Vascular Endothelial Growth Factor Activates PI3K/Akt/Forkhead Signaling in Endothelial Cells

Md. Ruhul Abid, Shaodong Guo, Takashi Minami, Katherine C. Spokes, Kohjiro Ueki, Carsten Skurk, Kenneth Walsh, William C. Aird

Objective—Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor that promotes endothelial cell (EC) survival, migration, and permeability. The forkhead transcription factors FKHR, FKHRL1, and AFX are mammalian orthologues of DAF-16, a forkhead protein that controls longevity in Caenorhabditis elegans. In this study, we examined whether VEGF is coupled to phosphatidylinositol 3-kinase (PI3K)/Akt/forkhead in ECs.

Methods and Results—We demonstrate that human ECs express members of the forkhead family (FKHR, FKHRL1, and AFX) and that VEGF modulates the phosphorylation, subcellular localization, and transcriptional activity of one or more of these isoforms by a PI3K/Akt signaling pathway. VEGF inhibited EC apoptosis, promoted DNA synthesis and the G1-to-S transition, and reduced expression of the cyclin-dependent kinase inhibitor p27Kip1. Each of these effects was blocked by the PI3K inhibitor LY294002 or by a phosphorylation-resistant mutant of FKHRL1, but not by wild-type FKHRL1.

Conclusions—These results suggest that VEGF signaling in ECs is coupled to forkhead transcription factors through a PI3K/Akt-dependent pathway. (Arterioscler Thromb Vasc Biol. 2004;24:294-300.)

Key Words: vascular endothelial growth factor • forkhead • endothelial cells • PI3K

Vascular endothelial growth factor (VEGF) is an endothelial cell (EC)-specific mitogen and chemotactic agent that is involved in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, vascular protection, and hemostasis (reviewed in Ferrara1 and Dvorak2). In addition to its mitogenic and chemotactic effects, VEGF also promotes EC survival.3–5

The VEGF family of proteins binds to 3 receptor-type tyrosine kinases, Flt-1 (VEGF receptor-1), KDR/Flk-1 (VEGF receptor-2), and VEGFR-3 (reviewed in Claesson-Welsh6 and Veikkola et al7). VEGFR-1 and -2 are normally expressed in vascular ECs, whereas VEGFR-3 is expressed in the lymphatic endothelium. Of the VEGF receptors, KDR/Flk-1 is believed to play the most important role in mediating EC proliferation, migration, and permeability.8–12 VEGF has been shown to activate a number of different intracellular signaling pathways, including protein kinase C, mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK), p38 MAPK, phospholipase Cγ, and phosphatidylinositol 3-kinase (PI3K)/Akt/protein kinase B.13

Members of the winged helix, or forkhead, family of transcription factors share a conserved DNA-binding domain consisting of 100 amino acids.14 These proteins were first identified in mammalian cells as part of a chromosomal translocation in pediatric soft tumor alveolar rhabdomyosarcoma15 and in mixed-lineage leukemias, suggesting a possible role in cell proliferation and/or differentiation. The nematode Caenorhabditis elegans forkhead transcription factor DAF-16 was identified as a possible homologue of FKHR.36,17 Genetic studies showed that DAF-16 lies downstream of an insulin-like signaling pathway that includes insulin/insulin-like growth factor (IGF)-1–like receptor, PI3K, and Akt.18 More recently, 3 orthologues of DAF-16 (FKHR, FKHRL1, and AFX) were identified in mammalian cells and shown to be substrates for Akt (reviewed in Kops and Burgering19 and Tran et al20). These and subsequent studies uncovered a critical role for the forkhead family of transcription factors in coupling extracellular signals to downstream changes in gene expression. When cells are exposed to insulin/IGF-1 or serum, the PI3K/Akt pathway is activated, resulting in the phosphorylation and nuclear exclusion of forkhead transcription factors, downregulation of target-gene expression, and enhanced cell survival and proliferation.19 In contrast, the withdrawal of insulin/IGF-1 or serum leads to nuclear translocation of forkhead proteins and transcriptional activation of target genes, with subsequent cell-cycle arrest and/or apoptosis.

Given the established link between insulin/PI3K/Akt and forkhead transcription factors in non-ECs and between VEGF and PI3K/Akt signaling in ECs, we reasoned that VEGF signaling might be coupled to the downstream activity of

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forkhead proteins in the endothelium. In this study, we show that VEGF induces the phosphorylation and inactivation of the forkhead transcription factors via a PI3K/Akt-dependent mechanism. We propose that the forkhead family plays an important role in mediating VEGF signaling and function in ECs.

Methods

Cell Culture and Reagents
Human coronary artery ECs (HCAECs), human umbilical vein ECs (HUVECs), and human pulmonary artery ECs (HPAECs) were grown in endothelial growth medium-2-MV (EGM-2-MV BulletKit, Clonetics). ECs from passage 3 to 6 were used for all experiments. Cells were serum-starved in 0.5% fetal bovine serum and treated with 50 ng/mL human VEGF

Western Blot Analyses
The following antibodies were used: anti-p27

Adenoviruses
HCAECs were infected with adenoviruses encoding the cDNAs of β-galactosidase (β-Gal or Adv); dominant-negative T308A, S473A-Akt (dominant negative Akt), constitutively active Gag-Akt (CA-Akt), wild-type (WT)-FKHRL1, and triple-mutant (TM)-FKHRL1. The TM version of FKHRL1 contains T32A, S253A, and S315A and is resistant to agonist-induced phosphorylation.

Cotransfection Assays
Control vector (pECE), vectors expressing WT-FKHRL1 (pECE-FKHRL1) and TM-FKHRL1 (pECE-TM-FKHRL1), and the pGL3-based construct containing 3 forkhead consensus-binding elements coupled to the simian virus 40 minimal promoter (FKHRE-Luc reporter) were provided by Michael Greenberg, Children's Hospital, Boston, Mass. Cells were transfected with FuGENE 6 (Roche).

Cell Viability, DNA Synthesis, and Cell-Cycle Distribution Assays
Apoptosis was assayed by fluorescence-activated cell-sorting (FACS) analysis for FITC-labeled annexin-V binding; DNA synthesis by radiolabeled thymidine uptake; and cell-cycle analysis by FACS analysis of the propidium iodide (FITC)–labeled DNA content of transfected cells. Please see http://atvb.ahajournals.org for additional Methods.

Results

VEGF Induces Phosphorylation of the Forkhead Transcription Factors in ECs
Our first goal was to determine whether the VEGF/PI3K/Akt signaling axis is coupled to forkhead transcription factor phosphorylation in ECs. As expected, the addition of VEGF to HUVECs or HCAECs resulted in rapid tyrosine phosphorylation of endogenous VEGF receptor-2 (Flk-1/KDR) and Akt (Figure 1). Incubation of HCAECs or HUVECs with VEGF also resulted in the phosphorylation of endogenous FKHR at Ser-256 and of AFX at Ser-193, with maximal levels occurring between 5 and 15 minutes (Figure 2A shows HCAECs). In addition, VEGF induced phosphorylation of FKHR at Thr-24 (Figure 2B shows the results at 15 minutes). VEGF-mediated phosphorylation of FKHR Ser-256 and Thr-24 was inhibited by preincubation with either LY294002 (50 μmol/L; Figure 2B) or wortmannin (50 nmol/L; data not shown). Interestingly, incubation of ECs with LY294002 alone reduced the basal level of FKHR phosphorylation, suggesting the presence of basal PI3K/forkhead activity in ECs. Overexpression of CA-Akt resulted in increased phosphorylation of endogenous FKHR at Ser-256 and of AFX at Ser-193, in the absence or presence of VEGF, whereas overexpression of dominant-negative Akt blocked VEGF-mediated phosphorylation of FKHR and AFX (Figure 2C). In contrast, VEGF-induced phosphorylation of FKHR at Ser-256 was not inhibited by the MEK1/2 MAPK inhibitor PD98059 (50 μmol/L; Figure 2D). The available phosphospecific antibodies to FKHR yielded nonspecific signals in Western blot analyses of HCAECs and HUVECs. However, FKHR1 total protein and mRNA were demonstrated in 3 different types of primary human ECs (HCAECs, HPAECs, and HUVECs) by Western blots and reverse transcription–polymerase chain reaction assays, respectively. (Figure 1, available online at http://atvb.ahajournals.org, shows reverse transcription–polymerase chain reaction) Taken together, the aforementioned findings suggest that the forkhead transcription factors lie downstream of VEGF/PI3K/Akt signaling in ECs.

VEGF Induces Nuclear Exclusion of FKHR, FKHRL1, and AFX in ECs
Previous studies in non-ECs have shown that when phosphorylated, forkhead transcription factors are excluded from the
nucleus. To determine whether VEGF-mediated phosphorylation of forkhead proteins is associated with their cytoplasmic translocation, immunofluorescence studies were carried out in control and VEGF-treated HCAECs. The addition of VEGF to ECs resulted in significant translocation of endogenous FKHR, FKHRL1, and AFX from the nucleus to the cytoplasm at 15 and 30 minutes, compared with control. (Figure 3A shows quantification at 30 minutes. Figure II, available online at http://atvb.ahajournals.org, shows immunofluorescence.) At 30 minutes, VEGF reduced the proportion of FKHR-positive nuclei from 68±3.5% to 27±2.8%, the proportion of FKHRL1-positive nuclei from 86±7.8% to 40±5.2%, and the proportion of AFX-positive nuclei from 56±4.8% to 19±3.1% (Figure 3A). The nuclear-cytoplasmic ratio approached control levels at 60 minutes (Figure II). Consistent with the results of the Western blot analyses (see Figure 1), VEGF-mediated cytoplasmic translocation of FKHR (Figure 3B), FKHRL1, and AFX was blocked by preincubation with the PI3K inhibitor LY294002 or by transduction with virus expressing dominant-negative Akt (Figure 3C). In keeping with the phosphorylation results in Figure 1, addition of LY294002 alone resulted in increased nuclear localization of FKHR (Figure 3B), FKHRL1, and AFX (data not shown). Again, these findings are consistent with the presence of basal PI3K/forkhead signaling in ECs. Finally, adenovirally overexpressed WT-FKHRL1 was localized primarily in the cytoplasm, whereas TM-FKHRL1 (which is resistant to agonist-induced phosphorylation) was predominantly localized in the nucleus (Figure 3D). Taken together, the aforementioned findings suggest that VEGF-mediated, PI3K/Akt-dependent phosphorylation of forkhead proteins in ECs promotes their translocation from the nucleus to the cytoplasm.

VEGF-Mediated Phosphorylation of Forkhead Results in Decreased Transcriptional Activity

The nuclear export of phosphorylated forkhead proteins might limit access of these transcription factors to their target genes, resulting in decreased gene expression. To determine whether VEGF signaling results in changes in the transcriptional potential of forkhead on a heterologous promoter, we used cotransfection assays of HUVECs with an expression vector that contained either WT-FKHRL1 or TM-FKHRL1 cDNA, along with a forkhead-responsive luciferase reporter gene (ie, FKHRE-Luc). Under serum-starved conditions, overexpression of WT-FKHRL1 and TM-FKHRL1 resulted in a 4- and 5-fold induction of the reporter gene, respectively (Figure 4). The addition of VEGF resulted in partial (50%) inhibition of WT-FKHRL1–mediated, but not of TM-FKHRL1–mediated, transactivation of the FKHRE promoter (Figure 4). The effect of VEGF on transcriptional activity was blocked by LY294002 (Figure 4, but not by GF109203X (data not shown). These data suggest that VEGF/PI3K-mediated phosphorylation of forkhead is associated with a reduction in transcriptional activity.
VEGF Promotes EC Survival, Proliferation, and Cell-Cycle Progression via a PI3K/Forkhead Pathway

To establish the prosurvival effect of VEGF in ECs, HCAECs and HUVECs were serum-starved for 36 or 60 hours in the presence or absence of VEGF. The cells were then labeled with annexin-V–FITC and propidium iodide and analyzed by FACS. After a 36-hour starvation, VEGF significantly reduced the proportion of apoptotic cells, from 7±1.1% to 2±0.3% (Figure 5A shows HCAECs). The prosurvival effect of VEGF was retained after 60 hours of serum deprivation (Figure 5B). It is noteworthy that preincubation of ECs with the PI3K inhibitor LY294002 not only abrogated the effect of VEGF on cell survival but also resulted in significantly increased apoptosis in the absence or presence of VEGF or 5% serum (Figure 5A). These findings are consistent with the notion that basal PI3K activity is required for the survival of serum-starved ECs. To determine whether forkhead transcription factors play a role in mediating the effect of VEGF on EC survival, HCAECs were infected with adenoviruses expressing β-galactosidase (Adv), WT-FKHRL1, or TM-FKHRL1; serum-starved; and then treated in the absence or presence of VEGF. Overexpression of WT-FKHRL1 and TM-FKHRL1 resulted in significantly increased apoptosis of HCAECs starved for 36 or 60 hours (Figure 5A and 5B), as determined by FACS analysis of annexin-V–FITC-labeled cells. VEGF treatment significantly reduced the number of apoptotic cells in serum-starved HCAECs infected with Adv or WT-FKHRL1, but not with TM-FKHRL1 (Figure 5A and 5B).

To elucidate the effects of VEGF on cell proliferation and cell-cycle progression, subconfluent HCAECs and HUVECs were synchronized in G0/G1 by serum starvation, treated for 12 to 20 hours with or without VEGF or serum, and then assayed for radiolabeled thymidine uptake or FACS analysis of propidium iodide–labeled DNA content. The addition of VEGF resulted in increased DNA synthesis, with a >1.4-fold induction of thymidine uptake in HCAECs and a >4-fold induction in HUVECs (Figure 5C shows HCAECs). VEGF stimulation of DNA synthesis in HCAECs and HUVECs was abrogated by LY294002 (Figure 5C), suggesting that VEGF-mediated DNA synthesis occurs through a PI3K-dependent pathway. Treatment of HCAECs with VEGF for 12 hours induced a significant percentage of cells to enter the S phase (12.8±1.2%) compared with the control (7.2±0.9%; Figure 5D). The effect of VEGF on cell-cycle progression was inhibited by LY294002 (data not shown). Overexpression of TM-FKHRL1 resulted in a significant reduction of DNA synthesis and the G1-to-S transition (Figure 5C and 5D). Incubation with VEGF resulted in increased DNA synthesis (Figure 5C) and cell-cycle progression (Figure 5D) of HCAECs infected with Adv or WT-FKHRL1 but not with TM-FKHRL1. Taken together, these results suggest that VEGF promotes EC survival, proliferation, and cell-cycle progression and that these effects are dependent on PI3K-mediated inhibition of forkhead activity.

VEGF Signaling Mediates Downregulation of p27kip1 Expression Through a PI3K/Forkhead Signaling Pathway

Forkhead proteins have been shown to modulate cell-cycle progression by transcriptional regulation of the cyclin E/cyclin-dependent kinase (cdk)-2 inhibitor p27kip1 and G2-M regulator, growth arrest and DNA damage-response gene GADD45. Moreover, the p27kip1 promoter contains multiple forkhead-binding sites, and p27kip1 has been shown to lie downstream of forkhead transcription factors in a murine pre-B-cell line. These observations raise the possibility that VEGF/PI3K-mediated effects on the cell cycle might be mediated in part through the modulation of expression of p27kip1. To test this hypothesis, serum-starved cells were treated in the absence or presence of VEGF and assayed for p27kip1 mRNA by Northern blotting. As shown in Figure 6A, VEGF reduced the level of endogenous p27kip1 mRNA by 68±3.5% after 1 hour of treatment compared with control. Pretreatment with LY294002 blocked VEGF-induced downregulation of p27kip1, suggesting a role for PI3K in this process. Together with the proposed role of p27kip1 as a forkhead target gene, our findings raise the possibility that VEGF-mediated suppression of p27kip1 is mediated by the nuclear exclusion...
of forkhead transcription factors. Consistent with this hypothesis, the overexpression of nonphosphorylatable TM-FKHRL1, but not of WT-FKHRL1, significantly inhibited VEGF-mediated downregulation of p27<sup>kip1</sup> (Figure 6B). Under serum-enriched conditions, in which basal levels of p27<sup>kip1</sup> are reduced, the overexpression of TM-FKHRL1 alone resulted in the upregulation of p27<sup>kip1</sup> expression both at the mRNA (Figure 6C) and protein (Figure 6D) levels. These results suggest that the VEGF-induced modulation of expression of p27<sup>kip1</sup> that regulates the G<sub>1</sub>-to-S transition of the cell cycle is mediated, at least in part, through a PI3K/forkhead signaling pathway in ECs.

**Discussion**

PI3K/Akt signaling has been implicated in multiple cellular and organ functions, including growth, cell survival, glucose metabolism, and protein synthesis. PI3K/Akt serves as a signal transducer, channeling information from the cell surface, including growth factors, hormones, flow, and cytokines, to downstream changes in cellular phenotype (reviewed in Brazil et al<sup>33</sup>). In ECs, the PI3K/Akt signaling pathway is important in mediating cell survival, proliferation, migration, and angiogenesis (reviewed in Shiojima and Walsh<sup>34</sup>). Given the multifaceted function of PI3K/Akt in ECs, an important goal is to identify the repertoire of upstream mediators and the downstream targets of this pathway.

Previous studies of forkhead transcription factors have largely focused on insulin or IGF-1 as the stimulus, transformed cell lines, and/or the overexpression of exogenous forkhead protein. We have shown for the first time that VEGF regulates the phosphorylation, subcellular localization, and transcriptional activity of endogenous forkhead transcription factors in ECs through a PI3K/Akt-dependent mechanism. This conclusion is supported by several lines of evidence: (1) VEGF induced phosphorylation of endogenous FKHR/AFX, an effect that was dependent on PI3K and Akt; (2) the phosphorylation of endogenous FKHR, FKHRL1, and AFX resulted in their exclusion from the nucleus in a VEGF/PI3K/Akt-dependent manner; (3) a phosphorylation-resistant form of FKHRL1 (TM-FKHRL1), but not the WT version, was constitutively localized to the nucleus; and (4) in cotransfection studies, VEGF reduced the transactivation potential of WT, but not of TM, forkhead in a PI3K-dependent manner.

VEGF has been shown to enhance the survival of ECs under in vitro and in vivo conditions.<sup>3,5</sup> Several mechanisms of cytoprotection have been postulated, including activation of PI3K/Akt and secondary attenuation of p38 signaling; increased expression of survivin, Bcl-2, and A1; and/or reduced caspase-3 activity.<sup>13,25</sup> We have shown that VEGF failed to rescue LY294002- and TM-FKHRL1–infected HCAECs. In contrast, VEGF increased cell survival in Adv- or WT-FKHRL1–infected HCAECs. These results suggest that VEGF enhances EC survival at least in part by promoting the phosphorylation and inactivation/nuclear exclusion of FKHRL1. Interestingly, the overexpression of TM-FKHRL1 in HCAECs did not result in increased levels of Fas ligand and Bcl-2-interacting mediator of cell death (BIM) (data not shown), proapoptotic genes that have been implicated in forkhead death pathways in other cell types.<sup>28</sup> These findings are consistent with previous reports of EC resistance to Fas ligand–mediated apoptosis.<sup>29,30</sup> Although it is conceivable that forkhead-mediated inhibition of cell-cycle progression (eg, through activation of p27<sup>kip1</sup>) might contribute to cell death, further studies will be required to identify the relevant proapoptotic mechanisms.

In this study, the overexpression of FKHRL1 in ECs was also shown to result in cell-cycle arrest at G<sub>1</sub> and the inhibition of DNA synthesis. Progression through the cell cycle requires activation of CDKs through their association with regulatory subunits of cyclins. The activity of CDKs is attenuated by CDK inhibitors, such as p27<sup>kip1</sup>. There is a growing appreciation for the role of p27<sup>kip1</sup> as a cell-cycle...
regulator in vascular biology. For example, adenosvirally mediated overexpression of p27kip1 attenuated neointimal thickening in balloon-injured blood vessels. Moreover, cardiac myocytes isolated from mice that are null for p27kip1 display prolonged proliferation. Finally, the forced overexpression of p27kip1 in HUVECs resulted in the inhibition of DNA replication and tube formation. The in vivo relevance of these latter findings was supported by the demonstration of reduced flow recovery and capillary density in the hindlimb ischemia model. In the current report, the addition of VEGF to ECs resulted in reduced expression of p27kip1, whereas the forced overexpression of TM-FKHRL1 had the opposite effect. These data suggest that the effects of VEGF on cell-cycle progression and proliferation are contingent on the ability of the growth factor to phosphorylate FKHRL1 and to downregulate the expression of p27kip1.

Previous investigations in non-ECs have uncovered a variety of forkhead-responsive genes, in addition to Fas ligand, BIM, and p27kip1. These include IGF-binding protein-1, glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, pancreas/duodenum homeobox gene-1, mitochondrial 3-hydroxy-3-methylglutarlyl-coenzyme A synthase, tumor necrosis factor-related apoptosis-inducing ligand, GADD45, transforming growth factor-α, and B-cell lymphoma-6 transcriptional repressor. Studies are underway to determine whether VEGF alters the expression of one or more of these candidate genes in ECs. In addition, we are carrying out DNA microarray experiments of VEGF-treated ECs preincubated in the absence or presence of the PI3K inhibitor LY294002 or infected with the TM versions of the forkhead protein to systematically screen for forkhead-responsive genes. The results of these investigations will provide clues as to which other target genes are involved in mediating the effect of VEGF/PI3K/forkhead on cell survival, proliferation, and cell cycle progression and perhaps other EC functions.

Figure 6. VEGF signaling downregulates p27kip1 expression through a PI3K/forkhead signaling pathway. A, Northern blot analysis of endogenous p27kip1 in serum-starved HCAECs treated with or without 50 ng/mL VEGF. Cells were pretreated with or without LY294002 for 30 minutes as indicated. Ethidium bromide photo of the formaldehyde gel showing 28S RNA as a loading control is included. The bar graph (lower) shows relative intensity of the p27kip1 band compared with untreated control (arbitrarily fixed at 100%) and represents means ± SD of 3 independent Northern blots from 3 separate RNA preparations. B, Same as in A, except the cells were infected with replication-deficient adenoviruses expressing β-Gal (Adv), TM-FKHRL1 (TM), or WT-FKHRL1 (WT) and were treated with or without 50 ng/mL VEGF. C, Northern blot analysis of HCAECs infected with adenoviruses as indicated and grown in serum-enriched medium. The membrane was subsequently stripped and reprobed with 18S RNA as a loading control. A representative blot of multiple experiments is shown here. D, Western blot analysis of endogenous p27kip1 in HCAECs infected with Adv, WT-FKHRL1, or TM-FKHRL1. The membrane was subsequently stripped and reprobed with anti-β-actin antibody for loading control.

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