBu) was from Sigma Aldrich. Akt phosphorylation inhibitor IV and etoposide were from Merck, and LY294002 was from BIOMOL (Plymouth Meeting, PA). HA-14-1 was from Maybridge Chemical Co. (Tintagel, Cornwall, UK). Recombinant human VEGF-A (referred to as VEGF) was purchased from PeproTech EC Ltd (London, UK). Z-VAD-FMK and the Z-FA-FMK negative control peptide were from BD Biosciences. Myristoylated (myr) PKC peptide inhibitors (myr-]<PKC> (myr-Arg-Phe-Ala-Lys-Gly-Ala-Leu Arg-Gln-Lys-Asn-Val) and PKCe V1–2 (myr-Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr) were from Promega (Southampton, UK) and BIOMOL, respectively. Myr-PKCe (myr-Leu-His-Gln-Arg-Gly-Ala-Ile-Lys-Gln-Ala-Lys-Val-His-His-Lys-Cys) and myr-PKCζ (myr-Ser-Ile-Tyr-Arg-Gly-Ala-Arg-Trp-Arg-Lys-Leu) were from Merck. In all experiments EC monolayers were treated with appropriate vehicle controls.

**Cell Culture**—Human umbilical vein ECs (HUCVEC) were isolated and cultured as previously described (36). The use of human EC was approved by Hammersmith Hospitals Research Ethics Committee (Ref no. 06/Q0406/21).

**Adenoviral Infection**—Generation of adenoviral expression vectors for dominant-negative (DN) and constitutively active (CA) PKC isozymes and Akt has been described previously (19, 37). Adenoviruses were amplified in human embryonic kidney 293A cells, purified, and titrated as previously described (35). HUVEC were infected by incubation with adenovirus in serum-free M199 for 2 h at 37 °C. The media were replaced with M199, 10% FBS, and HUCVEC were incubated overnight before experimentation. Optimal multiplicity of infection (m.o.i.), expressed as infectious units (ifu) per cell, for each adenovirus was determined by Western blotting.

**Flow Cytometry**—Flow cytometry was performed as previously described (36). The results are expressed as the relative fluorescent intensity, representing mean fluorescent intensity (MFI) with test monoclonal Ab divided by the MFI using an isotype-matched irrelevant mAb. Cell viability was assessed by examination of EC monolayers using phase contrast microscopy, cell counting, and estimation of trypan blue exclusion. For intracellular flow cytometry, EC were fixed in 2% formaldehyde and permeabilized in 90% methanol. Primary antibody was added in phosphate-buffered saline, 0.5% bovine serum albumin and detected with an appropriate fluorescein isothiocyanate-labeled secondary antibody.

**Quantitative Real-time PCR**—Quantitative RT-PCR was carried out using an iCycler (Bio-Rad). DNase-1-digested total RNA (1 μg) was reverse-transcribed using 1 μl oligo-dT and Superscript reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. cDNA was amplified in a 25-μl reaction containing 5 μl of cDNA template, 12.5 μl of iSYBR supermix (Bio-Rad), and 0.5 μl sense and 0.5 μl antisense gene-specific primers. The volume was adjusted to 25 μl with double-distilled H2O. The primer sequences used were as follows: Bcl-2 forward 5′-GTCATGGGTGGAGAGGCT-3′, reverse 5′-GCCGTACAGTTCCACAAAGG-3′; tubulin forward 5′-CTGTTTGGCTCAGGTCTTCTT-3′; tubulin reverse 5′-TCTGATGATGGGACAGGTGA-3′. The cycling parameters were 3 min at 95 °C followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 20 s.

**Western Blotting**— Immunoblotting was performed as described (35). Membrane fractions were isolated using the ProteoExtract kit (Merck) according to the manufacturer’s protocol. Immunoblots were probed with primary Abs overnight at...
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4 °C followed by appropriate secondary reagents for 1 h at room temperature. Immunoblots were developed with a chemiluminescence substrate (Amersham Biosciences). To ensure equivalent sample loading, protein content was determined using the Bio-Rad D_{2} protein assay (Bio-Rad), and membranes were stripped and re-probed with a control antibody. Integrated density values were obtained with an Alpha Innotech Chemi-Imager 5500 (Alpha Innotech, San Leandro, CA).

Cell Survival, Proliferation, and Apoptosis Assays—EC apoptosis was induced by serum starvation (0.1% FBS for 24 h) or treatment with etoposide (50–150 μM). Analysis of EC survival and proliferation was performed using the Promega Cell-Titer96 [3-(4,5-dimethylthiazol-2-yl)]-5-(3-carboxymethoxyphenyl)]-2-(4-sulfophenyl)]-2H-tetrazolium] (MTS) assay according to the manufacturer’s instructions. The assay was quantified by recording absorbance (490 nm) using a 96-well enzyme-linked immunosorbent assay plate reader (Dynex Technologies, Worthing, UK). Percent cell death was calculated as follows: % cell death = 100 − (absorbance test/absorbance control × 100), where control represents EC cultured in M199, 20% FBS alone for the duration of the experiment. For the assessment of EC apoptosis, cell culture supernatant was collected, and apoptotic EC were pelleted by centrifugation and added to EC harvested by trypsinization. EC were fixed in 70% ethanol, washed and resuspended in phosphate-buffered saline, 50 mM EDTA, 0.1% Triton X-100, 20 units/ml RNase, and 50 μg/ml propidium iodide before analysis by flow cytometry. Apoptotic cells were identified as those falling within the sub-G_{1} gate and expressed as a percentage of total cells. Apoptosis was also quantified by nuclear staining of total cells. Apoptosis was also quantified by nuclear staining of total cells. Apoptosis was also quantified by nuclear staining of total cells.

Immunoprecipitation—HUVEC were lysed in 1% Nonidet P40, 20 mM Tris (pH 8), 130 mM NaCl, 10 mM NaF, 1 mM dithiothreitol, 0.1 mM Na_{3}V_{0}4, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5% protease inhibitor (Sigma). After centrifugation at 14,000 rpm, supernatants were collected, and apoptotic EC were pelleted by centrifugation and added to EC harvested by trypsinization. EC were fixed in 70% ethanol, washed and resuspended in phosphate-buffered saline, 50 mM EDTA, 0.1% Triton X-100, 20 units/ml RNase, and 50 μg/ml propidium iodide before analysis by flow cytometry. Apoptotic cells were identified as those falling within the sub-G_{1} gate and expressed as a percentage of total cells. Apoptosis was also quantified by nuclear staining of total cells.

RESULTS

PKC Activation Protects EC against Serum Starvation—The PKC family of isozymes plays an important role in the regulation of cell survival. Using a model of serum starvation in which HUVEC were cultured for 24 h in medium containing 0.1% FBS, we observed an increase in cell death of 50% when compared with the same cells cultured in 20% FBS. Pretreatment of HUVEC with PKC agonists PDBu and VEGF significantly reduced serum starvation-induced cell death (Fig. 1A). The role of PKC in this response was suggested by abrogation of both PDBu- and VEGF-mediated cytoprotection in the presence of GF109203X, an inhibitor of classical and novel PKC isozymes (38). Of note, trypan blue exclusion demonstrated 5–10% cell death in control EC cultured in 20% FBS (not shown). Thus, although VEGF and PDBu are able to significantly reduce the deleterious effects of serum starvation on EC, they do not protect completely.

We have previously reported that VEGF-induced EC protection against complement activation is dependent upon PKCa and PKCe (35). Recognizing that in addition to PKC isozymes, GF109203X may inhibit MAPKAP-K1/RSK and MSK1 (39), we used cell-permeable myristoylated inhibitory peptides against PKCa and PKCe to investigate the role of these isozymes in protection against apoptosis. Blockade of PKCe increased the susceptibility of HUVEC to serum starvation, whereas the PKCa inhibitory peptide had no significant effect (Fig. 1B). Likewise, inhibition of PKCe, but not PKCa, significantly reversed the protective effect of both VEGF (Fig. 1C) and PDBu (not shown), suggesting that activation of PKCe exerts an anti-apoptotic effect in human vascular EC. As with myr-PKCa, myr-PKCe and myr-PKCG had no inhibitory effect on VEGF-mediated protection against serum starvation (not shown).

PKCe Overexpression in EC Protects against Apoptosis—To assess the effect of PKCe overexpression on EC survival, HUVEC were cultured for 24 h in endothelial cell growth factor-free HUVEC medium containing either 20 or 0.1% FBS. Serum starvation resulted in 50% cell death (Fig. 2A). Infection of EC with CA-PKCe Adv 24 h before serum withdrawal demonstrated a dose-dependent cytoprotective effect, maximal at an m.o.i. of 20–50 (Fig. 2A). In contrast, the control β-galactosidase Adv was not protective, although at an m.o.i. of 10 and 20 β-galactosidase expression reduced serum starvation-induced cell death to a level that did not quite reach significance (Fig. 2A). This response may reflect a previously reported pro-survival action of adenosine transfection per se (40). An MTS cell proliferation and survival assay was used to exclude a role for EC proliferation in the protective effect observed after PKCe overexpression. Subconfluent HUVEC were plated in endothelial cell growth factor-free HUVEC medium containing 5% FBS and subsequently infected with CA-PKCe Adv (m.o.i. 10–25), β-galactosidase Adv, and GFP control Adv (m.o.i. 25). When compared with EC cultures in HUVEC medium alone, endothelial cell growth factor treatment increased the total EC count over 24 h, whereas no significant change was seen after infection with CA-PKCe Adv or control Adv (Fig. 2B). These data suggest that EC proliferation plays no significant part in PKCe-mediated cytoprotection against serum starvation.

We next sought to demonstrate that the effect of PKCe activation on EC survival was the consequence of protection against apoptosis using an etoposide-induced model of apoptosis. HUVEC were infected with CA-PKCe or β-galactosidase control Adv and treated 24 h later with etoposide for 16 h. As
seen in Fig. 2C, nuclear staining with Hoechst 33342 of β-galactosidase control Adv-infected EC treated with etoposide revealed numerous apoptotic cells, identified by characteristic DNA bright nuclear condensation and fragmentation. In contrast, overexpression of CA-PKCe protected EC against apoptosis, with few apoptotic cells detected (Fig. 2D). Quantification of three separate experiments revealed a significant reduction in apoptosis in PKCe overexpressing EC (Fig. 2E). Likewise, propidium iodide staining of fixed and permeabilized EC followed by flow-cytometric analysis and quantification of

FIGURE 1. VEGF-induced protection against apoptosis is PKCe-dependent. A, HUVEC were treated with VEGF (25 ng/ml), PDBu (50 ng/ml), or vehicle control (GF) in the presence or absence of PKC antagonist GF109203X (1 μM) before serum starvation (M199, 0.1% FBS) for 24 h. Cell cytotoxicity was quantified by MTS assay and expressed as percent cell death calculated as: % cell death = 100 – (absorbance test/absorbance control × 100), where control represents EC cultured in M199/20% FBS alone. B, HUVEC were serum-starved (SS) for 24 h in the presence or absence of specific myristoylated peptide antagonists of PKCe (Myr-PKCe) (50 μM) or PKCα (Myr-PKCa) (100 μM) alone or in combination. Percent cell death was quantified by MTS assay. C, HUVEC were pretreated with VEGF (25 ng/ml) or vehicle in the presence or absence of myr-PKCa or myr-PKCe alone or in combination and serum-starved for 24 h. Percent cell death was quantified by MTS assay. Data are expressed as the mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01.

FIGURE 2. PKCe overexpression protects HUVEC against apoptosis. A, HUVEC were infected with Adv expressing CA-PKCe or β-galactosidase control adenovirus m.o.i. 0–50 ifu/cell for 24 h before exposure to serum starvation (M199, 0.1% FBS) for 24 h. Percent cell death was quantified by MTS assay, expressed as a percentage of the mean absorbance for control EC cultured in M199, 20% FBS. B, HUVEC (5000 cells/96 well) were left untreated or infected with Adv expressing CA-PKCe (PKCe), β-galactosidase, or GFP (m.o.i. up to 25). The medium was replaced with M199, 5% FBS with or without endothelial cell growth factor (ECGF) (10 ng/ml), and EC were cultured for a further 24 h. Change in cell number was quantified by MTS assay and expressed as a percentage of the untreated control EC cultured in M199, 5% FBS alone. GFP, green fluorescent protein. C and D, HUVEC were infected with Adv CA-PKCe (PKCe) (D) and β-galactosidase (m.o.i. 20) (C) for 24 h before exposure to etoposide (100 μM) for 16 h. After permeabilization, EC were stained with Hoechst 33342. Apoptotic cells (indicated by arrows) were identified by characteristic DNA bright nuclear condensation and fragmentation. E shows cumulative data from three separate experiments, quantified by counting apoptotic nuclei in three high-power fields, expressed as a percentage of total cells. F, HUVEC were cultured in M199, 10% FBS (UT) or infected with Adv CA-PKCe or β-galactosidase (m.o.i. 20) for 24 h before exposure to etoposide for 16 h, permeabilization, and staining with propidium iodide. EC were analyzed by flow cytometry, and apoptotic cells falling in the sub-G1 gate are expressed as a percentage of control EC. Data are expressed as the mean ± S.E. (n = 3). *, p < 0.05; **, p < 0.01.
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**FIGURE 3. Activation of PKCe induces Bcl-2 expression in HUVEC.** A, HUVEC were left untreated (UT) or infected with an Adv CA-PKCe at an m.o.i. of up to 50 ifu/cell or β-galactosidase control Adv (β-gal) (m.o.i. 50) for 24 h before lysis and immunoblotting with Abs against the phosphorylated (p-) form of PKCe (Ser167), Bcl-2, or α-tubulin. B, HUVEC were left untreated (UT) or infected with Adv CA-PKCe (PKCe), CA-PKCa (PKCa), or β-galactosidase (m.o.i. 20) and after 24 h lysed and analyzed by immunoblotting with Abs against Bcl-2 and α-tubulin. C, HUVEC were infected with Adv CA-PKCe (PKCe) or β-galactosidase (m.o.i. 20) for up to 48 h and analyzed for expression of Bcl-2 by immunoblotting. D, HUVEC were infected with adenoviruses expressing CA-PKCe (PKCe) or β-galactosidase (m.o.i. 25) for 24 h before lysis and immunoblotting for expression of pro- and anti-apoptotic Bcl-2 family members and α-tubulin. The data shown was obtained from a minimum of two and up to four separate experiments.

sub-G1 DNA as a measure of apoptosis demonstrated a significant cytoprotective effect of CA-PKCe against etoposide-induced apoptosis (Fig. 2F).

**PKCe Activation Increases EC Bcl-2 Expression**—In light of evidence that exogenous stimuli such as VEGF, laminar shear stress, and integrin ligation activate PKCe (34, 41–43) and increase EC expression of the anti-apoptotic protein Bcl-2 (supplemental Fig. 1) (9, 44, 45), we explored the relationship between PKCe activation and Bcl-2 expression in human vascular EC. Initial immunoblotting experiments revealed a dose-dependent increase in the phosphorylation of PKCe after infection with CA-PKCe-Adv (Fig. 3A) and determined the optimal m.o.i. of 20–50 ifu/cell for the CA-PKCe-Adv. CA-PKCe specifically increased expression of PKCe but had no effect on PKCa, -δ, or -ζ protein levels (not shown). Furthermore, overexpression of CA-PKCe resulted in a parallel increase in the expression of Bcl-2, whereas the β-galactosidase-expressing control adenovirus failed to phosphorylate PKCe or induce Bcl-2 (Fig. 3A). The specificity of the PKCe-mediated effect was demonstrated by comparing overexpression of CA-PKCe and CA-PKCa, in which only the former induced Bcl-2 (Fig. 3B). Subsequent experiments demonstrated that the induction of Bcl-2 was sustained up to 48 h post-infection with CA-PKCe Adv (Fig. 3C).

Immunoblotting was also used to explore the effect of PKCe activation on other pro- and anti-apoptotic members of the Bcl-2 family. As seen in Fig. 3D and in contrast to Bcl-2 itself, no significant change in the expression of anti-apoptotic Bcl-XL or pro-apoptotic Bad, Bak, or Bax was seen at 24 h post-infection of HUVEC with CA-PKCe-Adv when compared with β-galactosidase Adv. This suggests that, at least in human vascular EC, activation of PKCe is specifically associated with induction of the anti-apoptotic protein Bcl-2.

The role of PKCe-induced Bcl-2 in the anti-apoptotic effect observed was investigated further using HA-14-1, a functional antagonist of Bcl-2 (46). HA-14-1 is a small molecular ligand that binds to the hydrophobic region of the BH3 domain of Bcl-2, which is essential for its function (46, 47). In initial experiments, HA-14-1 was titrated to a concentration (15 μM) that resulted in <10% apoptosis in resting EC (47). In subsequent experiments the cytoprotective effect of adenoviral-mediated overexpression of CA-PKCe was confirmed (Fig. 4A). Inclusion of HA-14-1 (15 μM) was sufficient to reverse this cytoprotective effect, resulting in etoposide-mediated cell death equivalent to that seen in control

β-galactosidase Adv-treated EC. These data support a significant functional role for Bcl-2 induction in the anti-apoptotic effects of PKCe in vascular EC.

**PKCe Activation Inhibits Cleavage of Caspase-3**—Anti-apoptotic members of the Bcl-2 family regulate the mitochondrial pathway of apoptosis, acting to prevent cytochrome c release and subsequent activation of caspases (48). To investigate the role of PKCe activation in modulating the caspase death pathway, we initially sought to confirm that caspase activation was responsible for etoposide-induced apoptosis in vascular EC. As seen in Fig. 4B, inclusion of the peptide Z-VAD-FMK, a broad-spectrum caspase inhibitor, protected EC against etoposide-induced cell death, a response that was not seen with the matched negative control peptide Z-FA-FMK.

Intracellular flow-cytometric analysis of caspase-3, using a monoclonal Ab against an activation-specific epitope expressed by proteolytically cleaved caspase-3, was used to further investigate the effect of PKCe-CA on caspase-mediated cell death. Caspase activation is a critical step in apoptosis, and along with caspase-6 and caspase-7, caspase-3 acts as a downstream effector or death caspase (48). As seen in Fig. 4C, EC cultured in normal HUVEC media expressed a low level of cleaved caspase-3, which was reduced after infection with the PKCe-CA Adv and unchanged by the β-galactosidase control Adv. Treatment of HUVEC with etoposide led to a significant increase in cleaved caspase-3, and this response was attenuated by overexpression of CA-PKCe (Fig. 4C). In contrast, overex-
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expression of β-galactosidase failed to alter etoposide-induced caspase-3 cleavage.

PKCe Acts Downstream of PI-3K—Proteomic analysis in cardiomyocytes suggests that PKCe may form a cytoprotective signaling complex with PI-3K and Akt (49), although the precise details of their functional relationship remain to be determined. Moreover, cell type-specific heterogeneity in the PKCe/PI-3K/Akt signaling hierarchy exists, with both linear and parallel pathways proposed (43, 50, 51). Because the PI-3K/Akt pathway plays a central role in vascular EC survival (9, 52), we sought to establish the relationship between PKCe, PI-3K/Akt, and Bcl-2 in vascular EC.

Initial experiments suggested the presence of independent pathways for the activation of PKCe and PI-3K/Akt by VEGF. Inhibition of PKCe by the myr-PKCe peptide failed to prevent VEGF-induced phosphorylation of Akt, a response that was inhibited by PI-3K antagonist LY290042 (Fig. 5A). Pretreatment of EC with LY290042 abrogated the protective effect of VEGF against serum starvation-induced EC death (Fig. 5B). As shown above (Fig. 3A), infection of EC with CA-PKCe-Adv protected against serum starvation. Moreover, overexpression of PKCe-CA was able to reverse the inhibitory effects of LY290042 on VEGF-mediated cytoprotection (Fig. 5B). LY290042 also inhibited VEGF-induced phosphorylation of PKCe at Ser729 in the membrane fraction (Fig. 5C). Together, these data suggest that PKCe acts downstream of PI-3K in this setting. However, the failure of myr-PKCe to prevent VEGF-induced phosphorylation of Akt (Fig. 5A) suggests the presence of a branched signaling pathway.

A similar approach was used to investigate the effects of a cell-permeable antagonist of Akt phosphorylation. Akt inhibition also abrogated the cytoprotective effects of VEGF in the face of serum starvation (Fig. 5D). However, in contrast to the inhibition of PI-3K, overexpression of CA-PKCe activation failed to protect against the effect of the Akt antagonist, suggesting that PKCe may act in parallel to Akt phosphorylation. To examine the effect of Akt inhibition on Bcl-2 induction, CA-PKCe was expressed in EC in the presence of the Akt antagonist, and induction of Bcl-2 was quantified. Inhibition of Akt attenuated CA-PKCe-induced Bcl-2 up-regulation, suggesting that Akt and PKCe are both required for optimal Bcl-2 induction (supplementary Fig. 2).

PKCe and Akt Act Co-operatively to Enhance Bcl-2 Expression—To begin to investigate the relationship between the activation of PKCe and the phosphorylation status of Akt, we infected EC with CA-PKCe-Adv and immunoblotted for phosphorylated Akt (Ser473). As seen in Fig. 6A, this did not result in a significant increase in the phosphorylation of Akt when compared with overexpression of β-galactosidase. To explore the PKCe/Akt relationship further,
immunoprecipitation analysis was performed in EC overexpressing CA-PKCe and CA-Akt. HUVEC infected with CA-PKCe and CA-Akt Adv alone or in combination were lysed, and lysates were immunoprecipitated with an anti-PKCe Ab before immunoblotting for Akt. As seen in Fig. 6B, an association between PKCe and Akt was seen in those cells expressing both CA-PKCe and CA-Akt, suggesting the presence of a direct physical interaction.

To investigate whether endogenous PKCe and Akt interact in a similar way to the overexpressed proteins, EC were treated for up to 20 min with VEGF before immunoprecipitation with anti-PKCe and immunoblotting for Akt. As seen in Fig. 6C, a significant association between PKCe and Akt was seen 20 min post-stimulation, suggesting the presence of a physiologically relevant interaction between the endogenous proteins in response to VEGF.

These data suggested that PKCe and Akt form a signaling complex and that activation of both is required for optimal induction of Bcl-2. To test this hypothesis, EC were infected with CA-PKCe and CA-Akt Adv alone or in combination. Surprisingly, despite inducing a robust phosphorylation of Akt (Fig. 6D), overexpression of CA-Akt alone led to a minimal increase in Bcl-2 expression at an m.o.i. of up to 40 ifu/cell (Fig. 6E). In contrast, CA-PKCe increased Bcl-2 significantly. However, co-infection of EC with CA-Akt-Adv (m.o.i. 40) and suboptimal CA-PKCe-Adv (m.o.i. 10) led to a synergistic increase in Bcl-2 expression equivalent to that seen with CA-PKCe-Adv (m.o.i. 20) alone (Fig. 6F). Thus, the formation of a PKCe/Akt signaling complex allows optimal Bcl-2 expression in primary human vascular EC.

**DISCUSSION**

The healthy vascular endothelium has a low proliferative index with minimal apoptosis and cell turnover. Vascular injury, induced by factors including reactive oxygen species, oxidized low density lipoprotein and pro-inflammatory cytokines, is associated with the early inflammatory lesion of atherosclerosis, and results in increased EC apoptosis (4). In contrast, unidirectional laminar shear stress and the biosynthesis of VEGF and nitric oxide are typically vasculoprotective. Indeed, it has been suggested
Inhibition studies confirmed a predominant role for PKCε in VEGF-mediated EC resistance to serum starvation. In light of the importance of PI-3K/Akt in EC survival (9, 52), we explored the relationship between PI-3K, Akt, and PKCε. Previous data from EC are limited and suggest that VEGF-mediated activation of PI-3K, PKCε, and phospholipase Cγ occurs largely independently of one another (33). Our data are consistent with this, leading us to propose a branched signaling relationship between PI-3K, PKCε, and Akt rather than a vertical linear pathway (Fig. 7). Evidence from a variety of different experiments supports this model.

The demonstration that expression of CA-PKCε reversed the inhibitory effects of PI-3K antagonist LY290042 on VEGF-mediated cytoprotection against serum starvation implies that PKCε acts downstream of PI-3K. However, the failure of the myristoylated PKCε inhibitory peptide to prevent VEGF-induced phosphorylation of Akt suggests that PKCε is not an intermediate in PI-3K to Akt signaling. Nevertheless, PI-3K may activate PKCε, as LY290042 reduced VEGF-mediated phosphorylation of PKCε at Ser729. Moreover, it has been proposed that phosphorylation at Ser729, which increases PKCε activity, is mediated via PDK-1 and is sensitive to PI-3K activity (56, 57). A similar combination of signaling events has been reported for platelet-derived growth factor, which activates PKCε via independent pathways involving phospholipase Cγ and PI-3K (58). We have previously identified a phospholipase Cγ-dependent pathway activating PKCε downstream of VEGFR2 (35) (Fig. 7). Moreover, the presence of cross-talk between signaling pathways involved in PKCε activation may help to explain conflicting results in the literature, along with the use of different cell types and approaches (43, 50, 51, 57–59).

PKCε and Akt form signaling complexes in cardiomyocytes (29), MCF-7 breast cancer cells, and glomerular mesangial cells (51). PKCε exerts both positive (60) and negative (61) effects on Akt activity. Our immunoprecipitation studies suggested that after activation PKCε forms a signaling complex with Akt. To the best of our knowledge this is the first demonstration of a PKCε/Akt signaling module in primary EC. Inhibition of Akt activity abrogated PKCε-induced expression of Bcl-2, but overexpression of CA-PKCε failed to reverse the inhibitory effects of the Akt antagonist on VEGF-mediated cytoprotection against serum starvation. Somewhat to our surprise, overexpression of CA-Akt failed to significantly induce expression of Bcl-2. However, co-expression of both CA-PKCε and CA-Akt led to a synergistic induction of Bcl-2, suggesting that PKCε and Akt act co-operatively and interdependently within the signaling module.

The balance between pro- and anti-apoptotic members of the Bcl-2 family is critical in determining cell fate (48). Thus, if pro-apoptotic BH3-only proteins including Bim, Bid, and Bad are present in sufficient amounts to bind to and overwhelm Bcl-2 and Bcl-XL, sequestered Bax and Bak are released, allowing the escape of mitochondrial cytochrome c (62). This in turn activates apoptotic protease-activating factor-1 and procaspase 9 forming the apoptosome, which cleaves downstream effector caspases 3, 6, and 7, resulting in DNA fragmentation and the characteristic morphological changes of apoptosis (48).

PKCε typically exerts an anti-apoptotic effect that may reflect cell type-specific interactions with Bcl-2 family members. Thus, PKCε activation increased Bcl-2 expression in erythro-
PKCe-induced Vascular Endothelial Cytoprotection

FIGURE 7. Schematic illustrating the role of PKCe and PI-3K/Akt in VEGF-induced Bcl-2 expression. VEGF activates endothelial cell PI-3K via ligation of VEGFR2. PI-3K has multiple downstream targets in vascular EC and may independently activate PKCe and Akt. Activation of PI-3K regulates serine 729 phosphorylation and activity of PKCe. PKCe and Akt form a signaling complex and act co-operatively and synergistically to induce transcription of Bcl-2. Increased expression of Bcl-2 protects EC against apoptosis induced by serum starvation or etoposide.

blasts (63) and interleukin-3-dependent myeloid cells (24). In Jurkat cells (25) and cardiomyocytes (30), PKCe inhibits apoptosis through phosphorylation and inactivation of Bad, whereas in prostate cancer cells PKCe activity inhibits Bax (64). In contrast, although PKCe is essential for the survival of glioma cells, this is mediated via activation of Akt, and depletion of PKCe had no effect on the expression of Bcl-2 or Bax (26). Our data suggest that induction of Bcl-2 is the principle mechanism underlying PKCe-mediated resistance to apoptosis in human vascular EC. PKCe activation specifically induced Bcl-2, with no detectable change in Bcl-XL, Bad, Bax, or Bak. However, it remains possible that PKCe-induced phosphorylation of Bad contributes to the anti-apoptotic effect.

The relationship between physiological stimuli of PKCe and vasculoprotection in vivo remains to be defined. However, PKCe plays a role in the downstream signaling of exogenous anti-apoptotic stimuli including VEGF (33), unidirectional laminar shear stress (41, 65), and integrin activation (57). Laminar shear increases interactions between VEGFR2, endothelial integrins, and the extracellular matrix (66), exerting anti-apoptotic effects. Our demonstration that PKCe-mediated induction of Bcl-2 protects vascular EC against apoptosis suggests that PKCe may be a pivotal component in the anti-apoptotic signaling pathways activated by factors including VEGF and laminar shear stress. Thus, it is of note that both VEGF (67) and PKCe (68) are protective against oxidant-induced injury.

In vivo models have demonstrated a key role for PKCe in ischemic pre-conditioning (31, 32, 49, 69) and resistance to oxidative stress (68, 70). Our data suggest that PKCe, through its interaction with Akt and induction of Bcl-2, plays another important role in vascular cytoprotection by contributing to the maintenance of endothelial homeostasis and vascular integrity. This mechanism may be reinforced by a direct functional relationship between PKCe, Akt, and endothelial nitric-oxide synthase recently identified in cardiomyocytes (50). Activation of these signaling complexes represents an attractive therapeutic target for conditions in which vascular injury and EC apoptosis play a pathogenic role including graft rejection (2), systemic lupus erythematosus (71), and atherosclerosis (4).

In conclusion, this study further delineates VEGF-activated signaling pathways and reveals a physical interaction between PKCe and Akt in human EC, resulting in co-operative induction of Bcl-2 and enhanced protection against apoptosis via inhibition of caspase-3 cleavage. Alongside the importance of PKCe in cardioprotection, our findings suggest that PKCe plays a pivotal role in coordinating cellular responses to pro-apoptotic stimuli in vascular endothelium. A detailed understanding of the mechanisms acting upstream and downstream of PKCe may facilitate the rational design of novel therapies by which vascular endothelium can be conditioned to minimize vascular injury, EC dysfunction and subsequent atherogenesis.

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