Vascular Endothelial Growth Factor Activation of Sterol Regulatory Element Binding Protein
A Potential Role in Angiogenesis

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Abstract—By stimulating the migration and proliferation of endothelial cells (ECs), vascular endothelial growth factor (VEGF) is a potent angiogenic factor. However, the molecular mechanism involved in the VEGF-induced angiogenesis remains elusive. We hypothesized that sterol regulatory element binding proteins (SREBPs), transcription factors governing cellular lipid homeostasis, play an important role in regulating angiogenesis in response to VEGF. VEGF activated SREBP1 and SREBP2 in ECs, as demonstrated by the increased SREBPs, their cleavage products, and the upregulation of the targeted genes. VEGF-induced SREBP activation depended on SREBP cleavage-activating protein (SCAP), because knocking down SCAP by RNA interference (RNAi) inhibited SREBP activation in response to VEGF. SREBP activation was also blocked by 25-hydroxycholesterol (25-HC). To verify the functional implication of SREBPs in VEGF-induced angiogenesis, we tested the role of SREBPs in EC migration and proliferation. SCAP RNAi or 25-HC inhibited VEGF-induced EC proliferation, with cell growth arrested at the G0/G1 phase and a concomitant decrease of the S phase. Blocking the PI3K-Akt pathway inhibited the VEGF-activated SREBPs, demonstrating that PI3K-Akt regulates SREBPs. Consistent with our in vitro data, SREBP1 was detected in newly developed microvasculatures in a rabbit skin partial-thickness wound-healing model. SREBP inhibition also markedly suppressed VEGF-induced angiogenesis in chick embryos. In summary, this study identifies SREBPs as the key molecules in regulating angiogenesis in response to VEGF. (Circ Res. 2004;95:471-478.)

Key Words: SREBP • migration • proliferation • angiogenesis • endothelial cells

Angiogenesis is the formation of new blood vessels from a preexisting vascular network. By stimulating endothelial cell (EC) migration and proliferation, vascular endothelial growth factor (VEGF) plays a central role in angiogenesis under both physiological and pathological conditions.1–5 VEGF is ubiquitous at sites of angiogenesis, and its levels associate well with the temporal and spatial events of blood vessel formation.6 This angiogenic factor binds to specific receptors on ECs and thus induces several proangiogenic cues, including the PI3K-Akt pathway that mediates the prosurvival effects of VEGF.7–10 In addition, the VEGF-enhanced EC migration and capillary-like tube formation depends on Akt phosphorylation.8,11,12 In contrast, Akt dephosphorylation is involved in the inhibition of EC migration by low-density lipoprotein (LDL).13 Despite the ample knowledge of VEGF in angiogenesis, the involved downstream molecular events remain unclear. Sandwiched between the extracellular matrix and the cytoskeleton, the plasma membrane is essential for activating the membrane-bound receptors and associated signaling molecules that are required for cell migration and proliferation. It has been shown that changes in the contents of membrane cholesterol or fatty acid alter the microviscosity of the plasma membrane, which in turn can regulate EC migration.14,15 Angiogenic factors, such as VEGF and bFGF, are known to regulate EC migration by modulating the membrane microviscosity. Therefore, changes in membrane lipids might be involved in the VEGF-regulated membrane microviscosity, which in turn is required for enhanced EC motility.

Sterol regulatory element-binding proteins (SREBPs) consist of SREBP1a, SREBP1c, and SREBP2 (see review).16 Both SREBP1a and 1c are encoded from the srebp1 gene with alternative transcription start sites, whereas SREBP2 is from a different gene. SREBP1 is responsible for the biosyntheses of cholesterol and fatty acid, whereas SREBP2 mainly mediates the biosynthesis of cholesterol. With a hairpin-like structure, SREBPs are endoplasmic reticulum (ER) membrane-bound transcription factors, with the N- and...
C-termini protruding into the cyttoplasm, whereas the central hydrophilic domain spans the ER lumen. In sterol-loaded cells, SREBPs form a complex with the ER membrane-bound SREBP cleavage-activating protein (SCAP). In cells deficient in lipids or sterols, SCAP escorts SREBPs from the ER to the Golgi, where they are cleaved by Site 1 and Site 2 proteases. Such a proteolytic cleavage causes the nuclear translocation of the N-terminal leucine-zipper transcription factor to direct the transcriptional activation of genes such as LDL receptor (LDLR), HMG CoA reductase (HMGCR), and fatty acid synthase (FAS).

In view of the key role of VEGF in angiogenesis and the importance of SREBPs in lipid regulation, we hypothesize that VEGF activates SREBPs, which controls the cellular lipid homeostasis required for EC migration and proliferation during angiogenesis. We show that VEGF activates SREBP1 and SREBP2 in ECs and that inhibition of these proteins markedly suppressed the VEGF-induced angiogenesis in vivo.

Materials and Methods

Primary human microvascular endothelial cells (hMVECs) derived from human lung microvessels were purchased from Clonetics (Cat no. CC-2527, San Diego, Calif). The cells were cultured in EGM2-MV medium containing EC growth supplements (Cambrex). Cultures within 5 passages were used in all experiments. Anti-SREBP1(2A4), Anti-SREBP1(K-10), anti-SCAP, and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology. Anti-PECAM-1 was from R&D Systems. Anti-GAPDH was from DMI Research Diagnostics. Rhodamine- or FITC-conjugated secondary antibodies were from Santa Cruz Biotechnology.

Cell Culture, Reagents, and Materials

Western Blot Analysis

Cells were lysed in lysis buffers, and the whole cell lysates were subjected to 10% SDS-PAGE and Western blotting according to standard protocols. The blots were probed with various primary antibodies. The HRP-conjugated secondary antibodies were then used to reveal the specific protein bands with ECL detection reagents. The intensities of the various protein bands were quantified by densitometry.

Quantitative Real-Time PCR

Total RNA was extracted from ECs and underwent quantitative analysis of mRNA expression of SREBPs and their target genes by use of real-time PCR with an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems). The reaction reagent SYBR Green II Master Mix Kit (Stratagene) was used according to the manufacturer’s instructions. Gene-specific mRNA was normalized to GAPDH mRNA as an internal control. The amounts of mRNA in VEGF-treated and control samples were calculated as fold change relative to those of control cells set as 1. Sequences of the primer sets used were as follows: SREBP1a, 5'-CACTTCTGGTCGCTCTTACATCTC-3' and 5'-TACACATGCTGTA-3'; SREBP1c, 5'-TTGCGTGGATGTTAAGG-3' and 5'-CTGGCAGCCCAATGTGGGTAA-3', and GAPDH, 5'-CAACTACA-3' and 5'-CTGGCAGCCCAATGTGGGTAA-3'.

RNA Interference

Small interference RNA (siRNA) duplexes specific for SCAP (SCAP-siRNA, 5'-AACCUCUCCGGACAGAAGUGA-3') or GL3 luciferase (GL3-luc-siRNA, 5'-TACACGTGAGTTCTCGGA-3') were transfected into the hMVECs with the use of Oligofectamine (Invitrogen). Cells grown to 90% confluence were transfected with siRNA (150 nmol/L) for 24 hours before further treatment or analysis.

EC Migration and Quantitative Pseudopodia Assays

An outward growth migration model19,20 was used to detect EC migration. A cell cloning cylinder, 6 mm in diameter, was set on a 35-mm cell culture dish, and 1.0×10⁴ hMVECs were seeded inside the cylinder in 100 µL EGM-MV growth medium. Four hours after seeding, the cylinder was removed, the edges of cells marked, and migration at the indicated times was measured as the distance away from the edge of the cell nest.

Pseudopodium formation was quantitatively measured according to published procedures.21 hMVECs pretreated with RNA interference (RNAi) or 25-HC were trypsinized, and the cell suspensions (6×10⁴, in 0.5 mL culture media containing 0.5% FBS) were placed into the upper chamber of a Costar Transwell chamber (12 mm, 3.0 µm pore size). Cells were allowed to attach for 2 hours and then stimulated with VEGF in the lower chamber to establish a chemotactgradient. The inserts were fixed 90 minutes after VEGF was added and stained with 0.5% crystal violet. The cell body on the upper surface was manually removed with a cotton swab, which left the pseudopodia remaining on the bottom. The dye in the pseudopodia was then eluted in 10% acetic acid, and optical density was measured at 600 nm.

EC Proliferation Assay

DNA synthesis was assessed by BrdU incorporation. In brief, cells seeded in the 96-well plates were subjected to different conditions (siRNAs or 25-HC). The cells were then treated with VEGF, and BrdU was added to the medium. BrdU incorporation was detected by ELISA. EC proliferation was also determined by flow cytometry. hMVECs cultured in EGM-MV medium with 2% FBS were transfected with siRNAs or pretreated with 25-HC (5 µg/mL) for 24 hours. The cells were then treated with VEGF for another 24 hours. After trypsinization and fixation, the cells were stained with propidium iodide. A total of 2×10⁵ cells were analyzed with a FACSscan flow cytometer. The cell cycle modeling and proportion of cells in the G1, S, and G2/M phases were determined accordingly.

Transient Transfection and Luciferase Activity Assay

hMVECs were transiently transfected with luciferase reporter plasmids of the LDLR promoter22 or FAS promoter (−1594 to +65),23 together with dominant-negative Akt (Akt-DN), constitutively active Akt (Akt-CA), or wild-type Akt (Akt-WT) plasmids24 and CMV-β-galactosidase plasmids (β-gal) by use of the Lipofectamine method (Gibco BRL). Twenty-four hours after transfection, the cells were lysed and luciferase activities measured. In experiments involving VEGF treatment, 20 hours after transfection cells were treated with VEGF for another 12 hours. The luciferase activity in various experiments was normalized to β-gal activity for transfection efficiency.

Rabbit Skin Wound Healing Model

A partial-thickness wound-healing skin model was created in 4-month-old New Zealand White male rabbits (Western Oregon Rabbit Co., Philomath, Ore). All experiments were approved by the UCR Institutional Animal Care and Use Committee. The rabbits were sedated and anesthetized with acemprazine (1 mg/kg, IM), ketamine (50 mg/kg, IM), and xylazine (5 mg/kg, IM). The rostral-dorsal hair was shaved, and partial-thickness skin wounds were made by use of a 5-mm Acupunch (Acuderm). The wound-healing tissue samples were collected by punching with a 10-mm Acupunch at...
different time points during wound healing. Normal skin tissue samples were also punched and used as controls. Frozen tissue sections (10 μm in thickness) were permeabilized by 0.2% Triton X-100 in PBS and incubated with the anti-SREBP1 (K-10) or anti-PECAM-1. The specimens were then incubated with Rhodamine- or FITC-conjugated secondary antibodies and viewed under a Nikon TE300 fluorescence microscope. The serial tissue sections were stained with hemotoxylin and eosin for morphological observation.

Chick Embryo Angiogenesis Assays
The angiogenesis assay in the chick chorioallantoic membranes (CAM) was performed as described.24 Fertilized eggs were incubated at 38°C. At day 4, a window was opened on the eggshell exposing the CAM, and the window was covered with tape for further incubation. Pellets prepared with 15 μL of 1% methylcellulose containing DMSO, VEGF (2.5 μg) and DMSO, or VEGF and 25-HC (5 μg) were applied onto the CAM at day 10. After an additional 4-day incubation, the CAMs were fixed in situ. The areas covered by the pellet, together with the surrounding areas, were excised from the eggs. Pictures were taken with a stereoscope, and the number of vessel branches in the pellet area was counted. In separate experiments, CAMs covered by the pellets were harvested at day 14 and pooled together, which was followed by protein extraction and Western blotting.

Statistical Analyses
The results were expressed as mean ± SD from at least three independent experiments. The data were analyzed by two-tailed Student t test. Values of P<0.05 were considered to be statistically significant.

Results
VEGF Activates SREBPs in ECs in SCAP-Dependent Manner
We first treated hMVECs with VEGF to determine its effect on the SREBP activation, namely, the cleavage of SREBP1 and SREBP2. SREBP1 was detected by an antibody recognizing its N-terminus (the mature form), whereas SREBP2 was detected by an antibody recognizing its C-terminus. As shown in Figure 1A, VEGF treatment caused the activation of SREBP1 and SREBP2, as demonstrated by the increased SREBP precursors and the cleaved products. As a result of SREBP activation in response to VEGF, the levels of mRNA encoding SREBP-targeted genes such as FAS, LDLR, and HMGCR were all increased (Figure 1B). In addition, SREBP1c and SREBP2 mRNA were also augmented (Figure 1B). However, SREBP1a was barely detected in hMVECs. SCAP is essential for the activation of SREBP1 and SREBP2 by escorting them from the ER to the Golgi. To investigate the involvement of SCAP in the VEGF activation of SREBPs, we designed a SCAP-specific siRNA that could reduce the SCAP protein level by 70% (Figure 2A). By blocking SCAP with the siRNA, activation of both SREBPs by VEGF was suppressed (Figure 2A). The transport of the SCAP-SREBP complex from the ER to the Golgi is suppressed in the presence of oxysterols such as 25-HC.26,27 Given the dependence of the VEGF-activated SREBPs on SCAP, we treated hMVECs with 25-HC before VEGF stimulation. As shown in Figure 2B, 25-HC also significantly blocked VEGF-activated SREBP1 and SREBP2. These results indicate that VEGF activation of SREBPs requires SCAP.

SREBPs Mediate VEGF-Induced EC Migration and Proliferation
Because VEGF is a potent stimulant for EC migration and proliferation, we investigated the role of SREBPs in these processes in response to VEGF. As shown in Figure 3A, SREBP suppression by either SCAP RNAi or 25-HC significantly inhibited both basal and VEGF-enhanced EC migration. Furthermore, both agents retarded the formation of pseudopodia (Figure 3B), which is the initial step for establishing EC polarity and the direction of migration. To investigate whether SREBPs are also required in EC proliferation in response to VEGF, we detected the EC proliferation in the presence or absence of SCAP RNAi or 25-HC. As shown in Figure 4, suppression of SREBPs by either SCAP RNAi or 25-HC significantly inhibited BrdU incorporation in both basal and VEGF-stimulated EC proliferation. Flow cytometry analysis of the EC cell cycle showed that both SCAP RNAi and 25-HC treatment led to a significant decrease in the S phase associated with cell cycle arrest in the G0/G1 phase.

PI3K-Akt Is Necessary for VEGF-Activated SREBPs in ECs
The activation of the PI3K-Akt pathway by VEGF is a key signaling event in angiogenesis. We thus tested the role of PI3K and Akt in the VEGF-activated SREBPs in hMVECs.
As shown in Figure 5A, treatment with LY294002, a PI3K inhibitor, attenuated the VEGF-activated SREBP1 and SREBP2. Transient transfection assays revealed that VEGF increased the expression of luciferase reporter driven by the LDLR or FAS promoter (ie, LDLR-Luc, FAS-Luc), which could be inhibited by LY294002 (Figure 5B). We also used a dominant-negative mutant of Akt to investigate the role of Akt in the SREBP-mediated transcription in response to VEGF. Cotransfection of LDLR-Luc or FAS-Luc with the dominant-negative mutant of Akt abolished the VEGF-increased luciferase activity (Figure 5B). To mimic the VEGF-activated Akt, we cotransfected ECs with a plasmid encoding a constitutively active form of Akt together with LDLR-Luc or FAS-Luc. Expression of the active form of Akt significantly increased the luciferase activities compared with cells expressing the wild-type Akt, whereas dominant-negative mutant of Akt suppressed the luciferase activities (Figure 5B). These results suggest that PI3K and Akt are implicated in the VEGF activation of SREBPs.

SREBP1 Is Increased in the Neovasculature of the Healing Skin

Neovascularization is a key feature of the wound-healing process. We used a rabbit-skin wound-healing model to investigate the expression of SREBP during angiogenesis in vivo. In this model, the neovasculature formation was significantly increased from 3 days through 14 days after wounding. The level of VEGF increased 24 hours after wounding, reached a peak at 3 days, and remained at a higher level until 14 days after wounding. As shown in Figure 6, SREBP1 was abundantly expressed in the newly formed microvasculatures of the healing skin at day 4 after wounding, compared with the basal level of SREBP1 in the skin tissue without wound. Colocalization of antigens recognized by anti-SREBP1 and anti-PECAM-1 indicate the abundance of SREBP1 in ECs.

25-HC Inhibits VEGF-Induced Angiogenesis in Chick Embryos

To address the involvement of SREBP in the angiogenesis specifically induced by VEGF in vivo, we used the CAM
assay to apply the exogenous VEGF directly and analyzed the resultant angiogenesis quantitatively. Local application of VEGF resulted in a 2.2-fold increase in blood vessel branches. Coapplication of 25-HC with VEGF significantly inhibited the VEGF-induced angiogenesis, which was comparable to results of control experiments in which only DMSO was applied (Figure 7A and 7B). Western blot analysis showed further that 25-HC significantly suppressed the VEGF-induced SREBP1 activation in CAM (Figure 7C).

**Discussion**

By interacting with its receptors on ECs and the subsequent activation of several signaling pathways (eg, PI3K-Akt), VEGF is a potent angiogenic factor. However, the molecular basis for the VEGF-induced EC migration and proliferation remains elusive. The major finding in this study is that the activation of SREBPs contributes to VEGF-induced angiogenesis, which is supported by the following: (1) VEGF activates both SREBP1 and SREBP2 and their target genes (eg, LDLR, HMGCr, and FAS) in cultured hMVECs (Figure 1); (2) both SCAP siRNA and 25-HC suppressed the VEGF-activated SREBPs (Figure 2) and that these agents blocked EC migration (Figure 3) and proliferation (Figure 4) in response to VEGF; (3) PI3K-Akt is crucial for VEGF-activated SREBPs (Figure 5); and (4) in vivo SREBP1 is increased in microvasculatures during wound-healing (Figure 6). In addition, we addressed the involvement of VEGF-induced SREBP in angiogenesis in vivo. CAM assay with exogenously applied VEGF showed that SREBP inhibition led to a significant suppression of VEGF-induced angiogenesis (Figure 7). These findings suggest that SREBPs are functionally involved in VEGF-induced angiogenic processes, particularly in the migration and proliferation of ECs.

The ER-to-Golgi transportation of the SREBP-SCAP complex is sensitive to changes in the cellular level of sterols. SCAP contains the sterol-sensing domain in its...
N-terminus and its C-terminal domain interacts with SREBPs. In the presence of sterols, SCAP undergoes a conformational change, causing the SCAP-SREBP complex to be retained in the ER and thus abrogating SREBP activation. Because of the activation of SREBPs, it is likely that VEGF caused a subtle modification of cholesterol or lipid homeostasis. Pseudopodia formation after polarization is one of the earliest subcellular events in EC migration in response to VEGF. Plasma membrane microviscosity increases at the leading edge during migration, and the cholesterol enrichment in the front edge is responsible for the generation of this microviscosity gradient, which affects the actin dynamics needed for EC migration. We demonstrated that blocking SREBP activation by SCAP RNAi or 25-HC greatly reduced EC pseudopodia formation, which suggests that functional SREBPs are involved in the initial process of EC migration. Cell body movement, membrane detachment, and retraction at the rear edge follow the extension of pseudopodia during cell migration. The property of the plasma membrane may play an essential role in these steps during migration. Because SREBPs are key regulators in the synthesis and homeostatic control of the lipid composition of the cell membrane, it is reasonable to postulate that SREBPs mediate the VEGF-induced EC migration through the regulation of membrane property, including the cholesterol content, the ratio of cholesterol to phospholipids, or the fatty acid composition in phospholipids. In addition, plasma membrane property is essential for the activation of growth factor receptors and membrane-bound signaling molecules, endocytic events required for movement, and interaction of transmembrane integrins with the cytoskeleton and extracellular matrix. Thus, SREBP regulation of membrane lipid components may be also important in the angiogenic signaling pathways associated with the plasma membrane. We have also investigated whether SREBP activation was sufficient for enhancing EC migration and proliferation by the use of adenovirus overexpressing the N-termini of SREBP1c or 2 in hMVECs. The constitutively expressed active forms of SREBP did not increase...
EC migration nor proliferation (data not shown). These results suggest that SREBPs are necessary and thus play a permissive role in EC migration and proliferation in response to VEGF.

It has been demonstrated that the expression of FASs and synthesis of fatty acids are significantly increased in cancer cells and rapidly proliferating normal cells such as those in proliferative endometrium and fetal tissues.\(^{32,33}\) SREBP1 up-regulation was also found in tumor cells of colorectal neoplasia, which is characterized by increased cellular lipogenesis.\(^{34}\) Furthermore, in prostate cancer cells, LDLR is elevated, and the feedback suppression of SREBP2 by cholesterol is lost.\(^{35}\) The newly synthesized phospholipids in cancer cells are incorporated into membrane microdomains that are raft-aggregates involved in key cellular processes, including signal transduction, intracellular trafficking, cell polarization, and cell migration.\(^{36}\) Data presented in Figure 4 showed that SREBP inhibition caused suppression of DNA synthesis and cell cycle arrest. Our results together with previous findings suggest that functional SREBPs are required during EC proliferation, possibly to meet the increased biosynthesis of the plasma membrane and organelle membranes for cell division.

Among the divergent signaling pathways elicited by VEGF, the PI3K-Akt pathway has been well studied for its pivotal role in angiogenesis.\(^{7,9,11–13,37,38}\) Activation of Akt plays an important role in the proangiogenic effects of HMG-CoA reductase inhibitors.\(^{12}\) On the other hand, HMG-CoA reductase inhibitors can activate SREBP1s.\(^{39,40}\) Thus, Akt may interplay with SREBPs during angiogenesis induced by statins. Indeed, Akt has been linked to SREBP activation. In hepatocytes, insulin upregulates SREBP1c, which can be enhanced by the active form of Akt and blocked by the pharmacological inhibition of Akt.\(^{41,42}\) Glucose also induces the upregulation of SREBP1 in a PI3K-Akt-dependent manner.\(^{43}\) We showed that the PI3K inhibitor and Akt-DN blocked the VEGF-induced SREBP activation in ECs (Figure 5). In contrast, the constitutively active form of Akt activated the LDLR and FAS promoters. VEGF may share a mechanism similar to that involved in activation of SREBP by insulin or glucose. One possibility is that the activated PI3K-Akt pathway enhances cell migration and proliferation, which increases the demand of cellular lipids, leading to SREBP activation. PI3K-Akt may also participate in the expression and/or activation of SREBPs by directly modulating the stability of SREBP proteins or the transcriptional activity of their N-termini.

In summary, this study reveals for the first time that SREBPs are essential regulators of angiogenesis in response to VEGF. Targeting SREBPs in therapeutic interventions may be useful for diseases involving impaired angiogenesis.

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